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Assessment of Tiron as a cardioprotective agent

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Assessment of Tiron as a cardioprotective agent

By

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Assesement of Tiron as a cardioprotective agent

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Abstract

The antioxidant Tiron has been shown to protect against reactive oxygen species (ROS) related injury in several experimental models. However, to date, there is little information on the effects of Tiron in the heart. Therefore, the focus of this thesis was to identify and examine the effects of Tiron on the myocardium in a non-clinical setting using 3-month-old, male Sprague-Dawley rats. In addition, the effect of Tiron against Doxorubicin, a potent anti-cancer drug, was also investigated in naïve and ischaemia/reperfusion (I/R) conditions in the myocardium. The anticancer properties of Doxorubicin in the co-treatment with Tiron were assessed in human leukaemia HL60 and human liver carcinoma HepG2 cancer cell lines.

The results of these studies showed that under normoxic conditions, treatment with low concentrations of Tiron (0.25-1mM) did not reveal a negative effect on cardiac viability. However, administration of Tiron at high concentrations significantly increased the infarct size and decreased cell viability, indicating a pro-oxidant effect in the myocardium in normoxic settings. In the context of simulated regional ischaemia and reperfusion in the isolated hearts, treatment with Tiron (0.25-2.5mM) showed a significant reduction in the infarct size. In addition, an improved cellular viability, an increased concentration of the pro-survival Akt and a decrease in caspase-3 activity was also reported in Tiron treated groups when compared to ischaemia/reperfusion (I/R) or hypoxia/reoxygenation (H/R) control group.

Concomitant treatment of Tiron with Doxorubicin in both normoxic and ischaemia/reperfusion (I/R) or hypoxia/reoxygenation (H/R) conditions attenuated myocardial injury, improved the cardiac function and cellular viability, reduced cellular oxidative stress and caspase-3 levels when compared to Doxorubicin treated groups. Interestingly, in human cancerous HL60 and HepG2 cell

lines, 24-hour treatment with Tiron (0.25-2.5mM) alone did not cause significant changes in the cell viability, caspase-3 and reactive oxygen species (ROS) levels. Co-administration of Tiron with Doxorubicin did not impact the cytotoxic effects of Doxorubicin, which were marked by a decrease in cell viability and an increase in caspase-3 activity.

In conclusion, the results presented in this thesis showed for the first time that Tiron exhibited cardioprotective properties in both myocardial ischaemia/reperfusion (MI/R) injury and against Doxorubicin induced-cardiotoxicity via its antioxidative properties. Besides the beneficial cardioprotective effects, Tiron did not alter the efficacy of Doxorubicin in human cancerous cell lines, highlighting the potential use of Tiron as an adjunctive therapy that selectively reduces the toxic side effects of Doxorubicin in the heart without altering its anticancer potency.

Publications and presentations from this PhD thesis

Chiuzbaian, O., Gharanei, M., Mee, C., Maddock, H., and Hatch, E. (2016): Antioxidant Tiron protects against Doxorubicin-Induced Cardiotoxicity. PA2 Online- E-Journal of the British Pharmacological Society 133P **Abstract and Poster presentation at British Pharmacological Society Annual Meeting meeting, December 2016. London, United Kingdom.**

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List of abbreviations

AAR	Area at risk
AIF	Apoptosis inducing factor
Apaf-1	Apoptotic activating factor-1
ATP	Adenosine triphosphate
BAEC	Bovine aortic endothelial cells
BP	Blood pressure
CAD	Coronary artery diseases
CAT	Catalase
CF	Coronary flow
CHD	Coronary heart disease
CHF	Congestive heart failure
CsA	Cyclosporine A
CVD	Cardiovascular diseases
DISC	Death inducing signalling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNR	Daunorubicin
DOX	Doxorubicin
Endo-G	Endonuclease G
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ETC	Electron transport chain
FACS	Fluorescence activator cell sorter
FADD	Fatty acid synthetase associated death domain
FADH2	Flavin adenine dinucleotide 2
FasR	Fatty acid synthetase receptor

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GSH-Px	Glutathione peroxidase
H/R	Hypoxia/reoxygenation
H_2O_2	Hydrogen peroxide
H9c2	Rat cardiomyoblasts
HDL	High density lipoproteins
HepG2	Human liver carcinoma cell line
Hfe	Human hereditary haemochromatosis
HF	Heart failure
HL-60	Human leukaemia cell line
HO	Hydroxyl radical
HOCl	Hypochloride
HR	Heart rate
I/R	Ischaemia/reperfusion
IHD	Ischaemic heart disease
iNOS	Inducible nitric oxide synthase
IRP-1	Iron regulatory protein 1
IS	Infarct size
KHB	Krebs Hensleit buffer
KO	Knock out
LDL	Low density lipoproteins
LV	Left ventricle
LVDP	Left ventricular diastolic pressure
LVEF	Left ventricular ejection fraction
MI	Myocardial infarction
MI/R	Myocardial ischaemia/reperfusion

MMP	Metalloproteinases
MnSOD	Manganese superoxide dismutase
MOMP	Mitochondrial outer membrane permeabilisation
MPTP	Mitochondrial permeability transition pore
MTT	Thyazolyl blue tetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
Nec-1; Nec-3; Nec-5	Necrostatin-1; Necrostatin-3; Necrostatin-5
NO	Nitric oxide
NOX1	Nicotinamide adenine dinucleotide phosphate 1
O_2^-	Superoxide radical
OMM	Outer mitochondrial membrane
PAD	Peripheral arterial disease
PARP	Poly [ADP-ribose] polymerase
PCA	Percutaneous coronary angioplasty
PDE-5	Phosphodiesterase-5
PI3K	Phosphoinositide-3 kinase
RIP1; RIP3	Receptor interacting protein kinase-1; -3
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Smac/Diablo	Second mitochondria-derived activator of caspases/Direct IAP-binding protein with low-PI
SOD	Superoxide dismutase
TBST	Tris-buffered Saline Tween 20
TNFR1	Tumour necrosis factor receptor-1
TNFα	Tumour necrosis factor α
TRAIL-R	Tumour necrosis factor related apoptosis inducing ligand receptor

TTC	2,2,3 triphenyltetrazolium chloride
Wort	Wortmannin

Chapter 1. Introduction

1.1 Cardiovascular diseases

Cardiovascular diseases (CVDs) represent the leading cause of mortality and morbidity in the world, accounting for 17.3 million of deaths each year (WHO, 2016). A recent statistical report published by British Heart Foundation showed that CVDs account for more than one quarter of all deaths in the UK (British Heart Foundation, 2015). One of the major forms of CVDs is coronary heart disease (CHD) which contributes to over 150,000 deaths of CVDs deaths each year (British Heart Foundation 2015). It has been projected that by the year 2030 the number of deaths caused by CVDs is expected to increase to more than 23.6 million each year (Mozaffarian et al. 2015).

CVDs encompasses a variety of diseases affecting the heart and blood vessels, such as CHD, cerebrovascular disease, hypertension (high blood pressure), peripheral arterial disease (PAD) rheumatic heart disease and deep vein thrombosis (Stewart, Manmathan and Wilkinson 2017).

The main risk factors associated with CVDs are smoking, obesity, diabetes mellitus, hypertension, increased levels of low density lipoproteins (LDL) and depleted levels of high density lipoproteins (HDL) (Danaei et al. 2009, Lucero et al. 2014).

In all forms of CVDs, the myocardium undergoes oxidative changes and the resulting reactive oxygen species (ROS) act as the determining factor in the extent of injury, however they may be employed in the diagnostic methods where they can be used as specific biomarkers (Kurian et al. 2016). Research has demonstrated that cardiomyocytes (the major cells of the heart) have

complex mechanisms comprised of enzymatic and non-enzymatic molecules that maintain the oxidative balance (Santos et al. 2011). It has been hypothesised that molecules which stabilise the free radicals produced in the tissue could be useful in treating several diseases (Kurian et al. 2016, Santos et al. 2011), and thus this concept provides the basis to utilise these molecules as adjunctive therapies in preventing oxidative stress in tissue injury in the heart.

1.2 Mitochondria

Mitochondria- the power house of the living cell, have an essential role in generating ATP synthesis via oxidative phosphorylation (Chen and Zweier 2014, Gustafsson and Gottlieb 2007b). In mammals, the process of mitochondrial energy transduction is regulated by the respiratory enzyme complexes I-IV and ATP synthase (F_0F_1ATPase) (Chen and Zweier 2014, Marín-García and Goldenthal 2002) (Figure 1.1). The process of energy transduction required for ATP synthesis is conducted by the electron transport chain (ETC) localised within the internal mitochondrial membrane and requires the oxidation of the proteins nicotinamide adenine dinucleotide phosphate (NADPH) and flavin adenine dinucleotide 2 (FADH2) (Chen and Zweier 2014, Hüttemann et al. 2007) (Figure 1.1). The ETC function is to regulate the electron flow from NADPH or succinate to O_2 via a number of electron reactions and complexes such as complexes I-IV, ubiquinone and cytochrome c (Chen and Zweier 2014, Hüttemann et al. 2007). Electron flow facilitated by ETC can trigger the translocation of the proton from the mitochondrial matrix area in to the cytoplasmic region, which in turn promotes the catalysis of ATP synthesis by the F_0F_1ATPase complex (Berg, Tymoczko and Stryer 2012, Chen and Zweier 2014) (Figure 1.1). This process is termed oxidative phosphorylation.

Figure 1.1 Schematic diagram illustrating the mechanism of reactive oxygen species generated by the mitochondrial transport chain (Chen and Zweier 2014). Complex I (NADH ubiquinone oxidoreductase) mediates the transport of the electrons from the mitochondrial NADH to ubiquinone (Q) leading to the translocation of the protons across the membrane and thereby creating a proton gradient. In complex II (succinate-CoQ reductase), additional electrons derived from succinate are received by ubiquinone via FADH, however the protons are not transferred across the intermembrane space. Complex III ($CoQH_2$-cytochrome c reductase) facilitates the transfer of the electrons received from the Q to cytochrome c. As a result, the electrons are passed to Complex IV (cytochrome c oxidase) via cytochrome c, which in turn reduces the O_2 to H_2O.

1.3 Free radicals and reactive oxygen species

Reactive oxygen species (ROS) are highly reactive radicals that are produced within the mitochondria by oxidative phosphorylation reaction and through exposure to specific

compounds and exogenous sources (Di Lisa et al, 2011; Teresa and Lunawati, 2011).These radicals are comprised of ions and molecules that are derived from the reduction of a single, uncoupled electron of O_2 to form the radical superoxide (O_2^-) (Sullivan and Chandel 2014). The conversion of two superoxide molecules can lead to the formation of one molecule of non-radical ROS molecule hydrogen peroxide (H_2O_2) and one water molecule by superoxide dismutases (SOD) (Sullivan and Chandel 2014, Thannickal and Fanburg 2000). The hydrogen peroxide form can also receive an additional electron in the presence of iron or copper by the Fenton reaction to form a hydroxyl radical (HO) (Sullivan and Chandel 2014, Thannickal and Fanburg 2000).

Reactive oxygen species can be divided in to two different categories: free oxygen radicals and non-radical ROS (Table 1.1). Free oxygen radicals consist of superoxide (O_2^-), nitric oxide (NO), hydroxyl radical (OH), thiyl peroxyl radicals (RSOO), disulfides (RSSR), sulfonyl radicals (ROS) and organic radicals (R) (Liou and Storz 2010, Thannickal and Fanburg 2000). Non-radical ROS are comprised of hydrogen peroxide (H_2O_2), ozone/trioxygen (O_3), organic hydroperoxides (ROOF), singlet oxygen ($^1O_2$), hypochloride (HOCl) and peroxynitrite (ONO^-), nitrosoperoxycarbonate anion ($O=NOOCO_2^-$), dinitrogen dioxide (N_2O_2), nitronium (NO_2^+), nitrocarbonate anion ($O_2NOCO_2^-$) and highly reactive lipid or carbohydrate derived carbonyl compounds (Genestra 2007).

Free oxygen radicals	Non-radical reactive oxygen species
Superoxide (O_2^-)	Hydrogen peroxide (H_2O_2)
Nitric oxide (NO)	Ozone/trioxygen (O_3),
Hydroxyl radical (OH)	Organic hydroperoxides (ROOF)
Thiyl peroxyl radicals (RSOO)	Singlet oxygen ($^1O_2$)
Disulfides (RSSR)	Hypochloride (HOCl)
Sulfonyl radicals (ROS)	Peroxynitrite (ONO^-)
Organic radicals (R)	Nitrosoperoxycarbonate anion ($O=NOOCO_2^-$),
	Dinitrogen dioxide (N_2O_2),
	Nitronium (NO_2^+)
	Nitrocarbonate anion ($O_2NOCO_2^-$)
	Highly reactive lipid or carbohydrate derived carbonyl compounds

Table 1.1 Types of reactive oxygen species (ROS)

Within the heart, mitochondria occupy approximately 30-40% of the cardiomyocyte volume and produce approximately 90% of the ATP, making them a major source of ROS production (Gustafsson and Gottlieb 2007a, Murphy and Steenbergen 2007). In normal physiological conditions, the electron transport of molecular oxygen is connected with the oxidative phosphorylation process required for ATP synthesis (Wallace 1999). However, it has been shown that oxidative phosphorylation represents the main endogenous source of ROS leading to the generation of toxic derivatives such as O_2^-, OH and H_2O_2 (Wallace 2005, Wallace 1999). The production of superoxide anions by mitochondria is mostly generated by the electron leakage from the ETC (Chen and Zweier 2014) (Figure 1). Under physiological circumstances of stage 4 of respiration process, the oxygen pressure within the mitochondria is low, decreasing the oxidative phosphorylation reaction (Chen and Zweier 2014). A reduction in the ratio of mitochondrial oxidative phosphorylation enhances the electron leakage from the ETC, leading to the generation of O_2^- anions, which in turn is converted to H_2O_2 by manganese superoxide dismutase (MnSOD). Then the H_2O_2 is further catalysed by glutathione peroxidase (GPx) and converted to H_2O (Chen and Zweier 2014).

It is well established that proteins within the mitochondrial ETC are abundant in metal particles (complexes II, III and IV) and in iron-sulfur clusters (complexes I,II and III) (Atkinson and Winge 2009, Xu, Barrientos and Andrews 2013). Within the mitochondria, oxidative stress can be significantly elevated in the presence of reduced metal species because H_2O_2 can react with the iron groups (Fe^{2+} and Fe^{3+}) and can be oxidised to hydroxyl radicals via Fenton reaction or Haber-Weiss reaction (Winterbourn 1995, Xu, Barrientos and Andrews 2013) (Figure 1.1).

Even though minor variations in the steady-state concentration of free radicals and ROS play a part in the intracellular signalling mechanisms in a normal cell, uncontrolled rises in ROS generate free radical-mediated chain reactions (oxidative stress) which extensively target proteins, lipids, polysaccharides and DNA (Diplock et al. 1998, LeDoux et al. 1999, Rubbo et al. 1994, Stadtman and Levine 2000, Teresa and Lunawati 2011, Valko et al. 2007b).

1.4 Oxidative stress in cardiovascular diseases

Oxidative stress can be described as an imbalance between oxidants and antioxidants, which leads to the accumulation of ROS resulting in cellular damage (Kurian et al. 2016). Generally, cells trigger an adaptive mechanism to defend them against deleterious effects of oxidative stress, however when the adaptive capacity of the cells is surpassed by uncontrolled oxidant concentration, the cell experiences exacerbated levels of oxidative stress (Kurian et al. 2016). Oxidative stress can be defined as an imbalance in the redox couples such as reduced to oxidised glutathione (GSH/GSSG) or NADPH/$NADP^+$ ratios (Kurian et al. 2016).

Extended research has shown that oxidative stress is involved in the pathophysiology of several cardiovascular diseases including coronary artery diseases, ischaemia-reperfusion injury and heart failure (Dusting and Triggle 2005, Witztum and Steinberg 2001).

1.5 Oxidative stress and coronary artery diseases

Coronary artery diseases (CAD) are a result of the accumulation of plaques which restricts the blood flow in the coronary arteries that supplies the myocardium (Graham et al. 2004). Plaques are deposits comprised of cholesterol, fatty acids, calcium and fibrin, a clotting agent (Kalanuria,

Nyguist and Ling, 2012). Several studies have shown that atherosclerosis and arteriosclerosis are both linked with oxidative stress and inflammation (Bonomini et al. 2008, Madamanchi, Vendrov and Runge 2005, Nojiri, Daida and Inaba 2004).

It has been indicated that the progression of atherosclerosis is triggered by endothelial injury, which in turn stimulates the aggregation of LDL located in the subendothelial membrane (Kattoor et al. 2017, Riccioni et al. 2003). The oxidation of LDL promotes the expression of vascular cell adhesion molecules on the surface of endothelial cells resulting in the accumulation of monocytes and T-lymphocytes into the sub endothelial membrane (Kattoor et al. 2017, Ketelhuth and Hansson 2011). This process triggers the activation of chemoattractant proteins which in turn facilitate the transport of the monocytes and T-lymphocytes into the innermost tunica of the arterial wall (Kattoor et al. 2017). The interaction between these cells and the chemoattractant proteins results in the transformation and differentiation of the monocytes into scavenger receptors, macrophages and modified lipoprotein particles (Kattoor et al. 2017, Singh and Jialal 2006). Due to their structure and appearance, the newly formed lipid overloaded macrophages (also called foam cells) represent the initial feature of atherosclerotic lesions (Singh and Jialal 2006, Stocker and Keaney Jr 2004). Furthermore, during the migration process of leukocytes within the innermost tunica of the artery, a number of cytokines and growth factors are released within the arterial wall (Kattoor et al. 2017, Singh and Jialal 2006, Stocker and Keaney Jr 2004). Along with ROS, these cytokines and growth factors promote the passage of smooth muscle cells and accumulation of collagen, resulting in the formation of atheromatous plaque (Kattoor et al. 2017, Singh and Jialal 2006).

Notably, ROS have an important role in stimulating the expression of scavenger receptors in smooth muscle cells and their differentiation into foam cells. In addition, ROS can promote the activation of matrix metalloproteinases (MMP), a group of enzymes implicated in the disintegration of the atheromatous plaque and the basement membranes of endothelial cells, causing physical disruption of the plaque (Kattoor et al. 2017, Libby, Ridker and Maseri 2002). This enables the expansion of plaque lesions within the artery walls, process that leads to stenosis of the arterial lumen, reduction of the blood flow, coronary thrombosis and complete blockage of the arteries (Falk 2006, Glass and Witztum 2001, Libby, Ridker and Maseri 2002).

Plaque rupture and coronary thrombosis have serious implications on the myocardium by depriving the muscle of blood and nutrients and causing acute clinical complications of ischaemic heart disease (IHD) such as myocardial infarction (Glass and Witztum 2001, Steinberg and Witztum 2010).

1.6 Myocardial infarction

Myocardial infarction (MI), more commonly described as heart attack, is a process that occurs due to formation of plaques in the in the inner walls of the arteries, leading to reduced blood flow to the heart. This process has severe implications, leading to irreversible damage of the heart and necrosis of the cardiomyocytes (Frangogiannis 2015, Lu et al. 2015). The symptoms of myocardial infarction comprise of chest pain, shortness of breath, arrhythmia, anxiety and nausea leading to permanent damage of the heart muscle, and ultimately to heart failure (Frangogiannis 2015, Lu et al. 2015).

1.7 Myocardial ischaemia

Under normal conditions the myocardium pumps blood around the body, providing organs with the oxygen and nutrients required for cellular homeostasis (Balaban 2006). However, following the occlusion of the vessels which supply the myocardium with blood, several areas of the myocardium are restricted of oxygen and nutrients, leading to structural and metabolic changes (Farber, Chien and Mittnacht 1981; Hausenloy and Yellon, 2013). This process is described as myocardial ischaemia. The ultrastructural changes to the cells during prolonged ischaemic conditions include cell swelling and rupture, decreased levels of cellular ATP, changes in the free radical mediated injury, elevated levels of Ca^{2+} within the mitochondria, increased levels of lactic acid (Figure 1.2). Furthermore, these alterations can lead to cell death which may occur via several mechanisms such as: necrosis, apoptosis and autophagy (Farber, Chien and Mittnacht 1981, Hausenloy and Yellon 2013, Kalogeris et al. 2012)

Figure 1.2 Schematic diagram illustrating the main components of the acute myocardial ischaema-reperfusion injury (Hausenloy and Yellon 2013). During ischaemia, due to the lack of oxygen, the cell metabolism is converted to anaerobic respiration, resulting in the production of lactatate and a decrease in the intracellular pH. This process leads to significant changes in the Na^+-H^+ exchanger, leading to the overload of Ca^{2+} ions. The Na^+-K^+ ATPase activity stops during ischaemia, leading to the excessive intracellular Na^+ overload. The acidic environment during ischaemia counteracts the opening of MPTP and prevents cardiomyocyte herpcontracture. During reperfusion, the Na^+-H+ pump is activated resulting in the wash-out of lactic acid and restoration of the physiological pH. In addition, the ETC is reactivated leading to the formation of ROS, which subsequently triggers the opening of MPTP, and facilitates SR damage, thereby contributing to intracellular Ca^{2+} overload and cell membrane damage.

1.8 Myocardial reperfusion

Reperfusion is a vital process which facilitates cellular recovery and maintenance of function; neutralises the lactic acid accumulation during ischaemia by activating the Na^+-H^+ pump and Na^+-HCO^- symporter and restores the physiological pH level to normal values (Hausenloy et al. 2005, Hausenloy and Yellon 2013) (Figure 1.2). Even though early reperfusion is one of the best

approaches to reduce ischaemic damage, the process of restoring blood flow to the ischaemic myocardium can result in additional reperfusion-induced damage and exacerbated production of ROS (Yellon and Hausenloy 2007). However, reperfusion of an ischaemic myocardium can generate two different outcomes on the myocardium:

1) a beneficial effect- characterised by an attempt to rescue the myocardium and reduce the infarct size by 50% (Yellon and Hausenloy 2007)

2) a detrimental consequence- which results in cardiomyocyte injury and death, a phenomenon called “reperfusion injury” (Braunwald and Kloner 1985, Verma et al. 2002, Yellon and Hausenloy 2007).

Detrimental consequences of reperfusion injury are associated with arrhythmias, production of ROS, activation and release of myocardial cytokines, microvascular obstruction, myocardial stunning and lethal myocardial reperfusion injury (Eltzschig and Collard 2004, Hausenloy and Yellon 2013, Kalogeris et al. 2012). Previous studies have described the involvement of ROS and reactive nitrogen species (RNS) in ischaemia and reperfusion injury (Granger and Kvietys 2015, Guarnieri, Flamigni and Caldarera 1980, Tompkins et al. 2006). Factors that have been linked with cardiac dysfunction and death are comprised of oxidative stress, disruption of mitochondrial ion pumps homeostasis, mitochondrial permeability transition pore (MPTP) opening, myocyte hypercontracture and cytochrome c release (Perrelli, Pagliaro and Penna 2011, Sanada, Komuro and Kitakaze 2011, Yellon and Hausenloy 2007).

Even though the cellular homeostasis of the ischaemic area has been re-established after reperfusion, myocardial stunning often follows. Myocardial stunning is a short span effect

characterised by a temporary mechanical dysfunction that develops after ischaemia and reperfusion without causing any irreversible histological injury (Bolli and Marbán 1999; Guarrici et al, 2018). During this process, the left ventricular section of the myocardium is affected, resulting in a visible post-ischaemic contractile dysfunction (Bolli and Marbán 1999).

It has been shown that myocardial stunning activates the production of toxic hydroxyl radicals, which cause sarcoplasmic reticulum damage, also results in imbalances in Ca^{2+} membrane homeostasis and in turn increases the injury provoked by the free radicals (Pomblum et al. 2010).

Despite being a reversible effect of post-ischaemic injury, the clinical relevance of myocardial stunning remains controversial. Therefore, researchers have been focusing in identifying mechanisms and pathways associated with stunning, however, reperfusion therapies have been shown to be more relevant in the treatment of myocardial ischaemia/reperfusion injury (Pomblum et al. 2010). Results from both pre-clinical experimental studies and clinical trials have shown that therapeutic agents such as cyclosporin A (CsA) and carperitide (an atrial natriuretic peptide) could be employed in order to limit the damaging effects associated with MI/R injury (Kitakaze et al. 2007, Mewton et al. 2010, Piot et al. 2008). For example, results from a human clinical trial using a single dose of CsA in patients prior to primary percutaneous intervention for an acute MI revealed that administration of CsA caused a 29% reduction in the myocardial infarction size and significantly decreased the left ventricular end-diastolic volume at 5 days and 6 months after infarction (Mewton et al. 2010). In additional, experimental studies using animal models of MI/R injury have shown that administration of antioxidant therapies could be promising agents in reducing myocardial damage caused by oxidative stress (Burstein et al. 2007,

Hung, Su and Chen 2004, Qu et al. 2016, Yim and Ko 1999). Relevant examples in this case are flavonoids- a group of natural occurring compounds that possess strong antioxidant and cardioprotective properties (Akhlaghi and Bandy 2009a, Liebgott et al. 2000, Testai et al. 2013). Several studies have suggested that flavonoids may exhibit protective effects against ischaemia-reperfusion by scavenging ROS such as superoxide anion radicals (Chun, Kim and Lee 2003, Huk et al. 1998), peorxynitrite (Pollard et al. 2006) and peroxyl radicals (Nakao, Takio and Ono 1998). For instance, in vivo investigations carried out with Yulangsan flavonoid in the Sprague-Dawly rat model of iscahemia/reperfusion injury have shown that pre-treatment with Yulangsan exhibited potent cardioprotective effects that were marked by a significant decrease in the myocardial infarction, an improved activity of endogenous antioxidants SOD and GSH-Px and an enhanced expression of ANT1 mRNA, a protein present within the myocardial mitochondrial spaces (Zhang et al. 2014). In addition, it was revealed that Yulangsan administration was associated with a remarkable decrease in the levels of caspase-3, indicating the anti-apoptotic effects of this antioxidant (Zhang et al. 2014).

1.9 Oxidative stress in myocardial ischaemia/reperfusion (I/R) injury

Myocardial injury induced by ischaemia-reperfusion of the heart has been partly linked with the excessive production of ROS (Kalogeris et al. 2012, Levraut et al. 2003). Under normal physiological conditions, ROS is essential in mediating several cellular signaling pathways that control cell growth, development and stress adaptation (Sugamura and Keaney Jr 2011). However, exposure to environmental insults, such as hypoxia, leads to enhanced production of

ROS, overwhelming the endogenous antioxidant system and causing tissue dysfunction (Kalogeris et al. 2012, Zhou, Chuang and Zuo 2015, Zhu and Zuo 2013). A range of free radicals are produced within cells by several mechanisms, although the majority are generated in the mitochondria upon reperfusion, leading to the formation of superoxide anions, hydrogen peroxide and hydroxyl radicals (Raedschelders, Ansley and Chen 2012, Sugamura and Keaney Jr 2011, Zhou, Chuang and Zuo 2015).

Under normal conditions, xanthine oxidase enzyme is present in the dehydrogenase form and there is a little production of O_2^- (Raghuvanshi et al. 2007). However, under ischaemic conditions, the reduced ATP availability causes loss in the Ca^{2+} membrane gradient. An enhanced concentration of Ca^{2+} leads to the activation of Ca^{2+} dependent proteases (calpains), which activate the translation of xanthine dehydrogenase to xanthine oxidase, therefore resulting in the production of ROS and hydrogen peroxide (Raghuvanshi et al. 2007).

In normal physiological conditions, the free radical production in the myocardium is regulated by free radical scavengers that provide an antioxidant defense system and prevent ROS and RNS induced injury (Rodrigo et al. 2013a, Venardos and Kaye 2007). The free radical scavengers found in the heart are comprised of SOD, glutathione peroxidase (GSH-Px), catalase (CAT) which are located within mitochondria and cytosol. However, in ischaemic conditions, the antioxidant enzyme function is disrupted and a leakage of these enzymes in the extracellular matrix occurs (Venardos and Kaye 2007). During reperfusion, these enzymes are washed out, and the antioxidant defense control is lost, leading to exacerbated production of ROS. Therefore, the

uncontrolled production of free radicals overpowers the activity of these enzymes resulting in cell injury and loss of cell viability (Rahman 2007, Venardos and Kaye 2007).

In an early experimental model, using electromagnetic resonance Zweier et al, (1987), identified a spectral peak of free radicals in the heart during the first 10 seconds of reperfusion (Zweier, Flaherty and Weisfeldt 1987). In a different study, Ambrosio et al, (1991) noted that the spectral of carbon and oxygen radicals peaked quickly at 15-20 seconds upon reperfusion (Ambrosio, Zweier and Flaherty 1991).

Previous experimental models using genetically modified animals have exhibited the importance of endogenous antioxidant SOD in protecting the myocardium against I/R injury (Chen et al. 1998; Zhongyi et al, 2009). In this investigation, Zhongyi et al, (2009) have shown that overexpression of MnSOD in transgenic hearts resulted in a reduction in the infarct size and a decrease in lactate dehydrogenase release (Chen et al. 1998).

Research has also shown that exogenous antioxidants such as SOD mimetics (Masini et al. 2002), Fisetin (Shanmugam et al. 2018), Resveratrol (Shen et al. 2006), Kaempferide (Wang et al. 2017) and other flavonoids (Akhlaghi and Bandy 2009b) can reduce the levels of ROS within the cells, therefore ameliorating the damaging effects of ROS. However, a number of clinical trials carried out with antioxidants such as vitamin C, E and β-carotene in patients post MI have shown that treatment with these antioxidants did not improve the damaging effects of ROS, causing an increase in the risk of congestive heart failure (Marchioli et al. 2006; Hennekens et al. 1996, Lee et al. 1999).

1.10 Metabolic changes of cardiomyocytes in myocardial ischaemia/reperfusion injury (I/R)

Cardiac fatty acid and glucose metabolism are highly mediated mechanisms that supply the myocardium with required amount of energy (Frank et al. 2012). During ischaemia, the anaerobic metabolism characterized by enhanced rate of glycolysis which leads to the production of lactic acid and hydrogen ions (H^+), resulting in a significant decline in intracellular pH leading to acidosis (Frank et al. 2012, Rosano et al. 2008). Furthermore, the rapid depletion of ATP causes a reduced activity of the Na^+/K^+ ATPase ionic pump, which in turns causes an imbalanced increase in the intracellular Na^+ ions, leading to Ca^{2+} overload (Halestrap, Clarke and Khaliulin 2007, Rosano et al. 2008).

1.11 Calcium overload of cardiomyocytes in myocardial ischaemia/reperfusion injury

Calcium overload is a common mechanism that is triggered during ischaemia and reperfusion in the myocardial cells, and it can result in further injury to the myocardium (C Bompotis et al. 2016).

During ischaemia, the anaerobic glycolysis causes a reduction of the intracellular pH and deactivates the troponin C sensitivity to Ca^{2+} (Mani et al. 2015, Sanada, Komuro and Kitakaze 2011). In order to protect the rapid decrease in pH, excessive production of intracellular H^+ ions is activated by Na^+/H^+ exchanger pump, which in turn results in a sustained rise of Na^+ ions (C Bompotis et al. 2016, Garcia-Dorado et al. 2012). In addition, the rapid depletion of ATP hampers

the activity of ATPases, such as Na^+/K^+ ATPase, ATP-dependent Ca^{2+} reuptake and active Ca^{2+} excretion, leading to an overload of Ca^{2+} ions in the cell (Garcia-Dorado et al. 2012).

During reperfusion, the rapid increase of pH to basal levels results in the formation of excessive H^+ gradient across the plasma membrane, activating a strong Na^+/H^+ exchange and a massive increase in Na^+ concentrations (Sanada, Komuro and Kitakaze 2011). This imbalance in the ion exchanger pump stimulates the passive, inverted mechanism of the Na^+/Ca^{2+} exchanger pump, which absorbs the Na^+ accumulation via the excretion pathway, and causes overload of intracellular Ca^{2+} (Sanada, Komuro and Kitakaze 2011, Santulli et al. 2015).

Changes in the concentrations of intracellular Ca^{2+} ions have been suggested to have various negative effects such as the stimulation of intracellular calcium proteases and phospholipases, which leads to fragile cellular structure and hypercontracture, and may even initiate the cellular death pathways (Buja 2005, Santulli et al. 2015).

1.12 Cell death of cardiomyocytes

Cell death has an essential function in many physiological processes including differential tissue development during embryogenesis, tissue homeostasis, ageing and destruction and removal of damaged cells (Tait, Ichim and Green 2014).

Cell death can be divided into the following groups: necrosis, apoptosis and autophagy (Tait, Ichim and Green 2014). Countless studies have reported a link between the myocardial I/R injury and the major pathways of cell death (Eefting et al. 2004, Kim and Kang 2010, van Empel et al. 2005).

1.13 Necrosis of cardiomyocytes

Necrosis can be described as an uncontrolled, degradative process that usually affects multiple types of cells (Elmore 2007). This process is usually triggered in response to physical and chemical insults, including hypoxia, ischaemia, hypoglycemia and steep changes in the temperature homeostasis (Galluzzi et al. 2014, Konstantinidis, Whelan and Kitsis 2012, Vanlangenakker et al. 2008). The features of the necrotic cell death include fissure of the plasma membrane, cell and cytoplasmic organelles swelling, disintegration of mitochondria and the subsequent loss and release of the cellular content within the cytoplasmic area of the cell (Elmore 2007, Vanlangenakker et al. 2008). For decades, necrosis has been considered the only process implicated in the cardiomyocyte death following myocardial ischaemia and reperfusion injury (Buja and Willerson, 1981; Jennings and Reimer, 1981). However, since then significant research in this area elucidated that apoptosis, autophagy and oncosis are also responsible for the fate of cardiomyocytes following an ischaemic insult (Kostin et al, 2003; Buja and Vela, 2008). It has been suggested that necrosis may account for approximately 35% to 50% of the cardiomyocyte death following an episode of acute myocardial infarction (Buja and Weerasinghe, 2010). The main cause of necrosis in the reperfused myocardium is via the formation and opening of the MPTP which activates mitochondrial dysfunction and cardiomyocyte necrosis (Hausenloy and Yellon, 2013; Hausenloy, 2012).

Necrosis has been considered an uncontrolled process, however recent reports have exhibited that necrosis may be linked to a number of signaling pathways and mechanisms, a process called necroptosis (Konstantinidis, Whelan and Kitsis 2012, Takahashi et al. 2012, Vanlangenakker et al.

2008). Studies have indicated that necroptosis could be associated with the activation of death receptors such as TNFR1 signaling system, T-cell receptor, FasR, and TNF-related apoptosis inducing ligand receptor (TRAIL-R) and could have an important role in cardiac dysfunction (Zhe-Wei, Li-Sha and Yue-Chun, 2018; Adameova et al, 2016). Furthermore, it has been suggested that oxidative stress and a number of anticancer drugs might induce necroptotic cell death (Davis et al, 2010; Han et al, 2007). Several studies have shown that necroptosis could be stimulated by the inhibition or absence of the caspase-8 mediated apoptosis and relies on the activation of the receptor-interacting protein kinase 1 and 3 (RIP1 and RIP3) and pseudokinase MLKL resulting in the formation of the necrosome complex (Vandenabeele et al, 2010; Li et al, 2012; Zhe-Wei, Li-Sha and Yue-Chun, 2018).

Recently, a number of experimental studies have discovered that necrostatins 1, -3 and -5 (Nec-1, Nec-3, Nec-5) which target and inhibit the kinase domain of RIPK1 could be employed in preventing the cell death by necroptosis in myocardial I/R injury (Takahashi et al, 2012; Kleinbongard et al, 2011; Lin et al, 2004). Furthermore, several in-vivo and ex-vivo studies using the model of acute myocardial infarction have shown that administration of necrostatins has been associated with a reduction in the infarct ratio, improvement of the haemodynamic parameters and contractile function of the heart (Koshinuma et al, 2014; Luedde et al, 2014; Oerlemans et al, 2012; Smith et al, 2007). Specifically, the study carried out by Koshinuma et al, (2014) in the ex-vivo model of guinea pigs exposed to 30 minutes of global ischaemia and 4 hours of reperfusion aimed to investigate if necroptosis was implicated in the I/R injury. The study showed that concurrent administration of the Nec-1 and Z-VAD inhibitors 5 minutes prior ischaemia and 30 minutes throughout reperfusion resulted in a decrease in the infarct size and

amelioration of left ventricular diastolic pressure (LVDP) and left ventricular ejection fraction (LVEF) haemodynamic parameters when compared to control group (Koshinuma et al, 2014). In addition, in a different study carried out in the mouse in vivo model subjected to 30 minutes of regional ischaemia, it has been demonstrated that administration of Nec-1 5 minutes before reperfusion showed a reduction in the infarct size, oxidative stress along with a decrease in the activation of necrosis (Oerlemans et al, 2012). Furthermore, within the same study, the apoptotic cell death was also assessed, and the results showed no significant differences between the untreated and Nec-1 treated groups (Oerlemans et al, 2012).

1.14 Apoptosis of cardiomyocytes

Apoptosis or programmed cell death is an energy-dependant, highly conserved biochemical process that occurs under physiological and pathological circumstances in response to endogenous and exogenous stimuli (Elmore 2007, Fulda 2010, Majno and Joris 1995). The morphology associated with this process include cell shrinkage and pyknosis, cytoplasmic condensation and cellular fragmentation into membrane-enclosed “apoptotic bodies” (Fink and Cookson 2005, Fulda 2010, Konstantinidis, Whelan and Kitsis 2012). The apoptotic bodies are rapidly phagocytised by macrophages, parenchymal cells and neoplastic cells and are eliminated by the phagolysosomes (Elmore 2007, Konstantinidis, Whelan and Kitsis 2012). Apoptosis can be triggered through the stimulation of a death receptor or via the mitochondrial pathway (Buja 2005).

Apoptosis is associated with a number of molecular, biochemical and morphological processes such as gene activation (programmed cell death), stimulation of caspases, mitochondrial changes

including membrane potential loss, activation of the membrane permeability transition and release of cytochrome C (Buja 2005, Eefting et al. 2004, Fink and Cookson 2005, Fulda 2010).

Several studies have suggested that the process of apoptosis is activated in myocardial I/R injury. Furthermore, the involvement of apoptosis following ischaemia and reperfusion has been previously demonstrated in animal experimental models (Anversa et al. 1998, Dumont et al. 2000, Gottlieb et al. 1994, Mocanu, Baxter and Yellon 2000). Findings have suggested that extended periods of myocardial ischaemia are linked with a rise in necrosis, while reperfusion of the myocardium leads to an increase in the apoptotic process (Dumont et al. 2000, Gottlieb et al. 1994). An early study carried out by Fliss and Gattinger, 1996 on Sprague-Dawley rats revealed that apoptosis can be triggered during the first 2 hours after continuous coronary occlusion and exhibited an increase after 45 minutes of ischaemia and 1 hour of reperfusion (Fliss and Gattinger 1996). Although, in a rabbit experimental model, Gottlieb and his colleagues, 1994 have showed that apoptosis occurs after 30 min of ischaemia followed by 4 hours of reperfusion in the rabbit myocardial tissue, but not in the permanent ischaemic area (Gottlieb et al. 1994). This finding would imply that apoptosis is induced during reperfusion stage. In addition, a study undertaken in dog hearts by Zhao et al, (2000) demonstrated that apoptosis was observed in the myocardium subjected to a short period of ischaemia and reperfusion, but not in the ischaemic tissue only (Zhao et al. 2000)

The process of apoptosis occurs via two molecular signalling pathways: the extrinsic and the intrinsic signalling pathway (Winter et al. 2014a) (Figure 1.3).

Figure 1.3 The intrinsic and extrinsic apoptotic signalling pathways (Sarvothaman et al. 2015). The extrinsic pathway is triggered by the binding of the death receptors to the death ligands. This interaction leads to the formation of the death inducing signalling complex (DISC), which in turn binds to the procaspase-8 and stimulates the autocatalytic activation of caspase-8, and subsequently triggers the protease cascade, leading to apoptosis. The intrinsic pathway is activated by a number of stress factors such as DNA damage, hypoxia, ER stress and growth factor deprivation. The death signal is detected by BH3 only protein which in turn interacts with BAX and BAK. This process leads to the conformational change of BAX and BAK, initiating the formation of the mitochondrial pores and enhancing the permeability of the outer mitochondrial membrane, promoting the release of apoptogenic proteins (cytochrome C and SMAC/Diablo complex, etc). The released cytochrome C connects with the apoptotogenic activator factor-1 (Apaf-1) and enables the formation of the apoptosome complex, which in turn recruits and triggers pro-caspase-9. The activated caspase-9 initiates the activation of caspase-3,-6 and -7 leading to apoptosis.

1.14.1 Extrinsic pathway of apoptosis

The extrinsic pathway is stimulated by cell surface death receptors such as Fas, tumour necrosis factor (TNFα) and TRAIL receptors that bind to extracellular death ligands (Kiechle and Zhang 2002). The activation of the death ligand leads to the oligomerisation of the receptors and recruitment of Fas-associated death domain (FADD) and procaspase 8, forming the death-inducing signalling complex (DISC) (Jin and El-Deiry 2005, Kiechle and Zhang 2002, Winter et al. 2014b). This complex binds to the procaspase 8 and triggers the auto-catalytic activation of caspase 8, which in turn stimulates the protease cascade (caspase-3, -6 and -7), leading to apoptosis (Elmore 2007, Jin and El-Deiry 2005, Kiechle and Zhang 2002) (Figure 1.3).

1.14.2 Intrinsic pathway of apoptosis

The intrinsic pathway, also known as the mitochondrial-mediated apoptosis can be triggered by several endogenous and exogenous stimuli including ischaemia, oxidative stress, DNA damage and endoplasmic reticulum (ER) stress (Galluzzi, Kepp and Kroemer 2014, Loreto et al. 2014). This pathway plays a vital role in the development and in removal of damaged cells (Loreto et al. 2014). The intrinsic pathway is mainly controlled by Bcl-2 family proteins (Chipuk et al. 2010). The Bcl-2 protein complex is comprised of both antiapoptotic proteins (Bcl-2, Bcl-X_L, and Mcl-1) and pro-apoptotic proteins (Bax, Bak and BH3 only proteins) (Kim and Kang 2010, Konstantinidis, Whelan and Kitsis 2012, Zhang et al. 2009a). In normal, physiological conditions, Bax is located in the cytosol, however in response to exogenous stimuli, Bax translocates from the cytosol into the outer mitochondrial membrane (OMM) of the cardiomyocytes (Kim and Kang 2010, Konstantinidis, Whelan and Kitsis 2012, Tait and Green 2010, Zhang et al. 2009a). Furthermore,

the stress stimuli promote the conformational change of Bak, which resides to the outer mitochondrial membrane (OMM) (Cheng et al. 2003, Konstantinidis, Whelan and Kitsis 2012, Zhang et al. 2009a). The apoptotic stimuli that activate Bax and Bak are sensed and converted by BH3 only proteins (Konstantinidis, Whelan and Kitsis 2012). This process results in the formation of mitochondrial pores and enhances the permeability of the external mitochondrial membrane (Sarvothaman et al. 2015). The permeabilisation of the OMM promotes the release of the apoptogenic proteins (cytochrome C and SMAC/Diablo complex, Apoptosis Inducing Factor (AIF), Omi/HtrA2 and Endonuclease G (Endo G) from the mitochondria to the cytosol (Keoni and Brown 2015, Konstantinidis, Whelan and Kitsis 2012, Sarvothaman et al. 2015, Tait and Green 2010, Zhang et al. 2009a). The released cytochrome c connects with the apoptotic activating factor-1 (Apaf-1) and facilitates the formation of the apoptosome complex, which in turn recruits and triggers pro-caspase 9 (Keoni and Brown 2015, Sarvothaman et al. 2015, Zhang et al. 2009a). As a result, caspase-9 initiates the activation of downstream effector caspases-3, -6 and -7 leading to apoptosis of cardiomyocytes (Reed 2000, Slee et al. 1999) (Figure 1.3).

It is well established that intrinsic pathway occurs mainly in mitochondria, however recent studies have highlighted that ER and other organelles including lysosomes and nucleus may be linked with mitochondrial death signalling pathway (Crow et al. 2004, Danial and Korsmeyer 2004). This process is triggered mainly by intracellular Ca^{2+} overload. The concentrations of Ca^{2+} within the ER are increased by Bax and Bak, which can reside in the ER as well as in the mitochondria (Scorrano et al. 2003, Zong et al. 2003). It has been shown that exacerbated levels of cytoplasmic Ca^{2+} may initiate a number of apoptotic processes, including the opening of the MPTP and the release of cytochrome c (Halestrap, McStay and Clarke 2002). The

permeabilization of the membrane facilitates the release of AIF, Endo-G, Smac/DIABLO complex, Bax, Bak and HtrA2/Omi (Elmore 2007, Scorrano et al. 2003). Another mechanism by which intrinsic apoptotic pathway may be triggered is via the nucleus and implicates the activation of poly[ADP-ribose] polymerase (PARP) complex and recruitment of Apoptotic Inducing Factor (AIF) as a response to oxidative stress (Yu et al. 2002). In the nucleus, AIF initiates DNA degradation and activates the mitochondria to release cytochrome c- processes that induce apoptosis (Crow et al. 2004, Yu et al. 2002).

1.15 Autophagy of cardiomyocytes

Autophagy, also known as the third pathway of the cell death, is a stricly controlled catabolic intracellular process in which lysosomes control the degradation and removal of the damaged and dysfunctional proteins and organelles (Lavandero et al. 2015; Przyklenk et al. 2012). As opposed to the other cell death pathways (apoptosis and necrosis) the main mechanism of autophagy involves the consumption of the cellular components to produce energy instead of utilising it (Ma et al. 2012; Lavandero et al. 2015). Several studies have shown that during conditions of stress such as exacerbated increase in ROS levels, DNA damage and nutrient starvation the process of autophagy can be triggered in order to supply nutrients and energy for the maintenance of cell metabolism. However, excessive or uncontrolled autophagy can lead to apoptotic cell death (Eisenberg-Lerner et al. 2009; Lavandero et al. 2015; Antunes et al. 2018).

In the context of myocardial ischaemia, a number of investigations have suggested that autophagy exhibits an important role in cardioprotection and this effect occurs via mechanisms that inhibit apoptosis, protecting against cardiomyocyte damage (Eisenberg-Lerner et al. 2009;

Ma et al. 2014). Furthermore, in support of the role of autophagy in cardioprotection, the study undertaken by Nishida et al. (2009) in mice has shown that inactivation of Atg5 gene (a vital mediator of autophagy) caused ventricular dysfunction and dilation (Nishida et al. 2009). In addition, a number of studies have shown that activation of autophagy by rapamycin (which prevents the mammalian target of rapamycin (mTOR) and subsequently phosphoinositol-3 kinase (PI3K) and Akt stimulation) revealed distinctive cardioprotection against both I/R injury and Doxorubicin, effects that were indicated by an improvement in cell viability, a decline in the ROS levels and apoptosis, and an increased mitochondrial function (Shishi et al. 2012; Yang, Wang and Peng, 2010).

1.16 Cardioprotective strategies

The concept of cardioprotection includes management of cellular pathways and events by pharmacotherapies and other strategies during ischaemia and reperfusion to diminish myocardial cell death (Gerczuk and Kloner 2012).

During the last decade, research has been focused on finding novel, effective therapeutic strategies that could limit the myocardial injury and reduce the mortality and morbidity rates in patients with IHD (Fox et al. 2007, Hamilton 2007, Rodrigo et al. 2013b). Systemic thrombolysis and percutaneous coronary angioplasty (PCA), are two of the approaches that have been clinically used to salvage the myocardium, with the second approach being the most effective as it enables the blood reperfusion of the cardiac area affected by coronary artery occlusion

(Rodrigo et al. 2013b). However, as a result of this procedure, the ischaemic area of the myocardium is reperfused, facilitating the over production of ROS, which leads to myocardial tissue damage and activation of cell death signalling pathways (Maxwell 1997, Rodrigo et al. 2013b).

In addition, a variety of pharmacological therapies have been used to reduce the adverse effects of reperfusion injury (Spath, Mills and Cruden 2016). Pharmacological therapies that improve cardiac remodelling and are currently clinically available include β- blockade, statins and inhibitors of the renin-angiotensin-aldosterone axis (Spath, Mills and Cruden 2016). Furthermore, several reports have highlighted that the use of antioxidants could ameliorate the oxidant stress associated with myocardial IR injury (Hamilton 2007, Maxwell 1997).

1.17 Role of antioxidants in cardioprotection

Antioxidants (also named free radical scavengers) are substances that directly scavenge ROS or indirectly functions to up-regulate the antioxidant defence mechanism and to prevent oxidation and ROS production (Halliwell 2007, Khlebnikov et al. 2007). The antioxidant defence mechanism in the heart is comprised of endogenous enzymatic antioxidants such as superoxide dismutase (SOD), catalase, glutathione peroxidase, which enable the removal of excessive ROS including superoxide anions (O_2^-), hydroxyl radicals (OH), alkoxyl radicals (RO) and peroxyradicals (ROO) (Bouayed and Bohn 2010, Ratnam et al. 2006a). However, the endogenous antioxidant defence mechanism is incomplete without the exogenous antioxidants such as vitamin C, vitamin E, β carotene and polyphenols (Bouayed and Bohn 2010). These compounds have an important role

in sustaining the redox homeostasis between the oxidant and antioxidant in the heart (Hedayati et al. 2017, Moser and Chun 2016, Salvayre, Negre-Salvayre and Camaré 2016)

Cumulative evidence has shown that treatment with vitamin C, vitamin E and β carotene exhibited cardioprotective properties against oxidative-stress mediated myocardial injury (Hamilton 2007, Jackson et al. 1998, Qin et al. 2006, Upston et al. 2001). Furthermore, in a rabbit experimental model, Qin et al, 2006 have demonstrated that combined administration of vitamin E and C for 11 weeks in rabbits 1 week after myocardial infarction resulted in decreased ROS production, increased the anti-apoptotic Bcl-2 protein, decreased the pro-apoptotic protein Bax, inhibited the cytochrome c release, diminished the activity of caspase-3 and caspase 9, and reduced myocyte apoptosis when compared to control (Qin et al. 2006). Furthermore, the combined treatment prevented the downregulation of sarcoplasmic reticulum (SR) Ca^{2+}ATPase, an IR-associated alteration that contributes to Ca^{2+} overload related with IR injury (Qin et al. 2006). Although much work has documented the effects of these antioxidants, many randomised clinical trials and observational studies with vitamin C, E and β carotene concentrating on the prevention of cardiovascular diseases revealed disappointing outcomes (Hennekens et al. 1996, Lee et al. 1999, Virtamo et al. 1998). The results from HOPE clinical trial have shown that treatment with vitamin E increases the incidence of congestive heart failure (McQueen et al. 2005). In another clinical trial that included over 8000 post-MI patients without congestive heart failure at baseline, it has been observed that administration with vitamin E in these patients resulted in a 50% increase in the risk of progressing to chronic heart failure (Marchioli et al. 2006). However, the outcomes from Cambridge Heart Antioxidant Study have revealed that patients

with coronary atherosclerosis treated with vitamin E exhibited a significant decline in the rate of non-fatal MI (Stephens et al. 1996).

In the recent years, various experimental studies investigated the cardioprotective properties of the anti-ageing antioxidants (Petrovski, Gurusamy and Das 2011, Santhakumar, Bulmer and Singh 2014, Santhakumar et al. 2015). An example in this case is Resveratrol, a compound found in the polyphenols from grapes and red wine (Hung et al. 2000a, Magyar et al. 2012, Wu and Hsieh 2011). The cardioprotective properties exerted by Resveratrol include vasorelaxation, anti-inflammatory response and ROS scavenging (Das, Mukherjee and Ray 2011, Das et al. 1999). A study carried out by Hung et al. (2000) has emphasised the potent cardioprotective properties of Resveratrol against myocardial IR injury in rat experimental models (Hung et al. 2000b). This study has demonstrated that pre-treatment with Resveratrol exerted potent protective effects against IR induced arrhythmias and mortality, reducing the myocardial infarction ratio, ventricular tachycardia and ventricular fibrillation (Hung et al. 2000b). Furthermore, tests carried out on the enzyme activity have revealed that pre-treatment with Resveratrol increased the levels of NO, and significantly decreased the lactate dehydrogenase activity levels (Hung et al. 2000b). In addition, a number of experimental studies have reported that treatment with Resveratrol reduced the platelet aggregation and inflammation (Delmas, Jannin and Latruffe 2005), inhibited the activation of NFkB factor (Holmes-McNary and Baldwin 2000), inhibited the oxidation of LDL (Wu and Hsieh 2011) and promoted the neovascularization of the infarcted myocardial tissue linked with rises in thioredoxin, haemoxygenase-1 and vascular growth factor levels (Fukuda et al. 2006, Kaga et al. 2005).

Several signaling pathways have been suggested to be implicated in the process of cell survival and death. Investigations designed to distinguish these pathways may be useful to determine and emphasise the importance of the pathways linked with cardioprotection and cardiotoxicity.

1.18 Intracellular signalling pathways involved in cardioprotection

The maintenance of cellular homeostasis plays a vital role in the regulation of normal cellular processes, and it is determined by the capacity of the cells to distinguish and react to stimuli appropriately (Gomes and Blenis 2015). This is achieved through the ability of the cells to convert the intracellular and extracellular cues into cellular responses such as cell division and cell death (Gomes and Blenis 2015). This process involves the activation of a metabolic network of signaling pathways that mediate the cellular processes such as cell proliferation, growth, migration and apoptosis (Gomes and Blenis 2015). These cues are transferred to the cells through the cell surface receptors which bind to the appropriate ligand leading to conformational changes followed by the phosphorylation or dephosphorylation of proteins that culminate in the activation or inhibition of signal transduction pathways (Mockridge, Marber and Heads 2000).

Within the myocardial ischaemia/reperfusion injury setting, it has been highlighted that activation of protective signaling cascades is vital in rescuing the cardiac myocytes from reversibly damaged myocardium, preventing the progression to irreversible myocardial damage (Rossello and Yellon 2018). Cumulative reports have shown that the pro-survival kinases play an important role in cellular protection and growth in both healthy and I/R cells (Hausenloy and Yellon 2007, Rossello and Yellon 2018). Investigations carried out by Hausenloy and his co-workers in 2004

have demonstrated that cardioprotection from acute myocardial I/R damage occurs via the recruitment of pro-survival kinases Akt and extracellular signal-regulated protein kinases 1 and 2 (ERK1/2)- also referred as RISK pathway (Hausenloy and Yellon 2004). Further investigations carried out by the same group have revealed that RISK pathway includes the activation of phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) (PI3K/Akt) cascade, c-Jun N-terminal kinase (JNK) cascade and extracellular regulatory kinase ERK1/2 pathway.

1.18.1 Phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) (PI3K/Akt) signaling pathway involved in cardioprotection

The serine/threonine kinase Akt (also known as protein kinase B or PKB) has a critical function in regulating various functions of cell physiology including metabolism, growth, proliferation, transcription, survival and protein synthesis in normal and cancer cells (Bozulic and Hemmings 2009). In addition, cumulative evidence has reported that PI3K/Akt signalling pathway has an important role in cardiac physiology and has been associated with a protective role against both myocardial I/R injury and I/R preconditioning.

Akt is comprised of 3 subunits: one central catalytic domain (Thr308), an N-terminal plekstrin homology (PH) domain and a C-terminal regulatory domain (Ser473) (Mullonkal and Toledo-Pereyra 2007).

The first step in the process of activating the Akt/PKB pathway is the activation of PI3K lipid enzymes. The stimulation of PI3K requires the interaction and binding of specific growth factors and cytokines to G coupled protein receptors, triggering the phosphorylation of catalytic subunits phospholipid phosphoinositol-4,5 biphosphate (PIP_2), which in turn stimulates the activation of

the membrane phospholipid phosphoinositol-(3,4,5)-phosphate (PIP_3). A fraction of this is converted to PIP_2 by inositol phosphatase activity (Mullonkal and Toledo-Pereyra 2007). Following the activation of PI3K, the amino-terminal PH domain (PH) interacts and binds to PIP_3 facilitating the recruitment of Akt to plasma membrane from the cytosol (Fujio et al. 2000). This process triggers the phosphorylation of Akt at its Thr308 and Ser473, resulting in the full activation of Akt protein (Fujio et al. 2000).

Stimulation of pro-survival Akt/PKB signalling pathway has been associated with a number of cellular responses and protein interactions within the heart. Examples of the downstream effects of Akt include regulation of the glucose metabolism, by phosphorylating glycogen synthase kinase-3 (GSK-3), regulation the nitric oxide synthesis in endothelial cells and control of the gene transcription (Abel and Doenst 2011, Fulton et al. 1999, Isner and Asahara 1999, Shiojima and Walsh 2002).

Several studies indicated that within the mitochondria, Akt activation supports the survival of cardiomyocytes against apoptosis by phosphorylating and activating the anti-apoptotic proteins (Bcl-2, Bcl-X_L,) and inhibiting the promotion of pro-apoptotic proteins (Bax, Bak and BH3-only proteins) (Armstrong 2004, Kim et al. 2012, Matsui et al. 2001). The subsequent activation results in the inhibition of the mitochondrial outer membrane permeabilisation (MOMP), which in turn prevents the release of cytochrome c and AIF into the cytosol (Forini, Nicolini and Iervasi 2015, Kim et al. 2012). Further to this, studies have suggested the ability of Akt to inhibit apoptosis, by supressing the activation of caspase-3 signalling cascade and prevent the apoptosome formation (Su et al. 2011). Although short term activation of Akt can have beneficial effects on the

myocardium, sustained Akt stimulation might have a negative impact on the heart, increasing the cardiac contractility and promoting heart failure (O'Neill and Abel 2005, Wende et al. 2015).

1.19 Anticancer therapies and cardiotoxicity

Many anticancer therapies have demonstrated to be extremely cardiotoxic and have been associated with exacerbated production of ROS in the cardiac tissue (McGowan et al. 2017, Volkova and Russell 2011). Relevant example in this case are anthracyclines, an effective group of anticancer drugs, which induce cardiac toxicity through various mechanisms of action (Volkova and Russell 2011).

1.19.1 Anthracyclines chemotherapy and its side effects

Anthracyclines are one of the most effective anticancer agents, playing an important role in treating several forms of solid and liquid malignancies (McGowan et al. 2017, Weiss 1992). The first anthracyclines Doxorubicin (DOX) and Daunorubicin (DNR) were first isolated in early 1960s from soil bacterium Streptomyces peucetius (Grein 1987). The revolutionary discovery of these drugs had a massive impact in the antibiotic overproduction techniques of the day (Rimal et al. 2015). Furthermore, anthracyclines are currently listed in the in the World Health Organization (WHO) model list of essential medicines (Organization May 2015).

Currently, cancer affects more than 1 in 3 people in their lifetime, and along with cardiovascular diseases, they represent the leading causes of mortality in developed countries. Findings from an analysis of population-based cancer registry data carried out by Coleman et al, (2011) has revealed that ten-year cancer survival trend is approximately 50% among the most common types of cancer, and approximately an 80% survival in lymphoma, melanoma and uterine

malignancies (Coleman et al. 2011) These trends indicate a major progress in the survival ratios in the developed countries (Coleman et al. 2011). Furthermore, in the United Kingdom, since the 1960s, a two-fold increase was noted in the ten-year or more cancer survival rates in adults, and a three-fold increase in children (Cancer Research 2014). However, the effective use of DOX has been limited by its dose-limiting toxicities such as nausea, vomiting, extravasation, hematopoietic suppression, and nevertheless cardiotoxic reactions (Mitry and Edwards 2016, Octavia et al. 2012).

1.19.2 Anti-tumour activity of Doxorubicin

Despite expensive research and clinical use of anthracyclines, the mechanisms of action of these anti-tumour agents in cancer cells remain unknown and continue to be a matter of debate. Various hypotheses were suggested, including inhibition and synthesis of macromolecules which intercalates into DNA, causing inhibition of DNA replication and synthesis (Minotti et al. 2004, Schott and Robert 1989a). Another hypothesis of DOX activity includes generation of reactive oxygen species, which plays an important role in producing DNA damage and causing lipid peroxidation (Benchekroun et al. 1993, Minotti et al. 2004). Furthermore, several investigations suggested that DOX treatment can activate DNA binding and alkylation, DNA strands separation and direct membrane activity (Benchekroun et al. 1993, Minotti et al. 2004, Schott and Robert 1989b). One of the major mechanisms of DOX-induced cytotoxic effects is the inhibition of topoisomerase II activity which triggers the initiation of DNA damage and stimulation of apoptosis (Minotti et al. 2004, Ramachandran et al. 1993). There are two types of toposiomerase II enzymes: topoisomerase IIα and topoisomerase IIβ isoforoms. Topoisomerase IIα is well established marker of cell proliferation, and it is overexpressed in cancer tissue, but is not present

in healthy tissue (Zhang et al. 2012; Lyu et al. 2006). Therefore, topoisomerase IIα isoform is recognised as an important molecular target for Doxorubicin antiproliferative activity (Zhang et al. 2012). Within the heart, the topoisomerase II isoforom expressed in adult cardiomyocytes is topoisomerase IIβ. This isoform represents a target for Doxorubicin, having the ability to induce DNA double strand breaks, resulting in cardiomyocyte cell death (Lyu et al. 2007; Zhang et al. 2012).

Although DOX is considered one of the most effective anti-tumour compounds, countless investigations have shown that its use has been associated with a series of cardiac events, including progression to heart failure (Groarke and Nohria 2015, Volkova and Russell 2011, Von Hoff et al. 1979). For example, the study carried out by

1.19.3 Cardiotoxic effects of Doxorubicin treatment

Although, DOX is one of the most effective anti-cancer therapies, there are numerous off target effects, which can affect healthy tissues in other organs (Groarke and Nohria 2015, Volkova and Russell 2011). Serious side effects caused by DOX cumulative toxicity include damage to cardiac myocytes leading to dilative cardiomyopathy and congestive heart failure (CHF) (Groarke and Nohria 2015, Volkova and Russell 2011, Von Hoff et al. 1979). Early clinical trials carried out by Von Hoff et al, (1979) which included over 4000 patients subjected to DOX treatment have revealed that patients developed clinical effects of CHF (Von Hoff et al. 1979). This study has demonstrated that cumulative dose of DOX plays a crucial role in the frequency of CHF, observed at the administration of cumulative doses of 550 mg/m^2 (Von Hoff et al. 1979). Furthermore, investigations carried out in three clinical trials reported that an increased dose of DOX resulted

in an increased level of risk of congestive heart failure (Swain, Whaley and Ewer 2003). The incidence of DOX-related CHF in patients receiving a dose of 400 mg/m^2 was 5%, with a higher percentage of 26% at 550 mg/m^2, and 48% at 700 mg/m^2 (Sawin et al, 2003). The prevalence of DOX induced CHF has been shown to be dependent on age (Figure 1.4) (Swain, Whaley and Ewer 2003). Investigations carried out demonstrated that cumulative DOX-related CHF has a higher risk of incidence in patients over the age of 60, and a lower risk of CHF prevalence in younger patients (Barrett-Lee et al. 2009, Grann et al. 2006, Swain, Whaley and Ewer 2003). Furthermore, gender difference has also been noted as one of the risk features of DOX-related cardiotoxic effects (Lipshultz et al. 1995). A study carried out by Lipshultz et al, (1995) showed that female patients exhibited severe cardiotoxic events with more depressed contractility (Lipshultz et al. 1995).

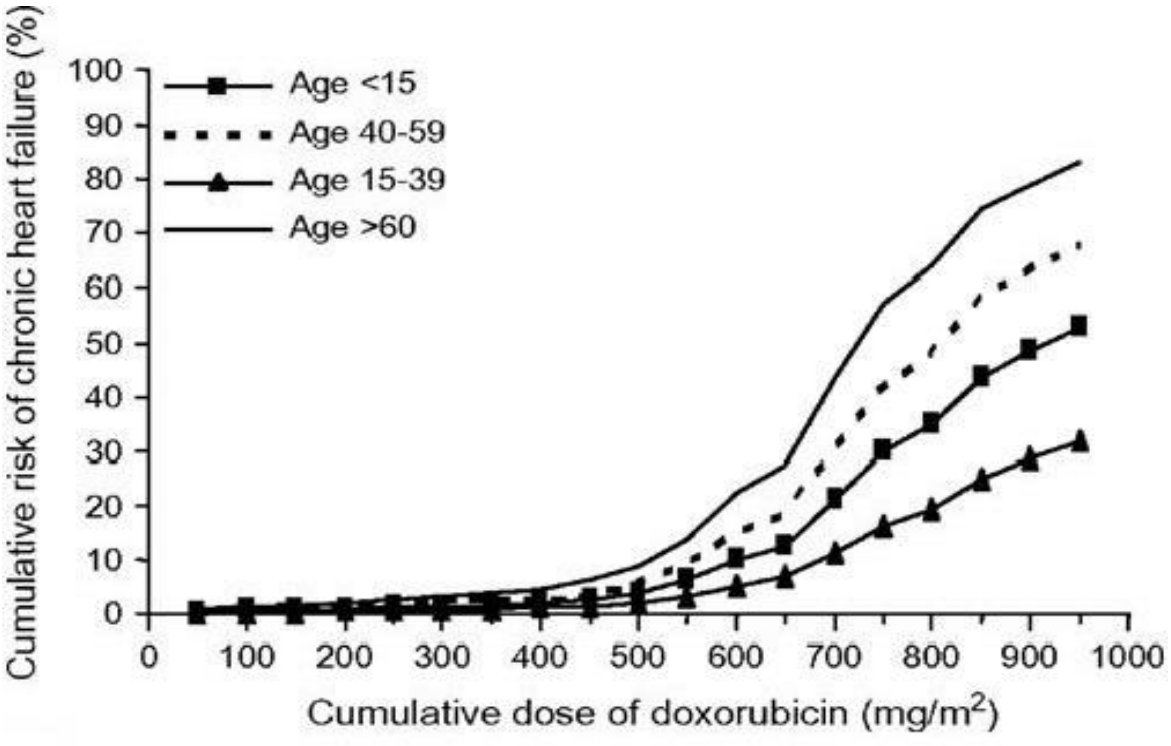


Figure 1.4 The percentage of DOX-related CHF dependant on age (Barrett-Lee et al. 2009. The risk of developing CHF in Doxorubicin treated patients is age dependant and increases significantly at cumulative doses of 500 mg/m^2 and higher.

Several studies have revealed that a concentration of 1-2µM DOX in plasma concentration appears to be the most effective concentration used in the in vitro studies (Camaggi et al. 1988). Severe acute and chronic adverse effects of DOX administration have been reported (Mitry and Edwards 2016). The acute side effects include arrhythmias, hypotension, tachycardia, arrhythmia and depression of the contractile function (Shakir and Rasul 2009a, Zucchi and Danesi 2003). Chronic adverse effects of DOX administration appear to develop or degrade over an extended time interval (Ganz et al. 2008, Hooning et al. 2007, Longhi et al. 2007). The main adverse reactions of anthracycline intake comprise of congestive heart failure and cardiomyopathy (Cardinale et al. 2010, Hooning et al. 2007, Shakir and Rasul 2009a). There are countless reports

which demonstrate that the cardiovascular events of anthracycline intake are dependent on dosage and irreversible (Drafts et al. 2013, Minotti et al. 2004, Salvatorelli et al. 2018).

1.19.4 Mechanisms of Doxorubicin-induced cardiotoxicity

Cardiotoxic effects of DOX were first studied during early clinical trials in the late 1970s. These studies revealed cardiac disturbances that could be a result of DOX repeated administration (Ganz et al. 2008, Torres and Simic 2012, Von Hoff et al. 1979). DOX-induced cardiac injury is a complex process and involves different pathways, of which some of these pathways are linked with the metabolism and the anti-tumour activity of the drug (Torres and Simic 2012). The mechanisms that are responsible for DOX-induced cardiac events are: free radical stress, calcium overloading and mitochondrial dysfunction (Octavia et al. 2012, Torres and Simic 2012). The proposed mechanisms are illustrated in Figure 1.5.

Figure 1.5 Proposed mechanisms of DOX induced cardiac toxicity (Renu et al. 2017).

1.19.5 Free radical stress induced by Doxorubicin

Even though DOX induced cardiac toxicity is a multifactorial process, formation of reactive oxygen species (ROS) plays an important role in activating the oxidative mitochondrial damage (Simunek et al. 2009, Torres and Simic 2012, Xu et al. 2001). The elevated level of free radical stress produced by DOX is linked to the molecular aspects which facilitate the initiation of redox reaction and consequently free radical cascade (Torres and Simic 2012, Xu et al. 2001).

DOX is a member of the quinone group of chemicals, which comprises of naphtacenequinone nucleus connected via a glycosidic bond to an amino sugar, daunosamine (Guin, Das and Mandal 2011). Reduction of quinone can occur in either one-electron or two-electron reduction. One electron of the tetracyclic ring of DOX results in the formation of a free radical semiquionone (Guin, Das and Mandal 2011, Montaigne, Hurt and Neviere 2012). This radical is stable in anoxic conditions, but in neutral environment, the unpaired electron is donated to oxygen, which results in the formation of superoxide radicals (Berthiaume and Wallace 2007, Guin, Das and Mandal 2011, Montaigne, Hurt and Neviere 2012). This reaction is catalysed by cytochrome P450, cytochrome P450 reductase, ubiquinone oxireductase, cytochrome b5 reductase and xanthine dehydrogenase, endothelial nitric oxide synthase enzymes (Berthiaume and Wallace 2007, Minotti et al. 2004). This sequence of reaction, also called “redox cycle” can have a damaging effect, due to the formation of various superoxide radicals (Berthiaume and Wallace 2007, Hahn, Lenihan and Ky 2014, Montaigne, Hurt and Neviere 2012). The redox cycle of DOX administration has been observed in cytoplasm, mitochondria and sarcoplasmic reticulum (Berthiaume and Wallace 2007, Hahn, Lenihan and Ky 2014).

Activation of myocardial oxidative stress represents one of the most important mechanisms of anthracycline-induced cardiac toxicity (Simunek et al. 2009). ROS promotion as well as drug binding to the nucleus, leads to DNA, mitochondria and myocytes cell death (Montaigne, Hurt and Neviere 2012). Several investigations have demonstrated that ROS production can lead to various cardiotoxic effects, such as: reduced expression of cardiac proteins, imbalance in cellular and mitochondrial calcium homeostasis, activation of mitochondrial DNA lesions, disturbances in mitochondrial bioenergetics and ATP system, deprivation of cytoskeleton and myofilament

proteins (Gilleron et al. 2009, Sawyer et al. 2010, Simunek et al. 2009, Tokarska-Schlattner et al. 2006). Several investigations and experiments carried out with isolated cardiac myocytes supported the involvement of oxidative stress in anthracyclines cardiac toxicity (Asensio-López et al. 2017, Ludke et al. 2017, Zhang et al. 2009a). It has been previously demonstrated that isolated cardiac myocytes are exhibiting production of mitochondrial ROS when exposed to anthracyclines treatment (Zhang et al. 2009a, Zhao and Zhang 2017).

1.19.6 Mitochondrial dependent ROS induced by Doxorubicin

It is well established that mitochondria are the most exposed and progressively damaged organelles of DOX-induced cardiotoxic effects (Chang et al. 2011, Octavia et al. 2012). One possible explanation for this effect could be associated with the cationic form of DOX that is preserved in the inner membrane of the mitochondria, leading to formation of an almost irreversibly complex with cardiolipin-a phospholipid of the inner mitochondrial membrane (Goormaghtigh et al. 1980). The cardiolipin is responsible for maintaining the functionality and homeostasis of the proteins from the electron-transport chain (Goormaghtigh et al. 1980), and since DOX disturbs the cardiolipin-protein interaction, elevated levels of superoxide anions O_2^- are formed (Schlame, Rua and Greenberg 2000). In a different investigation, Kashfi et al, (1990), have postulated that several membrane proteins that control the transport of carnitine, could be disrupted by DOX, causing a reduction in the mitochondrial function (Kashfi et al. 1990). The disturbances in mitochondrial function result in structural changes such as mitochondrial swelling and disruption of homeostasis within the mitochondria (Cole et al. 2006). Several reports have reported the damaging effects of DOX-induced myocardial injury, however, these studies tested high concentrations of DOX (Minotti et al. 1998, Papadopoulou et al. 1999). Nevertheless, clinical

concentrations of DOX could have a negative impact on the mitochondrial function, but these reactions are less damaging. Carvalho et al, (2010) have reported that in a rat model of DOX-induced cardiotoxicity, the oxidation of long-chain fatty acid in the mitochondria exhibited a significant reduction, whereas, the glucose metabolism was significantly enhanced, suggesting that an overall change from the aerobic to anaerobic respiration occurs (Carvalho et al. 2010).

Due to DOX accumulation in the mitochondria of cardiomyocytes, one electron reduction appears to be another possible pathway involved in the quinone reduction in the heart (Minotti et al. 2004, Montaigne, Hurt and Neviere 2012). Furthermore, quinones have a high activity, which can interact and redox cycle with their semiquionone radicals, resulting in formation of ROS such as H_2O_2 and hydroxyl radical (Minotti et al. 2004, Montaigne, Hurt and Neviere 2012).

Moreover, early studies suggested that cultured cells and animal models expressed antioxidant protection against anthracyclines induced cardiotoxicity (Yen et al. 1999). It has been highlighted that upregulation of MnSOD has been revealed to increase the cell survival rate in the presence of DOX via its function as a free radical scavenger in the mitochondria (Pani et al. 2000). Furthermore, Kim et al, (2006) have shown that calceolarioside, a natural substance from traditional Chinese medicine, offered protection against DOX-induced apoptosis by increasing the expression of SOD and hindered the mitochondrial death signalling (Kim et al. 2006a). Also, an investigation carried out in the rat model of DOX-induced cardiomyopathy, showed that MnSOD transgenic mice exhibited protection against DOX-induced ultrastructural mitochondrial injury in the heart (Yen et al. 1999).

1.19.7 Nitric oxide synthase and Doxorubicin induced cardiotoxicity

Besides ROS, RNS have a significant role in DOX induced cardiotoxicity (Fogli, Nieri and Breschi 2004).

The effect of DOX on the nitric oxide (NO) signalling pathway has been previously investigated in in vivo and in in vitro studies. In an in vivo model using M-mode and Doppler echocardiography to assess left ventricular performance in CF-1 mice, Weinstein et al, (2000) have shown that administration of DOX 5 days post treatment caused a reduction in the cardiac contractility and a significant increase was detected in the myocardial inducible nitric oxide synthase (iNOS) and 3-nitrotyrosine formation when compared to control (Weinstein, Mihm and Bauer 2000). These results indicate that iNOS production and the formation of $ONOO^-$, a harmful by-product in cardiomyocytes could be linked with a crucial process of DOX-induced cell damage (Fogli, Nieri and Breschi 2004). Several experimental studies have reported that absence of iNOS has been associated with protection against DOX-induced cardiotoxicity (Cole et al. 2006, Mukhopadhyay et al. 2009).

Furthermore, the role of endothelial nitric oxide synthase (eNOS) in DOX-induced cardiac toxicity has been previously investigated (Kalivendi et al. 2001, Vásquez-Vivar et al. 1997). It has been demonstrated that DOX binding to the reductase domain of eNOS leads to the formation of the semiquinone and O_2^- generation (Vásquez-Vivar et al. 1997). It has been postulated that DOX-induced apoptosis is associated with an enhanced transcription of eNOS (Kalivendi et al. 2001). Furthermore, in an in vitro experimental model of bovine aortic endothelial cells (BAEC), Kalivendi and his colleagues, (2001) have confirmed that administration of DOX caused a

significant increase in the eNOS transcription and protein expression and pre-treatment with antisense eNOS oligonucleotide prevented DOX-induced apoptosis (Kalivendi et al. 2001).

1.19.8 Nicotinamide adenine dinucleotide phosphate oxidase and Doxorubicin induced cardiac dysfunction

The nicotinamide adenine dinucleotide phosphate (NADPH) oxidases represent a class of numerous subunit enzymes that produce $O_2^{\cdot-}$ by reducing one electron of oxygen utilising NADPH as an electron resource (Bedard and Krause 2007). The NADPH oxidase is encompassed of two membrane-bound subunits (gp91 and p22) and four cytoplasmic subunits (p40, p47, p67 and G protein Rac1 or 2) (Bedard and Krause 2007). There are a number of seven Nox isoforms that are existent in non-phagocytic cells, along with vascular endothelial cells, smooth muscle cells, fibroblasts, and cardiac myocytes (Chen, Craige and Keaney Jr 2009).

Previous investigations have postulated that NADPH oxidases are associated with elevated levels of ROS upon DOX treatment (Deng et al. 2007; Gilleron et al. 2009). Deng et al, (2007), have shown that DOX and NADPH can generate superoxide radicals without any enzymatic activity (Deng et al. 2007). They have also demonstrated that gp91phox KO mice were resistant to cardiotoxic effects caused by DOX treatment, whereas the wild-type mice exhibited the opposite effects (Deng et al. 2007). A recent study carried out by Iwata and co-workers, (2016), has demonstrated that a non-phagocytic isoform of NAPDH oxidase, NOX1 in the heart was up-regulated in DOX-treated wild-type mice leading to an increase in ROS (Iwata, Matsuno and Yabe-Nishimura 2016). Furthermore, in NOX1 deficient mice, DOX-induced cardiac damage and the activity of pro-inflammatory cytokines were significantly reduced (Iwata, Matsuno and Yabe-

Nishimura 2016). Additionally, in vitro experiments in H9c2 rat cardiomyoblasts carried out by Gilleron et al, (2009) highlighted the importance of NADPH oxidases in the progression of DOX-induced cardiac dysfunction (Gilleron et al. 2009). The study demonstrated that treatment with DOX resulted in a rapid generation of superoxide radicals through NADPH oxidase activation (Gilleron et al. 2009), and the use of NADPH inhibitors resulted in a decrease in DOX-induced ROS generation, improving cell survival (Gilleron et al. 2009).

1.19.9 Iron and Doxorubicin induced cardiotoxicity

A different proposed mechanism of anthracyclines ROS production may be via the redox cycle of the aglycone groups along with the anthracycline-iron complexes (Montaigne, Hurt and Neviere 2012, Torres and Simic 2012).

Iron is an essential element used for several vital cellular processes within the body such as oxygen transport, energy generation and cell cycle (Gammella et al. 2014, Gammella et al. 2015). Previous studies have revealed that iron can play an important role in DOX-induced cardiotoxicity (Ichikawa et al. 2014). Iron is an essential element used for several vital cellular processes within the body such as oxygen transport, energy generation and cell cycle (Gammella et al. 2014, Gammella et al. 2015). Previous studies have revealed that iron can play an important role in DOX-induced cardiotoxicity (Ichikawa et al. 2014). However, in normal, physiological conditions, the concentration of free iron is not sufficient to interact with DOX to cause cardiotoxic effects (Dos Santos and Dos Santos Goldenberg, 2018). On the other hand, it has been postulated that DOX causes an iron-regulated increase in ROS, also called “ROS and iron hypothesis” (Ichikawa et al. 2014, Myers 1998). According to this theory, there are 2 major pathways by which iron could

generate ROS production in DOX-treated cells: the first pathway is via the Fenton and Haber-Weiss reactions, whereas, the second one includes activation and formation of DOX-iron complexes (Simunek et al. 2009). The ability of DOX-aglycone complex to undergo oxidation leads to the formation of superoxide free radicals ($O2^-$), which is rapidly converted into H_2O_2 spontaneously or it could be regulated by SOD enzyme activity (Ichikawa et al. 2014, Simunek et al. 2009, Xu, Persson and Richardson 2005). It is well established that H_2O_2 molecules are relatively stable molecules, and under biological conditions, the exacerbated levels of this molecule are removed by the CAT and GPx enzyme complex (Simunek et al. 2009). However, H_2O_2 molecules could lead to the formation of extremely toxic hydroxyl radicals' activity (Ichikawa et al. 2014, Simunek et al. 2009, Xu, Persson and Richardson 2005). This reaction is also called Fenton-Weiss reaction, and it is initiated in the presence of heavy metals, such as iron (Simunek et al. 2009). Taking into consideration the catalytic role that free cellular iron plays in the production of harmful hydroxyl radicals, organisms have specific proteins intended for iron acquisition, passage and storage as well as complex mechanisms that mediate the levels of intracellular iron homeostasis (Ichikawa et al. 2014, Simunek et al. 2009).

The labile pool of cytosolic iron (which matches to the iron transported between the transporter ferritin and the storage protein ferritin) is detected by the iron regulatory proteins (IRPs). The main role of these proteins is to regulate and control the expression of transferrin receptor and ferritin at the translation level, preserving the intracellular free iron pool at a low concentration (Simunek et al. 2009; Dos Santos and Dos Santos Goldenberg, 2018; Myers 1998). In an early study undertaken by Thomas and Aust (1986) it has been demonstrated that DOX enhanced the concentration of free, redox-active iron by stimulating the production of $O2^-$ radicals, which

subsequently facilitated the reductive discharge of iron from ferritin (Thomas and Aust, 1986). The resultant hydroxyl radical produced in this manner is highly reactive, cannot be removed by the enzymatic systems and it can produce an impairment in the macromolecules function such as lipid peroxidation, protein and nucleic acid mutations (Halliwell and Gutteridge, 2007).

The second pathway by which iron could stimulate ROS production in DOX treated cells involves the activation of DOX-iron compounds. Previous studies have demonstrated that DOX can react with the iron leading to the generation of reactive DOX-iron compounds such as doxorubicinol (Ichikawa et al. 2014, Minotti, Cairo and Monti 1999, Montaigne, Hurt and Neviere 2012).

Earlier experimental evidence has demonstrated that the effects of DOX on iron uptake may be regulated via the proteins that separate and bind the intracellular iron. A proposed mechanism was described by Minotti et al, (1998), and includes doxorubicinol metabolite producing complexes with aconitase/IRP-1 (iron regulatory protein), a mechanism that increases transferrin mRNA stability and counteracts the translation of iron sequestration proteins (Minotti et al. 1998). In addition, a reduction in IRP-1 results in an enhanced production of free iron, which could have an impact on the ROS formation (Minotti et al. 1998). In a different experimental study, using Hfe (human hereditary hemochromatosis)-deficient mice, Miranda et al, (2003) have shown that these mice exhibited a higher predisposition of DOX-induced cardiotoxicity (Miranda et al. 2003). Furthermore, experimental approaches carried out in a rodent model have shown that Sprague-Dawley rats exposed to an iron-rich chow diet for 10-14 weeks exhibited a significant increase in DOX-induced cardiac dysfunction (Panjrath et al. 2007). These findings highlight the vital role that iron plays in DOX-induced cardiac toxicity.

1.19.10 Doxorubicin treatment induced apoptosis

It has been previously demonstrated that cardiac toxicity induced by anthracyclines involves two mechanisms: oxidative stress and apoptosis (Montaigne, Hurt and Neviere 2012, Sawyer et al. 2010, Simunek et al. 2009, Zhang et al. 2009b). The majority of the cellular events caused by ROS production are involved in the process of cardiac myocytes death, which has been revealed as being one of the major mechanisms of anthracyclines induced cardiomyopathy (Montaigne, Hurt and Neviere 2012, Sawyer et al. 2010, Simunek et al. 2009, Zhang et al. 2009b). Several studies carried out in animal experimental in vivo and in vitro models have shown that cardiac myocyte death involves the activation of the apoptotic and necrotic pathways, phenomenon that occurs after in vivo and in vitro treatment with anthracyclines (Toldo et al. 2013, Zhao and Zhang 2017). The mechanism of apoptosis in the heart caused by anthracyclines administration reveals the involvement of mitochondrial pathways, which involves the release of Bax proteins, cytochrome c and activation of caspase-3 (Kotamraju et al. 2000, Montaigne, Hurt and Neviere 2012, Zhao and Zhang 2017).

Research has also suggested that elevated levels of ROS produced by DOX administration might disrupt the calcium homeostasis balance by altering the Ca^{2+} gradient (Mitry and Edwards 2016, Pecoraro et al. 2017, Zhang et al. 2009a). It has been proposed that DOX might interact with ryanodine receptor, stimulating uncontrolled concentrations of calcium to be released from the sarcoplasmic reticulum (Angsutararux, Luanpitpong and Issaragrisil 2015, Kim et al. 2006, Saeki et al. 2002, Zhou et al. 2001). In addition, studies have shown that DOX could also disrupt the Na^+/K^+ pump located on the sarcolemma, affecting the Na^+ concentrations required for Ca^{2+} ions to flow into the sarcolemma of cardiac myocytes (Zhou et al. 2001). Alterations of these pumps

and channels disturb the calcium homeostasis leading to calcium overload phenomenon, which in turn results in mitochondrial dysfunction and apoptosis (Kim et al. 2006, Zhou et al. 2001).

1.20 Cardioprotection against Doxorubicin induced cardiotoxicity

As mentioned before, cardiac toxicity is one of the most common side effects associated with anthracyclines, so the improvement in life expectancy after treatment with anthracyclines might be compromised by life threatening cardiovascular diseases and risk factors such as: hypertension, myocardial dysfunction, arrhythmias, myocardial infarction, cardiomyopathy and congestive heart failure (Rahman, Yusuf and Ewer 2007, Shakir and Rasul 2009b)

Several studies have been carried out in order to find adjunctive therapies that would offer protection against DOX induced cardiotoxicity (Della Torre et al. 1999, Erboga et al. 2016, Lou et al. 2015, QuanJun et al. 2017). For instance, experimental animal studies carried out by Osman et al, (2013), have revealed that treatment with Resveratrol exhibited an increase in the cytotoxic activity of DOX in cancer cells, as well as offering protection against DOX-induced cardiotoxicity (Osman et al. 2013). The histopathological analysis of the heart following the co-administration of Resveratrol and DOX in male Wistar rats indicated that Resveratrol exhibited cardioprotective properties against DOX-induced cardiotoxicity by preserving the normal structure of the muscle fibres (Osman et al. 2013). Furthermore, co-treatment with Resveratrol and DOX in Ehrlich ascites carcinoma bearing mice showed that Resveratrol increased the survival rate percentage to 70% long term survivors compared to 10% long survivors when DOX treatment alone was administered (Osman et al. 2013). In addition, findings from the same study have shown that

Resveratrol increased the cytotoxicity of DOX against Ehrlich ascites carcinoma cells (Osman et al. 2013).

In the last decade, clinical trials and meta-analysis studies have examined the cardioprotective effects of Dexrazoxane against DOX-induced cardiotoxicity (Lipshultz et al. 2004, Venturini et al. 1996). Dexrazoxane, and FDA approved drug and an iron-chelator compound, has been found to reduce the cardiotoxic effects of anthracyclines administration in patients with breast cancer, sarcoma and lung cancer (Langer 2014). It has been suggested that protection of Dexrazoxane against DOX induced-cardiotoxicity involves the binding of free iron in the cell, diminishing the intracellular concentrations of iron associated with DOX, therefore inhibiting the production of free radicals, which could cause injury to the mitochondria and other cellular components (Della Torre et al. 1999, Lebrecht et al. 2007, Wouters et al. 2005). Despite the beneficial cardioprotective properties of Dexrazoxane against DOX-induced cardiotoxic effects in adults exposed to anthracycline treatment (Swain et al, 1997), several studies and clinical trials carried out in paediatric patients reported that treatment with Dexrazoxane resulted in a threefold increase in the incidence of secondary malignancies in children with acute lymophoblastic leukaemia and myelodysplastic syndrome (Salzer et al. 2010, Tebbi et al. 2007). According to the authors, the cohort studies were not designed to monitor the cardiac effects of Dexrazoxane in these patients.

Other cardioprotective agents that have been reported to protect against DOX associated cardiac dysfunction in animal studies include phenolic compounds (Razavi-Azarkhiavi et al. 2016), phosphodiesterase-5 inhibitors (PDE-5) (Koka and Kukreja 2011), green tea extract (Khan et al.

2014), phytochemicals (Abushouk et al. 2017) and many other compounds. For instance, the investigation conducted by Khan et al, (2014) in the in vivo Wistar albino rat model exposed to green tea extract for 30 days and DOX administered once in the 29th day of the study showed that oral treatment with green tea extract protected against DOX induced cardiotoxicity, by restoring the activities of glutathione transferase, superoxide dismutase, glutathione peroxidase and catalase and enhancing the heart antioxidant defense system (Khan et al, 2014). In addition, in a similar manner, in the same in vivo model, Singh et al, (2007) have demonstrated that treatment with different doses of Arjunolic acid, (a phytochemical antioxidant) 6 days a week for 4 weeks and a single dose of DOX accelerated the cardiac defense mechanism and reduced the levels of creatine kinase-MB and decreased the lipid peroxidation when compared to DOX treated animals (Singh et al, 2008). Furthermore, within the same study, the electron microscope myocardial histological analysis showed that animals treated with DOX displayed mitochondrial swelling, focal dilation of smooth endoplasmic reticulum and lipid inclusions, whereas the concomitant treatment of Arjunic acid reduced DOX-induced histological alterations (Singh et al, 2008).

Several investigations have suggested the involvement of PI3K/Akt pathway as a strategy to prevent DOX-induced cardiotoxic effects (Maulik et el, 2018; Deres et al. 2005, Gao et al. 2016, Yu et al. 2017). In a recent in vitro study Maulik et al, (2018) have shown that simulated ischaemia preconditioning protects the isolated cardiomyocytes against DOX induced cell damage in a PI3K/Akt dependant manner. It has been shown that cardioprotective effects of simulated ischaemia preconditioning were associated with an increase in the Akt phosphorylation levels in the cells, and the PI3K inhibitor LY294002 abolished the increase in Akt phosphorylation and

protection against DOX (Maulik et al, 2018). Furthermore, in the Kumming mice experimental model, Wei et al, (2017) have investigated the cardioprotective effects of Apigenin antioxidant (a natural flavonoid) against Adriamycin induced cardiotoxicity and the involvement of PI3K/Akt signalling pathway (Yu et al. 2017). It has been highlighted that treatment with Apigenin protected against Adriamycin induced apoptosis by reducing the Bax/Bcl-2 ratio production and activating the PI3K/Akt pathway, suggesting that the cardioprotective properties of Apigenin against Adriamycin-induced cardiotoxicity could partly be attributed to Akt pathway activation (Yu et al. 2017).

1.21 Tiron

Tiron is a vitamin E analogue, a SOD mimic, a selective cell permeable scavenger and a substrate in electron chain processes (Herscher et al. 1994, Krishna et al. 1992). Its chemical term is sodium 4,5- dihydroxybenzene- 1,3- disulfonate, and the chemical formula is $C_6H_4Na_2O_8S_2$ (NIH-PubChem) (Figure 1.6). Tiron is available in a white powder form, highly soluble in water, with a melting point above 300^{o}C. It is also used as a colorimetric reagent for iron, manganese, titanium and molybdenum and it generates water-soluble coloured mixtures with metal salts (Santa Cruz Biotechnology, 2010).

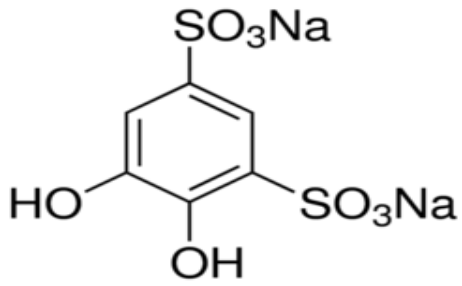


Figure 1.6 The chemical structure of Tiron (NIH-PubChem)

Early experimental studies used Tiron as a metal ion chelating reagent for the assessment and purification of metal ion particles (Krishna et al. 1992, Poeggeler et al. 2002) as well as a treatment of heavy metal poisoning such as lead (Pocock and Simons 1987), beryllium (Sharma, Johri and Shukla 2000), uranium (Basinger and Jones 1981) and vanadium (Domingo 1994).

The antioxidant properties of Tiron have been previously assessed in comparison with a number of other antioxidants. For example, the study undertaken by Oyewole et al. (2014) aimed to investigate and compare the effects of mitochondria targeted antioxidant MitoQ and the mitochondria-localised antioxidant Tiron with a number of cellular antioxidants (Oyewole et al. 2014). The study has shown that antioxidant Tiron provided superior protection against both H_2O_2 induced mitochondrial damaged and UV radiation in the *in vitro* skin cell model when compared to MitoQ and a number of other untargeted antioxidants including curcumin, resveratrol and N-acetyl cysteine (Oyewole et al. 2014).

A possible explanation for the greater protection conferred by Tiron in this study could be associated with its antioxidative and iron chelation properties (Oyewole et al. 2014). Although both Tiron and MitoQ are mitochondria targeted antioxidants, their mechanism of targeting the mitochondria is different. For example, MitoQ, a derived ubiquinone conjugated to

triphenylphosphonium (a lipophilic cation) facilitates the accumulation and passage of this molecule within the inner mitochondrial membrane as a result of the electrochemical gradient (Oyewole and Birch-Machin, 2015; Gottwald et al. 2018).

In comparison with MitoQ, Tiron is a mitochondria-localised antioxidant which belongs to the group of spin traps and instead of being developed to target the mitochondria, has the ability to permeabilise the cell membrane and accumulate within the organelle (Oyewole and Birch-Machin, 2015; Vorobeja and Pinegin, 2016).

In addition to this, a number of studies investigating the effects of oxidative stress in a number of cellular models have compared Tiron in response to different exogenous antioxidants (Lin et al. 2012; Vorobeja and Pinegin, 2016). For instance, the *in vitro* study undertaken by Vorobeja and Pinegin (2016) in human neutrophils has revealed that Tiron provided the most effective scavenger activity against phorbol 12-myristate 13-acetate (PMA) induced ROS when compared to Trolox and Tiron (Vorobeja and Pinegin, 2016). In a previous study, Resveratrol and Vitamin C demonstrated significant ROS protection against the chemotherapeutic drug temozolomide (Lin et al. 2012). When compared to Tiron, the ROS protection generated with these antioxidants was similar however, in some assays Tiron demonstrated to offer a greater protection against ROS production (Lin et al. 2012). Furthermore, within the same study, co-administration of Tiron with the anticancer drug temozolomide in human glioblastoma (U87MG) cells was marked by a decrease in the autophagy and a subsequent activation of apoptosis, indicating that the combination of Tiron and temozolomide has a synergistic effect (Lin et al. 2012).

1.22 The superoxide scavenger properties of Tiron

In the recent decades, several investigations have been focusing on the superoxide scavenger properties of Tiron in mediating a number of biological reactions (Arimura et al. 2001, MacKenzie and Martin 1998, Münzel et al. 1999, Seki et al. 1999). For example, in a study carried out by Bryan and his colleagues in 2001 on middle cerebral artery of Long-Evans rats, Tiron, at a concentration of 10mM/L was able to significantly reduce the shear stress-induced constrictions in the vessels with intact endothelium and in vessels in which the endothelium had been disconnected (Bryan, Steenberg and Marrelli 2001). Furthermore, in a different report, carried out on electrically stimulated rat diaphragms, Tiron was used at a concentration of 32mM to assess the specificity of the fluorescent assay for ROS on the hypothesis that the effects of Tiron were mainly associated with its active properties to act as an intracellular scavenger of superoxide anion (Supinski et al. 1999).

The effects of Tiron on Na^+-K^+-ATPase on K^+ basolateral channels in the Sprague-Dawley rat cortical collecting duct have also been examined (Lu and Wang 1998, Wei, Lu and Wang 2001). It has been revealed that Tiron, at a concentration of 10mM, significantly reduced the inhibitory effect of increased concentrations of calcium (400nM) on the small-conductance K^+ basolateral channel activity (Lu and Wang 1998). In addition, treatment with the same concentration of Tiron significantly reversed the inhibitory effect of strophanthidin- an inhibitor used to block the activity of Na^+-K^+-ATPase on the basolateral K^+ channel (Wei, Lu and Wang 2001). Based on these findings with Tiron, the authors of these studies suggested that the inhibitory effect of elevated

concentrations of calcium was facilitated by the interaction of NO with superoxide anions (Lu and Wang 1998, Wei, Lu and Wang 2001).

An early investigation carried out by Herscher et al, (1994) demonstrated that administration of Tiron at a final concentration of 5mM protected the Chinese V79 (lung fibroblasts) hamster cells against SR 4233 (an experimental anti-cancer drug exploiting hypoxia in solid tumours) aerobic cytotoxicity in clonogenic assay (Brown 1993, Herscher et al. 1994).

In addition, the effects of Tiron unconnected to its properties as a scavenger of superoxide anions have also been reported (Ghosh, Wang and McNeill 2002). In an experimental model of rat mesenteric bed, the effects of Tiron (10mM) on calcium binding and vascular responses were studied (Ghosh, Wang and McNeill 2002). It has been reported that in competition assays in buffered solutions in the absence of tissue, Tiron significantly reduced the fluorescence of fura-FF-an assay used to quantify the amount of Ca^{2+} binding (Ghosh, Wang and McNeill 2002). Furthermore, in the rat perfused mesenteric bed preconstricted with KCl, Tiron exhibited a vasodilator response, reducing the perfusion pressure of the mesenteric bed (Ghosh, Wang and McNeill 2002). In addition, this study has also tested the effects of Tiron on Ca^{2+} induced responses. It has been shown that in the presence of KCl in the perfusate, increasing concentrations of Ca^{2+} resulted in an increased of perfusion pressure of the mesenteric bed. Addition of Tiron in the perfusate shifted the concentration-response curve to Ca^{2+} to the right, indicating the Tiron binds to the Ca^{2+} (Ghosh, Wang and McNeill 2002). It was also noted that in freshly collected blood from the rats, Tiron caused an increase in the clotting time (Ghosh, Wang and McNeill 2002).

1.23 The effects of Tiron in cardiovascular system

In the recent years, cumulative evidence has highlighted the ROS scavenger properties of Tiron in cardiovascular system (Arimura et al. 2001, Bosch et al. 2011, Fleming et al. 2001, Klawitter et al. 2002, Matsumoto et al. 2003, Wambi-Kiéssé and Katusic 1999a).

The experiments carried out by Wambi-Kiesse and Katusic, (1999) in rings of isolated canine basilar arteries highlighted the pharmacological properties of Tiron in abolishing the impairment of NOS-mediated relaxations after the inhibition of Cu/Zn SOD activity. In this study, Tiron significantly attenuated the inhibitory effect of diethylduthuicarbamic acid, a Cu^{+} chelator, and enhanced the relaxations to bradykinin in control rings (Wambi-Kiéssé and Katusic 1999b). Furthermore, in a in vivo canine model of tachycardia-induced HF, it has been reported that treatment with Tiron (7mmol/min) significantly inhibited the formation of oxidative free radicals in myocardium and coronary arteries and improved the endothelium vasodilation by enhancing the activity of NO in the dog model of pacing induced HF (Arimura et al. 2001). In addition, in a different experimental model of New Zeeland White rabbits, microinjection of Tiron into the pressor area of the rostral ventrolateral medulla decreased the cardiovascular effects to air-jet stress rabbits (De Matteo, Head and Mayorov 2006). Also, treatment with Tiron significantly reduced the blood pressure (BP) and heart rate (HR) responses to stress in the air-jet stress rabbits (De Matteo, Head and Mayorov 2006).

A more recent study carried out on hypoxic rat diaphragm muscle strips, a model similar to ischaemia in the heart, revealed that treatment with the superoxide scavenger Tiron (10mM)

inhibited the contracture during hypoxia and protected the contractile function of the skeletal muscle (Wright et al. 2005).

1.24 Anti-cancer effects of Tiron

Besides the known action in scavenging the ROS, Tiron has shown potent anti-cancer activity against human promyelotic HL60-leukaemia cell line in in vitro studies (Kim et al. 2006b). It has been shown that treatment with Tiron at concentrations ranging between 0.1mM and 3mM on HL-60 cells resulted in a growth inhibition and differentiation in the cells, induced severe DNA damage and activated the apoptotic cell death (Kim et al. 2006b).

1.25 Aims, objectives and hypotheses

1.25.1 Aims

The aims of this thesis were to:

- Determine the effects of Tiron in normoxic conditions in the Langendorff heart model using male Sprague-Dawley rats.
- Determine the effects of Tiron in I/R conditions in the Langendorff heart model using male Sprague-Dawley rats.
- Investigate the ability of Tiron to offer cardioprotection against anti-cancer therapies without affecting the antineoplastic effect in HL60 and HepG2 cells;
- Establish whether Tiron is capable to reduce the myocardial injury caused by I/R and treatment with anti-cancer therapies.

1.25.2 Objectives

The objectives of this thesis were to:

- Examine the effects of increasing concentrations of Tiron in normoxic settings in the isolated rat hearts and adult ventricular myocytes;
- Identify whether Tiron offered protection in the myocardium in the model of I/R and respectively H/R conditions;
- Investigate the effects of Tiron on ROS levels and PI3K/Akt intracellular signaling pathway in heart tissue;

- Determine the cardioprotective properties generated by co-treatment of Tiron with the anti-cancer drug Doxorubicin in naïve and stressed hearts;
- Elucidate the potential mechanism of protection generated by Tiron against Doxorubicin induced cardiotoxicity
- Test the effects of Tiron in HepG2 and HL60 cell lines when co-administered with Doxorubicin and their effects on cell viability, ROS levels and caspase-3 activity.

1.25.3 Hypotheses

The hypotheses of this thesis are defined below.

- Administration of Tiron will not elicit an effect in the heart in normoxic conditions.
- Treatment with Tiron will offer cardioprotection in the model of I/R injury, and this effect will be observed by a decrease in the infarct size, an improvement in the cardiac function and an increase in the Akt survival pathway.
- Tiron will protect against the cardiotoxic effects of Doxorubicin treatment in both normoxic and I/R settings by reducing myocardial injury and the levels of the apoptosis marker caspase-3.
- In HL60 and HepG2 cell lines, co-administration of Tiron and Doxorubicin will reduce the cell viability and cause an increase in the intracellular ROS and cleaved-caspase-3 activity.

Chapter 2. Material and methods

2.1 Experimental animals

Sprague-Dawley male rats with a body weight of 350±50 g were used for all experiments. The animals were obtained from Charles River (UK), had unrestricted access to food and water receiving human care assistance in concordance with the Guidance in the Operation of the Animals (Scientific Procedures) Act 1986 (The Stationary Office, London, UK).

2.2 Chemical and drugs used

Tiron (4,5-dihydroxy-1.3-benzenedisulfonic acid disodium salt monohydrate) was purchased from Sigma Aldrich (Dorset, UK) and prepared freshly every day prior to each experiment by dissolving in ultrapure water to a stock concentration of 60mM.

Doxorubicin hydrochloride ((7S,9S)-7-[(2R,4S,5S,6S)-4-amino-5-hydroxy-6-methyloxan-2-yl] oxy-6,9,11-trihydroxy-9-(2-hydroxyacetyl)-4-methoxy-8,10-dihydro-7H-tetracene-5,12-naphthacenedione hydrochloride and Wortmannin 11-(Acetyloxy)-1,6b,7,8,9a,10,11,11b-octahydro-1-(methoxymethyl)-9a,11b-dimethyl-3H-furo[4,3,2-de]indeno[4,5,-h]-2-h]-2-benzopyran-3,6,9-trione) were purchased from Tocris (Bristol, UK). DOX was dissolved in ultra-pure water to a stock concentration of 1mM; Wortmannin was dissolved in dimethyl sulfoxide (DMSO), with a final concentration of DMSO less than 0.02%. The dissolved drugs were aliquoted and stored in the freezer at -20˚C.

All salts and chemicals used to prepare the buffers for Langendorff technique and cardiomyocyte isolation were purchased from Fischer Scientific (Loughborough, UK). All antibodies were

purchased from Cell Signalling (UK). The equipment and the reagents used for Western blot technique were purchased from Bio-Rad (Watford, UK). DCFDA (Cellular reactive oxygen species detection assay) kit was purchased from Abcam (Cambridge, UK).

2.3 Cell lines and culture conditions

As previously mentioned in the introduction chapter 1 (Section 1.9.1), DOX is a potent anti-cancer therapy used in both solid and liquid malignancies. Therefore, DOX in combination with Tiron was investigated in cell lines (HepG2 and HL60) derived from both solid and liquid malignancies.

In order to investigate the mechanisms of Doxorubicin in combination with the antioxidant Tiron, human hepatocellular carcinoma (HepG2) and human leukaemia (HL60) cell lines were used as relevant cell models.

HepG2 cells are immortalised cell lines that maintain the characteristics of hepatocytes, as well as the activities of enzymes that control the stimulation and detoxification of DNA-reactive carcinogens (Cao et al. 2007).

HL60 cells are immortalised cell lines that display a myeloblastic/promyelocytic morphology (Birnie, 1988). This cell line represents a practical model in studying the induction of cell death pathways by anti-cancer agents and topoisomerase II inhibitors such as Doxorubicin (Zare Mirakabadi et al. 2012; Kluza et al. 2000).

HepG2 cell lines were obtained from American Type Culture Collection (ATCC) and maintained in monolayer cultures at 37^{o}C in a humidified atmosphere containing 5% CO_2 in Dulbecco Modified Eagle Medium (DMEM) (Fisher Scientific, UK) supplemented with 10% foetal bovine serum (FBS)

(Fisher Scientific, UK), 2mM L-glutamine (Fisher Scientific, UK) and 1% Penicillin-Streptomycin (10,000 U/mL) (Fisher Scientific, UK).

HL60 cell lines were supplied from the European Collection of Cell Cultures (ECACC) and grown in a suspension culture. Cells were cultured in RPMI 1640 media (Fisher Scientific, UK) supplemented with 10% FBS (Fisher Scientific, UK), 1% L-glutamine (Fisher Scientific, UK) and 1% Penicillin-Streptomycin (10,000 U/mL) (Fisher Scientific, UK) and maintained at 37^{o}C in a humidified atmosphere containing 5% CO_2.

2.4 Buffers and reagents

Krebs Henseleit buffer (KHB) containing 118.5mM NaCl, 25 mM $NaHCO_3$, 4.8mM KCl, 1.2mM $MgSO_4$, 1.2mM KH_2PO_4, 12mM glucose and 1.7mM $CaCl_2$ was prepared freshly every day prior to each experiment. KHB was gassed with 95% O_2, 5% CO_2, maintained at 37^{o}C, at a pH of 7.4 (Bell et al, 2011).

Modified calcium free Krebs Hensleit buffer used for isolation of adult rat ventricular cardiomyocytes containing 116mM NaCl, 5.4mM KCl, 0.4mM $MgSO_4$, 10mM glucose, 20mM taurine, 5mM sodium pyruvate, 25mM $NaHCO_3$ and 11.75mM KH_2PO_4 was prepared freshly every day prior to each experiment. Modified KHB was gassed with 95% O_2, 5% CO_2, maintained at 37^{o}C, at a pH of 7.4.

Restoration buffer containing 116 mM NaCl, 5.4mM KCl, 0.4mM $MgSO_4 7H_2O$, 10mM glucose, 20 mM taurine, 5mM sodium pyruvate, 25 mM $NaHCO_3$, 11.75mM KH_2PO_4, 1% bovine serum

albumin (BSA), 1% Pen-Strep, 5mM creatine monohydrate and 100mM $CaCl_2$, pH 7.4 was used to re-suspend the ventricular cardiomyocytes following the isolation protocol.

Esumi ischaemic buffer containing 11.95 mM KCl, 1.04mM $MgCl_2$, 1.7mM $CaCl_2$, 4mM HEPES, 10mM deoxyglucose and 20mM sodium pyruvate, pH 6.2, was used to induce hypoxia in isolated rat ventricular cardiomyocytes.

2.5 Langendorff technique

2.5.1 Heart extraction procedure and isolated heart perfusion technique

The animals were sacrificed by cervical dislocation as outlined in Schedule 1 Home Office Procedure. The heart was removed using the process of thoracotomy as described below (Bell et al, 2011).

A trans-abdominal skin incision was carried out at the xyphoid-sternum and expanded to the lateral ends of the right and left costal sides (Bell et al, 2011). The surgical procedure was continued to through the ribs at the left and right anterior axillary lines to generate the clamshell thoracotomy (Bell et al, 2011). Next, the pericardium was opened, the thoracic cage was detached, and the heart was exposed (Bell et al, 2011).

The heart was promptly removed by excising the descending aorta, the inferior vena cava followed by the superior vena cava and pulmonary arteries (Skrzypiec-Spring et al, 2007). Immediately after excision, the heart was transferred in ice cold KHB (see buffers and reagents section 2.2.1) in order to prevent any ischaemic damage of the organ (Skrzypiec-Spring et al, 2007, Bell et al, 2011). The heart was rapidly mounted on the Langendorff-perfusion apparatus.

The aortic cannula was carefully inserted into the aorta, and the aortic clip was positioned around it to secure the heart while establishing the coronary perfusion with KHB (see buffers and reagents section 2.2.1) (Skrzypiec-Spring et al, 2007, Bell et al, 2011, Gharanei et al, 2013).

Next, a thread was fastened several times around the aorta in order to secure the heart on the Langendorff perfusion circuit before removing the aortic clip (Skrzypiec-Spring et al, 2007, Bell et al, 2011). The perfusion buffer was administered in the retrograde route via the aorta, at a constant hydrostatic pressure and stable flow rate (Skrzypiec-Spring et al, 2007, Bell et al, 2011).

Furthermore, it was essential to take extra care to prevent mechanical fissure of the aortic valves or obstruct the coronary ostia by ensuring the cannula was not inserted too deep into the aorta (Bell et al, 2011). Once the heart was stabilised on the Langendorff perfusion circuit, the temperature of the organ was maintained between 36.5^{o} C and 37.5 ^{o} C by a water-jacketed reservoir surrounding the heart at a temperature of 37 ^{O}C (Bell et al, 2011).

The optimal time of stabilisation the heart function was confirmed to be within 5-10 minutes after the cannulation process, the contractile strength and heart rate functions were established within seconds (Skrzypiec-Spring et al, 2007, Bell et al, 2011).

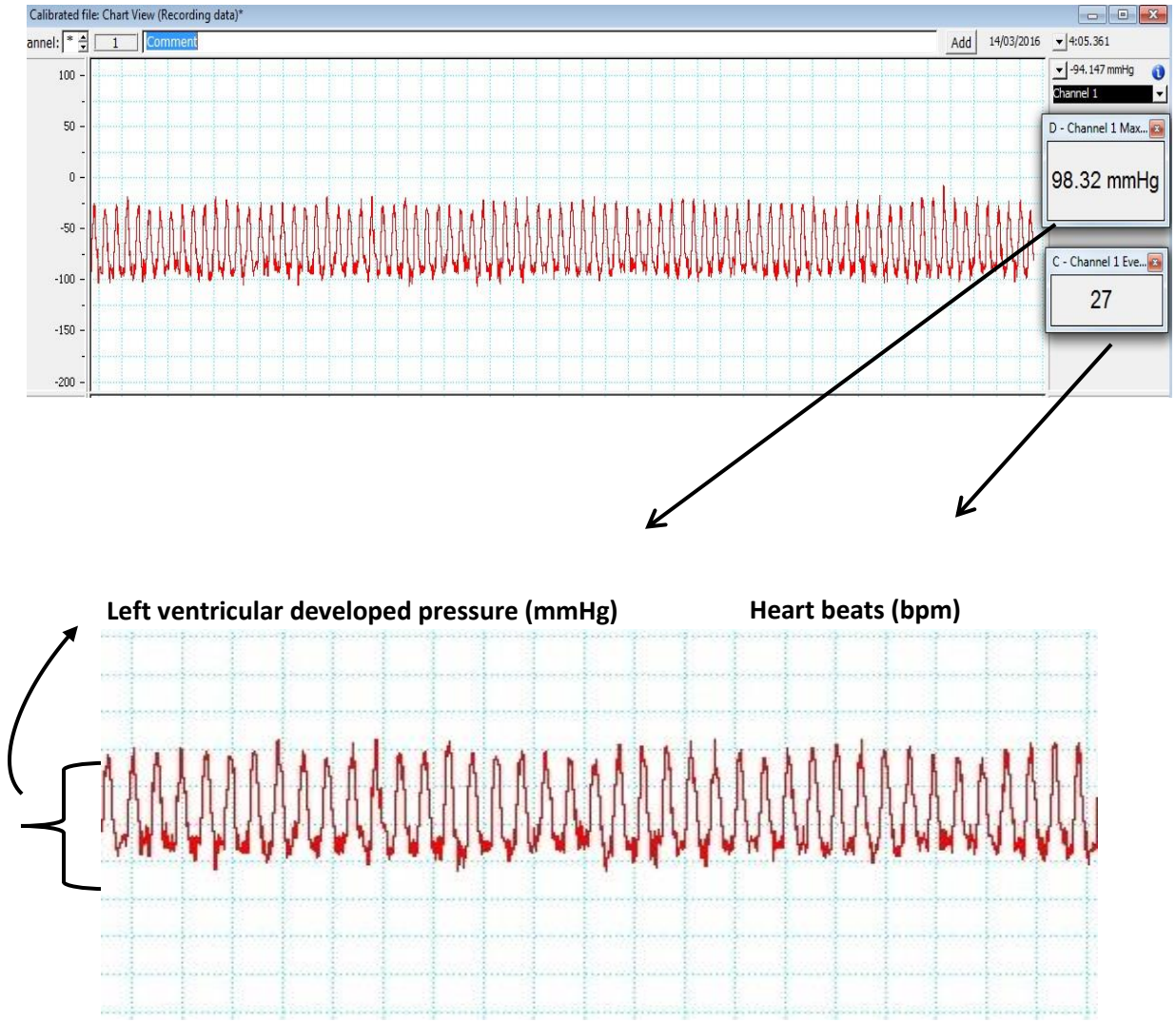
2.5.2 Evaluation of cardiac parameters: CF (coronary flow), LVDP (left ventricular developed pressure) and HR (heart rate)

The pressure transducer was calibrated prior to each experiment using the LabChart 7 with PowerLAB (AD Instruments Ltd. Chalgrove, UK) software. Prior to each experiment, the pressure transducer was set to the zero point via the bridge amp. The force transducer nominal value was

recorded by applying pressure to the force transducer and digital resolution scale was set by changing the range values within the bridge amp. Once the force transducer values and range were set, the units conversion was established by applying the desired calibration values and recording these values in the software with the applicable units (mmHg) required for the experiment.

As soon as the heart contraction was observed, the left atrium was removed and an iso-volumic, latex balloon was inserted into the left ventricle and inflated with water to set a pre-load of 5-10mmHg (Skrzypiec-Spring et al, 2007, Bell et al, 2011). The iso-volumic balloon was connected to a pressure transducer through which the LVDP and HR were constantly recorded and examined using the Power Lab system (AD Instruments, Chalgrove, UK) (Figure 2.1) (Gharanei et al, 2013).

The LVDP, HR parameters were recorded at regular time intervals. In order to measure CF rate, the effluent was collected for 1 min at each time point and recorded at regular intervals. All three parameters were recorded every 5 minutes throughout stabilisation and ischaemia period, and every 15 minutes during reperfusion.



***F*igure 2.1** Langendorff trace illustrating the heart beats and left ventricular developed pressure using the Power Lab system

Haemodynamic values were graphed using Microsoft Excel for analysis. LVDP and HR were calculated as the percentage of the mean stabilisation period, whereas CF was adjusted by the heart weight and calculated as the percentage of the mean stabilisation period. Hearts that had a high coronary flow rate (>27 ml/min) or arrhythmic episodes during the 20 minutes stabilisation period were excluded from this study.

2.5.3 Induction of ischaemia

Regional ischaemia was induced by ligation, using a surgical needle to obstruct the main left coronary artery, the occlusion point being established above the coronary artery branch. Next, the ends of the suture were passed through two plastic tubes to form a tight snare. Ischaemia was induced by tightening the snare, and the reperfusion was initiated by removal of the plastic tubes and release of the threads. After reperfusion, the surgical threads were tightened to obstruct the left coronary artery and 1 ml of saline solution containing 0.25% Evans blue was administered slowly via the aorta to delineate the viable areas (Figure 2.2).

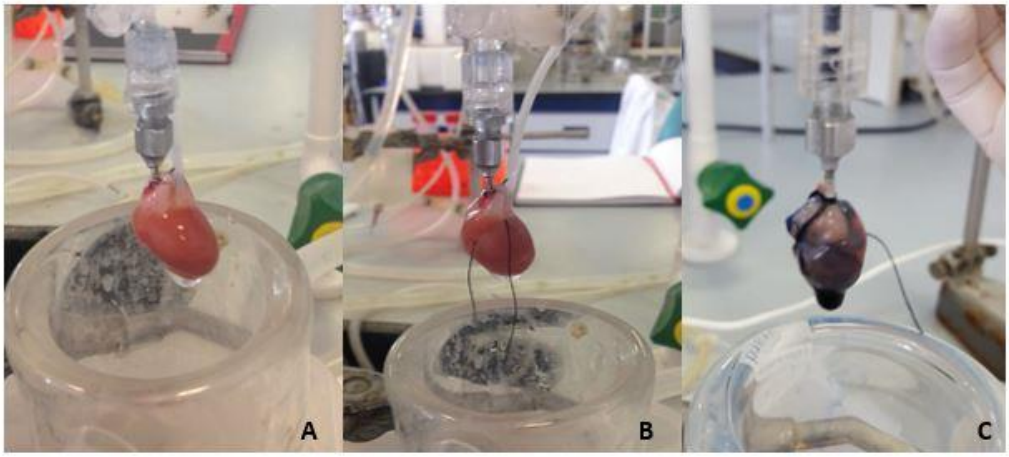


Figure 2.2 Sprague-Dawley rat heart mounted on the Langendorff setup illustrating the stabilisation period before ischaemia (A); heart during reperfusion after regional ischaemia was induced by tightening the snare to ligate the left anterior descending aorta (B), end of reperfusion when the surgical thread was tightened, and Evans blue was perfused via the aortic cannula (C).

2.5.4 Infarct size and area at risk analysis

At the end of reperfusion period, all hearts were decannulated, weighed and frozen at -20˚C. The frozen hearts were sectioned into 2 mm thick transverse slices and incubated in triphenyltetrazolium chloride (TTC) solution (1% of TTC in phosphate buffer, pH 7.3, 37˚C) (Bell et al, 2011). The TTC staining method is dependent on the capacity of intracellular dehydrogenase enzymes to react with tetrazolium salts, resulting in production of formazan (Bell et al, 2011). As a result, the viable cells, containing preserved NADPH are stained in brick-red colour, whilst the non-viable cells (infarct cells) are represented by a pale white-yellow colour (Bell et al, 2011). Next, the tissue slices were incubated in 10% formaldehyde for 24 hours prior to analysis. This step enabled a distinct contrast between the infarct and the risk areas.

For measurement, transversal sections of the heart were positioned between two glass plates in order to expand the tissue and fixed with clips. The viable, non-risk area (dark blue), risk area (brick-red) and infarct (pale white-yellow) areas were traced on an acetate paper and each trace was examined using Image Tool program (Version 3.1, Rockford, USA). The ratio of the infarct area within the risk area was evaluated for each heart. The viable, risk and infarct area were traced in a blinded manner an analysed by an unconnected individual to ensure no bias. A selection of samples was double analysed to ensure accuracy (Figure 2.3).

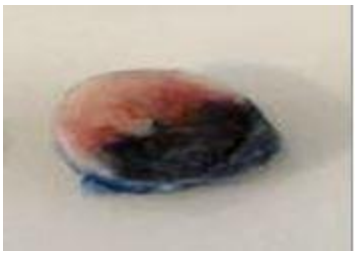


Figure 2.3 TTC stained transversal section of the left ventricle showing the non-risk area (dark blue), viable risk area (brick-red) and infarcted area (pale white-yellow)

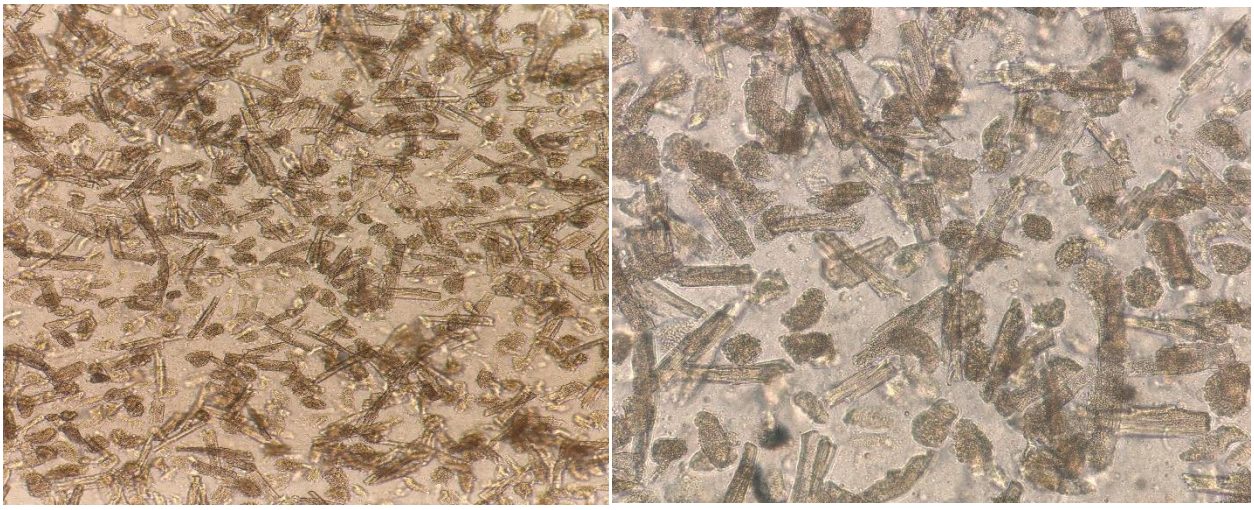
The infarct size (IS) for each group was calculated as a percentage of the are at risk (AAR) (IS/AAR%). AAR is defined as the area devoid of Evans blue that corresponds to the entire myocardial perfusion bed distal to the occluded left anterior descending artery (Redford et al, 2012; Murry, Jennings and Reimer, 1986). It is well established that the AAR represents a possible source of variance in infarct size, therefore it is important to assess and quantify it to confirm that there is no substantial difference among the experimental groups. The AAR was quantified for all hearts subjected to I/R experimental protocol and it was expressed as a percentage of the left ventricle (LV) (AAR/LV%). The hearts that had an AAR of less than 30% or over 45% of the ventricular volume were excluded from this study.

The infarct size and area at risk were calculated as a percentage and the graphs were plotted in Microsoft Excel. Statistical analysis was performed using IBM Statistical Package for the Social Sciences (SPSS) programme, and the data was analysed using one-way ANOVA, with Tukey post hoc test and mean±SEM.

2.6 Adult rat ventricular cardiomyocyte isolation

Adult ventricular rat cardiomyocytes were harvested and isolated as previously described (Maddock et al, 2002; Gharanei et al, 2013). Adult Sprague Dawley rats (350±50g) were sacrificed by cervical dislocation as in the Langendorff method. The hearts were quickly excised and mounted on a modified constant flow Langendorff apparatus and perfused in a retrograde manner. The hearts were initially perfused with modified calcium free buffer KHB (see buffers and reagents section 2.2.1) in order to stop the contractions of the heart. Next, the hearts underwent the perfusion stage with modified KH digestion buffer (1mg/ml Gibco's type II Collaginase isolated from Clostridium histolyticum, 1mM $CaCl_2$). During the perfusion phase with collagenase (Gibco, UK), the effluent was recirculated throughout the experiment in order to help obtain a successful isolation subjecting the tissue to the same steady decline in the enzyme activity (Louch et al, 2011). Once the enzyme digestion of the heart was complete, the ventricles were fragmented and incubated for 5 minutes in an orbital shaker containing digestion buffer. The supernatant was centrifuged for 2 minutes at 600 rpm and the pellet obtained was gently re-suspended in fresh restoration buffer (See buffers and reagents section 2.2.1).

The cell viability was strictly monitored and assessed by visualising the cells under the inverted microscope. In order to avoid calcium overload, the final concentration of calcium was gradually increased to establish a final concentration of 1.25 mM. Only isolations with above 60% cell viability were progressed to the experimental procedure.



a) b)

Figure 2.4 Viable rat ventricular cardiomyocytes observed under the inverted microscope: a) 10x magnification; b) 20x magnification

2.7 Hypoxia induction in isolated rat ventricular myocytes

Isolated adult rat ventricular cardiomyocytes were centrifuged for 2 minutes at 600 rpm, the supernatant was gently removed and the pellet containing viable cardiac myocytes was gently redistributed in Esumi ischaemic buffer (see buffers and reagents section 2.2.1) and transferred into culture dish and incubated for 1 hour in New Brunswick hypoxic chamber at 37^{o}C, in 5%CO_2 and 0.01-1% O_2 to induce hypoxia.

2.8 Assessment of cell viability using the colorimetric MTT assay and Trypan blue staining

2.8.1 Colorimetric MTT assay

Assessment of cell viability using the colorimetric MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)] assay was used to evaluate the cell viability (Gomex et al, 1997).

Briefly, MTT assay is presented as a yellow tetrazole and is utilised to analyse the enzymatic function of NAD(P)H-dependent cellular oxidoreductase enzymes (Riss et al, 2013; Gomex et al, 1997). These enzymes reduce the MTT yellow dye into purple formazan pigment, which represents the number of viable cells with active metabolism (Riss et al, 2013; Gomex et al, 1997). The absorbance of formazan pigment can be assessed at specific wavelengths using a spectrophotometer device. This technique was used in several investigations to assess the effects of various compounds on cell viability and death.

The MTT (Sigma-Aldrich, UK) was dissolved in PBS (phosphate buffer saline) to a concentration of 5mg/ml and stored in the fridge at 4˚C in a light protected container before use in cell viability asssay.

2.8.2 Cell treatment

Cells were counted using a haemocytometer in order to determine the cell concentration required for each assay. Drug concentrations of Tiron and DOX were prepared by serial dilution of the drug stock in restoration buffer (Tiron: 0.25-2.5mM; DOX:1µM).

Next, 100µl suspension cells containing 10,000 cells/well were incubated in the absence and presence of the drug treatment in a 96 well flat-bottomed microtitre plate (ThermoFisher Scientific, UK) for 24 hours in a Nuair incubator in an atmosphere of 37˚C, 21%O_2 and 5% CO_2.

2.8.3 MTT experimental protocol

After 24 hours incubation with drug treatment, 50µl of 5mg/ml MTT assay was added to each well containing 10,000 cells/well and incubated further for 2 hours covered in foil. Following the MTT incubation period, 50µl of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, UK) was administered to each well and incubated at room temperature for 10 minutes on a plate shaker. The absorbance was read at 450nm wavelength on the Tecan Sunrise plate reader (Tecan UK Ltd, UK).

2.8.4 Data analysis

The values obtained were calculated as a percentage in MTT reductase activity of the mean absorbance of the control group and the graphs were plotted in Microsoft Excel. Statistical analysis was performed using IBM SPSS programme and the data were analysed using one-way analysis of variance (ANOVA) with Tukey post-hoc test to identify group differences.

2.8.5 Trypan blue staining experimental protocol

Wells containing 100,000 cells/well in 1 ml suspension were incubated in the presence and absence of the drug treatment in 24 well flat-bottomed plate (ThermoFisher Scientific, UK) for 24 hours in a Nuair incubator an atmosphere of 37˚C, 21% O_2 and 5% CO_2. Following the incubation period, a sample of cell suspension was transferred into a labelled Eppendorf tube containing Trypan Blue. Cell viability was calculated as a percentage of the viable cells divided by total number of cells. Statistical analysis was performed using IBM SPSS programme and the data

were analysed using one-way analysis of variance (ANOVA) with Tukey post-hoc test to identify group differences.

2.9 Flow cytometric analysis

2.9.1 Flow cytometric experimental protocol using Caspase-3 antibodies

Flow cytometric technique was used to evaluate the caspase-3 levels following drug treatment. The isolated cardiomyocytes (100,000 cells/ml) were incubated for 24 hours in a 24 well flat-bottomed plate in the presence and absence of Tiron (0.25-2.5mM) and DOX (1µM) at 37˚C, 21% O_2 and 5% CO_2. Following drug treatment incubation period, the cells were transferred into labelled Eppendorf tubes and centrifuged at 1200 rpm for 2 minutes. The pellet obtained was re-suspended in phosphate buffer saline solution (PBS) (140 mM NaCl, 5mM KCl, 1.8mM CaCl2) and fixed with 6% formaldehyde for 10 minutes at room temperature. Subsequently, the cells were permeabilised by incubation in ice cold 90% methanol for 30 minutes at 4˚C and stored at -20˚C before analysis.

Cells were pelleted at 1200 rpm for 2 minutes and washed twice with incubation buffer (0.5% BSA in PBS) at room temperature. Next, cells were probed with the cleaved-caspase 3 antibodies (Active/cleaved Caspase-3 Assay kit, Novus Biologicals/Bio-Techne, UK) at 1:100 dilutions in incubation buffer (in accordance with the manufacturer instructions) and incubated at 37^{o}C for 1 hour. Following incubation, the cells were washed 2x with wash buffer and re-suspended in wash buffer for analysis (in accordance with the manufacturer instructions). Analysis was performed using FL-2 channel on the BD Accuri C6 Plus flow cytometer (Ex/Em=540/570nm).

Statistical differences were calculated using IBM SPSS programme and the data were analysed using one-way analysis of variance (ANOVA) with Tukey post-hoc to identify group differences. Significance was considered at $P<0.05$.

2.9.2 Flow cytometric experimental protocol using Cellular Reactive Oxygen Species Detection Assay (DCFDA) kit

Flow cytometric analysis was used to assess the reactive oxygen species activity within a cell. DCFDA- Cellular Reactive Oxygen Species Detection assay kit comprises of the cell reagent 2',7'-dichlouroflorescein diacetate, labelled as a fluorogenic dye that quantifies hydroxyl, peroxyl and other reactive oxygen species (ROS) action within the cell. Following cell diffusion, DCFDA dye is deacetylated by the cellular esterases to a non-fluorescent product, which is further oxidised by ROS into DCF (2', 7'-dichlouroflorescien). This compound has a high fluorescence which can be detected by the flow cytometer (FL-1 channel; Ex=485nm and Em=535nm).

The isolated cardiomyocytes (100,000 cells/ml) were incubated for 24 hours in a 24 well flat-bottomed plate in the presence and absence of desired drug concentration, at an atmosphere of 37˚C, 21% O_2 and 5% CO_2. Positive control samples using 2mM H_2O_2 (hydrogen peroxide) were incubated at room temperature for 1 hour covered in foil, to avoid H_2O_2 decompose. Following the incubation period, 25µM DCFDA assay was aliquoted in each sample and incubated for 30 minutes at 37˚C. Once the incubation stage was completed, the cells were transferred in labelled Eppendorf tubes and analysed using FL-1 channel on the BD Accuri C6 Plus flow cytometer. Data obtained was calculated as a relative change in ROS fluorescence activity of the mean absorbance of the control group. Data sets were assessed for group differences using the IBM SPSS

programme using one-way analysis of variance (ANOVA) with Tukey post-hoc test. Significance was considered at $P<0.05$.

2.10 Western blotting

Western blotting is a widely used technique designed to detect and separate specific proteins based on their molecular weight from a given sample of proteins extracted from tissue or cells (Mahmood and Yang, 2012; Kim, 2017). This technique is divided into four stages. These are: 1) electrophoresis- the separation of proteins depending on molecule size, structure and charge; 2) transfer of the proteins onto a polyvinylidene membrane (PVDF); 3) incubation with the primary and secondary antibody to detect the proteins of interest; 4) visualisation of the detected protein bands and analysis of their relative abundance.

2.10.1 Left ventricular tissue collection

Tissue samples used for western blot technique were collected from Langendorff experiment.

In normoxic studies, the hearts were subjected to 20-minute stabilisation period, followed by 135 minutes of perfusion with KH buffer/ drug (see each section for the drug treatment protocol). In ischaemia reperfusion (IR) studies, the hearts were subjected to 20 minutes of stabilisation period, followed by 35 minutes of ischaemia and 20 minutes of reperfusion with KH buffer/drug (see each section for the drug treatment protocol). The 20 minutes reperfusion period for the IR study was chosen because the ischaemia reperfusion injury has been demonstrated to cause damage in the first 10 minutes of the reperfusion process (Hausenloy et al, 2002).

At the end of the perfusion/reperfusion period, the hearts were removed from the Langendorff setup and the left ventricle was trimmed away using a sterile scalpel. The tissue was immediately wrapped in tin foil, labelled accordingly, snap frozen in liquid nitrogen, and then stored at -80˚C.

2.10.2Protein extraction

Protein extraction from the snap frozen samples was carried out quickly in order to avoid the tissue thawing. 50 mg of the frozen tissue sample was cut and placed into a sterile cryogenic vial containing 300µl of lysis buffer (0.1M NaCl, 10mM Tris Base, 1mM EDTA (pH 8.0), 2mM Sodium phosphate, 2mM NaF, 2mM β-glycerophosphate, 1 protease cocktail tablet/100ml lysis buffer and 1 PhosSTOP tablet/10 ml lysis buffer). Samples were homogenised using an IKA Ultra-Turrax Labortechnik T25 WERKE homogeniser and then the homogenised samples were centrifuged at 11,000 rpm, at 4˚C for 10 minutes in order to pellet the insoluble fragments. The supernatants from each sample containing the protein content, were collected and aliquoted in 1.5 ml labelled Eppendorf tubes. The protein concentration of each sample was measured using the Pierce™ BCA assay kit (ThermoFisher Scientific, UK) (as explained in section 2.8.4). The samples obtained were further diluted (2X) in sample buffer (250mM Tris-HCl pH 6.8, 10% glycerol, 0.0006% bromophenol blue, 4% SDS, 2% β-mercaptoethanol-pH 6.8) and incubated for 5 minutes at 100˚C before being stored at -80˚C. All samples were defrosted at room temperature before use and diluted with sample buffer to obtain a protein concentration of 60µg for each sample for gel loading.

2.10.3Protein quantification using the BCA protein assay

The BCA protein assay is an accurate technique used for the colorimetric detection and measurement of total protein (Walker, 1994; Olson and Markwell, 2007). This technique is based on the chemical reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline environment using a higly precise and selective colorimetric exposure of the cuprous cation by bicinchoninic acid. The purple colour is the result from the reaction of two particles of BCA with one Cu^{1+} ion.

In order to determine the protein concentration in the samples, a standard curve was created using bovine serum albumin (BSA) as a reference reagent. A set of standards were prepared from 2mg/ml BSA ampule provided with the assay. The standards were prepared as dilutions with a range from 2000µl/ml to 25µl/ml, using distilled H_2O as diluent. 25µl of each standard was added into a 96 well flat-bottomed microtitre plate, and a blank standard was required. 2µl of the unknown sample protein was pipetted to distilled H_2O to obtain a dilution of 1:12.5. The working reagent (WR) was prepared by adding 50 parts of BCA reagent A with 1 part of reagent B (reagent A and B provided in the kit). 200µl of the WR was pipetted to each well and mixed thoroughly on a plate shaker for 30 seconds. The plate was covered in foil to eliminate light interference and maximise the rate of reaction, and incubated for 30 minutes at 37˚C. The plate was cooled at the room temperature. The absorbance was measured at 562nm using a plate reader. The responses of the standards were used to calculate and plot a standard curve. Absorbance values of the unknown samples were inserted into the standard curve formula to determine their protein concentration.

2.10.4Gel electrophoresis

For this step, Bio-Rad Ready Gel precast gradient gels (4-15% Tris/Glycine) (Bio-Rad,UK) were used for all western blot experiments. The gels were placed in the Mini-PROTEAN 3 electrode assembly unit with the short plates facing inward. The Mini-PROTEAN 3 electrode assembly unit was placed into the electrophoresis apparatus (Bio-Rad, UK) and the inner chamber was filled with approximately 125 ml of 1x running buffer (14.42 g/l glycine, 1.0 g/l SDS, 3.03g/l TrisBase) and approximately 200 ml of 1x running buffer was added in the external chamber. Then, the combs were removed, and the wells of each gel were loaded with 60µg of the protein sample. One well in each gel was loaded with 2.5µl of biotinylated protein ladder (Cell Signalling Technologies, UK). Once the samples were loaded in all the wells, the gel was run for 60 minutes at 130 V using a Power-Pac 3000 (Bio-Rad, UK).

2.10.5 Protein transfer procedure

Once the separation of proteins using electrophoresis was completed, the gel was carefully removed from the glass plates and placed into the Trans-Blot Turbo transfer packs (Bio-Rad, UK), which is comprised of filter paper, Hybond-P Polyvinyl Diflouride (PVDF) membrane and buffer. The transfer cassette was assembled and firmly closed, according to the manufacturer instructions (Bio-Rad, UK) and placed into the Trans-Blot Turbo transfer system (Bio-Rad, UK). The settings required for the transfer were set on the transfer system at 25V, 1.3A for 7 minutes for two gels.

2.10.6 Immunoblotting

Following the protein transfer procedure, the membrane was transferred into freshly prepared blocking buffer (5% w/v milk in 1X TrisBase Tween20 (TBS-T) buffer (2.42 g/l Tris-Base, 8g/l NaCl, 1ml/l Tween20) with the proteins facing upwards. The membranes were incubated for 1 hour at the room temperature on an orbital shaker. Once the incubation period was completed, the membranes were washed 3 times for 5 minutes with 1X TBS-T buffer and then incubated with the primary antibody 1:1000 diluted in the antibody buffer (5% w/v BSA in 1XTBS-T) on an orbital shaker for 14-18 hours at 4˚C.

The membranes were then washed 3x 5 minutes in 1X TBS-T buffer and incubated with secondary antibody Anti-rabbit antibody HRP (1:10000 dilution in the antibody buffer) linked IgG and HRP linked with anti-biotin antibody (used to visualize the biotinylated protein marker) for 1 hour on an orbital shaker. Following the last stage of incubation, the membrane was washed for 3 times for 5 minutes in 1X TBS-T before analysis.

2.10.7Visualisation of the band density

The membranes were placed on acetate paper and 1ml of Super Signal West Femto Maximum Sensitivity Substrate solution (ThermoFisher Scientific, UK) (which was prepared by mixing reagent A and reagent B using a 1:1 dilution), was added onto the membrane. Super Signal West Femto maximum Sensitivity Substrate is an ultra-sensitive substrate that contains luminol, a widely used chemiluminescent reagent. Luminol is oxidized in the presence of horseradish peroxidase (HRP) enzyme resulting in an excited state product called 3-aminophthalate. This product declines to a lower energy state, releasing photons of light.

The Super Signal West Femto solution was allowed to cover the surface of the membrane for 5 minutes and then was placed into the Chemi-Doc imaging device (Bio-Rad, UK) to be read. Images of the membrane were captured, and the protein band density was calculated using Quantity One software. Data was presented as mean±SEM. Statistical analysis differences were calculated using IBM SPSS programme ANOVA with Tukey post-hoc test. Significance was considered at $P<0.05$.

2.11 Statistical analysis performed for all techniques

All data obtained in this thesis was plotted in Microsoft Excel and presented as mean±SEM. The statistical analysis performed to quantify the significance was calculated using IBM Statistical Package for the Social Sciences (SPSS) programme package with the One-Way Analysis of variance (ANOVA) with Tukey post-hoc test. Statistical significance was considered if the P value was less than 0.05 ($P<0.05$).

Chapter 3. Understanding the preclinical profile of the antioxidant Tiron and the potential as a cardioprotective agent

3.1 Introduction

Ischaemia reperfusion injury plays a fundamental role in the pathology of ischaemic heart disease (Braunersreuther and Jaquet 2012, Cadenas 2018). Although restoration of blood flow after an ischaemic event is vital to salvage the myocardium and to limit cardiac dysfunction, reperfusion itself can exacerbate myocardial injury (Braunersreuther and Jaquet 2012, Cadenas 2018, Hausenloy and Yellon 2013).

The hypothesis that excessive ROS production is a contributing factor to myocardial I/R injury is supported by many experimental studies and clinical investigations (Granger and Kvietys, 2015; Zweier and Talukder, 2006; Kurian et al. 2016). As mentioned in Chapter 1, in normal physiological conditions, free radical homeostasis is controlled by endogenous enzymatic antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase and thioredoxin peroxidase which are primarily located in the mitochondria or cytosol (Venardos and Kaye, 2007; Dhalla et al, 2000; Zweier et al, 2006). However, during an ischaemic insult, there is a loss of antioxidant enzyme function and a leakage in the extracellular matrix (Venardos and Kaye, 2007; Hausenloy and Yellon, 2013). During reperfusion, these antioxidants are further washed out, leading to a depletion in the myocardial antioxidant defence system and uncontrolled production of ROS (Blaustein et al, 1989; Steare and Yellon, 1993; Haramaki et al, 1998; Chen et al, 1998; Kang, 1999; Leichtweis and Ji, 2001; Jones et al, 2003; Venardos and Kaye,

2007; Kim et al, 2013). Cumulative evidence shows that the excess ROS can result in cellular damage via several pathways, including direct damage to the membranes and proteins (Maddaford and Pierce, 1997; Monassier, 2008) or indirect by activating mitochondrial induced cell death pathways (Cadenas 2018, Carden and Granger 2000, Hausenloy 2012, Konstantinidis, Whelan and Kitsis 2012, Zhou et al. 2018). Several studies have shown that targeting ROS production with various antioxidants exhibited cardioprotective effects in I/R associated injury by reducing the oxidative stress and improving the cardiac function from I/R injury (Zheng et al, 2018; Li et al, 2018; Zhang et al, 2017; Akhlaghi and Bandy, 2009; Britto et al, 2018). For example, Zhang et al, (2017) investigated the effects of Illexsaponin A (a natural Chinese antioxidant) therapy in the in vivo and in vitro studies using Sprague-Dawley rat model of myocardial I/R injury. In this study it was found that Illesxsaponin A treatment in the rat model of I/R injury preserved the cardiac function by reducing the myocardial infarction and decreasing the ratio of serum dehydrogenase (LDH), creatine kinase-MB (CK-MB) and aspartate transaminase (AST) markers of myocardial injury (Zhang et al, 2017). Furthermore, in the same investigation, the in vitro studies using neonatal rat cardiomyocytes model of H/R have shown that treatment with Illexsaponin A at reoxygenation increased the expression of anti-apoptotic proteins Bcl-2, decreased the activation of caspase-3, and activated PI3K/Akt signaling pathway, suggesting that apoptosis and oxidative stress have an important role in I/R injury (Wang et al. 2017). Therefore, examining the alterations of apoptosis, oxidative stress injury along with their effects on the signaling pathways is vital in finding therapeutic approaches for the treatment of I/R-associated cardiovascular diseases.

It has been shown that recruitment of PI3Ks and their downstream target Akt has been implicated in the regulation of cell survival, inflammatory responses and apoptosis (Chang et al. 2017b, Zhang et al. 2016a; Aoyagi and Matsui, 2011; Catalucci and Condorelli, 2006; Walsh, 2006). Several investigations indicated that PI3K/Akt pathway may act as an endogenous negative feedback regulator, that produces a compensatory mechanism to prevent pro-inflammatory responses and apoptotic events in response to deleterious insults (Guha and Mackman 2002, Hua et al. 2007, Wang et al. 2017 Jiani et al, 2011). In an investigation carried out in H9c2 cell model of simulated H/R, Tang et al (2017) have reported that the activation of PI3k/Akt signalling pathway in the cardiac cells is associated with the inhibition of apoptosis and caspase-3 activation, indicating that stimulation of this pathway plays an important role in protecting the myocardium from I/R injury (Tang, Yang and Zhang, 2017). In addition, a number of investigations have shown that activation of PI3k/Akt signaling pathway reduces the myocardial reperfusion injury by preventing cardiac myocytes apoptosis caused by oxidative stress (Liu et al. 2017; Jiang et al. 2016 Jiani et al, 2011).

Several studies have confirmed the beneficial effects of therapeutic agents aimed to reduce or prevent the harmful effects of ROS (Adlam et al. 2005, Hosseinzadeh, Modaghegh and Saffari 2009, Zhou et al. 2018). These therapeutic strategies include the use of different antioxidants such as superoxide dismutase mimetics (Masini et al. 2002), ROS scavengers such as Trolox (Sagach et al. 2002), Resveratrol (Das, Santani and Dhalla 2007, Magyar et al. 2012; Tomé-Carneiro et al. 2013) and various iron and metal chelators (Korkmaz et al. 2013, Powell et al. 1994).As described in chapter 1 Section 1.22-1.23, several studies have examined the protective

effects of the antioxidant Tiron in several experimental models (Wambi-Kiéssé and Katusic, 1999; Arimura et al. 2001; Fleming et al.2001).

Tiron (4,5 dihydroxy-1,3 benzene disulfonic acid)- a superoxidase dismutase mimetic, has been reported to be an effective chelator of a number of metal ions and a substrate for various enzymatic reactions (Herscher et al. 1994, Krishna et al. 1992). In an investigation carried out by Oyewole et al. (2014), the protective effects of Tiron were compared to other well-known antioxidants in reduction of ROS related injury and skin ageing in human dermal fibroblasts cell model (Oyewole et al. 2014). It was revealed that Tiron exerted a higher degree of protection against ROS induced mitochondrial DNA damage when compared to MitoQ antioxidant (Oyewole et al. 2014).

The study carried out by El-Sherbeeny et al (2016) in the model of chronic asthma, has demonstrated that administration of Tiron in sensitised ovalbumin (OVA) challenged mice was able to ameliorate the OVA-induced oxidative stress by increasing the levels of enzymatic antioxidants glutathione (GSH) and superoxide dismutase (SOD) levels (El-Sherbeeny, Hassan and Ateyya 2016), highlighting the potential of Tiron as an adjunctive therapy in chronic asthma. Furthermore, the ROS scavenger effects of Tiron have also been studied in rodent vascular models, suggesting that antioxidant properties of Tiron were linked to its ability to act as both an extracellular and intracellular scavenger of superoxide anion (Bryan, Steenberg and Marrelli 2001, Supinski et al. 1999).

One study has reported that in a model of rat cardiac muscle cells (H9c2) exposed to simulated hypoxia and reoxygenation conditions, treatment with Tiron was able to significantly reduce the

production of intracellular levels of ROS as a result of simulated I/R conditions (Borchi et al. 2010). Furthermore, in the same cellular model of H9c2, Tiron inhibited the apoptotic rate in oxygen-glucose deprivation/recovery apoptosis model of H9c2 cells (Chang et al. 2017a). In addition, in an investigation conducted on vascular diameters during ischaemia/reperfusion injury in rat heart, Hoshino et al, (2005) reported that the vasoconstriction response in arterioles was reversed by treatment with Tiron (Hoshino et al. 2005). This study further highlights the potential role of Tiron as a cardioprotective agent.

To date there has been relatively little research into any protective effects afforded by Tiron within the cardiac tissue or in specific in the heart, under normal physiological or I/R conditions. Therefore, the aim of this study was to determine the effects of increasing concentrations of Tiron (0-10mM) in normoxic conditions in isolated rat hearts and adult primary ventricular myocytes and to identify if Tiron protected the myocardium in the model of I/R injury in isolated rat hearts and H/R conditions in adult primary ventricular cardiomyocytes by reduction in myocardial infarct size and increased cardiomyocyte viability. This study will also investigate the possible role of Akt signalling pathway in the protective effects of Tiron in isolated rat hearts and primary ventricular myocytes in the models of naïve and I/R injury to elucidate the mechanism of protection.

3.2 Methods

3.2.1 Chemicals

Tiron was purchased from Sigma Aldrich, UK and Wortmannin was supplied from Tocris Cookson (Bristol, UK). The working concentrations of both drugs were freshly prepared each day as previously described in Chapter 2, Section 2.2.

3.2.2 Animals

Sprague-Dawley rats (350±50g) were sacrificed by cervical dislocation as outlined in Schedule 1 Home Office Procedure using the process of thoracotomy as previously described in Chapter 2, section 2.3.

3.2.3 Isolated perfused rat heart preparation (Langendorff protocol)

The treatment protocol is illustrated in Figure 3.1. The Langendorff normoxic experiments were carried out for 155 minutes in total. In the Normoxic experiments, all hearts were exposed to 20 minutes stabilisation period, followed by 135 minutes perfusion with KH buffer.

The hearts were randomly allocated to the following treatment groups:

a) Rat hearts perfused with KH buffer for 155 minutes (Normoxic control)

b) Rat hearts perfused with KH buffer for 20 minutes, followed by 135 minutes of perfusion with Tiron (0.25mM; 0.5mM; 1mM; 2.5mM and 10mM).

For the studies using the Langendorff model of I/R (details in section 2.6), the experiments were carried out for 175 minutes in total. In these studies, hearts were allowed to stabilise for a period

of 20 minutes, subsequently to 35 minutes of regional ischaemia and 120 minutes of reperfusion. Hearts were also randomly allocated to the following treatment groups:

a) Rat hearts perfused with KH buffer for 20 minutes, 35 minutes of simulated regional ischaemia and 120 minutes of reperfusion with KH buffer (I/R control)

b) Rat hearts perfused with KH buffer for 20 minutes, 35 minutes of regional ischaemia and 120 minutes of reperfusion stage with Tiron (0.25mM, 0.5mM, 1mM and 2.5mM).

During the course of all experiments, the stability of the hearts were monitored by recording HR, LVDP and CF parameters at regular intervals (as previously described in Section 2.3.2).

At the end of reperfusion period, the infarct to risk ratio was assessed using the Evans blue triphenyltetrazolium chloride (TTC) staining procedures as previously described in Chapter 2, Section 2.3.5. The area at risk (AAR) was calculated as a percentage of the left ventricle. Infarct to risk ratio was presented as a percentage of the area at risk for the treatment groups.

For Western blot experiments, all hearts in normoxic studies were treated in the same manner as described in the treatment protocol and Wortmannin (100nM) was administered throughout perfusion in the presence or absence of Tiron (0.25mM). In I/R studies, all hearts were subjected to 20 minutes of stabilisation period, followed by 35 minutes of ischaemia and 20 minutes of reperfusion in the presence or absence of Tiron (0.25mM) and Wortmannin (100nM). At the end of the experimental protocol, the hearts were removed from the Langendorff and the left ventricle was snap frozen in liquid nitrogen, before storage at -80˚C for further analysis as described in Chapter 2, section 2.8).

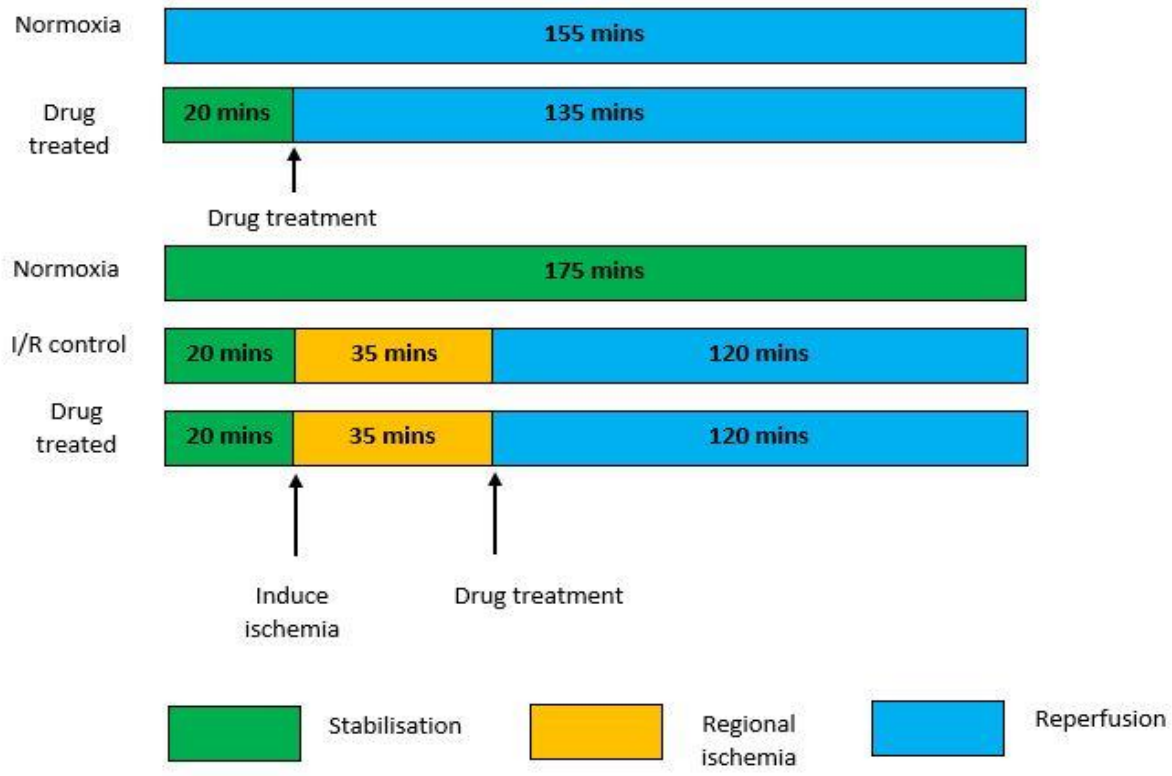


Figure 3.1 Treatment protocol for infarct/risk ratio asessement and Western blot tissue collection

3.2.4 Isolation of adult rat ventricular cardiomyocytes

Adult rat ventricular cardiomyocytes were isolated by enzymatic digestion as described previously (Maddock et al, 2002, Gharanei et al, 2013) (Section 2.4). Details of the heart digestion protocol and conditions required for the succesful isolation of primary adult rat ventricular cardiomyoctes are described in detail in Chapter 2, Section 2.4.

3.2.5 Induction of hypoxia and reoxygenation conditions in adult rat ventricular myocytes

As previously descried in Chapter 2, Section 2.5, freshly isolated rat ventricular myocytes were incubated in Esumi hypoxic buffer for 1 hour in a hypoxic chamber at 37^{o}C, in 5%CO_2 and 0.01-1% O_2 to induce hypoxia.

3.2.6 Drug treatment protocol in adult rat ventricular myocytes

Isolated rat ventricular cardiomyocytes were exposed to drug treatment with Tiron (0-2.5mM) in both normoxic and hypoxia/reoxygenation conditions as detailed below:

1) isolated myocytes exposed to Tiron (0-2.5mM) for 24 hours in normoxic conditions at 37^{o}C, 5% CO_2 and 95% O_2

2) isolated myocytes exposed to 1 hour of hypoxia conditions, followed by treatment with Tiron at the onset of re-oxygenation for 3 hours at 37^{o}C, 5% CO_2 and 95% O_2.

3.2.7 Cell viability assessment using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)] assay

Cell viability was assessed using MTT (MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)]) (Sigma-Aldrich, UK) as previously described in Chapter 2, section 2.6.3.

3.2.8 Cell viability assessment using Trypan blue staining

Trypan blue staining was performed as previously described in Chapter 2, Section 2.6.5. Cell viability was calculated as a percentage of the viable cells divided by total number of cells.

3.2.9 Analysis of adult rat ventricular myocytes using Caspase 3 staining

Following drug treatment protocol (as described in section 3.2.6) rat ventricular myocytes were probed with the cleaved-caspase-3 antibody (Active/cleaved Caspase-3 Assay kit, Novus Biologicals/Bio-Techne, UK) (as previously detailed in Chapter 2 Section 2.7.1). Data obtained was normalised to cell only control by subtracting the mean fluorescence background recorded in untreated samples. Data was presented as a relative change in fluorescence activity.

3.2.10 Analysis of adult ventricular cardiomyocytes using Cellular Reactive Oxygen Species Detection (DCFDA) Assay

DCFDA assay (Abcam, UK) was used to assess the intracellular levels of ROS in the isolated adult ventricular cardiomyocytes. Following drug treatment as previously detailed in section 3.2.6, isolated ventricular cardiomyocytes were incubated with DCFDA assay in accordance with the manufacturer instructions. The assay was carried out as described in Chapter 2, section 2.7.2. Data was normalised to cell only control. The values obtained were calculated as a relative change in ROS fluorescence activity of the mean absorbance of the control group.

3.2.11 Western blot analysis

Western blot analysis was conducted as detailed in Chapter 2 Section 2.8. Protein concentration was assessed using the BCA assay (ThermoFisher) according to the manufacturer instructions (as previously described in Chapter 2, Section 2.8.3). Membranes were probed for the phosphorylated and total form of the monoclonal rabbit Akt (Ser473) protein (1:1000, Cell Signalling, UK). The protein detection was carried out using enhanced chemi-luminescence (ECL) assay and the protein density bands were visualised using Bio-Rad Quantity One programme as

detailed in Chapter 2, Section 2.8.7. The relative variations in the levels of phosphorylated monoclonal rabbit Akt (Ser473) protein were normalised to the total form of monoclonal rabbit Akt protein. In all immunoblotting experiments, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal loading control to ensure the uniform loading of gel samples as well as the uniform transfer of the proteins during the immunoblotting stage.

3.2.12 Data analysis

All data is presented as mean± SEM. Haemodynamic parameters, heart rate, left ventricular developed pressure and coronary flow were analysed by one-way ANOVA for each time point. All other data was analysed using one-way analysis of variance (ANOVA) with Tukey post-hoc tests to identify group differences. P values that had p less than 0.05 were considered statistically significant.

3.3 Results

3.3.1 The effects of Tiron administration (0.25mM-10mM) on the infarct size in the naïve Langendorff hearts.

There was no significant difference in infarct to risk ratio upon treatment with Tiron (0.25-1mM) compared to control group (Tiron 0.25mM:9.34±0.52% vs. 8.48±0.48%; Tiron 0.5mM: 9.94±0.18% vs. 8.48±0.48%; Tiron 1mM: 11.29±0.48% vs. 8.48±0.48%). A significant increase in infarct to risk ratio was observed at concentrations of 2.5mM and above (Tiron 2.5mM: 23.37±1.18% vs. 8.48±0.48%; Tiron 5mM: 35.03±4.68% vs. 8.48±0.48%, respectively Tiron 10mM: 56.71±2.72% vs. 8.48±0.48%) (Figure 3.2).

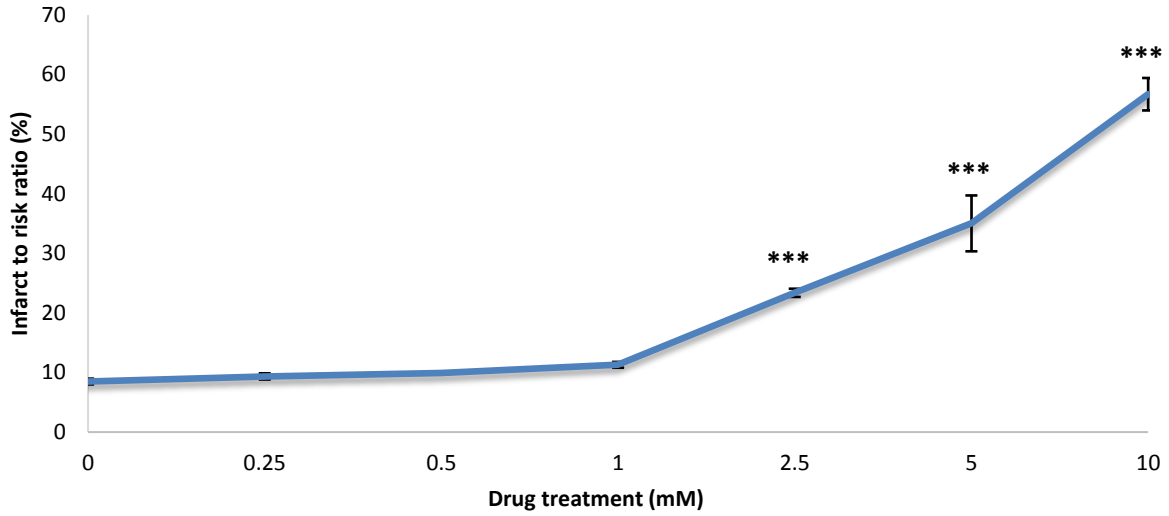


Figure 3.2 The effects of Tiron administration (0.25mM-10mM) on the infarct to risk ratio analysis on isolated naïve Langendorff heart model. Data are presented as mean±SEM of 6 experiments; ***p<0.0001 vs. control.

3.3.2 The effects of Tiron administration (0.25mM-10mM) on the haemodynamic parameters CF, HR and LVDP from the naïve Langendorff hearts.

Data obtained revealed that there was no significant effect with any of the concentrations of Tiron tested (0.25-10mM) on coronary flow (CF), heart rate (HR) and left ventricular developed pressure (LVDP) when compared to normoxic control (Figure 3.3 A, B and C).

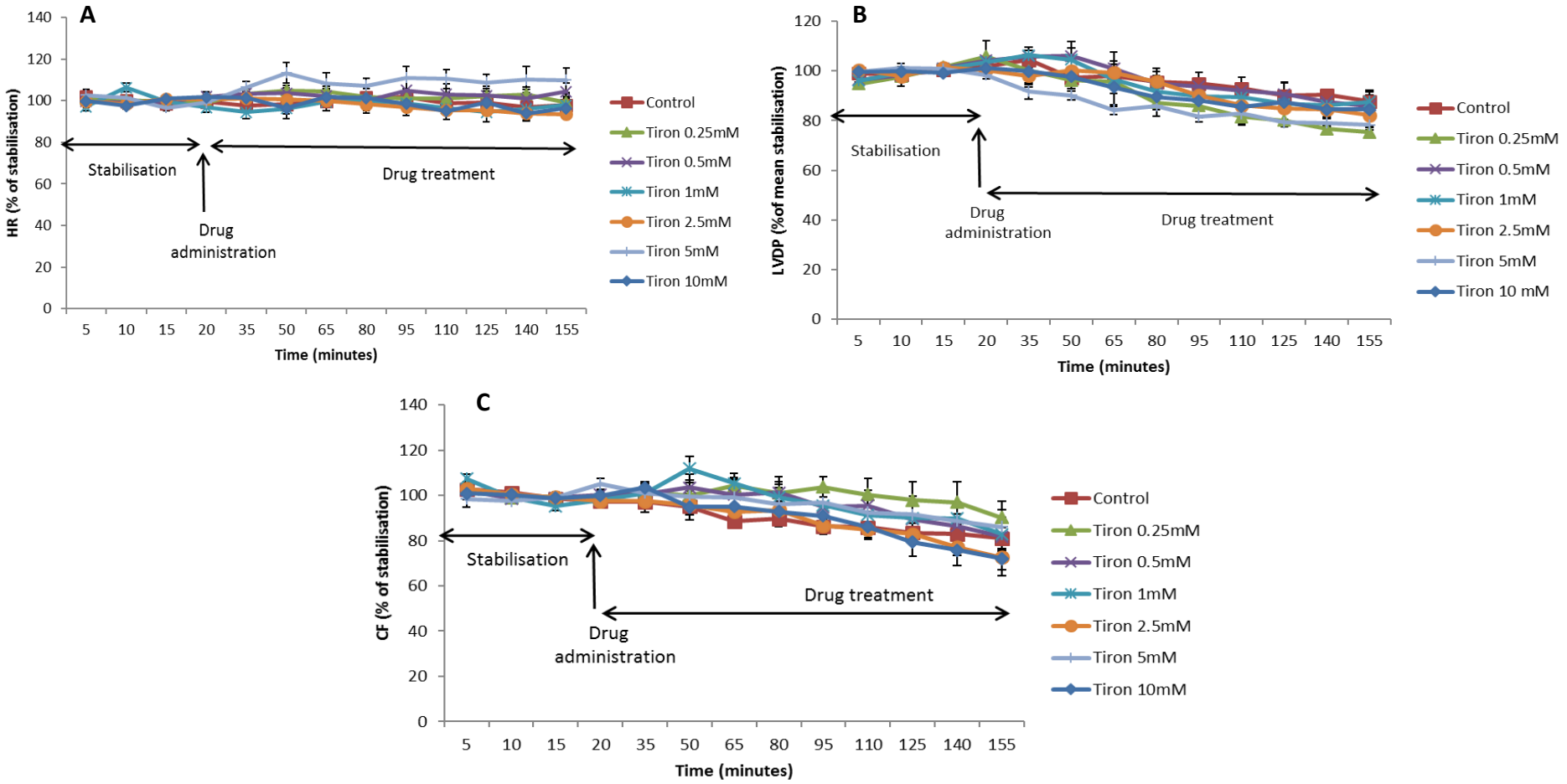


Figure 3.3 The effects of Tiron administration (0-10mM) on HR (A), LVDP (B) and CF (C) represented as a percentage of the mean stabilisation: Control (red); Tiron 0.25mM (green); Tiron 0.5mM (purple); Tiron 1mM (turquoise); Tiron 2.5mM (orange); Tiron 5mM (light blue); Tiron 10mM (blue). Hearts were subjected to 20 minutes of stabilisation, followed by 135 minutes of perfusion with KHB±Tiron (0-10mM). Data are presented as mean±SEM of 6 experiments.

3.3.3 The effects of increasing concentrations of Tiron on the viability of isolated rat ventricular cardiomyocytes

No significant effect on cell viability was observed upon cell treatment with with 0-1mM Tiron. A reduction in cell viability was observed upon cell treatment with Tiron 2.5mM when compared to control (73.01±3.9% vs. 100.00±0.0%, Figure 3.4). A similar trend was confirmed with Trypan blue staining (Figure 3.5).

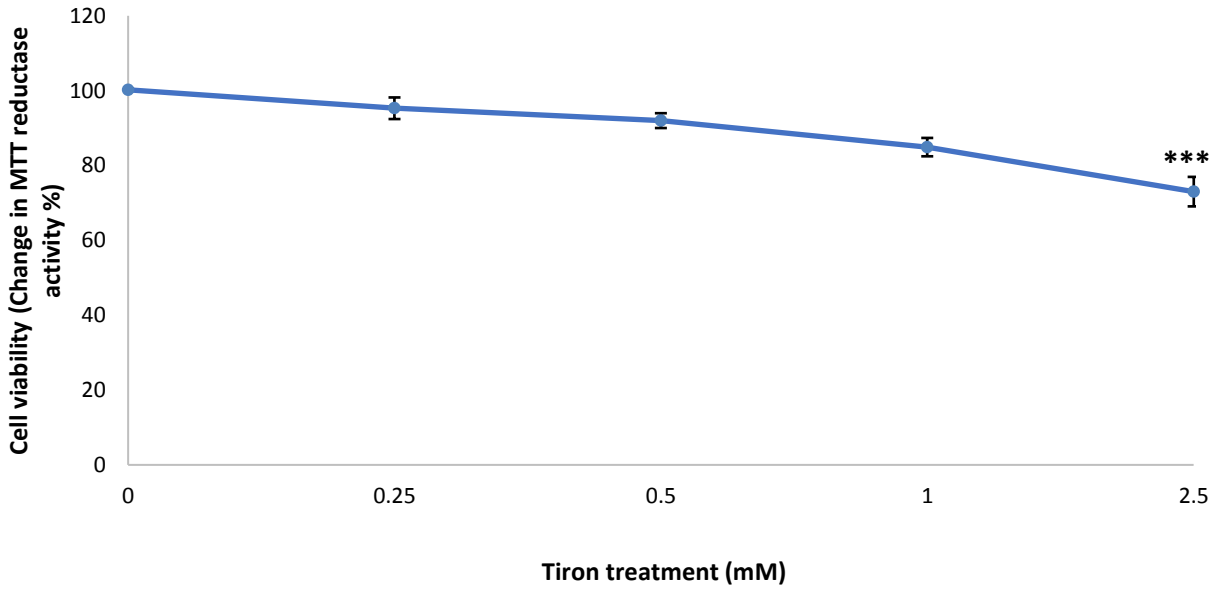


Figure 3.4 MTT analysis showing the viability of isolated adult rat ventricular cardiomyocytes in response to increasing concentrations of Tiron (0-2.5mM) (blue) (Control: 100±0.00%; Tiron 0.25mM: 95.29±2.88%; Tiron 0.5mM: 91.98±1.97%; Tiron 1mM: 84.90±2.45%; Tiron 2.5mM: 73.01±3.94%). Data are presented as mean±SEM, with a number of n=6 experiments, ***p<0.0001 vs. Control.

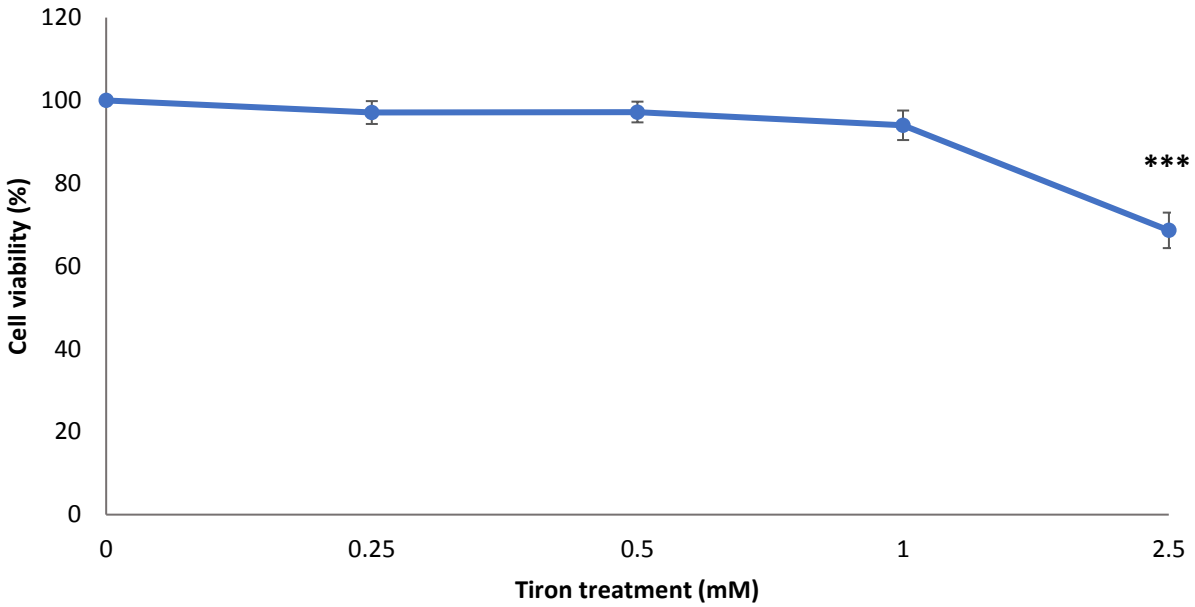


Figure 3.5 Trypan blue staining analysis showing the viability of isolated adult rat ventricular cardiomyocytes in response to increasing concentrations of Tiron (0-2.5mM) (blue). Data are presented as mean±SEM, with a number of n=6 experiments, ***p<0.0001 vs. Control.

3.3.4 The effects of Tiron administration (0.25mM) on the levels of intracellular ROS in isolated ventricular rat cardiomyocytes in normoxic conditions

Hydrogen peroxide (H_2O_2) (2mM) was used as a positive control to test the DCFDA assay and to induce ROS in the isolated adult rat ventricular cardiomyocytes (Figure 3.6a).

DCFDA assay showed a significant increase in reactive oxygen species with 2mM H_2O_2 treated samples when compared to control (907.04±39.79% vs. 100.97±1.49%) (Figure 3.6b). 24 hours pre-treatment with Tiron resulted in a significant decrease in the ROS levels when compared to H_2O_2 group (173.31±43.29% vs. 907.04±39.79%). In addition, pre-treatment with Tiron did not

cause a significant effect on the ROS when compared to control group (89.87±30.11% vs. 100.97±1.49%) (Figure 3.6A, B).

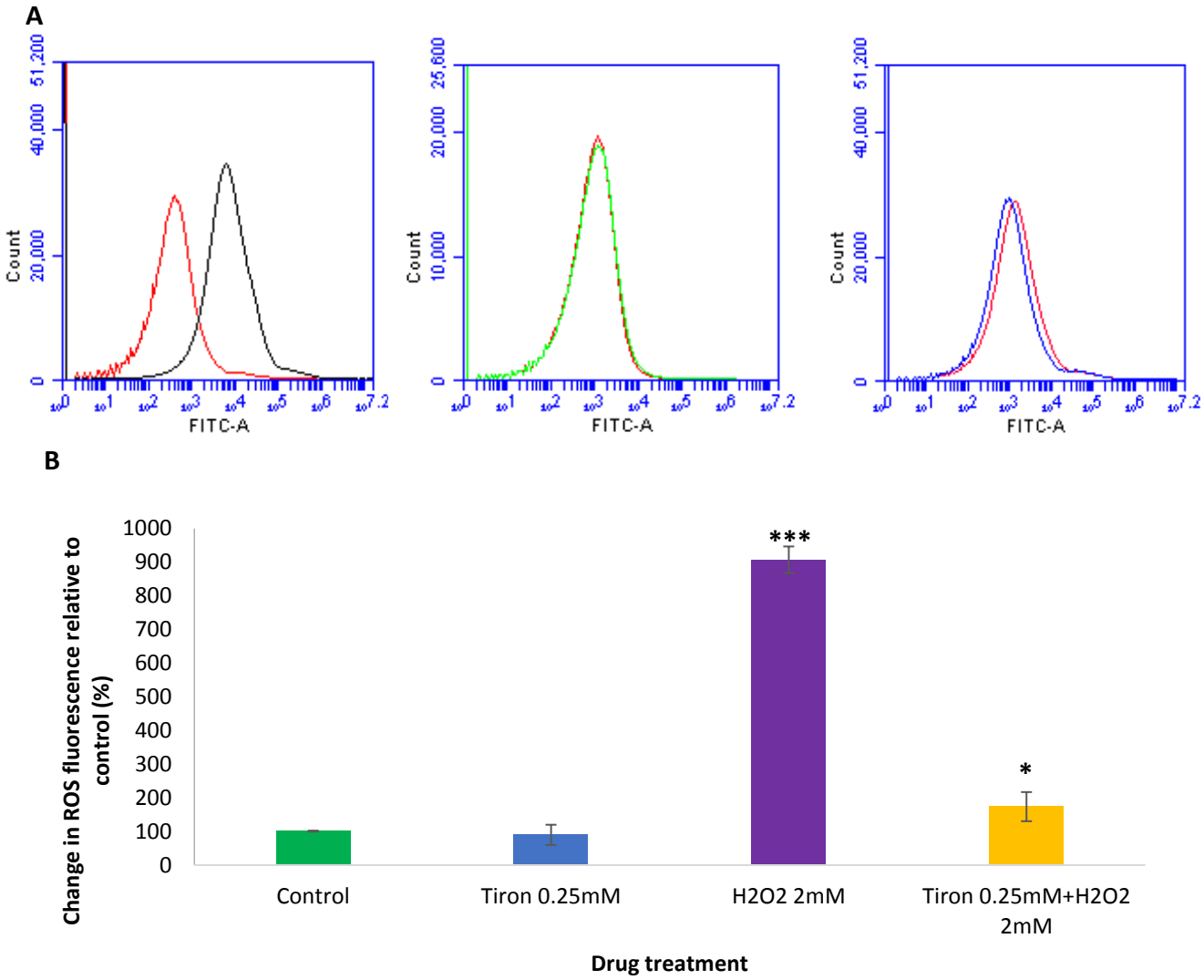
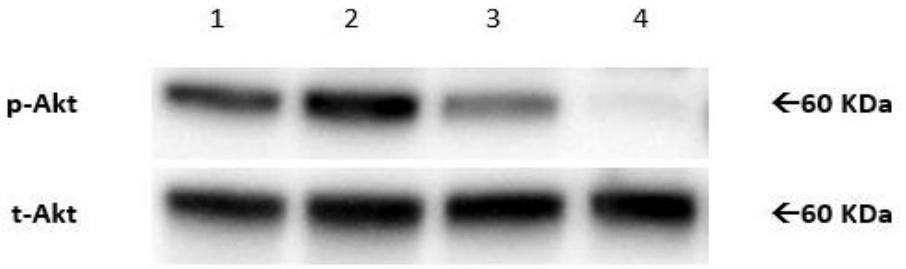


Figure 3.6 DCFDA flow cytometric scatter plots (A) and analysis (B) showing the change in ROS fluorescence relative to control in isolated adult rat ventricular cardiomyocytes in normoxic conditions in response to treatment with Tiron 0.25mM. Data are presented as mean±SEM, n=6 experiments, ***p<0.0001 vs. Normoxia. (Representation of the scatter plots: Control (red); H_2O_2 positive control (black); Tiron 0.25mM (green); Tiron 0.25mM+H_2O_2 (blue).

3.3.5 The effect of Tiron administration (0.25mM) on Akt signaling protein levels in cardiac tissue in normoxic conditions

Western blot analysis indicated that Tiron treated samples exhibited a slight increase in p-Akt levels when compared to control (36.72±2.32% vs. 40.47±1.60%). Co-administration of Tiron and Wortmannin did not cause a significant effect in the p-Akt levels when compared to control (36.90±6.81% vs. 32.62±3.67%). Administration of Wortmannin alone resulted in a decrease of p-Akt levels when compared to control group (11.00±5.22% vs. 32.62±3.67%), whereas the concomitant administration of Tiron and Wortmannin exhibited a significant increase in the p-Akt levels when compared to Wortmannin group alone (36.90±6.81% vs. 11.00±5.22%) (Figure 3.7).



1. Control; 2 Tiron (0.25mM); 3 Tiron (0.25mM)+Wortmannin (100nM);
4. Wortmannin (100nM).

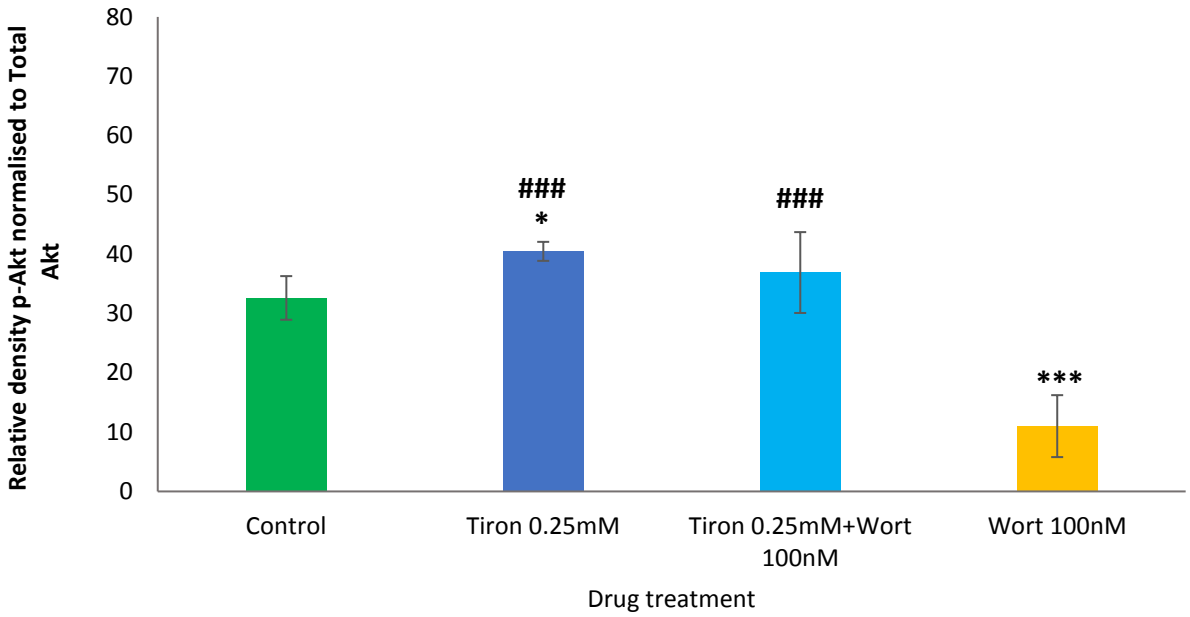


Figure 3.7 Western blot analysis showing the effects of Tiron 0.25mM, Wortmannin (100nM) and co-treatment on the levels of phosphorylated Akt at 120 minutes into perfusion phase. Data are presented as mean±SEM, with a number of n=6 experiments, ***p<0.0001 vs. control; ###p<0.0001 vs. Wortmannin.

3.3.6 The effects of Tiron administration (0.25mM-2.5mM) on the area at risk (AAR) and infarct size (IS) in the presence of ischaemia reperfusion injury in Langendorff hearts.

As illustrated in Figure 3.8, no significant difference was reported in the percentage area at risk (AAR)/left ventricular volume upon administration of Tiron (0.25mM-2.5mM) when compared to I/R control group.

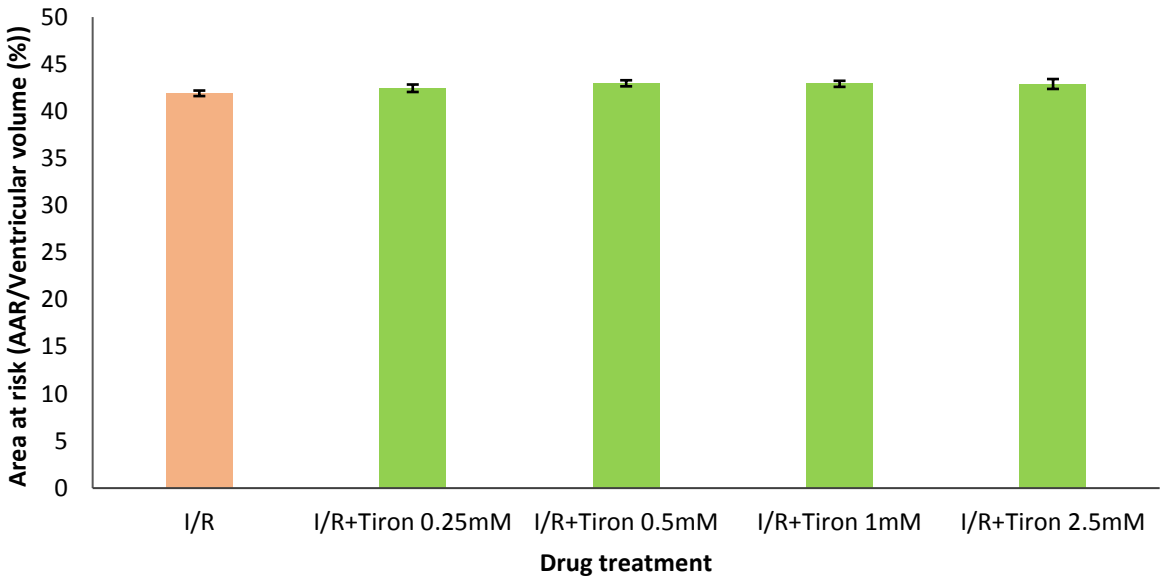


Figure 3.8 The effects of Tiron administration (0.25mM-2.5mM) on the percentage of area at risk of the ventricular volume on isolated Langendorff heart model of ischaemia and reperfusion injury. Hearts were exposed to 20 minutes stabilisation, followed by 35 minutes of regional ischaemia and 120 minutes of reperfusion with KHB±Tiron (0.25-2.5mM). Results were presented as mean±SEM, n=6 experiments.

Administration of Tiron (0.25-2.5mM) during reperfusion significantly ($p<0.05$) reduced the infarct size to risk ratio when compared to I/R control (Tiron 0.25mM: 24.05±0.68% vs. 47.72±0.91%; Tiron 0.5mM: 23.39±0.84% vs. 47.72±0.91%; Tiron 1mM: 17.24±1.04% vs. 47.72±0.91%; Tiron 2.5mM: 20.49±0.69% vs. 47.72±0.91%) (Figure 3.9).

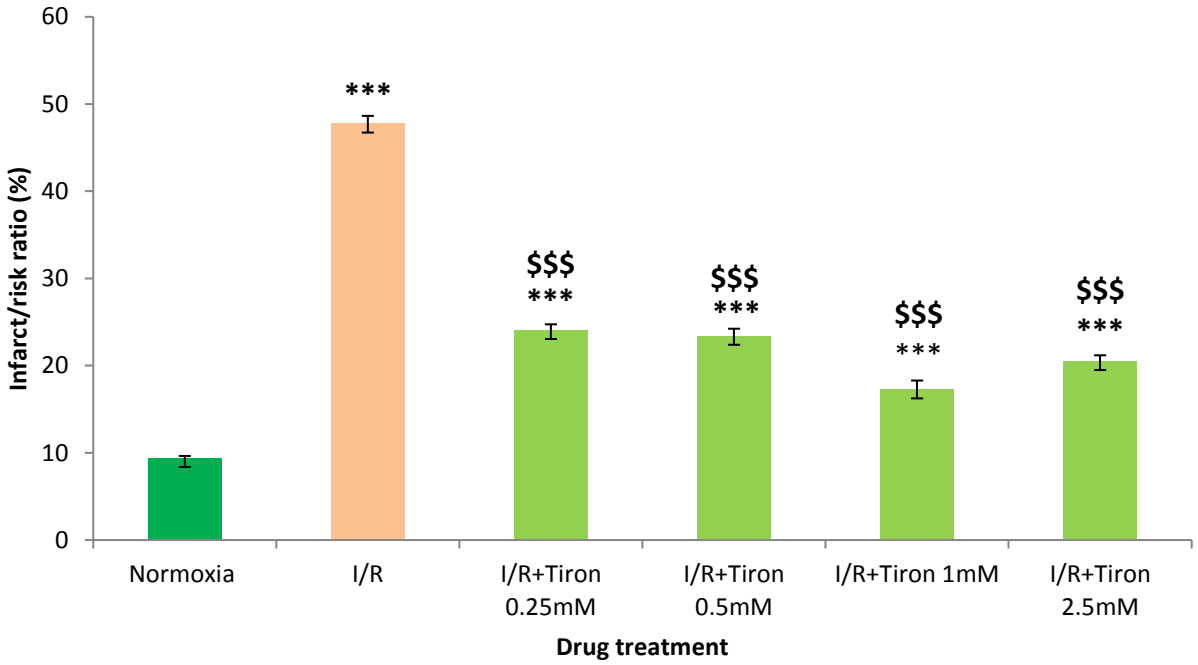


Figure 3.9 The effects of Tiron administration (0.25mM-2.5mM) on the infarct to risk ratio analysis on isolated Langendorff heart model of ischaemia reperfusion injury. Hearts were exposed to 20 minutes stabilisation, followed by 35 minutes of regional ischaemia and 120 minutes reperfusion with KHB±Tiron (0-2.5mM). Data are presented as mean±SEM of 6 experiments; $$$p<0.0001 vs. I/R control; ***p<0.0001 vs. Normoxia.

3.3.7 The effects of Tiron administration (0.25-2.5mM) on the haemodynamic parameters of the heart in ischaemia reperfusion studies

LVDP was reduced by approximately 40% of the stabilisation mean throughout ischaemia and improved during reperfusion but did not achieve the values at stabilisation (Figure 3.10B).

There was no significant difference observed in the HR, CF and LVDP values when treated with Tiron (0.25-2.5mM) at reperfusion (Figure 3.10A, B and C). No significant difference was identified within the groups at any point.

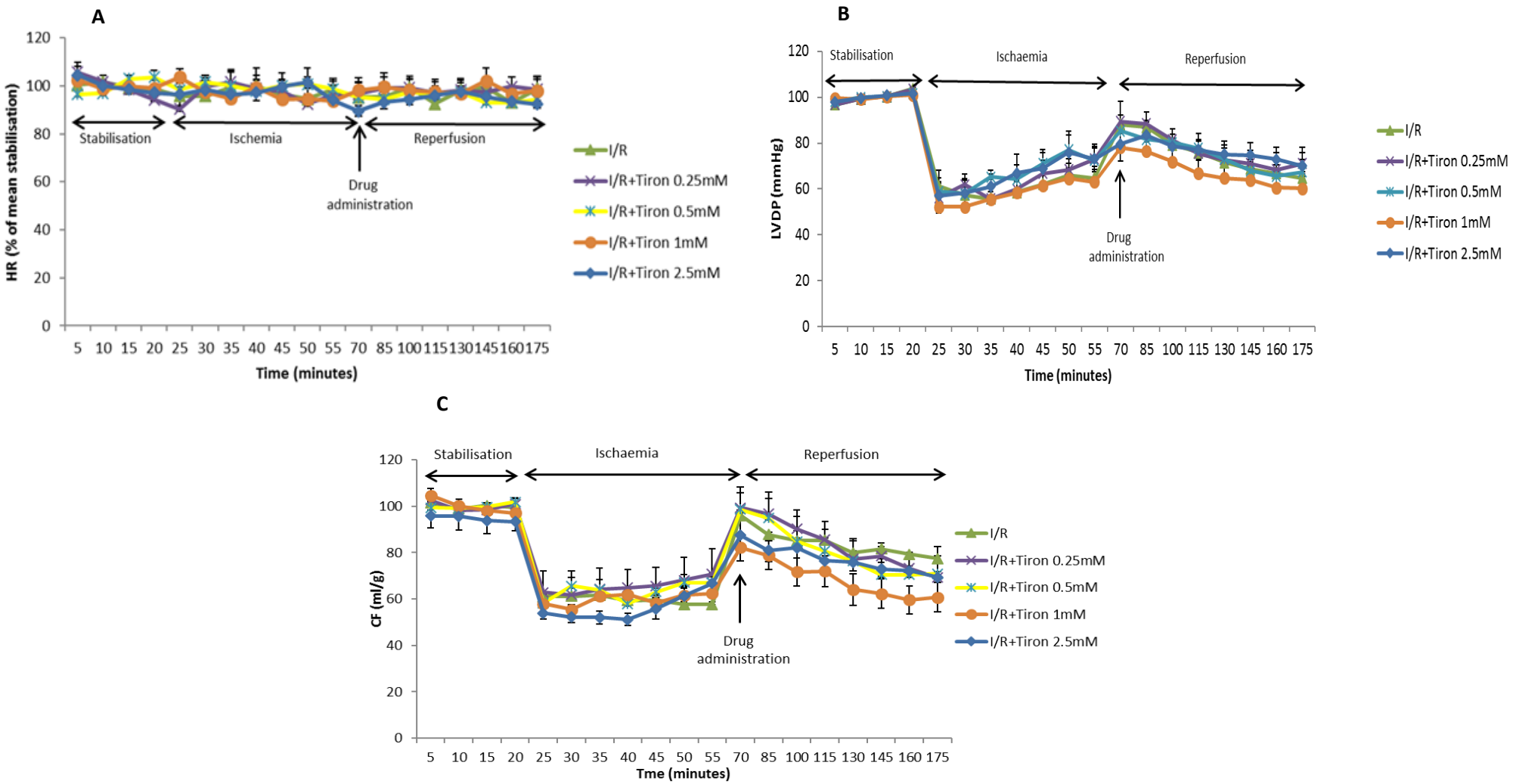


Figure 3.10 The effects of Tiron (0.25mM-2.5mM) on HR (A), LVDP (B) and CF (C). The hearts were subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion with KHB±Tiron (0-2.5mM). Data are presented as a percentage of average stabilisation: I/R (green); I/R+Tiron 0.25mM (purple); I/R+Tiron 0.5mM (yellow); I/R+Tiron 1mM (orange); I/R+Tiron 2.5mM (blue). Data points are plotted as mean±SEM of 6 experiments.

3.3.8 The effect of Tiron administration (0.25mM) on the viability of isolated rat ventricular cardiomyocytes subjected to H/R conditions

Isolated rat ventricular cardiomyocytes were exposed to 60 minutes of hypoxia and 180 minutes of reoxygenation, with Tiron (0.25mM) being administered during reoxygenation stage.

Cells exposed to H/R conditions resulted in a significant reduction in viability compared to normoxic control group (45.43±4.89% vs. 100±0.00%) (Figure 3.11).

An increase in the cell viability was observed upon H/R treatment and Tiron 0.25mM when compared to H/R group (60.94±2.55% vs. 45.43±4.89%, Figure 3.11). However, the significantly improved cell viability observed in Tiron treated samples did not achieve the values reported in normoxic control group (60.94±2.55% vs. 100±0.00%). This trend was confirmed by Trypan blue staining (57.73±1.15% vs. 44.34±0.93%; $p<0.05$; Figure 3.12). A proportional increase was detected in both assays.

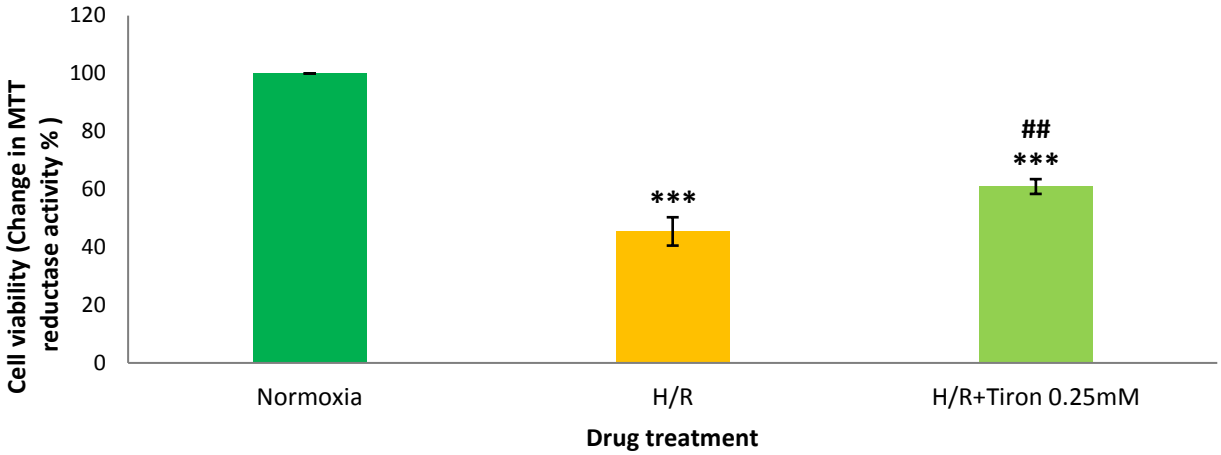


Figure 3.11 MTT analysis showing the viability of isolated adult rat ventricular cardiomyocytes subjected to H/R conditions in response to treatment with Tiron 0.25mM. Cells were exposed to 60 minutes of hypoxia and 180 minutes of reoxygenation in the presence/absence of Tiron (0.25mM). Data are presented as mean±SEM, with a number of n=6 experiments, ***p<0.0001 vs. Normoxia; ##<0.001 vs. H/R.

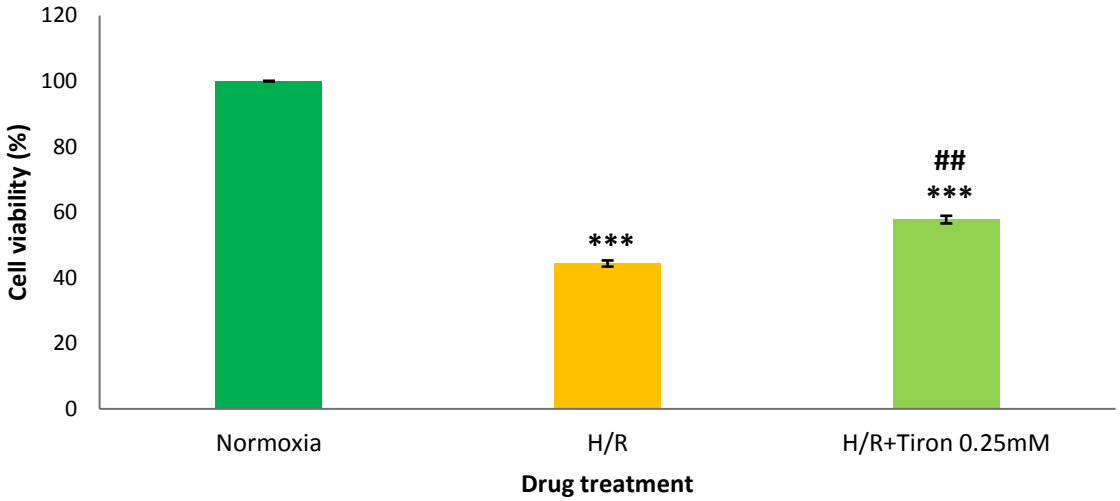


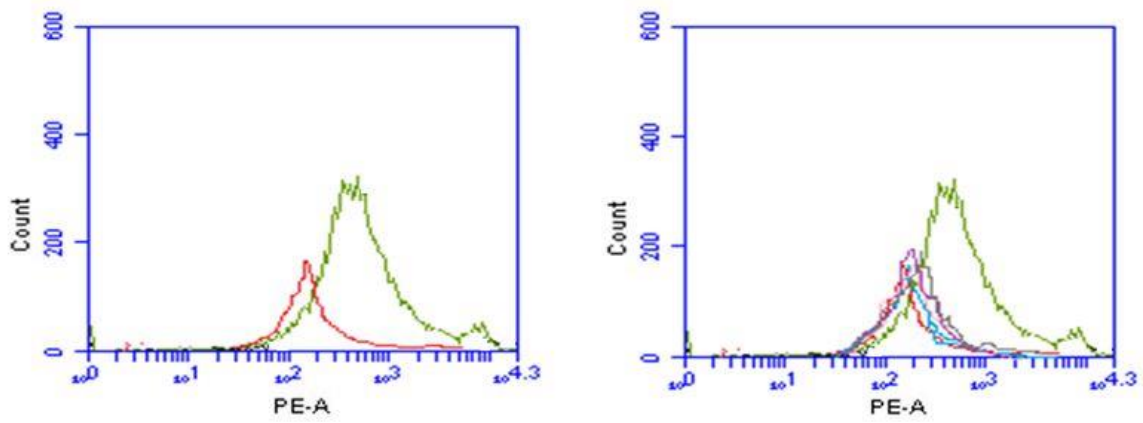
Figure 3.12 Trypan blue staining analysis showing the viability of isolated adult rat ventricular cardiomyocytes subjected to H/R conditions in response to treatment with Tiron 0.25mM. Data are presented as mean±SEM, n=6 experiments, ***p<0.0001 vs. Normoxia; ##<0.001 vs. H/R.

3.3.9 The effect of Tiron administration (0.25-2.5mM) on the cleaved-caspase 3 levels in isolated adult rat ventricular cardiomyocytes subjected to H/R conditions

Isolated primary rat ventricular cardiomyocytes were exposed to 60 minutes of hypoxia and 180 minutes of reoxygenation, with Tiron (0.25-2.5mM) being administered throughout reoxygenation period.

A significant increase could be observed in the levels of caspase-3 in the H/R group in comparison with the normoxic control group (454.31±8.21% vs. 100±0.0%) (as shown in Figure 3.13A and 3.13B). Treatment with Tiron (0.25-2.5mM) throughout the reoxygenation stage was associated with a significant decrease in the levels of caspase-3 when compared to H/R group (Tiron 0.25mM: 98.69±7.14% vs. 454.31±8.21%; Tiron 0.5mM: 105.23±6.67% vs. 454.31±8.21%; Tiron 1mM: 109.86±4.76 vs. 454.31±8.21%; Tiron 2.5mM: 123.59±8.58% vs. 454.31±8.21%; Figure 3.13B). No significant difference in the levels of cleaved-caspase-3 was detected in any of the Tiron treated groups when compared to normoxic control group (Figure 3.13B).

A



B

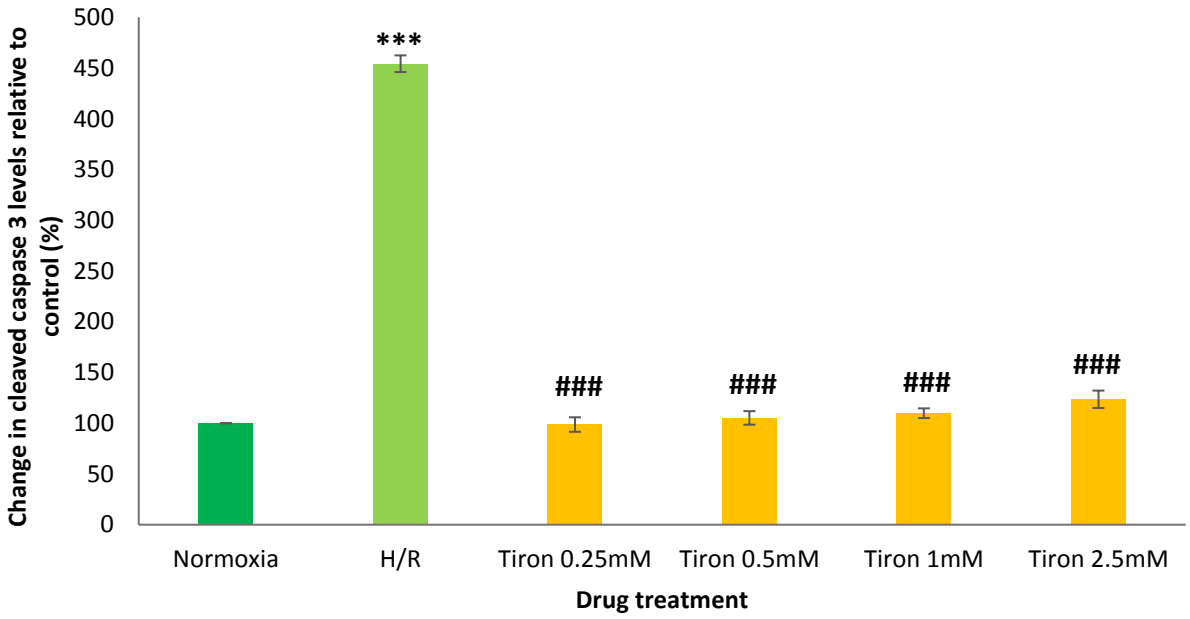


Figure 3.13 Cleaved caspase-3 levels flow cytometric plots (A) and analysis (B) in isolated adult rat ventricular cardiomyocytes subjected to H/R conditions in response to treatment with Tiron (0.25-2.5mM). Data are presented as mean±SEM, n=5-6 experiments, ***p<0.0001 vs. Normoxia; ###p<0.001 vs. H/R; Normoxic control (red); (Representation of the scatter plots: H/R control (green); Tiron 0.25mM (purple); Tiron 0.5mM (blue); Tiron 1mM (pink); Tiron 2.5mM (grey).

3.3.10 The effect of Tiron (0.25mM) on Akt signaling protein levels in cardiac tissue subjected to I/R conditions

An increase in p-Akt was observed with Tiron treated samples in I/R conditions when compared to I/R control (74.35±2.83% vs. 51.68±5.18%). Co-administration of Tiron and Wortmannin and administration of Wortmannin alone resulted in a decrease of p-Akt levels when compared to I/R control group (5.92±1.77% vs. 51.68±5.18%, respectively 3.25±0.75% vs. 51.68±5.18%, Figure 3.14).

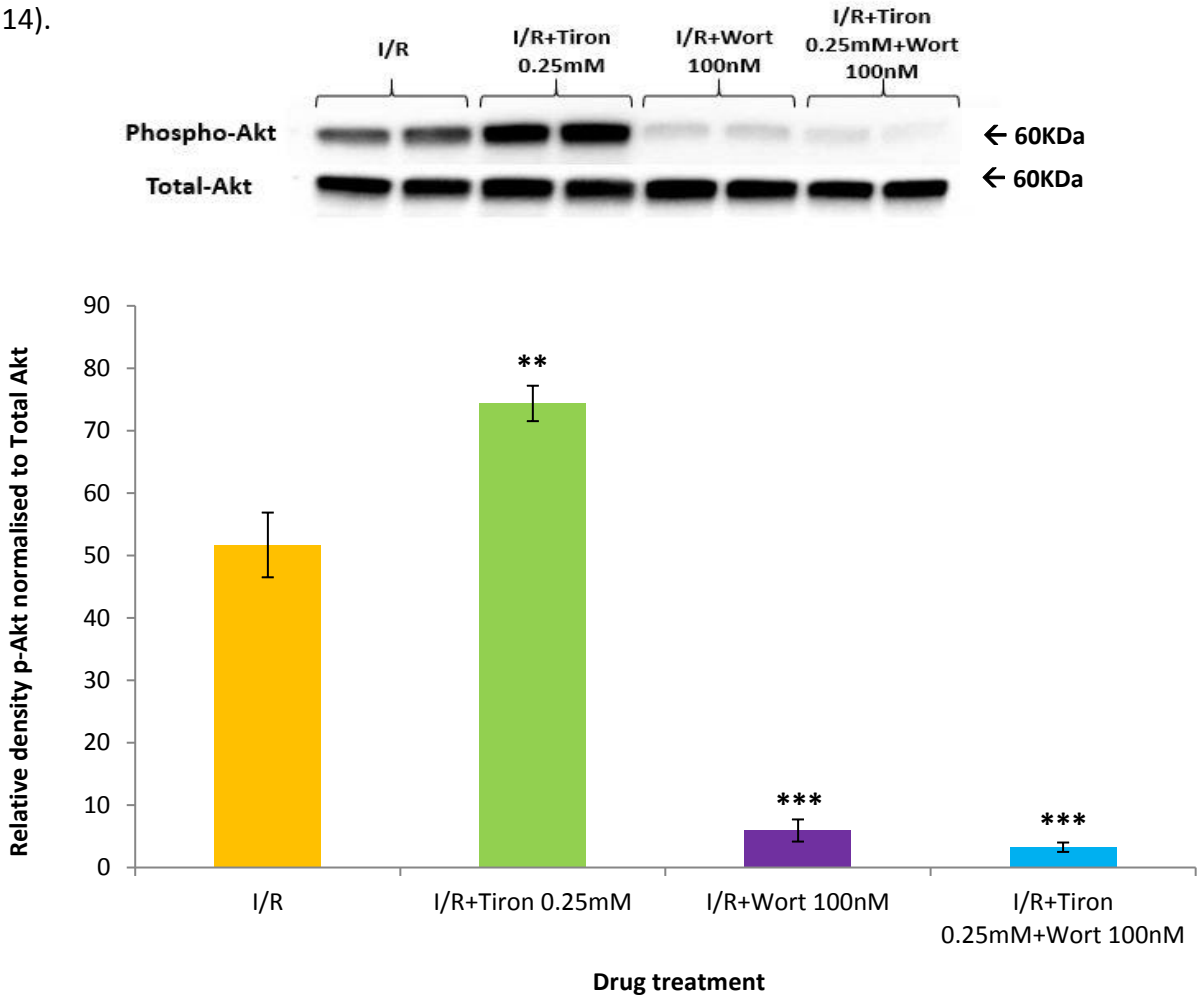


Figure 3.14 Western blot analysis showing the effects of Tiron 0.25mM and Wortmannin (100nM) and co-treatment on the levels of phosphorylated Akt at 20 minutes into reperfusion phase. Data are presented as mean±SEM, with a number of n=6 experiments, ***p<0.0001 vs. H/R.

3.4 Discussion

In the current investigation we have demonstrated that treatment with previously described concentrations of Tiron (0.25-1mM) (Borchi et al. 2010, Silva-Platas et al. 2016; Kim et al. 2006) in normoxic conditions did not negatively impact the haemodynamic parameters CF, HR, and LVDP and the infarct ratio in isolated rat hearts (Figure 3.3). However, administration of Tiron (2.5-10mM) resulted in an increase in the infarct size in the isolated perfused rat heart (Figure 3.2) indicating an increase in myocardial injury. The injury caused by high concentrations of Tiron could be associated with its pro-oxidant activity. Several reports have established that polyphenol antioxidants can exert beneficial effects, however high doses and alterations in the pH could impact the antioxidant/pro-oxidant activities and their chelating mechanism (Azam et al. 2004, De Marchi et al. 2009, Watjen et al. 2005). The study carried out by De Marchi et al, (2009) with the antioxidant quercetin in the isolated mitochondria and HCT116 cells revealed that high doses of this antioxidant enhance the production of superoxide anions (O_2^-) resulting in mitochondrial dysfunction and opening of the mitochondrial transition pore (De Marchi et al. 2009). In addition, this hypothesis was further confirmed in a rat hepatoma cell model (H4IIE), in which it was shown that low concentrations of quercetin exhibited antioxidant effects by reducing the H_2O_2 induced cytotoxicity and apoptosis, whereas high concentrations resulted in DNA damage and apoptosis (Watjen et al. 2005). In addition, in a rodent in vivo investigation, Ruiz et al, (2015) have shown that treatment with high concentrations of quercetin exerted a negative effect on the heart mitochondrial function, by promoting protein oxidation and decreasing the activity of Complex IV within the mitochondria (Ruiz et al. 2015). Furthermore, it has been shown that oxidative stress is a contributing factor in cardiomyocytes cell death and

this response is mediated by the opening of the mitochondrial transition pore leading to the stimulation of apoptosis and an increase in the myocardial injury (Gustafsson and Gottlieb, 2007; Chiong et al. 2011). Therefore, in agreement with these studies, it is reasonable to hypothesise that high concentrations of Tiron in normoxic conditions only may have a negative effect on the myocardium due to its pro-oxidant activity exerted by enhanced production of the superoxide anions and accumulation in the mitochondria, leading to cardiomyocytes cell death and subsequently an increase in the myocardial infarct size in the isolated perfused rat hearts.

The effects of increasing concentrations of Tiron in the model of I/R injury were also assessed. Our results have shown that Tiron possesses cardioprotective properties in a dose-dependent manner, as indicated by reduction in the induced myocardial I/R injury (Figure 3.9). Furthermore, Tiron was shown to have cardioprotective effects at low concentrations (0.25 mM), which was observed by a decrease in the infarct to risk ratio in the isolated rat hearts (Figure 3.9) and an increase in the cell viability in adult rat ventricular cardiomyocytes (Figure 3.11 and 3.12). Similarly, in a Langendorff model of simulated myocardial I/R injury, Ray et al, (2010) have demonstrated that the extract from Calendula officinalis exerted antioxidant and anti-inflammation properties reducing the myocardial infarct and improving the left ventricular cardiac function in the Sprague Dawley hearts (Ray et al. 2010). The results in our study showed that administration of Tiron did not cause any significant changes in the haemodynamic parameters during reperfusion when compared to control group (Figure 3.10). In a similar manner, a study carried out by Song et al, (2010) in isolated male Wistar rats exposed to 30 minutes of regional ischaemia and 2 hours of reperfusion showed that treatment with the polyphenol epigallocatechin gallate (EGCG), did not cause any significant alterations in the

haemodynamic parameters LVDP, CF and HR when compared to I/R control group, however a significant decrease in the infarct size was reported (Song et al, 2010). Comparable results were also reported by Badavi et al, (2017) in the Langendoff heart model of 30 minutes of no-flow global ischaemia and 1 hour of reperfusion (Badavi et al, 2017). It has been shown that different concentrations of EGCG did not impact the effects of LVDP when compared to I/R control group (Badavi et al, 2017). These results show that a reduction in the infarct size is not always linked with an improvement in the cardiac function which could be associated with the possibility that the salvaged tissue has not reached its normal values (Song et al, 2010; Lochner, Genade and Moolman, 2003).

Our results are in accordance with a previous study in which Tiron (500mg/kg) provided cellular protection by reducing superoxide production in the model of I/R injury and alleviated the MI/R injury in the in vivo model of adult mice (Jiao et al. 2009).

In this study, we have also investigated the effects of Tiron on intracellular levels of ROS in primary adult ventricular cardiac myocytes. We have demonstrated that pre-treatment with Tiron offered significant protection from H_2O_2 induced oxidative stress (Figure 3.6a and 3.6b). Previously, it has been reported that Tiron exhibited cytoprotection against H_2O_2 and superoxide anions produced as a result of oxidative damage (Krishna et al. 1992). A study carried out by Habenar et al, (2016) showed that in HL-1 cardiomyoblast-like cells, addition of Tiron decreased H/R-induced apoptosis by 60% and reduced the H/R induced ROS production by 91% when compared to control group (Habener et al. 2016). Furthermore, in a different study, Tiron was reported to improve the endothelial dysfunction of the coronary circulation and reduce the

oxygen free radical formation in myocardial tissue in dog-model of pacing-induced heart failure (Arimura et al. 2001). A study carried out by Borchi et al, (2010) in human failing hearts revealed that Tiron significantly reduced NADPH-dependent superoxide generation in failing ventricles. Thus, in agreement with previous published articles, our results show that treatment with Tiron reduces the oxidative stress induced by myocardial I/R injury and offers cytoprotection against oxidative induced damage.

It is well established that myocardial apoptosis plays a contributing factor to myocardial I/R injury (Badalzadeh, Mokhtari and Yavari 2015, Eefting et al. 2004, Keoni and Brown 2015, Kim and Kang 2010, McCully et al. 2004). Inhibiting the mechanism of apoptosis may reduce the I/R injury and might delay the incidence of heart failure (Oikawa et al. 2013, Zhang et al. 2017a). Several studies have noted that cardiomyocytes apoptotic cell death may be triggered during ischaemia/hypoxia however, the injury produced to the cells could be exacerbated during reperfusion (Granger and Kvietys 2015, Kalogeris et al. 2012, Kurian et al. 2016, Zhou, Chuang and Zuo 2015).

Apoptosis is divided into 2 distinct signalling pathways: the intrinsic pathway, also named the mitochondrial cell death pathway and the extrinsic pathway also called death receptor pathway (Badalzadeh, Mokhtari and Yavari 2015, Fulda 2010, Kim and Kang 2010). Several investigations have shown that upon activation of either intrinsic or extrinsic pathway, the initiator caspases is activated, triggering the executioner caspases -3, -6 and -7 (S Singh and M Kang 2011, Zhang et al. 2017b). It has also been reported that activation of caspase-3 could be associated with mitochondrial oxidative stress, which results in increased permeability to the membrane and

release of cytochrome c into the cytosol, producing the apoptosomes complexes (Eefting et al. 2004, Gottlieb et al. 1994, Krijnen et al. 2002, Ng, Wan and Yim 2005).

Previous studies have reported the antioxidant activity of Tiron in a number of pathological conditions (Arimura et al. 2001, Borchi et al. 2010, De Matteo, Head and Mayorov 2006, Habener et al. 2016, Jiao et al. 2009). However, few investigations stated whether Tiron could exhibit similar protective effects against myocardial ischaemia/ reperfusion injury.

Our study demonstrated that adult primary ventricular cardiomyocytes exposed to H/R conditions showed an increase in the levels of cleaved caspase 3 compared to the normoxic control group (454.31±8.21% vs. 100±0.00%) and administration of Tiron (0.25-2.5mM) inhibited the activation of caspase 3 levels, protecting the heart and salvaging the cardiac myocytes from undergoing the process of apoptosis (Figure 3.13b.). In this study, Tiron (0.25mM) significantly improved cell viability in isolated adult rat ventricular myocytes when compared to H/R control group (shown in Figure 3.11 and Figure 3.12). As mentioned before, it has been reported that cardiomyocytes exposed to hypoxic conditions exhibited the phenomenon of nuclear shrinkage, which has previously been linked with the activation hypoxic caspase-dependent cell death (Shinzawa and Tsujimoto 2003). The levels of caspase-3 in Tiron treated myocytes were significantly decreased when compared to H/R control group, indicating that Tiron cardioprotection could be attributed with a reduction in the apoptosis. This outcome is further supported by another study, in which Tiron has been demonstrated to inhibit apoptosis, by decreasing the levels of caspase-3 in an in vivo model of adult mice subjected to myocardial I/R injury (Jiao et al. 2009).

The effects of Tiron (0.25mM) and Wortmannin on PI3K/Akt pathway were also investigated in normoxic conditions. Treatment with Tiron (0.25mM) perfused for 135 minutes caused a slight increase in the levels of p-Akt when compared to control group and administration with Wortmannin, a PI3K inhibitor decreased the p-Akt levels when compared to control group (Figure 3.7). Furthermore, concomitant administration of Tiron and Wortmannin reversed the decrease in p-Akt pathway when compared to Wortmannin treated samples. To date there are no studies that investigated the effects of antioxidants and Wortmannin in the p-Akt pathway in the normoxic studies. However, it could be hypothesised that the mild increase in the p-Akt pathway in Tiron treated hearts could be associated with a protective mechanism elicited by Tiron. Although Langendorff heart preparation is a technique that could maintain the heart viable for couple of hours, studies have shown that a decrease of approximately 5-10% per hour was observed in the contractile function of the heart and it should be considered a dying preparation (Bell et al, 2011; Sutherland and Hearse, 2000). This could potentially have an impact on the myocardium and could trigger the activation of Akt survival pathway, a well-known pathway involved in cell survival, therefore explaining the possible increase in the p-Akt levels in Tiron treated hearts and the abrogation of p-Akt by Wortmannin. Interestingly, our results show that concomitant administration of Tiron and Wortmannin reversed the decrease in p-Akt levels, indicating that in normoxic conditions Tiron could act via a PI3K/Akt independent pathway, however due to lack of reports in this particular setting, this hypothesis requires further elucidation.

In this study, we also examined if treatment with Tiron (0.25mM) at the onset of reperfusion had any effects on PI3K/Akt signalling pathway. Treatment with Tiron (0.25mM) during reperfusion

of the ischaemic myocardium for 20 minutes resulted in the phosphorylation and activation of Akt signalling pathway when compared to I/R control group (Figure 3.14). Previous studies have indicated that Akt signalling is a crucial survival pathway that promotes the survival of cardiomyocytes against I/R induced injury and apoptosis (Fujio et al. 2000, Hers, Vincent and Tavaré 2011, Mullonkal and Toledo-Pereyra 2007). Furthermore, a number of investigations carried out in in vivo and in vitro animal models have shown that activation of PI3K/Akt pathway and regulation of the anti-apoptotic (Bcl-2, Bcl-X_L andMcl-1) and pro-apoptotic proteins (Bax, Bak and BH3 only proteins) represents one of the key processes in myocardial protection against ischaemia/reperfusion injury in the acute phase (Raphael et al. 2006, Tsang et al. 2004, Uchiyama et al. 2004, Xu et al. 2013). It has also been revealed that Akt pathway might be directly associated with the stimulation of a protecting process that inhibits the apoptotic cell death in cardiomyocytes (Fujio et al. 2000, Xu et al. 2013). In addition, the activation of PI3K leads to an increase in the Akt protein expression levels, thus, inhibiting the activation of apoptotic related proteins such as caspase-3 and Bax, will result in a decrease in the myocardial apoptosis. Wortmannin is a non-specific inhibitor of PI3K related proteins, that can inhibit the phosphorylation of Akt, a downstream target of PI3K signalling pathway (Eisenreich and Rauch 2011, McNamara and Degterev 2011). In this study we have shown that using Wortmannin (100nM) completely blocked the increase of Tiron-induced Akt phosphorylation in I/R injury model (Figure 3.12). Previous rodent experimental models carried out with different antioxidants have shown similar results (Chen et al. 2016, Wang et al. 2017). For example, Wang et al. (2017) have demonstrated that the antioxidant kaempferide (a natural occurring flavonoid) attenuated the myocardial I/R injury through the activation of PI3K/Akt pathway (Wang et al. 2017).

Therefore, in agreement with these studies, our findings suggest that the cardioprotective properties of Tiron are most likely as a result of its stimulation on PI3k/Akt pathway. However, in this study we have only investigated the effects of Tiron via the PI3K/Akt. It is yet unclear whether the other survival signalling pathways such as Erk1/2 and JNK contribute to the cardioprotective, anti-apoptotic mechanism of Tiron.

3.5 Summary of the findings

In summary, the present study represents the first evidence that Tiron can reduce the myocardial ischaemia reperfusion injury in Sprague Dawley rat model. Our results show that:

-Tiron offers cardioprotection by attenuating oxidative stress generated in the ischaemic reperfused myocardium;

-Tiron administration provoked the activation of Akt during ischaemia-reperfusion, effect that was diminished by Wortmannin administration.

-Tiron reduced H_2O_2 induced oxidative stress, prevented ROS generation in isolated ventricular cardiomyocytes and decreased caspase-3 activity, an effector enzyme of the apoptotic cell death cascade;

-The protective properties of Tiron may account not only to its antioxidant superoxide scavenging activities, but also to its ability to regulating PI3K/Akt pathway in myocardial I/R injury.

These findings emphasise the possibility that Tiron supplementation could offer protection against oxidative stress induced cardiovascular risk factors and myocardial injury.

Chapter 4.Investigating the effect of Tiron against Doxorubicin induced cardiotoxicity in normoxic conditions

4.1 Introduction

In the recent years, cancer treatment has progressed significantly by increasing the survival rate in several types of cancer (Dent 2013, McGowan et al. 2017, Shakir and Rasul 2009). Doxorubicin, an antineoplastic drug that belongs to anthracyclines family is one of the most effective therapy against a variety of solid tumors including breast cancer, small lung cancer ovarian cancer, lymphoma and leukaemia for adult and paediatric patients (Hershman et al. 2008, Hortobagyi 1997, Sepe, Ginsberg and Balis 2010, Zhao et al. 2017). Furthermore, Doxorubicin treatment has been frequently utilised in liver malignancies, being considered one of the most effective chemotherapeutic agents to treat hepatocellular carcinoma (Tam 2013). Unfortunately, its therapeutic potential in clinical use is limited by cumulative, dose-dependent and irreversible cardiotoxic effects (Cardinale et al. 2015, Chatterjee et al. 2010a, De Angelis et al. 2016, Sun et al. 2013a, Vejpongsa and Yeh 2014, Zhang et al. 2009a).

It has been shown that patients treated with Doxorubicin exhibit cardiomyopathy and severe congestive heart failure, occurring up to several years after cessation of Doxorubicin chemotherapy (Swain et al, 2003; Steinherz et al, 1991). In a retrospective meta-analysis of phase III clinical trials in patients with breast cancer and small cell lung carcinoma, Swain et al, (2003) have revealed that over 50% of the patients exposed to Doxorubicin treatment at a dose of 500 mg/m^2 reported cardiac events such as variations in left ventricular ejection fraction (LVEF) and congestive heart failure (Swain et al, 2003). Furthermore, in a different study carried out in 201

long-term survivors of paediatric cancers that were exposed to anthracycline treatment, showed that 23% of the patients included in the study had abnormal changes in the cardiac function 4 to 20 years after cessation of Doxorubicin treatment (Steinherz et al, 1991).

Despite continuous research elucidating the intracellular pathways associated with Doxorubicin-induced cardiotoxicity, the exact mechanism of myocardial injury is still elusive. Several studies have indicated that Doxorubicin-induced cardiotoxicity is a multifactorial process and includes the activation of several intracellular signaling pathways such as oxidative stress, mitochondrial damage and activation of inflammation and apoptosis-related signaling pathways (Chatterjee et al. 2010b, Henriksen 2017, Menna and Salvatorelli 2017, Mistiaen 2016, Shi, Abdelwahid and Wei 2011, Takemura and Fujiwara 2007, Toldo et al. 2013, Zhang et al. 2012). As highlighted in Chapter 1, a number of investigations have indicated that cardiotoxic effects associated with Doxorubicin involve the induction of oxidative stress by quinone-hydroquinone moiety of the anthracyclines structures (Chen et al. 2007, Chularojmontri, Gerdprasert and Wattanapitayakul 2013, Štěrba et al. 2013). In addition, it has been shown that long term administration of Doxorubicin or high doses may cause a reduction in the levels of endogenous antioxidant enzymes, that are responsible for scavenging ROS (Doroshow, Locker and Myers 1980, Lehenbauer Ludke et al. 2009, Wallace 2003). This process results in an imbalance in the prooxidant-antioxidant homeostasis, which leads to exacerbated production of ROS and oxidative stress (Lehenbauer Ludke et al. 2009, Wallace 2003). Several investigations indicated that oxidative stress induced by Doxorubicin may directly or indirectly cause damage to the cardiac mitochondria and stimulate a number of intracellular signaling pathways that lead to

cardiomyocytes apoptosis and heart failure (Kalyanaraman et al. 2002, Octavia et al. 2012, Sun et al. 2016, Thorn et al. 2011, Zhang et al. 2009b, Zhou, Palmeira and Wallace 2001).

In recent years, considerable research efforts have focused on finding therapeutic strategies that could reduce the toxic effects of Doxorubicin and protect the heart without affecting its antitumor properties (Gu, Hu and Zhang 2015, Hamza et al. 2016, Shaker et al. 2018). Several studies proposed that selective blocking of ROS action by specific antioxidants could offer significant protection against Doxorubicin induced oxidative stress damage in cardiac tissue (Chang et al. 2011, Panchuk et al. 2016, Ratnam et al. 2006).

As mentioned in Chapter 1, (Sections 1.20-1.22), Tiron a vitamin E analogue and mitochondria-targeted antioxidant has been found to permeabilise the mitochondrial membrane and accumulate within the organelle (Oyewole et al. 2014). Furthermore, Tiron functions as a direct hydroxyl radical and superoxide scavenger (El-Sherbeeny, Hassan and Ateyya 2016, Habener et al. 2016, Monticone et al. 2014; Herscher et al. 1994, Krishna et al. 1992). It is both a beneficial protective antioxidant and an effective non-toxic metal chelator that has been commonly used in oxidative stress associated studies (Arimura et al. 2001, MacKenzie and Martin 1998, Münzel et al. 1999, Seki et al. 1999, Poeggeler et al. 2002, Krishna et al. 1992). Conversely, it has reported that treatment with Tiron triggered the apoptotic cell death in human promyelotic HL-60 leukaemia cell line (Kim et al. 2006).

Chapter 3 highlighted the potential protective capabilities of Tiron in the heart against myocardial ischaemia reperfusion injury, by attenuating oxidative stress generated during reperfusion and activating the Akt survival pathway. As previous studies have shown that Doxorubicin induces

mitochondrial dysfunction and cardiotoxicity, it is important to investigate the cardioprotective properties of Tiron when used in combination with Doxorubicin.

Therefore, the focus of this chapter is: To assess the cardioprotective effects of Tiron and Doxorubicin administered during perfusion in isolated perfused rat hearts and primary adult rat ventricular cardiomyocytes in naïve conditions; to investigate the intracellular pathways associated with the protective effects of Tiron treatment observed in the heart tissue. In addition the effects of Tiron on the anti-proliferative effects of Doxorubicin in human HL-60 leukaemia cell lines and HepG2 liver carcinoma cells was also studied.

4.2 Methods

4.2.1 Chemicals

As stated previously, Tiron was purchased from Sigma Aldrich (UK), Doxorubicin hydrochloride and Wortmannin were both supplied from Tocris Cookson (Bristol) and prepared in the same manner as described in Chapter 2 Section 2.2

4.2.2 Animals:

Adult male Sprague-Dawley rats (350±50g) were supplied by Charles River (UK). Animals received human care assistance in accordance with the guidance in the operation of the animals (Scientific Procedure) Act 1986.

4.2.3 Isolated perfused rat heart preparation (Langendorff protocol):

Following sacrifice by cervical dislocation and the excision using the process of thoracotomy, rat hearts were mounted on the Langendorff perfusion apparatus (as explained in detail in Chapter 2 Section 2.3.1). All hearts were subjected to a retrograde perfusion, and each experiment was conducted for 155 minutes. The hearts were randomly allocated to the following treatment groups a) hearts perfused with Krebs-Hensleit (KHB) buffer for 155 minutes; b) hearts perfused with KHB buffer for 20 minutes, followed by 135 minutes of drug perfusion with Tiron (0.25mM, 0.5mM, 1mM) c) hearts perfused with KHB buffer 20 minutes followed by 135 minutes of perfusion with Doxorubicin (1μM) and c) hearts perfused with KH buffer for 20 minutes followed by 135 minutes of drug perfusion with Tiron (0.25mM,0.5mM and 1mM) in the presence of Doxorubicin (1μM). The concentration of 1μM Doxorubicin has been previously shown to be in the clinical effective range (Gewirtz, 1999).

The infarct ratio analysis using tryphenyltetrazolium chloride (TTC) solution technique was conducted as detailed in chapter 2, Section 2.3.4. Infarct ratio analysis was presented as a percentage of the area at risk for the treatment groups. Data presented for coronary flow assessment was adjusted by the heart weight and calculated as a percentage of mean stabilisation.

As previously described in Chapter 2, section 2.8.1, for Western blot experiments assessing the levels of p-Akt (Ser473), all hearts were treated in the same manner as described in the Langendorff protocol, and Wortmannin (100nM) was administered throughout perfusion in the presence or absence of Tiron (0.25mM), Doxorubicin (1μM) and combined treatment of

Doxorubicin(1μM) and Tiron (0.25mM). At the end of the experimental protocol, the hearts were removed from the Langendorff apparatus and left ventricle was collected and snap-frozen in liquid nitrogen and stored at -80^{o}C. Western blot analysis was described in detail in section 2.8.

4.2.4 Isolation of adult ventricular cardiomyocytes

Adult rat ventricular cardiomyocytes were isolated by enzymatic digestion as described previously (Maddock et al, 2002, Gharanei et al, 2013). Details of the heart digestion protocol and conditions required for the succesful isolation of primary adult ventricular rat myocytes are described in detail in section 2.4 of Chapter 2.

4.2.5 Cell treatment protocol

Isolated ventricular cardiomyocytes, HL-60 leukaemia cells and HepG2 liver carcinoma cells were exposed to drug treatment with Tiron (0.25-1mM) and Doxorubicin (1μM) for 24 hours at 37˚C, 5% CO_2 and 95% O_2 prior to the assessment of cell viability, caspase 3 levels and reactive oxygen species, which are described in detail below in section 4.2.6-4.2.9.

4.2.6 Assessment of cell viability using the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)] assay

The cell viability for isolated adult rat ventricular cardiomyocytes, HL-60 and HepG2 was carried out using the MTT colorimetric assay as previously detailed in Chapter 2, section 2.6.3. All cells were subjected to drug treatment conditions as stated in section 4.2.5.

4.2.7 Assessment of cell viability using Trypan Blue staining

Trypan Blue assay staining was carried out in accordance with the protocol described in Section 2.6.5. Drug treatment was administered to primary isolated rat ventricular mycoytes, HL-60 and HepG2 cells as described in section 4.2.5. Following the cell treatment protocol, cell viability was assessed as a ratio of the viable cells divided by the total number of cells.

4.2.8 Assessment of primary adult ventricular cardiomyocytes, HL-60 and HepG2 cells using caspase-3 staining

Upon completing the drug treatment protocol (section 4.2.5) permeabilisation of the cells and preparation for the staining procedure (Details in Chapter 2 Section 2.7.1), isolated rat ventricular myocytes and HepG2 cells were probed with cleaved-caspase-3 antibody (Active/cleaved Caspase-3 Assay kit, Novus Biologicals/Bio-Techne, UK) in accordance with the manufacturer instructions (Details in Section 2.7.1). Flow cytometric analysis was completed using FL-2 channel of BD Accuri C6 Plus Flow cytometer (Ex/Em=540/570nm).

For the flow cytometric analysis of cleaved-caspase 3 activity, HL-60 cells were incubated with cleaved caspase-3 rabbit monoclonal antibody (Alexa Flour 488 Conjugate, Cell Signalling, UK) at 1:100 dilutions in incubation buffer at room temperature for 1 hour (in accordance with the manufacturer instructions). Following incubation, the cells were washed with incubation buffer and re-suspended in PBS prior to analysis using FL-1 channel on the BD Accuri C6 Plus flow cytometer (Ex/Em=488/535nm).

Data was normalised to cell only control by deducting the mean fluorescence background of the untreated samples. The values obtained were calculated as a relative change in fluorescence activity.

4.2.9 Assessment of primary adult ventricular cardiomyocytes, HL-60 and HepG2 cells using Cellular Reactive Oxygen Species Detection (DCFDA) Assay

Following drug treatment (Section 4.2.5), isolated ventricular cardiomyocytes, HL60 cells and HepG2 cells were incubated with DCFDA assay (Abcam, UK) as stated in the manufacturer instructions and in accordance with Section 2.7.2. Hydrogen peroxide (2-8mM) was used as a positive control to test the kit. The values obtained were calculated as a relative change in ROS fluorescence activity of the mean absorbance of the control group.

4.2.10 Western blot analysis

Western blot analysis was performed as previously described in Chapter 2 Section 2.8. Following the separation stage and the protein transfer procedure, the mermbranes were probed for the phosphorylated and total form of the monoclonal rabbit Akt (Ser473) protein (Cell Signalling, UK) as stated in Section 2.8.6. Protein detection was quantified using Super Signal West Femto Maximum Substrate Solution (ThermoFisher Scientific, UK) as an enhanced chemi-luminescence assay (Detailed in Chapter 2, Section 2.8.7) and the protein density bands were visualised using Bio-Rad Quantity One programme. The relative variations in the level of phosphorylated monoclonal rabbit Akt (Ser473) protein were normalised to the total form of monoclonal rabbit

Akt protein. GAPDH was used as an internal loading control in all experiments as previously explained in Section 3.2.11.

4.2.11 Data analysis:

All data is presented as mean±SEM. Data presented for the CF parameter was adjusted by the heart weight and calculated as a percentage of mean stabilisation. Haemodynamic parameters, HR, LVDP and CF were analysed by one-way ANOVA for each time point. All other data was analysed using one-way analysis of variance (ANOVA) with Tukey post-hoc tests to identify group differences. Significance was considered at $p<0.05$.

4.3 Results

4.3.1 The effects of the co-administration of Tiron (0.25mM-1mM) and Doxorubicin (1µM) on the infarct size in the naïve Langendorff hearts.

Treatment with Doxorubicin resulted in a significant increase in the infarct to risk ratio when compare to control group ($p<0.0001$) (31.27±2.55% vs. 8.48±0.48%). It was observed that co-administration of Tiron (0.25mM-1mM) and Doxorubicin (1µM) resulted in a significant decrease in the infarct size to risk ratio when compared to Doxorubicin group ($p<0.0001$) (Doxorubicin vs. Tiron 0.25mM: 31.27±2.55% vs. 14.79±0.67%; Doxorubicin vs. Tiron 0.5mM 31.27±2.55% vs. 15.59±0.58%; Doxorubicin vs. Tiron 1mM: 31.27±2.55% vs.24.02±1.79%) (As shown in Figure 4.1).

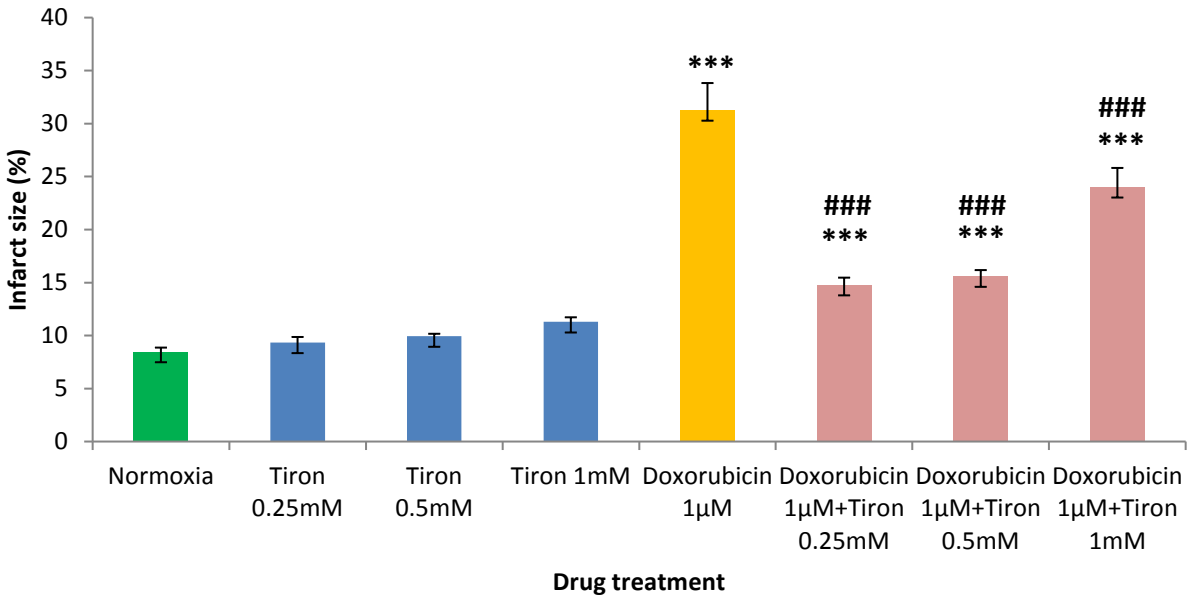


Figure 4.1 The effects of Tiron (0.25-1mM) and Doxorubicin (1µM) on the infarct ratio analysis on isolated naïve Langendorff hearts model. Hearts were subjected for 20 minutes of stabilisation, followed by 135 minutes of perfusion with KHB in the absence or in the presence of drug treatment. Data are presented as mean±SEM of 6 experiments; ***p<0.0001 vs. control; ###<0.0001 vs. Doxorubicin.

4.3.2 The effects of co-administration of Tiron (0.25-1mM) and Doxorubicin (1µM) on the haemodynamic parameters from the naïve Langendorff hearts

The effects of Tiron and Doxorubicin on the heart rate are illustrated in Figure 4.2. Treatment with Doxorubicin resulted in a significant reduction in the heart rate when compared to control group from 45 minutes into drug perfusion (at 65 minutes: 83.13±1.31% vs. 99.90±1.99%; respectively, p<0.05). Concomitant treatment of Tiron (0.25-1mM) with Doxorubicin (1µM) attenuated the reduction in HR observed with Doxorubicin group alone (at 65 minutes: Tiron 0.25mM+Dox: 99.94±1.77% vs. 83.13±1.31%; Tiron 0.5mM+Dox: 96.63±7.29% vs. 83.13±1.31%;

Tiron 1mM+Dox: 91.29±6.11% vs. 83.13±1.31%; p<0.05). No significant change was observed in co-treated groups when compared to normoxic control group.

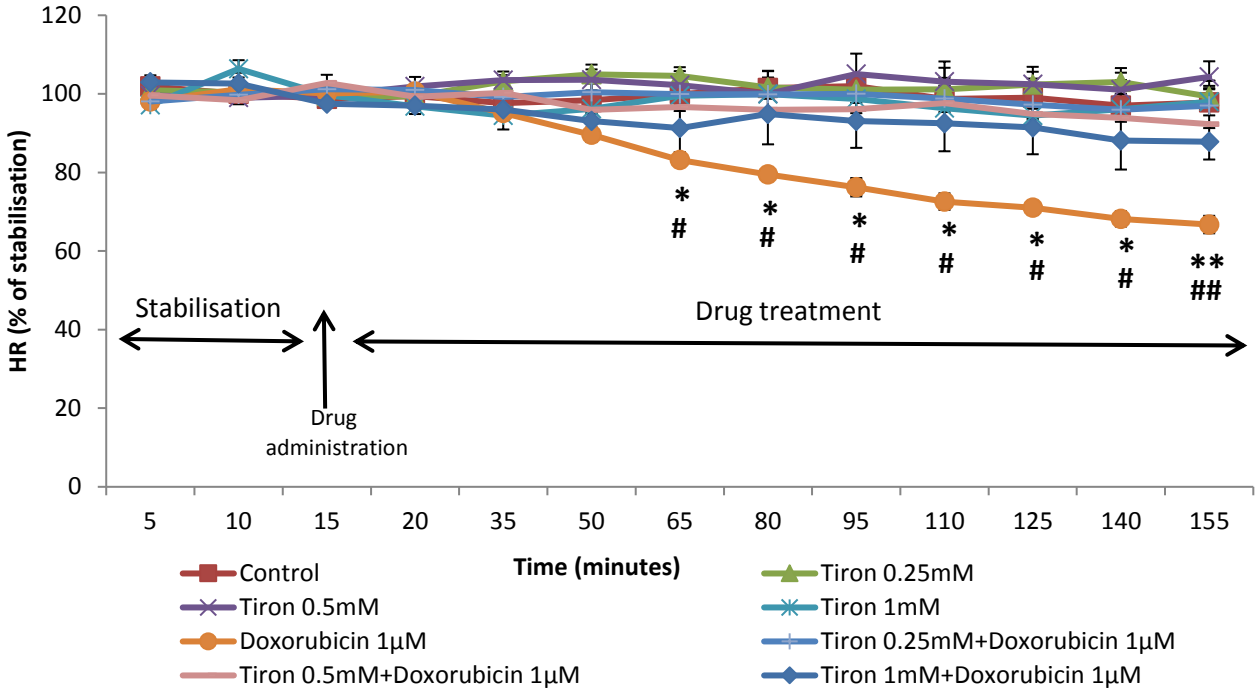


Figure 4.2 The effects of Tiron (0.25-1mM), Doxorubicin (1µM), and co-administration of Tiron (0.25-1mM) and Doxorubicin (1µM) on the heart rate (HR) represented as a percentage of the mean stabilisation. Data are represented as mean±SEM of n=6 experiments, *p<0.05 vs. normoxic control, $^{\#}$p<0.05 vs. Tiron (0.25-1mM) and Doxorubicin (1µM).

The effects of Tiron and Doxorubicin on LVDP parameters are shown in Figure 4.3. In Doxorubicin treated hearts there was a significant reduction in LVDP parameters when compared to control group from 45 minutes into drug perfusion (at 65 minutes: 77.37±5.74% vs. 96.78±5.77%; p<0.05). A profound decrease in the LVDP values was observed at 140 minutes (64.88±5.37% vs. 90.35±2.35%) and 155 minutes into perfusion with Doxorubicin treatment when compared to control (63.14±5.30% vs. 87.71±2.43%). Co-administration with Tiron (0.25-1mM) significantly

improved the reduction in LVDP observed with Doxorubicin group alone (at 65 minutes: Tiron 0.25mM+Dox: 97.87±6.32 vs. 77.37±5.74%; Tiron 0.5mM+Dox: 94.83±7.17% vs. 77.37±5.74%; Tiron 1mM+Dox: 99.15±5.65% vs. 77.37±5.74%; $p<0.05$). There was no significant effect detected on LVDP values on combined treatment groups when compared to normoxic control.

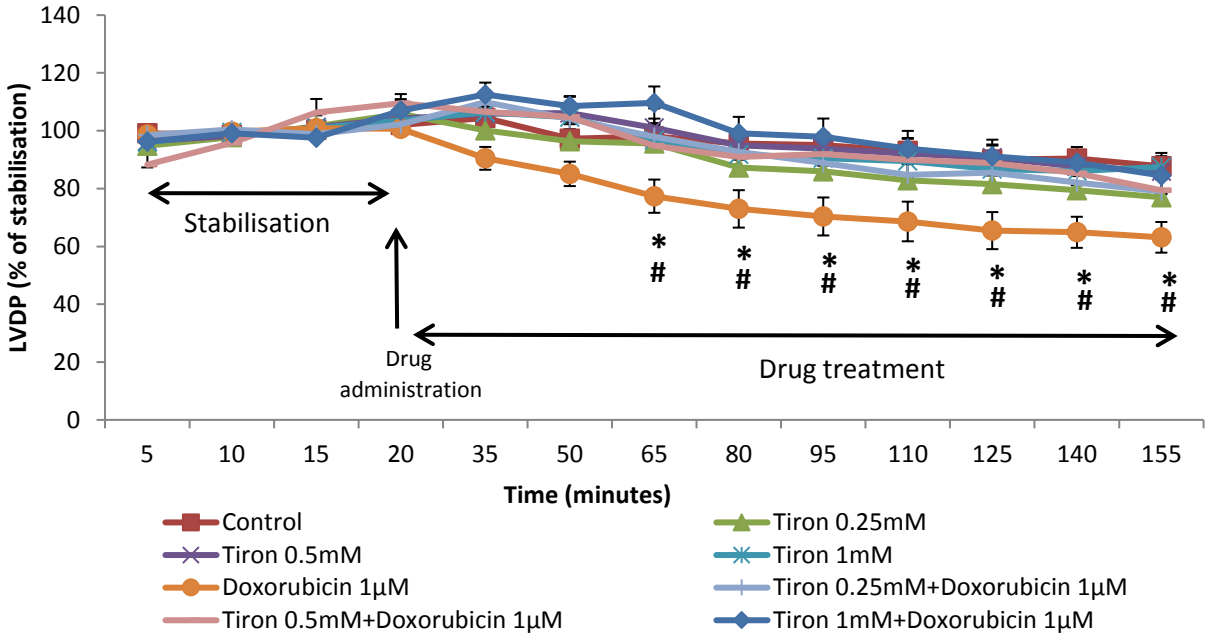


Figure 4.3 The effects of Tiron (0.25-1mM), Doxorubicin (1µM) and co-treatment of Tiron (0.25-1mM) and Doxorubicin (1µM) on the left ventricular diastolic pressure (LVDP) represented as a percentage of mean stabilisation. Data are presented as mean±SEM of n=6 experiments, *$p<0.05$ vs. normoxic control, $^{\#}p<0.05$ vs. Tiron (0.25-1mM) and Doxorubicin (1µM).

As observed in Figure 4.4 a substantial decrease could be detected in CF in Doxorubicin (1µM) treated hearts when compared to normoxic control group from 45 minutes into perfusion (at 65 minutes: 79.42±5.33% vs. 95.91±2.63%; $p<0.05$). Furthermore, co-administration of Tiron (0.25-1mM) and Doxorubicin (1µM) showed a significant increase in the CF when compared to Doxorubicin group from 45 minutes into drug perfusion (at 65 minutes: Tiron 0.25mM+Dox:

104.30±6.39% vs. 79.42± 5.33%; Tiron 0.5mM+Dox: 103.84±5.57% vs. 79.42±5.33%; Tiron 1mM +Doxorubicin: 92.45±8.11% vs.79.42±5.33%; p<0.05). Also, it has been observed that the CF values were reversed to control group in the co-treated groups.

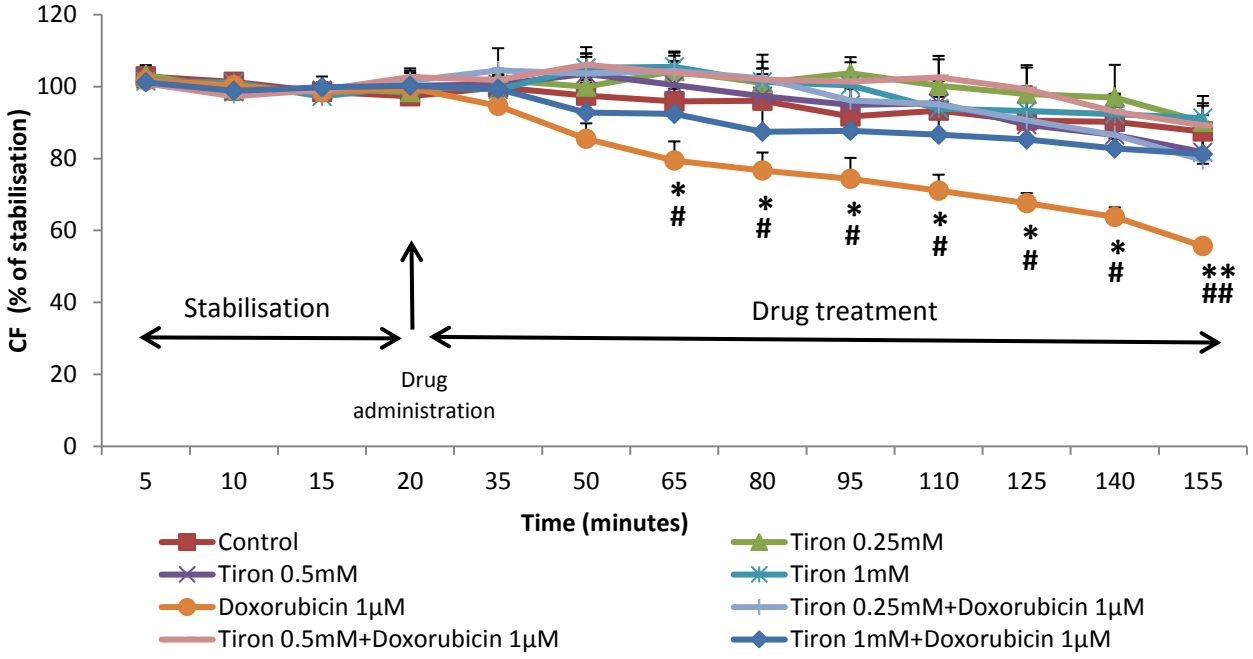


Figure 4.4 The effects of treatment with Tiron (0.25-1mM), Doxorubicin (1µM) and co-treatment of Tiron (0.25-1mM) and Doxorubicin (1µM) on coronary flow (CF), data represented as percentage of mean stabilisation of n=6 experiments, *p<0.05 vs. normoxic control; #p<0.05 vs. Tiron (0.25-1mM) and Doxorubicin (1µM).

4.3.3 The effects of co-administration of Tiron (0.25mM), Doxorubicin (1µM) and Wortmannin (100nM) on the haemodynamic parameters from the naïve Langendorff hearts used for tissue collection

The effects of Tiron (0.25mM), Doxorubicin (1µM) and Wortmannin (100nM) on the coronary flow are illustrated in Figure 4.5. As observed in the previous graphs, treatment with Doxorubicin resulted in a significant decrease in the CF rate when compared to normoxic control group (65

minutes: 73.94±6.02% vs. 96.90±7.62%, respectively *p<0.05). Parallel treatment with Tiron (0.25mM), Wortmannin (100nM) and Doxorubicin (1µM) resulted in a significant improvement in the CF rate when compared to Doxorubicin treated group (at 65 minutes: Tiron 0.25mM+Doxorubicin 1µM: 101.63±5.05% vs. 73.94±6.02%; Wortmannin 100nM+Doxorubicin 1µM: 95.03±2.44% vs. 73.94±6.02%; Tiron 0.25mM+Doxorubicin 1µM+Wortmannin 100nM: 94.31±1.77% vs. 73.94±6.02% #p<0.05). Furthermore, perfusion with Tiron or Wortmannin alone or in combination resulted in no significant changes in CF rate when compared to normoxic control group.

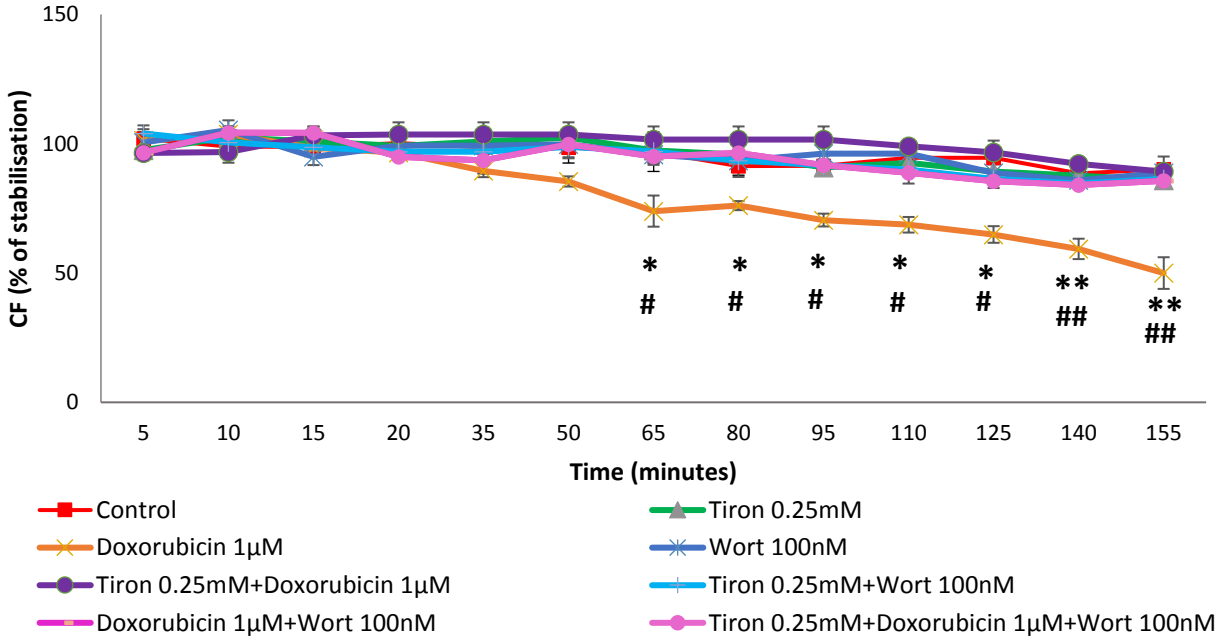


Figure 4.5 The effects of treatment with Tiron (0.25mM), Doxorubicin (1µM), Wortmannin (100nM) and co-administration of Tiron (0.25mM) and/or Wortmannin (100nM) with Doxorubicin (1µM) on coronary flow (CF), data represented as percentage of mean stabilisation of n=6 experiments, *p<0.05 vs. normoxic control; #p<0.05 vs. Tiron (0.25mM)±Wortmannin (100nM) and Doxorubicin (1µM).

Treatment with Doxorubicin (1µM) resulted in significant decrease in the LVDP when compared to normoxic control group from 45 minutes into drug perfusion (at 65 minutes: 73.72±4.95% vs. 102.29±2.48%; $p<0.05$) (Figure 4.6). Interestingly, administration of Tiron (0.25mM), Wortmannin (100nM) and Doxorubicin (1µM) significantly reversed the LVDP decline reported in Doxorubicin treated groups (at 65 minutes: Tiron 0.25mM+Doxorubicin 1µM: 102.26±4.98% vs. 73.72±4.95%; Wortmannin 100nM+Doxorubicin 1µM: 102.74±3.04% vs. 73.72±4.95%; Tiron 0.25mM +Wortmannin 100nM +Doxorubicin 1µM: 103.42±5.32% vs. 73.72±4.95%, respectively $^{\#}p<0.05$). As observed in Figure 4.6, no significant alterations in the LVDP values were observed upon perfusion with Tiron or Wortmannin alone or in combination.

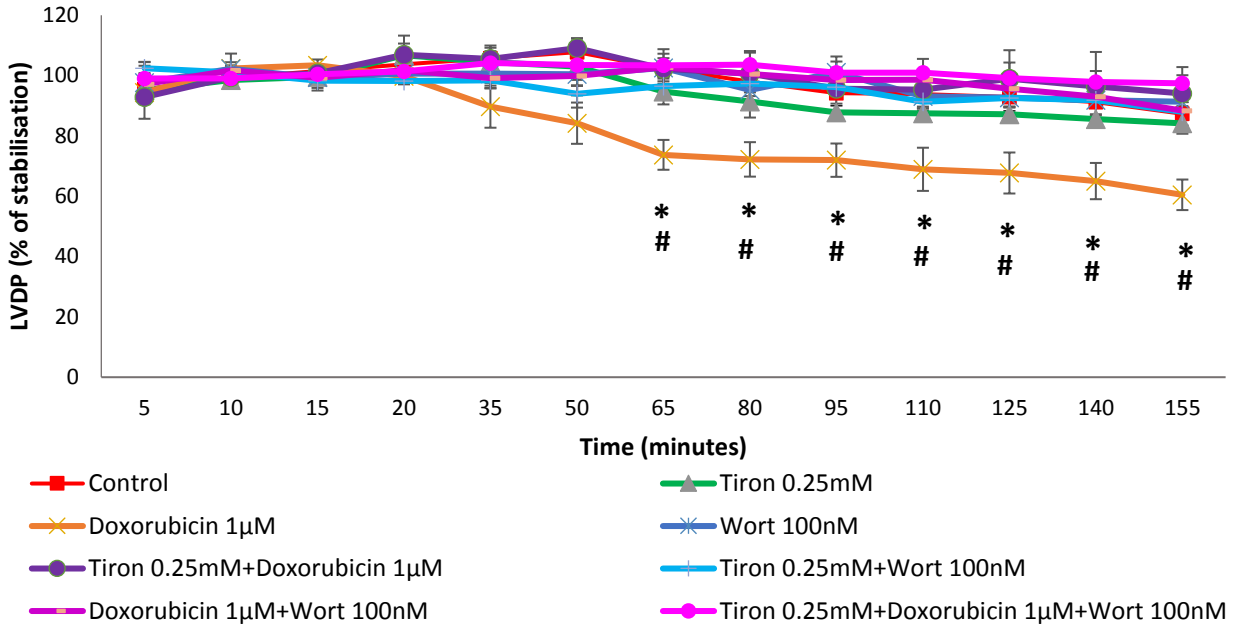


Figure 4.6 The effects of treatment with Tiron (0.25mM), Doxorubicin (1µM), Wortmannin (100nM) and co-administration of Tiron (0.25mM) and/or Wortmannin (100nM) with Doxorubicin (1µM) on left ventricular diastolic pressure (LVDP), data represented as percentage of mean stabilisation of n=6 experiments, $^{*}p<0.05$ vs. normoxic control; $^{\#}p<0.05$ vs. Tiron (0.25mM)±Wortmannin (100nM) and Doxorubicin (1µM).

As observed in Figure 4.7 and similarly to the previous graphs, treatment with Doxorubicin (1µM) throughout the perfusion period significantly reduced the HR when compared to normoxic control group. This decline could be observed from 60 minutes into drug perfusion (at 80 minutes: 84.59±2.43% vs. 99.44±3.58%, respectively $p<0.05$). Furthermore, addition of Tiron (0.25mM), Wortmannin (100nM) with Doxorubicin (1µM) completely abrogated the HR decrease observed in Doxorubicin treated hearts (at 80 minutes: Tiron 0.25mM+Doxorubicin 1µM: 108.91±1.24% vs. 84.59±2.43%; Wortmannin 100nM+Doxorubicin 1µM: 99.64±3.30% vs. 84.59±2.43%; Tiron 0.25mM+Doxorubicin 1µM+Wortmannin 100nM: 98.29±1.57% vs. 84.59±2.43%, respectively $^{\#}p<0.05$). Also, treatment with Tiron or Wortmannin alone or in combination did not significantly alter the HR values when compared to control group.

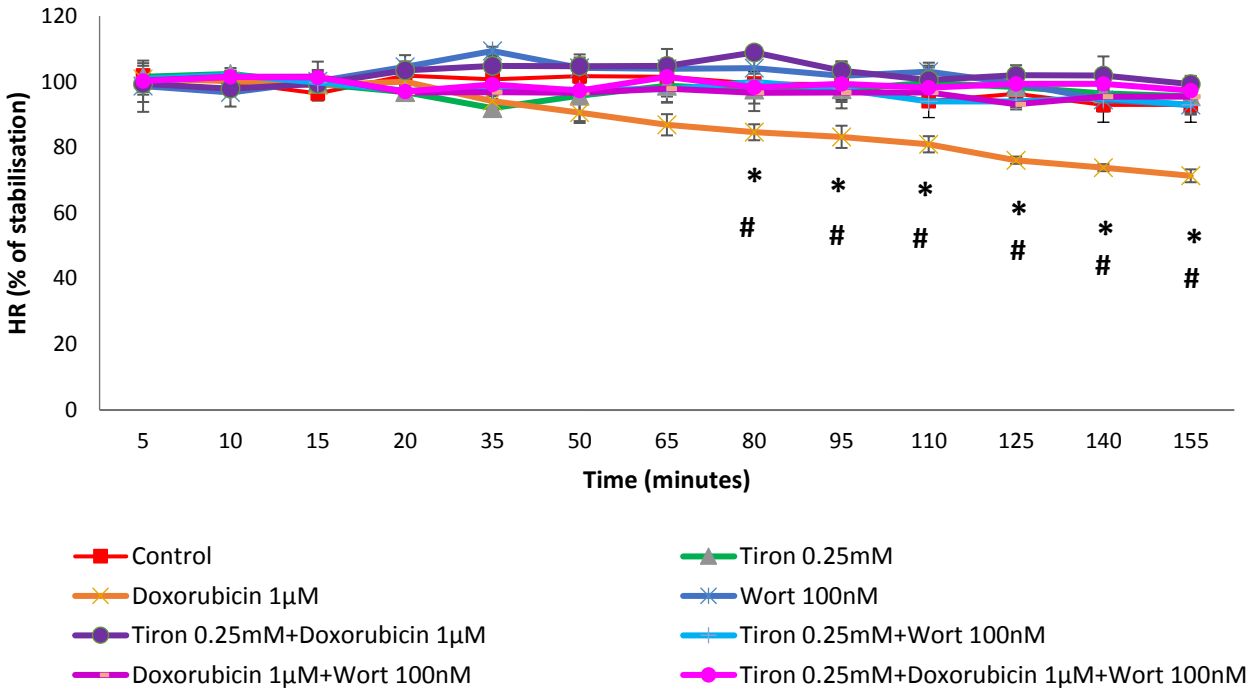


Figure 4.7 The effects of treatment with Tiron (0.25mM), Doxorubicin (1µM), Wortmannin (100nM) and co-administration of Tiron (0.25mM) and/or Wortmannin (100nM) with Doxorubicin (1µM) on left ventricular diastolic pressure (LVDP), data represented as percentage of mean stabilisation of n=6 experiments, *$p<0.05$ vs. normoxic control; $^{\#}p<0.05$ vs. Tiron (0.25mM)±Wortmannin (100nM) and Doxorubicin (1µM).

4.3.4 The effects of Doxorubicin and Tiron on the viability of isolated rat ventricular cardiomyocytes using MTT assay

Treatment with Doxorubicin (1µM) caused a significant decrease in cell viability of isolated rat ventricular cardiomyocytes when compared to control (71.03±2.41% vs. 100±0.00%). Co-administration of Doxorubicin (1µM) and Tiron (0.25-1mM) was able to significantly reduce the cardiac damage induced by Doxorubicin at cellular level in isolated ventricular cardiomyocytes

(Tiron 0.25mM+Dox: 84.48±3.44% vs. 71.03±2.41%; Tiron 0.5mM+Dox: 87.81±3.64% vs. 71.03±2.41%; Tiron 1mM+Dox: 82.22±6.81% vs. 71.03±2.41%; Figure 4.8). Administration of Tiron (0.25-1mM) in the presence of Doxorubicin significantly improved cell viability significantly, although the results obtained did not achieve the values reported in the control group. A similar trend was confirmed by Trypan Blue staining (Figure 4.9).

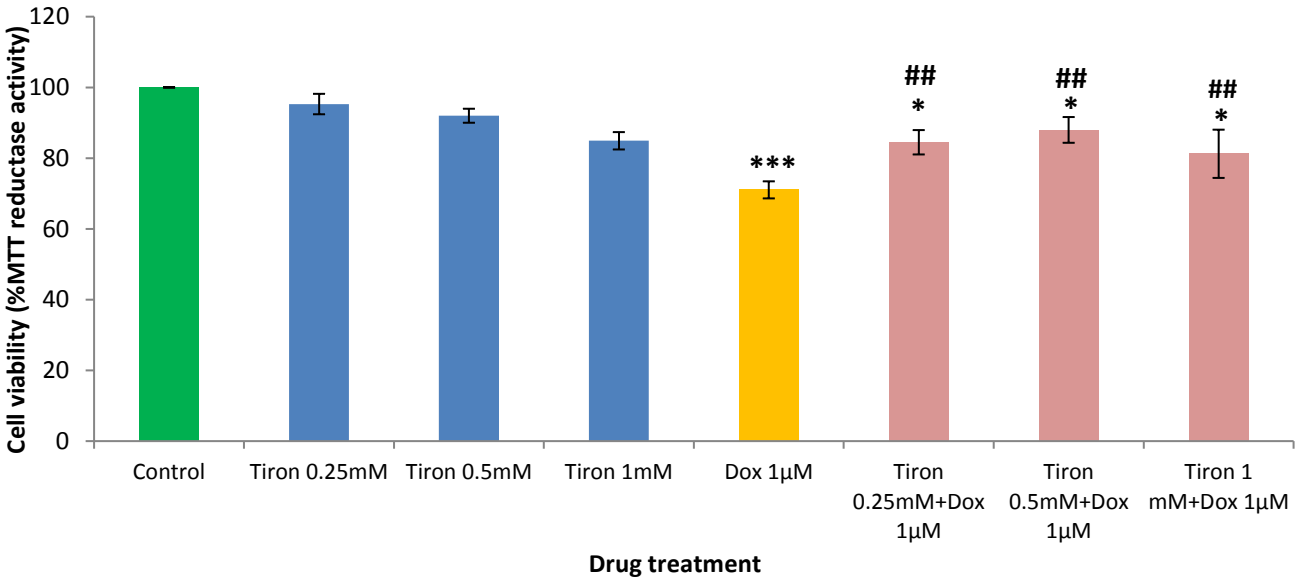


Figure 4.8 MTT analysis showing the viability of isolated rat ventricular cardiomyocytes in response to drug treatment of Tiron (0.25-1mM), Doxorubicin (1µM), and co-treatment of Tiron (0.25-1mM) and Doxorubicin (1µM). Data are presented as a mean±SEM, with a number of n=6 experiments, ***p<0.0001 vs. Control; ##p<0.001 vs. Doxorubicin.

Treatment with Doxorubicin (1µM) resulted in a significant reduction in cell viability as highlighted by an increase in the number of dead cells stained with Trypan Blue when compared to control group (55.81±3.79% vs. 100±0.00%) (Figure 4.9). Co-treatment with Tiron (0.25-1mM) in Doxorubicin (1µM) treated cardiomyocytes significantly increased the cell viability when compared to Doxorubicin treated group (Tiron 0.25mM+Dox 1µM: 80.75±3.80% vs.

55.81±3.79%; Tiron 0.5mM+Dox 1µM: 87.37±4.06% vs. 55.81±3.79%; Tiron 1mM+Dox 1µM: 80.52±4.42% vs. 55.81±3.79%).

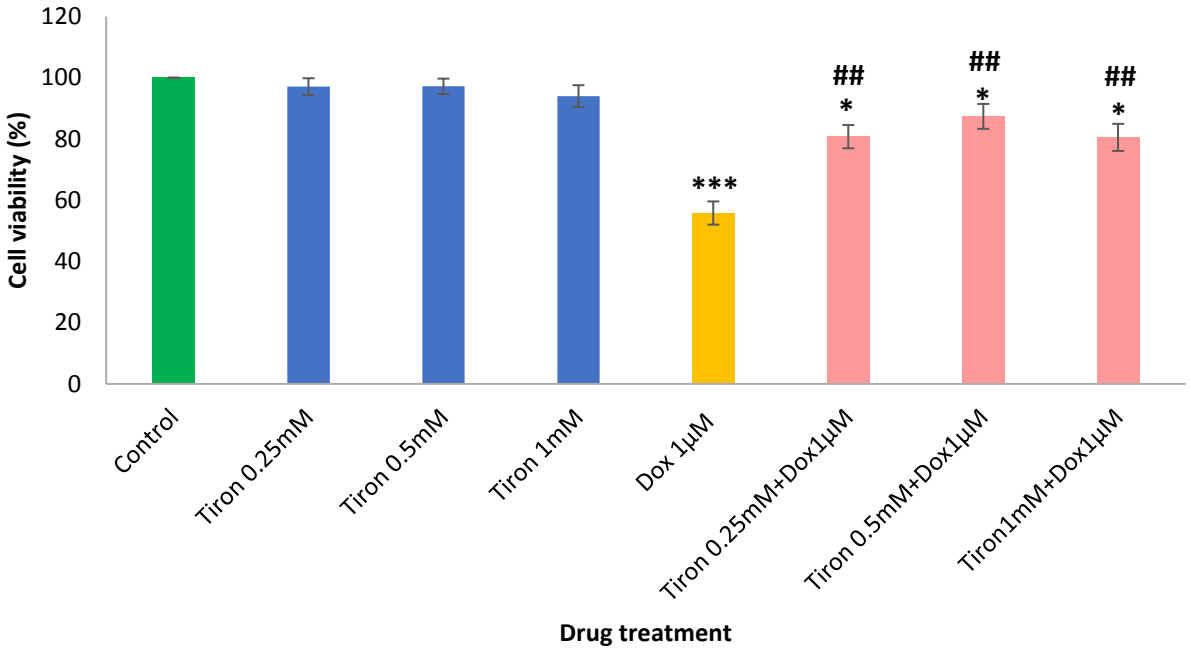


Figure 4.9 Trypan blue staining analysis showing the viability of isolated rat ventricular cardiomyocytes in response to treatment with Tiron (0.25-1mM), Doxorubicin(1µM) and Tiron (0.25-1mM) and Doxorubicin (1µM). Data are presented as an average±SEM, of n=6 experiments, ***p<0.0001 vs. Control. $^{\#\#}$p<0.001 vs. Doxorubicin.

4.3.5 The effects of Tiron (0.25mM) and Doxorubicin (1µM) on reactive oxygen species levels in isolated rat ventricular cardiomyocytes in normoxic conditions

Doxorubicin induced-cardiotoxicity has previously been associated to cardiomyocyte damage caused by increased intracellular levels of ROS. Our experimental results carried out with DCFDA assay showed that 24 hours treatment with Doxorubicin resulted in a substantial increase in

reactive oxygen species when compared to control (707.66±12.10% vs. 107.81±11.96%; respectively, $p<0.0001$). Furthermore, co-treatment with Tiron (0.25mM) and Doxorubicin caused a significant decrease in ROS levels when compared to Doxorubicin group (107.81±11.96% vs. 707.66±12.10% $p<0.0001$), which demonstrates that Tiron can effectively inhibit the production of Doxorubicin-induced-ROS. In addition, no significant increase in ROS levels was observed upon treatment with Doxorubicin and Tiron when compared to control group (107.81±11.96% vs. 107.81±11.96%) (Figure 4.10).

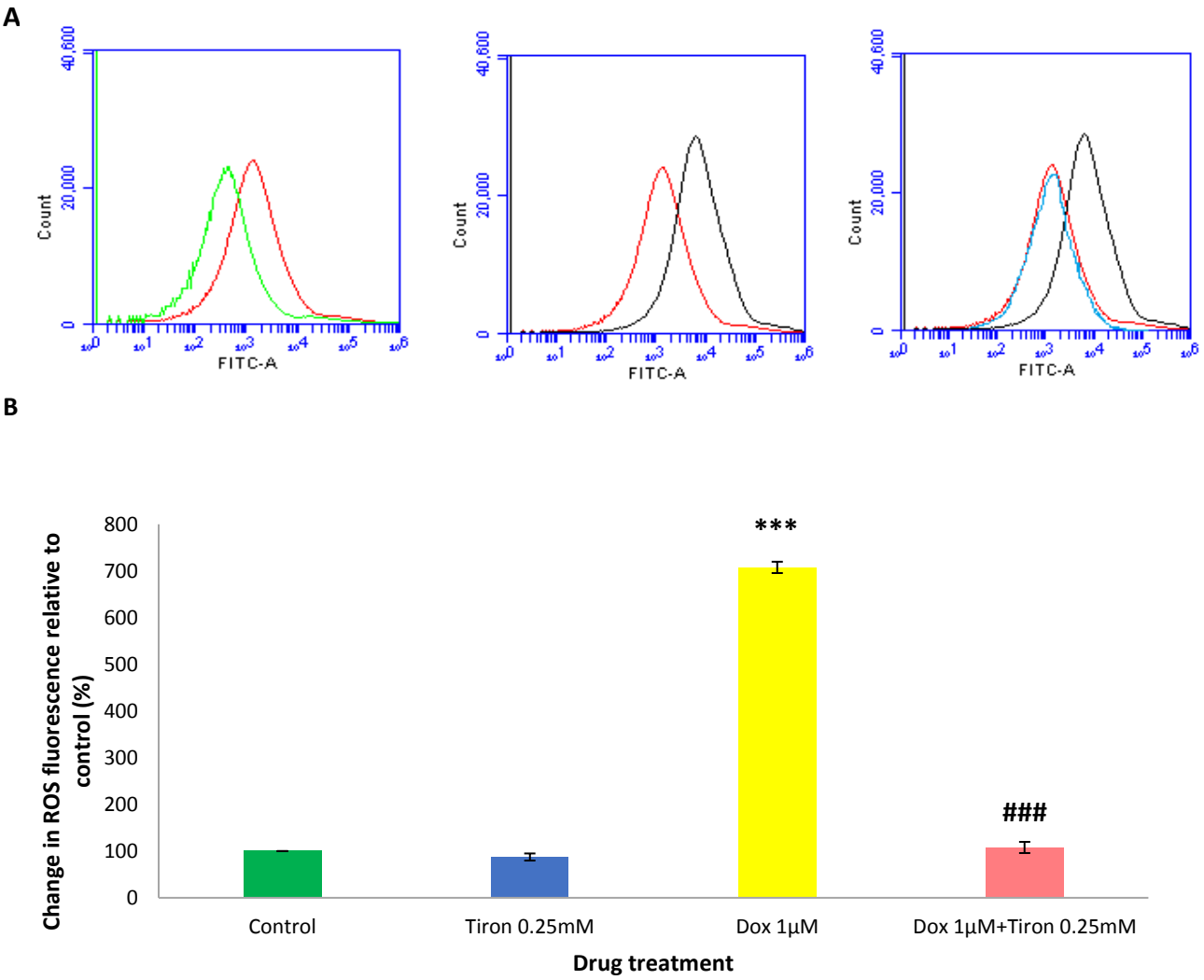


Figure 4.10 DCFDA flow cytometric scatter plots (A) and analysis B showing the change in ROS fluorescence relative to control in isolated adult rat ventricular cardiomyocytes in normoxic conditions in response to treatment with Tiron 0.25mM and Doxorubicin 1µM. Data are presented as mean±SEM, with a number of n=6 experiments, ***p<0.0001 vs. Normoxia; ###p<0.0001 vs. Doxorubicin. (Representation of the scatter plots: Control (red) Tiron 0.25mM (green) DOX (black); Tiron 0.25mM+DOX (1µM) (blue).

4.3.6 The effects of co-treatment of Tiron (0.25-1mM) and Doxorubicin (1μM) on the cleaved caspase-3 levels in isolated adult rat ventricular myocytes

Treating the isolated cardiomyocytes with Tiron for 24 hours in normoxic conditions did not cause an effect on the levels of cleaved-caspase 3 when compared to control, untreated cells (Tiron 0.25mM: 100.25±5.61% vs. 100±0.0%; Tiron 0.5mM: 98.23±1.37% vs. 100±0.0%; Tiron 1mM: 100.59±2.98% vs. 100±0.0%). However, upon treatment with Doxorubicin (1μM), the levels of cleaved caspase-3 were significantly increased when compared to control group (231.34±8.41% vs. 100±0.00%) (Figure 4.11). In addition, co-administration of Tiron (0.25-1mM) with Doxorubicin significantly reversed Doxorubicin-induced increase in the cleaved-caspase-3 (Tiron 0.25mM+Doxorubicin: 105.79±6.79% vs. 231.34±8.41%; Tiron 0.5mM+Doxorubicin: 101.55±3.17% vs. 231.34±8.41%; Tiron 1mM+Doxorubicin: 105.38±5.59% vs. 231.34±8.41%). No difference in cleaved-caspase-3 levels could be detected between the co-treated samples and control.

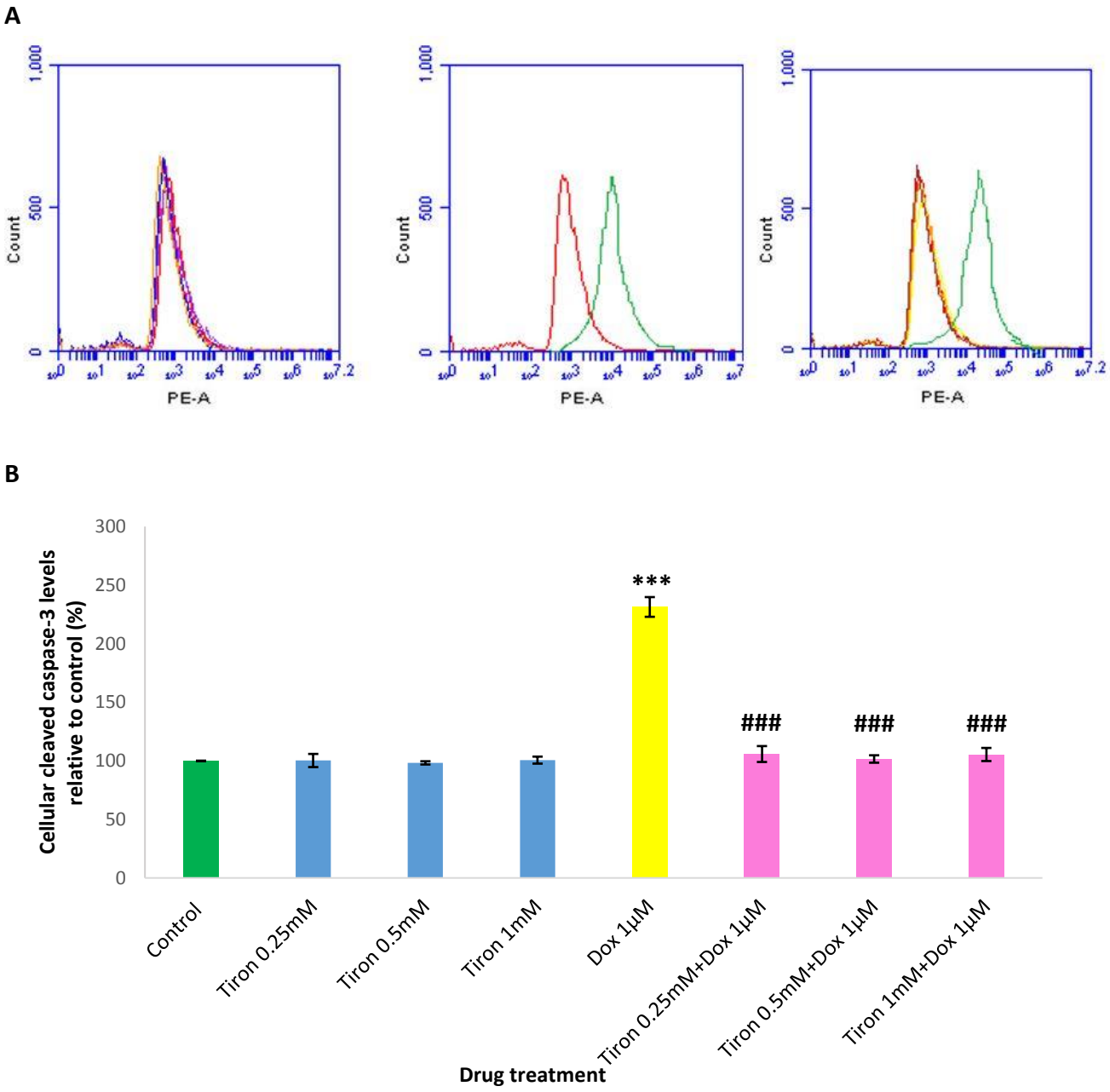


Figure 4.11 Cleaved caspase-3 levels flow cytometric plots (A) and analysis (B) in isolated adult rat ventricular cardiomyocytes in normoxic conditions in response to treatment with Tiron (0.25-1mM) and Doxorubicin (1µM). Data are presented as mean±SEM, n=6 experiments, ***p<0.0001 vs. Control; ###p<0.0001 vs. Doxorubicin (Representation of the scatter plots: Control (red); Tiron 0.25mM (pink); Tiron 0.5mM (purple); Tiron 1mM (blue); Doxorubicin (green); Tiron 0.25mM+ Doxorubicin (yellow); Tiron 0.5mM+Doxorubicin (orange); Tiron 1mM+ Doxorubicin (brown).

4.3.7 The effects of Tiron (0.25mM), Doxorubicin (1µM) and Wortmannin (100nM) on Akt signalling protein levels in cardiac tissue in naïve conditions.

Western blot analysis indicated that Doxorubicin (1µM) treated samples showed a significant up-regulation in the levels of p-Akt (Ser473) when compared to control group (69.46±4.79% vs. 32.62±3.67%) and Tiron treated group (69.46±4.79% vs. 40.47±1.60%) Furthermore, co-treatment of Tiron (0.25mM) with Doxorubcin (1µM) significantly decreased the levels of phoporylated Akt when compared to Doxorubicin group alone (39.27±2.14% vs. 69.46±4.79%). In addition, it was observed that co-administration of Tiron (0.25mM) with Doxorubicin (1µM) and Wortmannin (100nM) resulted in a significant downregulation in the levels of p-Akt when compared to Doxorubicin (24.89±4.94% vs. 69.46±4.79%) and control (24.89±4.94% vs. 32.62±3.67%) (Figure 4.12).

There was no significant difference detected in the levels of p-Akt levels between Tiron treated samples and co-treated samples with Doxorubicin and Tiron (40.47±4.79% vs. 39.27±2.14%).

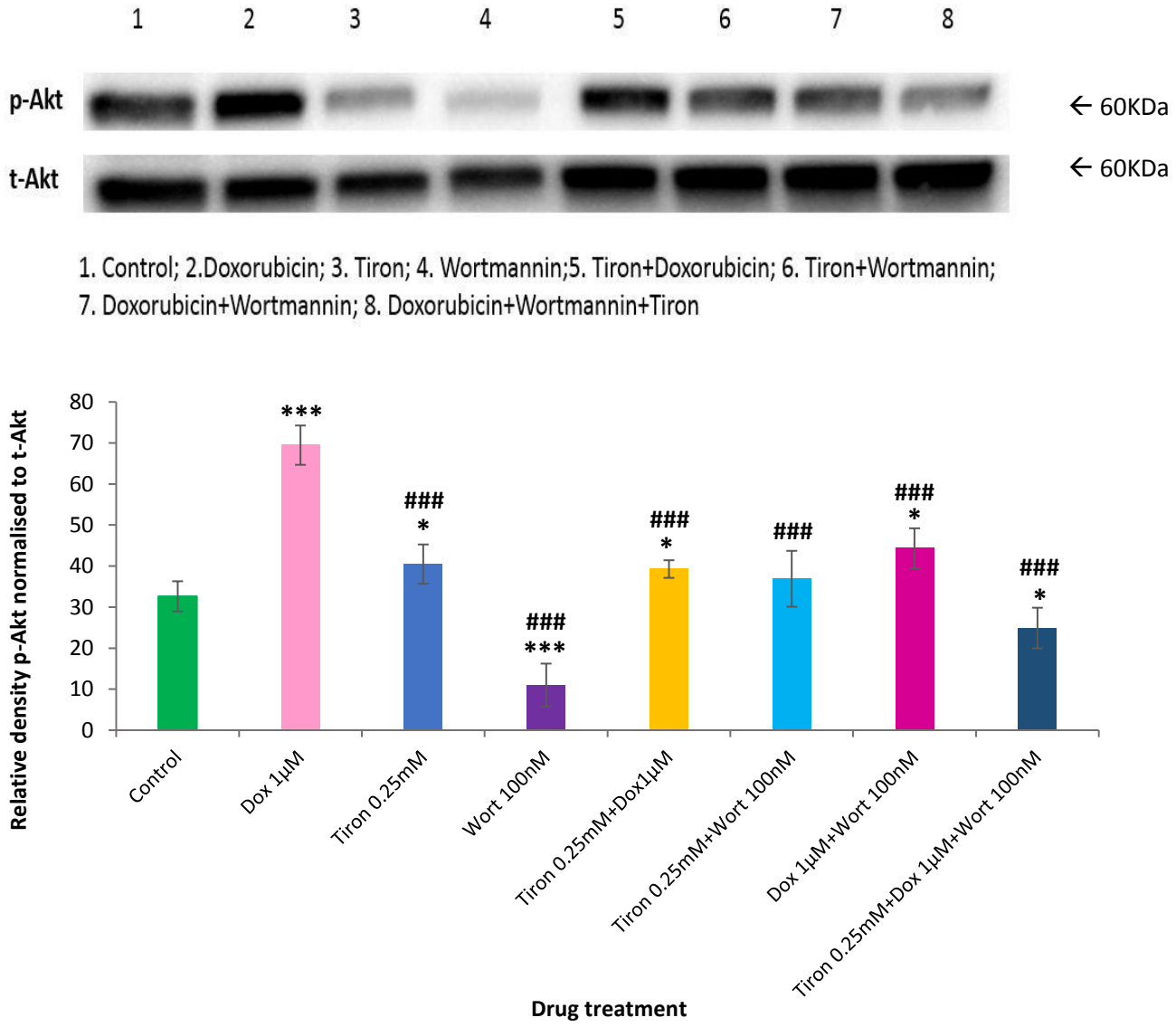


Figure 4.12 Western blot analysis showing the effects of Tiron (0.25mM), Doxorubicin (1µM), Wortmannin (100nM) and co-treatment on the levels of phosphorylated Akt at 120 minutes into perfusion phase. Data are presented as mean±SEM, with a number of n=6 experiments, ***p<0.0001 vs. control. $^{###}$p<0.0001 vs. Doxorubicin.

4.3.8 Co-incubation of Tiron (0.25mM and 0.5mM) with Doxorubicin (1μM) did not appear to affect the toxicity of Doxorubicin (1μM) in human cancerous cell lines HepG2 and HL60 cells.

The MTT colorimetric assay showed that Doxorubicin significantly reduced the viability in both HL60 cells and HepG2 cells when compared to non-treated control cells (HL60 cells: Doxorubicin: 32.61±3.74% vs. 100±0.00%; HepG2 cells: Doxorubicin: 73.63±2.98% vs. 100±0.00%) ($p<0.0001$). Co-administration of Tiron (0.25-1mM) with Doxorubicin (1μM) did not inhibit the anti-cancer properties of Doxorubicin group alone. Furthermore, treatment with Tiron (0.25-1mM) alone did not exhibit any effects on the cell viability of the human cancerous cell lines when compared to control group (Figure 4.13A and 4.14A). Again, this trend was confirmed by Trypan Blue staining (Figure 4.13B and 4.14B).

In Trypan blue staining, cells treated with Doxorubicin showed a significant decrease in the cell viability in both HL60 and HepG2 cells when compared to control cells (HL60 cells: Doxorubicin: 19.09±0.5% vs. 100±0.00%; HepG2 cells: 29.46±3.80% vs. 100±0.00%). Parallel treatment with Tiron and Doxorubicin did not impact the antineoplastic activity of Doxorubicin (Figure 4.13B and 4.14B).

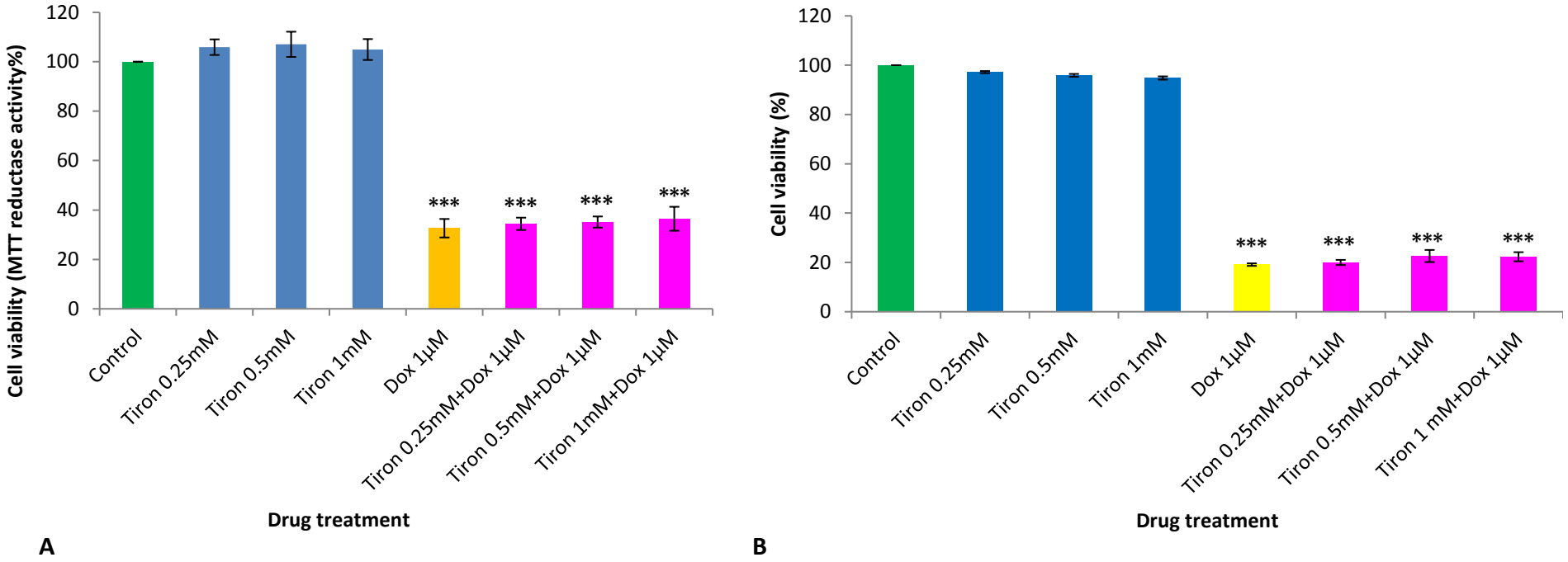


Figure 4.13 MTT cytotoxicity analysis (A) and Trypan Blue staining (B) showing the viability of human cancerous HL60 cells in response with treatment with Tiron (0.25mM and 0.5mM) (blue), Doxorubicin (1µM) (yellow) and Tiron (0.25mM and 0.5mM) with Doxorubicin (1µM). Data are presented as mean±SEM, with a number of n=6 experiments, ***p<0.0001 vs. Control

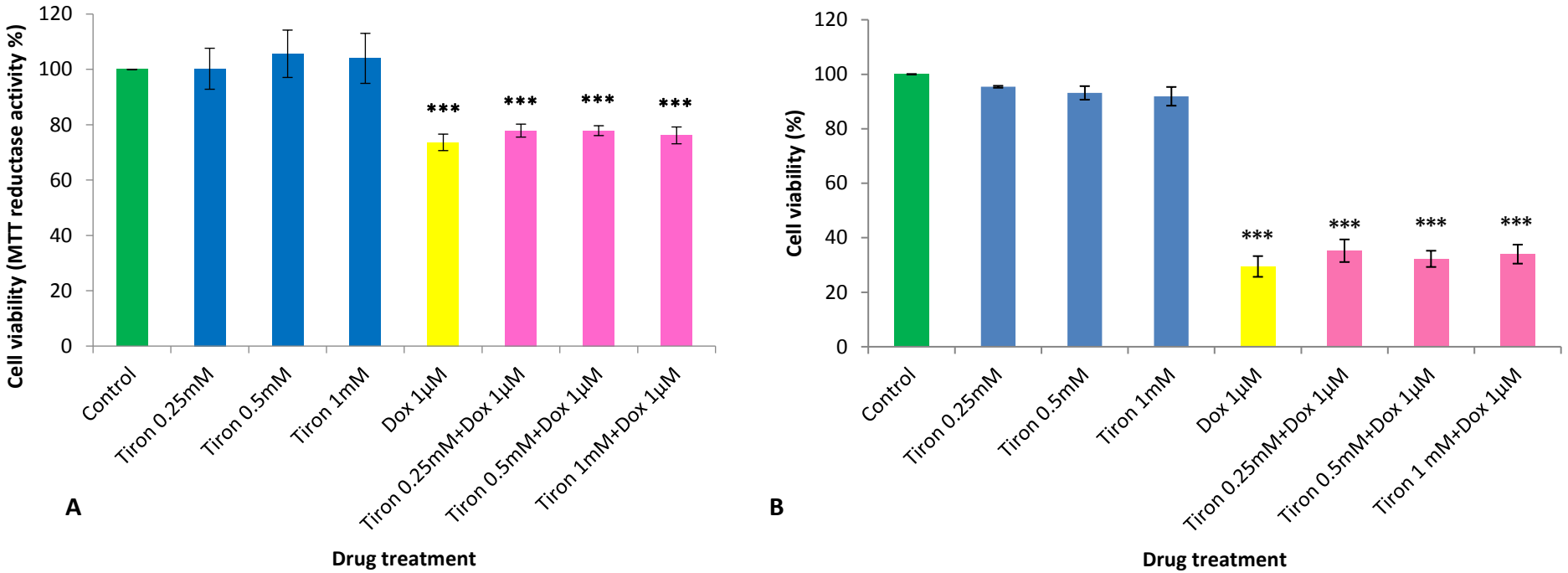


Figure 4.14 MTT cytotoxicity analysis (A) and Trypan blue staining (B) showing the viability of human cancerous HepG2 cells in response with treatment with Tiron (0.25mM-1mM) (blue), Doxorubicin (1µM) (yellow) and Tiron (0.25-1mM) with Doxorubicin (1µM). Data are presented as mean±SEM, with a number of n=6 experiments, ***p<0.0001 vs. Control.

4.3.9 The effects of Tiron (0.25-1mM) and Doxorubicin (1µM) on cleaved caspase-3 levels in human cancerous HL60 and HepG2 cell lines

Doxorubicin treated samples exhibited a substantial increase in the levels of cleaved-caspase 3 activity in HL-60 cells when compared to non-treated control cells (1059.29±11.66% vs. 100.0±0.0%) ($p<0.0001$). Co-administration of Tiron (0.25-1mM) with Doxorubicin (1µM) did not inhibit the increase of cleaved caspase-3 levels when compared to Doxorubicin group (Tiron 0.25mM + Dox 1µM: 1020.72±18.65% vs. 1059.29±11.66%; Tiron 0.5mM + Dox 1µM: 1009.99±9.90% vs. 1059.29±11.66; Tiron 1mM+ Dox 1µM: 1006.43±12.12% vs. 1059.29±11.66%). In addition, treatment with Tiron (0.25-1mM) alone did not impact the levels of cleaved caspase-3 of when compared to control group (Tiron 0.25mM: 92.97±4.02% vs. 100.0±0.00%; Tiron 0.5mM: 91.97±5.05% vs. 100.0±0.0%; Tiron 1mM: 100.04±4.67% vs. 100.0±0.00%) (Figure 4.15).

A similar trend could be observed in the levels of cleaved caspase 3 in HepG2 cells, however, the increase in Doxorubicin treated samples was not as profound as the one reported in HL60 cells (Doxorubicin vs. Control: 311.10±17.73% vs. 100.0±0.0%; Tiron 0.25mM+Doxorubicin vs. Doxorubicin: 310.28±18.65% vs. 311.10±17.73%; Tiron 0.5mM+Doxorubicin vs. Doxorubicin: 302.81±14.65% vs. 311.10±17.73%; Tiron 1mM+Doxorubicin vs. Doxorubicin: 304.22±19.28% vs. 311.10±17.73%) (Figure 4.16).

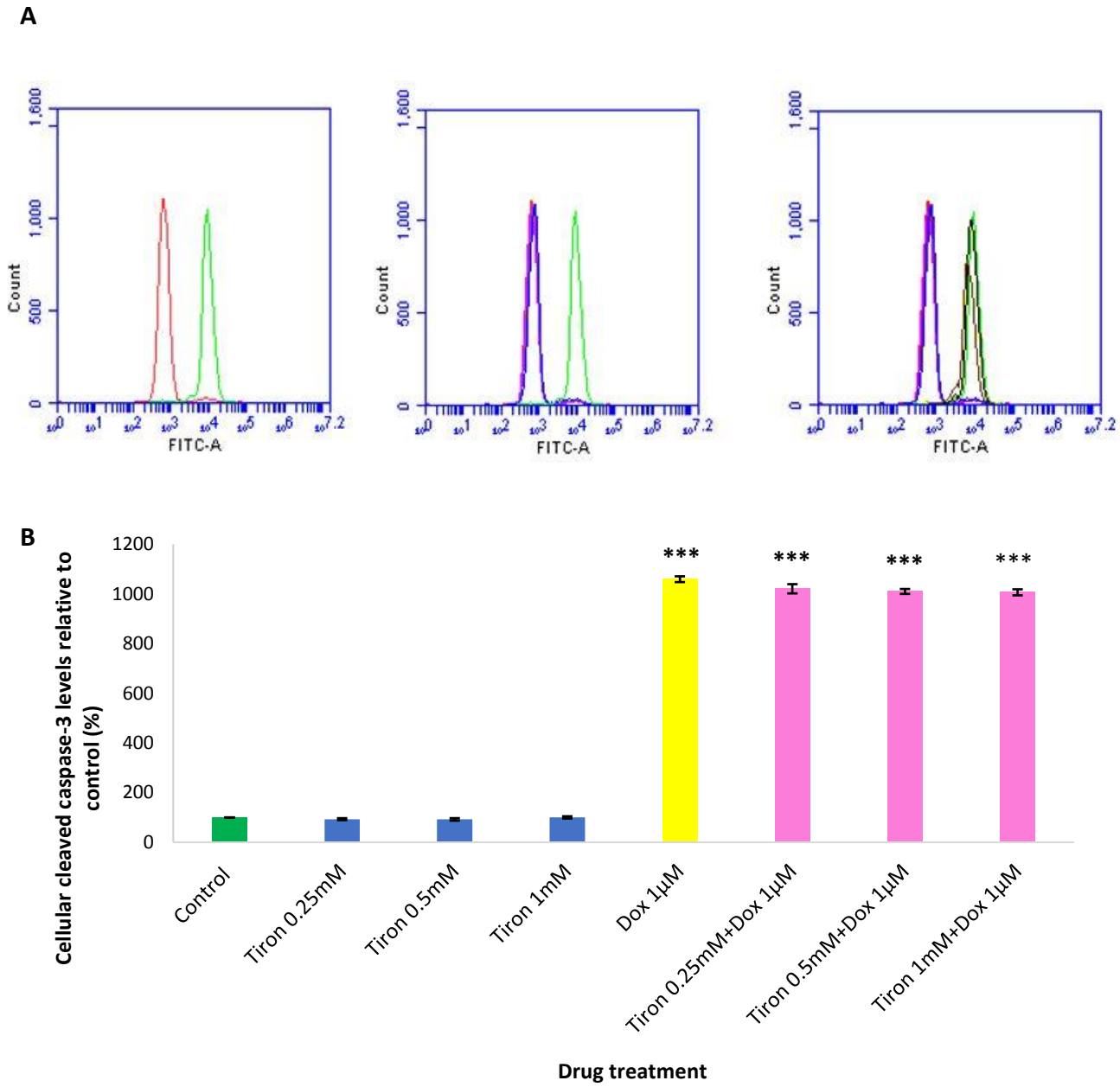


Figure 4.15 Flow cytometry scatter plots (A) and analysis (B) showing the change in cleaved caspase 3 fluorescence relative to control in human cancerous HL60 cells in response to treatment with Tiron (0.25-1mM) and Doxorubicin (1µM). Data are presented as mean±SEM, with a number of n=6 experiments, ***p<0.0001 vs. Control (Representation of the scatter plots: Control (red); Dox (green); Tiron 0.25mM (pink); Tiron 0.5mM (purple); Tiron 1mM (blue); Tiron (0.25mM)+Dox (yellow); Tiron 0.5mM+Dox (brown); Tiron 1mM+Dox (black).

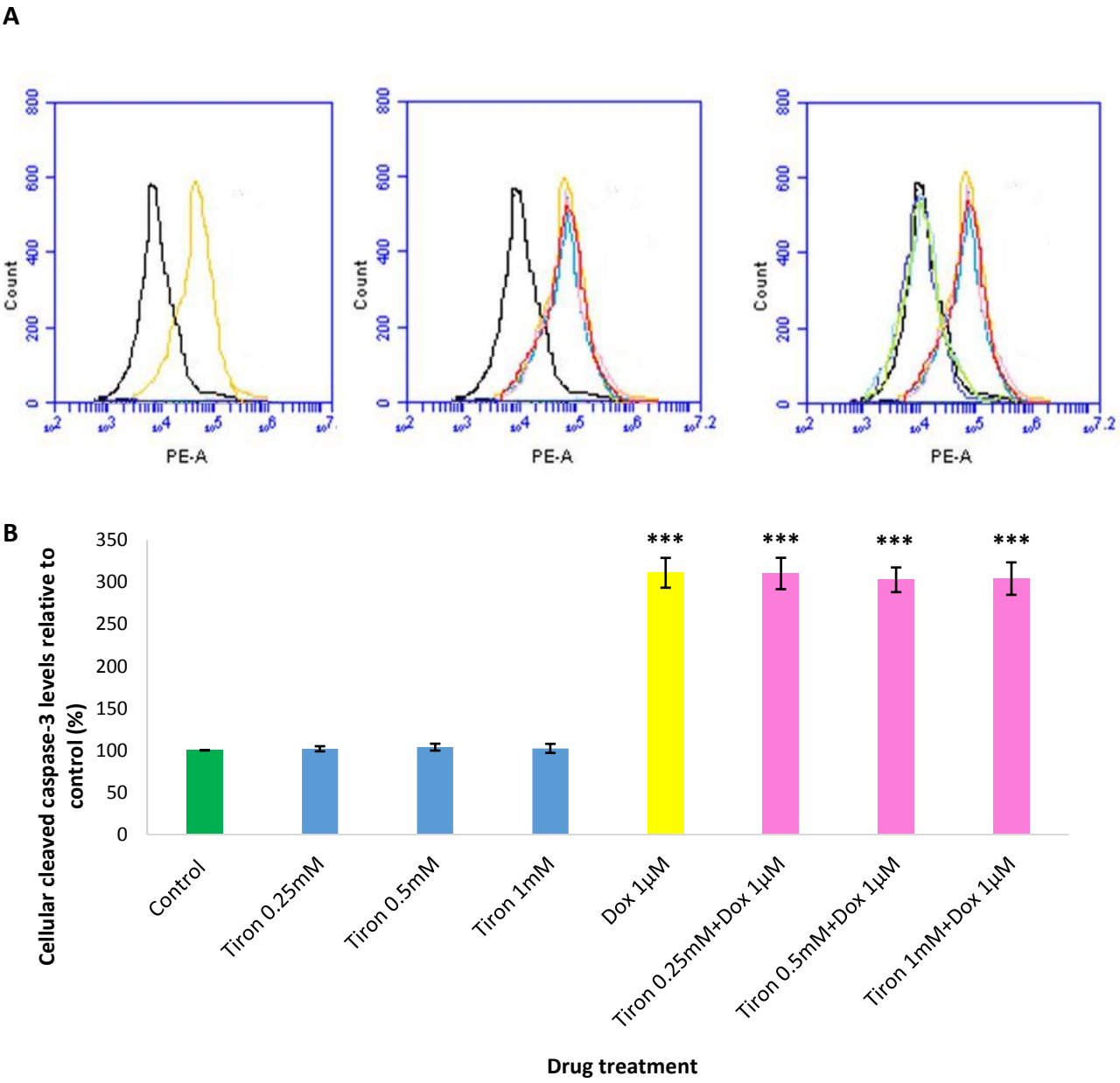


Figure 4.16 Flow cytometric scatter plots (A) and analysis (B) showing the change in cleaved caspase 3 fluorescence relative to control in human cancerous HepG2 cells in response to treatment with Tiron (0.25-1mM) and Doxorubicin (1µM). Data are presented as mean±SEM, with a number of n=6 experiments, ***p<0.0001 vs. Control (Representation of the scatter plots: Control (black); Dox (orange); Tiron 0.25mM (blue); Tiron 0.5mM (green); Tiron 1mM (yellow); Tiron 0.25mM+Dox (red); Tiron 0.5mM+Dox (pink); Tiron 1mM+Dox (turquoise).

4.3.10 The effects of Tiron (0.25-1mM) and Doxorubicin (1µM) on the levels of intracellular ROS in human cancerous HL60 and HepG2 cell lines

Flow cytometric analysis of DCFDA assay in human liver carcinoma HepG2 and HL60 cells showed that an increase in the levels of ROS was observed in Doxorubicin treated samples, however the values obtained were not significant when compared to control group (HL60 cells: 124.21±23.44% vs. 100±0.00%; HepG2 cells: 115.42± 14.46% vs. 100±0.00%) (Figure 4.17A and 4.17B). As observed in Figure 4.15A and 4.15B there was a trend of decrease in Tiron (0.25-1mM) treated samples, however, a significant value was not reached when compared to control, untreated group in both cell lines (HL60 cells: Tiron 0.25mM: 89.02±4.94% vs. 100±0.00%; Tiron 0.5mM: 93.49±14.13% vs. 100±0.00%; 94.10±12.53% vs. 100±0.00%; HepG2 cells: Tiron 0.25mM: 92.87±1.90% vs. 100±0.00%; Tiron 0.5mM: 87.84±6.42% vs. 100±0.00%; Tiron 1mM: 88.19±5.41% vs. 100±0.00%) . In addition, co-treatment with Tiron (0.25-1mM) with Doxorubicin (1µM) in cancer cell lines did not exhibit any significant effects on the levels of ROS produced by Doxorubicin alone (HL60 cells: Tiron 0.25mM+Doxorubicin 1µM: 116.58±22.58% vs. 124.21±23.44%; Tiron 0.5mM+ Doxorubicin 1µM: 111.37±17.36% vs. 124.21±23.44%; Tiron 1mM: 112.25±18.70% vs. 124.21±23.44%; HepG2 cells: Tiron 0.25mM+Doxorubicin 1µM: 91.26±10.38% vs. 115.42± 14.46%; Tiron 0.5mM+Doxorubicin 1µM: 94.40±13.26% vs. 115.42± 14.46%; Tiron 1mM+Doxorubicin 1µM: 107.09±15.32% vs. 115.42± 14.46%).

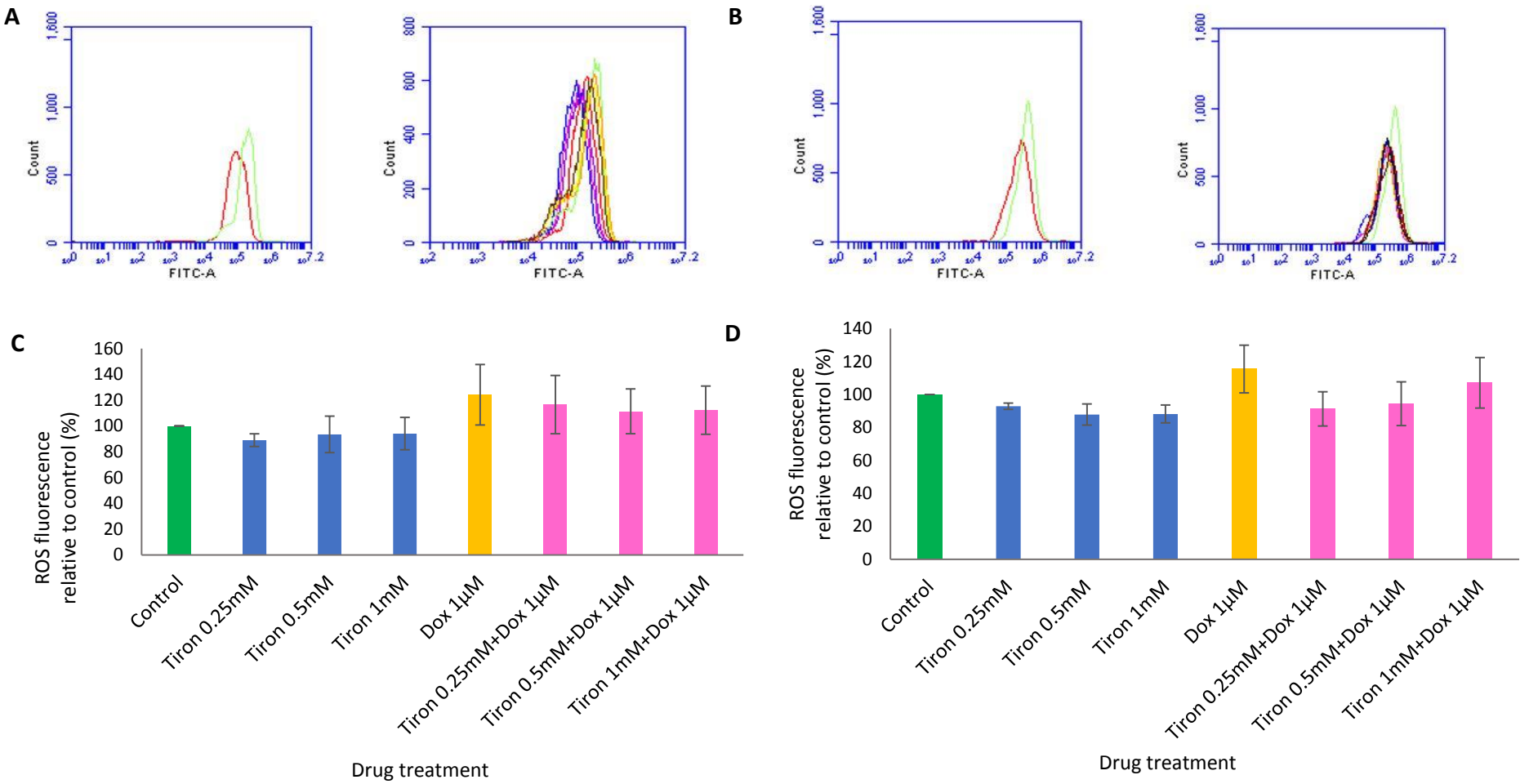


Figure 4.17 DCFDA analysis showing the change in ROS fluorescence relative to control in leukaemia HL-60 cells (A and C) and human liver carcinoma HepG2 cells (B and D) in response to treatment with Tiron (0.25-1mM) and Doxorubicin 1µM and Tiron (0.25-1mM). Data are presented as mean±SEM, with a number of n=6 experiments.

4.4 Discussion

Doxorubicin induced cardiotoxic effects have been previously reported in numerous investigations; however, the precise, underlying mechanisms responsible for Doxorubicin induced myocardial injury have not been fully elucidated. This has hindered the progression and development of a successful adjunctive treatment designed to prevent and inhibit the cardiotoxicity induced by Doxorubicin. The results in the current study show that Tiron, a vitamin E analogue and ROS scavenging antioxidant protects against Doxorubicin-induced cardiotoxicity without affecting the anti-tumour activity of Doxorubicin on HL60 leukaemia and HepG2 liver carcinoma cell lines.

4.4.1 Tiron mitigates Doxorubicin induced myocardial injury

Data presented in this study shows that rat perfused with Doxorubicin exhibited an increase in the infarct ratio (Figure 4.1), a decline in the haemodynamic parameters LVDP, HR and CF (Figure 4.2-4.4) and a decrease in cell viability in adult rat ventricular myocytes (Figure 4.8 and 4.9), indicating an increase in the myocardial dysfunction and oxidative damage. Our results are in agreement with previous in vitro and in vivo studies which reported that Doxorubicin treatment in the myocardium was associated with an increase in the myocardial infarction, a decline in the cardiac function (Gharanei et al. 2013), marked increase in the cardiac serum markers CK-MB and significant histological changes in the heart such as interstitial edema, fibrotic bands and severe cytoplasmic vascular degeneration (Abdel-Sattar et al. 2012). Furthermore, we have shown that Tiron possesses cardioprotective properties as suggested by a significant reduction in the infarct ratio in Doxorubicin treated hearts and an improved cell viability in rat ventricular

cardiomyocytes. A number of reports postulated that free radical scavenger therapy could diminish the cardiotoxic effects of Doxorubicin by accumulating in areas exposed to oxidative injury (Al‐Harthi et al. 2014; Ananthanarayanan et al. 2016; Li et al. 2010). For example, using in vivo model of male rats, Al-Harthi et al. (2014) investigated the effects of Resveratrol in Doxorubicin treated hearts and showed that concomitant treatment with Resveratrol and Doxorubicin decreased the plasma CPK and LDH activities, reversed the fragmentation and lysis of the myofibrils and preserved the mitochondrial structure (Al-Harthi et al. 2014). Furthermore, within the same study it has been reported that combined treatment with Resveratrol and Doxorubicin reversed the reduction in the glutathione levels and decreased the MDA levels expressed after Doxorubicin treatment, suggesting that Resveratrol exhibits its cardioprotective effects by inhibiting Doxorubicin-induced accumulation of ROS in the mitochondria of cardiac cells (Al-Harthi et al. 2014). In addition, a study carried out by Deres et al. (2005) in Langendorff heart models exposed to 60 minutes of drug treatment with Doxorubicin revealed that co-administration of low concentrations of the antioxidants H-2545 and H-2954 significantly attenuated the impairment in left ventricular functional parameters and reduced the levels of lipid peroxidation and myocardial oxidative damage (Deres et al. 2005a). In the previous chapter it was demonstrated that Tiron exhibited cardioprotective properties by scavenging ROS produced in the model of I/R conditions. Therefore, it is reasonable to hypothesise that Tiron's cardioprotective response against Doxorubicin toxicity may be attributed to its ability to accumulate in the mitochondria and scavenge free radicals.

We have also investigated the effects of concurrent administration of Tiron (0.25-1mM) and Doxorubicin (1µM) on the heart haemodynamic parameters. The results obtained show that

Langendorff perfused hearts with Doxorubicin exhibited a decrease in the LVDP, CF and HR values when compared with control, untreated hearts (Figure 4.2,4.3 and 4.4). The reduction in heart rate and LVDP after Doxorubicin treatment support the outcomes of previous studies (Cai et al. 2010, Deres et al. 2005, Gharanei et al. 2013, Pouna et al. 1995). In a previous investigation carried out by Liu et al, (2002) in a murine isolated heart model of Doxorubicin induced cardiotoxicity it was shown that concomitant administration of melatonin and Doxorubicin was able to reverse the decrease in HR and LVDP values observed in Doxorubicin treated hearts (Liu et al, 2002). The authors postulated that a potential explanation for the decrease in the HR and LVDP in Doxorubicin treated hearts could be associated with the ROS production that may induce disturbances in calcium homeostasis (Liu et al. 2002). It is well known that superoxide free radicals are produced during the redox cycling of Doxorubicin, and the resulting oxidants produce cellular alterations such as loss of calcium homeostasis, enlargement of the sarcoplasmic reticulum and myofilament degeneration (Hanna et al. 2014; Sag et al. 2011; Kim et al. 2006; Maillet et al. 2016). For example, the study carried out by Sag et al. (2011) on isolated ventricular myocytes from rat and mouse showed that exposure with Doxorubicin (10µM) was marked by an increase in ROS, a decrease in the Ca^{2+} transient amplitudes, an increase in the diastolic Ca^{2+} channel ions, and an increase in the Ca^{2+} leakage from the sarcoplasmic reticulum. Furthermore, within the same study, addition of melatonin (a ROS scavenger) showed to partially reverse the increase in diastolic Ca^{2+} ions and prevented the increase in the sarcoplasmic reticulum Ca^{2+} loss (Sag et al. 2011). Comparable findings were reported by Kim et al (2006), in which Doxorubicin induced Ca^{2+} release was inhibited by the addition of α-tocopherol and α-lipoic acid (Kim et al. 2006). In a similar manner it was revealed that administration of Tiron (100µM) in rat isolated

ventricular myocytes inhibited the enhanced production of ROS, restored the contractile function and abrogated the Ca^{2+} abnormalities observed in cells treated with diethyldithiocarbamic acid, a copper-zinc superoxide dismutase inhibitor (Luo et al. 2006). In our study, concomitant administration of Tiron and Doxorubicin abrogated the reduction in the LVDP and HR observed in Doxorubicin treated hearts and improved the left ventricular contractility and heart rate (Figure 4.2 and 4.3). Based on these findings, it is possible that reversal of Doxorubicin-induced decline on HR and LVDP parameters by Tiron are most likely linked with its ability to scavenge superoxide radicals by inhibiting Doxorubicin induced Ca^{2+} release, thus preserving the cardiac contractility and improving the heart function.

Our study has also shown that the CF haemodynamic values were severely compromised in isolated rat hearts treated with Doxorubicin, and co-administration of Tiron (0.25-1mM) with Doxorubicin attenuated the vasoconstrictor effect (Figure 4.4). One possible explanation of this effect could be linked with the vasoconstriction effect of Doxorubicin on cardiac function (Bar-Joseph et al. 2015). Previous investigations have indicated that Doxorubicin-induced cardiotoxicity is linked with increased levels of endothelin-1 and nitric oxide (Sayed-Ahmed et al. 2001; Yamashita et al. 1995; Bien et al. 2007). Endothelin-1, an endothelium-derived peptide exhibits potent vasoconstrictor effects in the cardiovascular system which can lead to hypertension, increased cardiac contractility, endothelial dysfunction and heart failure (Schiffrin, 2001 Ferro and Webb, 1997; Spieker et al. 2001; Kedzierski and Yanagisawa, 2001). Earlier investigations have shown that endothelin-1 stimulates ROS production and is implicated in triggering and maintaining endothelial dysfunction (Schiffrin, 2001) and endothelin receptor

blockade has been demonstrated to improve endothelial function in human coronary arteries (Verma et al. 2001).

Furthermore, it has shown that patients exposed to Doxorubicin treatment exhibited elevated levels of endothelin-1 (Sayed-Ahmed et al. 2001). In addition, the study carried out by Bien et al. (2007) revealed that increased levels of endothelin-1 were observed in both in vivo and in vitro cellular models (Bien et al. 2007). The results from the study have shown that administration of Doxorubicin in in vivo mice model resulted in a reduced cardiac function, which was marked by a decrease in the cardiac output and an increase in the lipid peroxidation and endothelin-1 peptide. Within the same study, the in vitro investigations using murine cardiomyocyte cell line HL-1 and adult rat cardiomyocytes confirmed that up-regulation of endothelin-1 is dependent on Doxorubicin. Moreover, administration of endothelin-1 antagonist bosentan significantly improved cardiac function and reversed the increase in lipid peroxidation (Bien et al. 2007). In a different investigation carried out in isolated small pulmonary artery preparations from Sprague-Dawley rats showed that exposure to chronic hypoxia resulted in a significant increase in the endothelin-1 induced vasoconstriction, ROS production and actin polymerization, whereas the incubation with Tiron abolished the endothelin-1-induced vasoconstriction and reduced the ROS formation and actin polymerization suggesting that Tiron might exhibit vasodilatation properties (Weise-Cross et al. 2017). Previously, it has been shown that in rat perfused mesenteric bed pre-constricted with KCl administration of Tiron resulted in a vasodilator response, diminishing the perfusion pressure of the mesenteric bed (Ghosh, Wang and McNeill 2002). Therefore, based on these studies, it is reasonable to suggest that Tiron-induced inhibition on the negative effects of

Doxorubicin on the CF haemodynamic parameter may be associated with its ROS scavenger and vasodilation properties.

4.4.2 Treatment with Tiron reduces Doxorubicin induced oxidative stress and caspase-3 activation in isolated rat ventricular myocytes

Although, the precise underlying pathways accountable for Doxorubicin induced myocardial injury have not been fully explained, increasing evidence has suggested mitochondrial dysfunction and oxidative stress as the two main mechanisms in Doxorubicin cardiac toxicity (Alpsoy et al. 2013, Geisberg and Sawyer 2010, Green and Leeuwenburgh 2002, Ichikawa et al. 2014, Minotti et al. 2004). As mentioned before, several investigations have shown that mitochondrial damage plays an important role in Doxorubicin induced cardiotoxicity, which may be linked to the ability of Doxorubicin to generate ROS leading to disturbances in the regulation of molecular signaling pathways, mitochondrial dysfunction and subsequent the activation of cardiomyocytes apoptotic cell death (Asensio-López et al. 2017, Shi, Abdelwahid and Wei 2011, Volkova and Russell 2011).

Considering that oxidative stress plays an important role in Doxorubicin-induced cardiotoxicity, the current study aimed to examine whether Tiron induced cardioprotection against Doxorubicin occurs via a ROS-dependent mechanism.

In the current study, we investigated the effects of combined treatment of Tiron and Doxorubicin on the intracellular levels of ROS in adult ventricular cardiomyocytes. We have shown that 24 hours treatment with Doxorubicin induced a significant increase the levels of ROS when compared to control group (as shown in Figure 4.10). These results are consistent with the

findings reported by other published research articles which highlight the oxidative stress implication in Doxorubicin-induced damage (Khan et al. 2006, Kotamraju et al. 2000a, L'Ecuyer et al. 2004, Spallarossa et al. 2004). In a previous in vivo investigation Khan et al, (2006) have shown that Doxorubicin treatment caused a significant increase in the oxidative stress which was marked by elevated levels of lipid peroxidation in the cardiac tissue (Khan et al, 2006). In addition, in a different study using H9c2 cell model, it was shown that exposure to 5µg/ml of Doxorubicin for 4 hours resulted in a significant increase in the levels of oxidative stress, which was emphasised by an increase in the DCFDA (2',7'-dichlorofluorescein diacetate) fluorescence when compared to control group (L'Ecuyer et al, 2004).

We have shown that co-administration of Tiron and Doxorubicin successfully reversed the oxidative stress induced by Doxorubicin (Figure 4.10). Studies have reported that Doxorubicin induced cardiotoxic effects are linked with the accumulation of Doxorubicin in the mitochondria and ROS generation (Kavazis et al. 2017, Kotamraju et al. 2000b, Varga et al. 2015). The heart is particularly predisposed to oxidative injury due to a lower level of antioxidant defense mechanism which protects the heart from ROS toxicity and oxidative stress. This may account for the high sensitivity of the myocardium to Doxorubicin induced oxidative injury (Aryal, Jeong and Rao 2014, Belmonte et al. 2015, Koti et al. 2009). Several reports have suggested that free radical scavengers have an important role in protecting the heart against Doxorubicin-induced ROS (Chang et al. 2011, Gao et al. 2016, Gu, Hu and Zhang 2015, Zhang, Yi and Huang 2017; Ruan et al. 2015). Ruan et al. (2015) tested the effects of Resveratrol in both Doxorubicin-induced neonatal rat cardiomyocytes and adult mice and found that Resveratrol offered significant protection against Doxorubicin-induced damaged (Ruan et al, 2015). The cardioprotective effects

of Resveratrol were indicated by an improved cell viability, a decrease in ROS generation, apoptosis markers caspase-3 and Bax and an increase in Bcl-2 expression when compared to Doxorubicin treated group (Ruan et al. 2015). Furthermore, a study carried out with antioxidant oxymatrine has revealed that pre-treatment with oxymatrine significantly attenuated doxorubicin-induced oxidative stress in the model of H9c2 rat cardiac muscle cells (Zhang, Yi and Huang 2017). In addition, in the same cellular model, Gao et al. (2016) have shown that ginkolide B (a major terpenoid component extract from Ginkgo biloba leaves) exhibited antioxidant effects by decreasing Doxorubicin induced apoptosis and ROS levels (Gao et al. 2016). Previously the study carried out by Almeida et al. (2018) highlighted the neuroprotective beneficial effects of Tiron in Doxorubicin and Mitoxantrone (anticancer drug) induced cytotoxicity (Almeida et al. 2018). Also, a number of studies have reported that the antiapoptotic effects of Tiron in hyperglycemic heart models occur via its beneficial effects on oxidative stress and are accompanied by a reduction in caspase-3, improved cell viability and contractility and a reduction in lipid peroxidation (Jiang et al. 2017; Zuo et al. 2011). As shown in previous chapter, Tiron exhibits protective properties against oxidative injury, and in accordance with these studies, it is reasonable to believe that the reduction in ROS is likely due to the superoxide and hydroxyl radical scavenger activity of Tiron.

As highlighted in the present study, Doxorubicin treatment compromised the cardiac function by activating caspase-3, a biomarker of apoptosis. It has been suggested that both intrinsic and extrinsic apoptotic cell death pathways may contribute to Doxorubicin induced cardiomyocyte apoptosis (Childs et al. 2002, Khan et al. 2006). Several experimental studies have shown that Doxorubicin induced cardiotoxicity causes mitochondrial dysfunction, which in turn activates

cytochrome c release and activation of caspase-3-well-known regulators of cell death (Childs et al. 2002, Khan et al. 2006; Gharanei et al. 2013). Gharanei et al. (2013) have showed that Doxorubicin induced cardiotoxicity disturbs mitochondrial activity and integrity, leading to the collapse of mitochondrial membrane potential and eventually cell death in isolated rat ventricular myocytes (Gharanei et al. 2013). These effects were abrogated by Cyclosporine-A, a mitochondrial membrane transition pore opening blocker. Furthermore, in a different study conducted by Montaigne et al. (2011) on human atrial trabeculae showed that Doxorubicin caused a reduction in the mitochondrial transmembrane potential and calcium retention capacity and impaired the contractile function. Administration of Cyclosporine A attenuated these effects, suggesting that Doxorubicin induced cardiotoxicity mechanism involves mitochondrial dysfunction in human hearts (Montaigne et al. 2011).

Our results show that rat ventricular myocytes exposed to concomitant administration of Tiron and Doxorubicin showed a decrease in the levels of cleaved caspase-3 when compared to Doxorubicin (Figure 4.11), indicating the ability of Tiron to salvage the cardiomyocytes exposed to Doxorubicin from undergoing the process of apoptotic cell death. Previously, the beneficial effects of Tiron on the cardiac mitochondrial metabolism have been also reported (Silva-Platas et al. 2016). The study by Silva-Platas et al. (2016) aimed to investigate the effects of novel chemotherapeutic copper-based drugs (Casiopeinas) on the cardiac mitochondria metabolism and energetics in isolated cardiomyocytes and found that treatment with Casiopeinas resulted in a significant decline in the mitochondrial membrane potential stimulating mitochondrial permeability transition pore opening (Silva-Platas et al. 2016). Interestingly, pretreatment with Tiron reversed the increase in ROS production and prevented the mitochondrial depolarization,

indicating its potential to prevent Casiopeinas-induced cardiotoxicity by inhibiting the mitochondrial permeability transition pore opening (Silva-Platas et al. 2016). Therefore, in agreement to these studies, our findings suggest that the protective effects of Tiron against Doxorubicin induced apoptosis can be attributed to its free radical scavenger properties and mitochondrial-targeted mechanism.

4.4.3 Administration of Tiron (0.25mM) reverses the increase in p-Akt levels observed in Doxorubicin treated hearts

In this study, western blot analysis was performed in order to identify if co-treatment with Tiron (0.25mM) and Doxorubicin (1µM) exhibited any effects on the PI3K/Akt signaling pathway. We have shown that treatment with Doxorubicin in isolated perfused hearts for 135 minutes resulted in phosphorylation and activation of Akt signaling pathway a downstream effector of PI3K, which is similar to previous findings (Gharanei et al. 2013; Merten et al. 2006; Deres et al. 2005). As mentioned before, PI3K/Akt pathway has been shown to play an important role in mediating the cell growth, proliferation and survival (Hausenloy and Yellon, 2013; Cantley, 2002). PI3K- a lipid kinase, which is positioned downstream of multiple receptor tyrosine kinases and G-coupled protein receptors, triggers the addition of a phosphate group to the free-3 position of the inositol ring of phosphatidylinositol or phosphoinositides (Chen et al., 2001). The subsequent products have been shown to be involved in stimulating signaling pathways implicated in cardiac hypertrophy (Chen et al., 2001). Stimulation of PI3K signaling pathway in the myocardium has been reported previously in in vivo experimental studies in pressure overload hypertrophy in mice (Naga et al., 2000). Furthermore, the study carried out by Shioi et al. (2000) in the transgenic

mice model expressing constitutively active or dominant-negative mutants of PI3K in the heart showed that cardiac specific expression of constitutively active PI3K caused an enlargement in the hearts, an effect associated with cardiac hypertrophy (Shioi et al. 2000). In addition, treatment with Doxorubicin (1µM) for 2 hours in H9c2 cardiomyocyte cell line resulted in an exacerbated upregulation of Akt pathway when compared to control untreated group (Merten et al., 2006). Indeed, these findings are confirmed by our results reported here: a significant increase in p-Akt levels were observed in Langendorff hearts perfused with Doxorubicin for 135 minutes (Figure 4.12). Within the same study, it has been revealed that administration of LY294002, a PI3K inhibitor successfully reversed Doxorubicin induced hypertrophy in H9c2 cells, by increasing the cellular volume and protein levels (Merten et al. 2006). In a similar way, we have shown that co-administration of Wortmannin (100nM) and Doxorubicin (1µM) significantly reduced the upregulation of Akt pathway. Although the infarct ratio was not assessed in this study upon treatment with Wortmannin and Doxorubicin, the cardiac function parameters HR, LVDP and CF showed a marked improvement in their values when compared to Doxorubicin group, suggesting that Wortmannin might exert protective effects against Doxorubicin induced hypertrophy and cardiac damage.

Interestingly, data presented in this study has shown that concomitant treatment with Tiron (0.25mM) and Doxorubicin (1µM) resulted in a marked decrease in the upregulation of Akt levels when compared to Doxorubicin treated group. Furthermore, addition of Wortmannin (100nM) in Tiron (0.25mM) and Doxorubicin (1µM) treated hearts enhanced the downregulation of Akt levels when compared to Doxorubicin treated group. Previous studies carried out in the Langendorff heart model have also investigated the levels of Akt in response to Doxorubicin at

the end of perfusion period (Deres et al. 2005). Our findings confirm the results obtained by Deres et al. (2005) which revealed a marked upregulation in the Akt levels when hearts were perfused with a supraclinical concentration of Doxorubicin (100μM) for 1 hour (Deres et al. 2005). Also, the outcomes from the same study have shown that concomitant treatment of Doxorubicin with the experimental antioxidants H-2545 and H-2954 resulted in a significant decrease in the p-Akt levels and improved cardiac function (Deres et al. 2005). In a similar manner, our results have shown that co-treatment of Doxorubicin with the cardioprotective antioxidant Tiron reversed the increase in p-Akt, improved cardiac function and decreased the infarct ratio, indicating a cardioprotective role of Tiron.

It has been postulated that the upregulation of Akt signaling pathway in Doxorubicin treated hearts could be associated with the exacerbated production of ROS by Doxorubicin (Deres et al. 2005a). Deres et al, (2005) suggested that the increased production of ROS within cardiac myocytes upon Doxorubicin treatment may trigger the activation of Akt cascade via the nitration of the receptor tyrosine kinase by peroxynitrite. Data presented in our study has shown that Tiron had the ability to scavenge the free radicals, decrease the caspase-3 activity produced by Doxorubicin and subsequently abrogated Akt activation. The beneficial effects of Tiron could be attributed to its ability to accumulate in the cell membrane and scavenge free radicals produced by Doxorubicin.

As mentioned before, concomitant administration of Wortmannin, Tiron and Doxorubicin significantly mitigated Akt activation observed in Doxorubicin treated group and improved the cardiac function of LVDP, HR and CF in Langendorff heart model, suggesting that it could

potentially enhance the cardioprotective effects of Tiron, by reducing the cardiac damage induced by Doxorubicin. Previous in vitro investigation using neonatal cardiomyocytes have shown that treatment with H_2O_2 resulted in a significant increase in PI3K phosphorylation, which was counteracted by the co-treatment with Wortmannin, indicating that Wortmannin prevents H_2O_2-induced cardiac hypertrophy in cardiomyocytes (Tu et al. 2002). Similar findings were also reported by Ushio-Fukai et al. (1999) in vascular smooth muscle cells. The outcomes from their study revealed that upon treatment with H_2O_2 a significant increase in Akt phosphorylation was reported, which was abrogated by the co-administration of either Wortmannin or LY294002 (Ushio-Fukai et al. 1999). Based on the findings which highlight the potential of Wortmannin to enhance the cardioprotective effects of Tiron against Doxorubicin induced toxicity in the myocardium, future studies assessing the effects of Doxorubicin, Tiron and Wortmannin in I/R conditions could provide a better understanding of the involvement of Wortmannin in cardioprotection.

4.4.4 Tiron does not interfere with the anticancer activity of Doxorubicin in HL60 and HepG2 cancer cell lines

Numerous studies have shown that ROS mediated cardiotoxicity by Doxorubicin can be reduced by the administration of antioxidants (Abushouk et al. 2017; Sun et al. 2016). However, the use of antioxidants as adjunctive therapies to prevent cardiotoxicity during cancer treatment with Doxorubicin resulted in a serious debate, as their administration can inhibit the anti-tumour properties of Doxorubicin in cancer cells (Vincent et al. 2013). Therefore, in order to investigate the potential implications of Tiron co-administration with Doxorubicin on the efficacy of

Doxorubicin, this study has also examined the effects of parallel administration of Tiron and Doxorubicin in human HL-60 leukemia and HepG2 liver carcinoma cell lines. The cell viability assays carried out in both cell lines indicate that concomitant treatment with Tiron and Doxorubicin did not affect the anticancer action of Doxorubicin in these cells at the concentrations studied (Figure 4.13 and 4.14). We have shown that treatment with Tiron alone did not induce an effect on the cell viability. These results are consistent with previous studies carried out with different antioxidants in several cancer cell models (Fulbright et al. 2015; Rai et al. 2016). In this study Fulbright et al, (2015) investigated the effects of N-acetylcysteine (NAC) on the cardiomyocytes and leukaemia cell lines exposed to Doxorubicin and reveled that antioxidant NAC acts as a cardioprotective agent without interfering with the cytotoxicity of Doxorubicin. Within this study it was highlighted that treatment with Doxorubicin for 24 hours in H9c2 (cardiomyocytes) cells and ML-1 (acute leukaemia) cells caused a significant decrease in cell viability and DNA fragmentation, whereas concomitant administration of NAC with Doxorubicin did not affect the cytotoxicity of doxorubicin in ML-1 cells. However, in H9c2 cells, NAC offered significant protection against DNA induced fragmentation (Fulbright et al, 2015). In addition, within the same investigation, the levels of cellular glutathione at the height of caspase activation were also assessed in both cell lines. It was revealed that cellular glutathione levels were not significantly altered before cell death upon treatment with Doxorubicin in both cell lines. However, treatment with the pancaspase z-VAD-fmk inhibitor showed that the glutathione levels in ML-1 cells were not altered in the presence of the inhibitor, suggesting that the acute leukaemia and cardiomyocyte cell lines exhibit different pathways of cell death induction and dependence upon oxidative stress. Based on these findings, our results show that Tiron offered

significant protection in isolated adult myocytes but did not protect HL60 and HepG2 cells from Doxorubicin induced cytotoxicity.

We have also investigated the effects of combined treatment of Tiron and Doxorubicin on the cleaved-caspase-3 levels in HL60 and HepG2 cells. We have shown that concomitant administration of Tiron and Doxorubicin did not affect the induction of caspase-3 triggered by Doxorubicin treatment in both cell lines (Figure 4.15 and 4.16) indicating that Tiron did not interfere with the anti-tumour activity of Doxorubicin. Similar findings were reported by Kumar et al. (2013), in which they showed that administration of Tiron for 12 and 24 hours did not produce any significant changes in cell viability, ROS levels and caspase-3 levels in HL60 cells. (Kumar et al. 2013). In addition, co-administration of Tiron with Ery5 (a polyphenolic compound) enhanced the cytotoxicity of Ery5 in HL60 cells by activating autophagic proteins without interfering with the caspase-3 and PARP-1 activation (Kumar et al. 2013). In contrast, a study carried out on HL-60 cells, Kim et al, (2006) demonstrated that prolonged treatment with Tiron (72 hours) exerted a potent anti-proliferative and apopotic effect on HL-60 leukemia cells, inducing activation of caspase-3 cascade, suggesting that anti-proliferative effects of Tiron in cancer cell lines might be time-dependent.

The effects of Tiron and Doxorubicin and their concomitant treatment on ROS activity in HL60 and HepG2 cells was also assessed. Doxorubicin treated samples exhibited a mild increase in ROS levels, however this increase did not reach significant levels, indicating that Doxorubicin apoptosis might occur by a ROS-independent mechanism. Although the anticancer activity of Doxorubicin involves the formation of ROS, evidence suggest that this mechanism is not a solely

contributor in the apoptosis (Ko et al, 2005; Hou et al, 2004; Lin et al, 2003). In a study carried out in HL60 cell lines Ko et al, (2005) highlighted ROS-independent cell death pathway which was generated by myricetin, a flavonoid agent with antioxidant properties (Ko et al, 2005). The results from this investigation have shown that apoptosis triggered by myricetin is linked with the cytochrome c release to the cytosol, caspase 3 and 9 activation, reduced Bcl-2 protein levels and increased Bax protein levels (Ko et al, 2005). Interestingly, it was shown that no significant production of intracellular ROS levels was detected by DCHF-DA assay, DPPH assay and plasmid digestion. Treatment with antioxidants NAC, catalase, superoxide dismutase and Tiron did not protect against myricetin induced apoptosis, suggesting that myricetin apoptotic cell death occurs via a ROS-independent mechanism (Ko et al, 2005). In addition, similar results were reported by Hou et al, (2004). The outcomes of the study have demonstrated that upon treatment with gossypol (a polyphenolic compound extracted from cotton seeds) in HL60 cells the intracellular levels of ROS had similar values to the untreated group (Hou et al, 2004). It was also reported that concomitant administration of antioxidants NAC and catalase with gossypol did not impact gossypol induced apoptosis which was marked by loss of mitochondrial membrane potential, caspase-3 activation and release of cytochrome c. (Hou et al, 2004). Our study showed a mild decrease in ROS levels in Tiron treated samples, however this decrease was not significant. Concomitant administration of Tiron and Doxorubicin did not have an impact on the ROS activity, indicating that Doxorubicin-induced elimination of malignant cells in the presence of Tiron was as effective as without the antioxidant.

As mentioned before, our study has shown that Tiron did not interfere with the anti-tumour activity of Doxorubicin, which was marked by a substantial increase in caspase-3 activity. The

study carried out by Kumar et al. (2013) showed that administration of Tiron for 12 and 24 hours did not elicit any significant effects in cell viability, ROS levels and caspase-3 levels in HL60 cells. (Kumar et al. 2013). In addition, within the same study it has been demonstrated that administration of Tiron (1mM) alone did not cause an effect in the cleaved-caspase-3 levels or PARP1 in HL60 cells, however a significant increase in the activity of autophagy related proteins ATG7 and ATG12 has been reported (Kumar et al. 2013). Furthermore, co-administration of Tiron in combination with Ery5 (a polyphenolic compound) resulted in a 2-fold increase in the cytotoxicity of Ery5 when compared to Tiron and Ery5 groups alone as well as a further enhancement in the autophagy-related proteins (Kumar et al. 2013). Also, within the same investigation, the role of Bax has been investigated in order to elucidate if this protein is implicated in autophagic cell death caused by Ery5. It has been revealed that treatment with Tiron resulted in a significant upregulation of Bax, along with an increase in the expression of autophagic indicator LC3B (Kumar et al. 2013). Based on these findings, and due to the fact that our results show that Tiron did not have an impact on Doxorubicin induced caspase-3 and ROS activity, it is reasonable to suggest that Doxorubicin induced apoptosis in HL60 and HepG2 cell lines may occur via a ROS independent mechanism via autophagy and apoptotic pathways. However, the effects of combined treatment of Tiron and Doxorubicin on autophagy and apoptosis in these cell lines require further elucidation. The Autophagy Antibody Sampler Panel (Abcam, UK), containing a collection of monoclonal rabbit antibodies against ATG7, ATG12 and LC3B could potentially identify and elucidate the involvement of autophagy in Tiron treatment.

4.5 Summary of the findings

In summary, the mechanism of action of Tiron against Doxorubicin-induced cardiotoxicity in ex-vivo heart model and isolated ventricular cardiomyocytes as presented by this data is schematically illustrated in Figure 4.18. Doxorubicin compromised cardiac cell survival and function through increased ROS production and activation of Akt pathway. This triggered the mitochondrial dependent apoptosis resulting in the activation of the pro-apoptotic marker caspase-3. Treatment with Tiron mitigated these effects caused by Doxorubicin via the down-regulation of PI3K/Akt pathway, which resulted in a decrease in the ROS production and caspase-3 levels and improved cardiac function. In addition, parallel treatment with Tiron and Doxorubicin in HL60 and HepG2 cells did not affect the cytotoxicity of Doxorubicin, which was marked by a decrease in cell viability and an increase in caspase-3 levels as represented in (Figure 4.19).

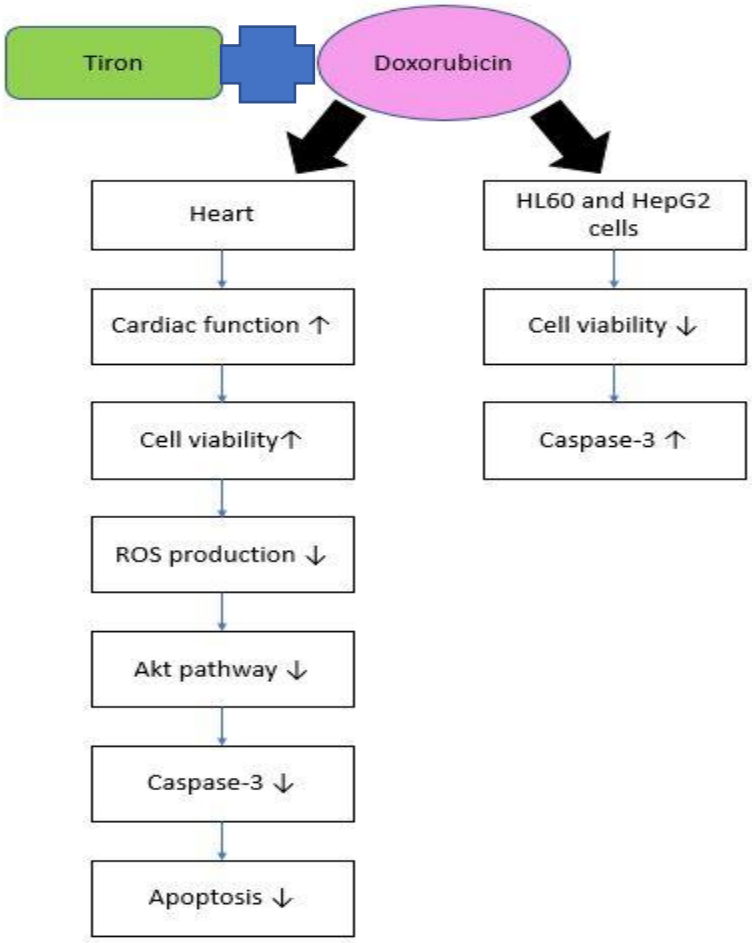


Figure 4.18 Schematic diagram illustrating the combined treatment of Tiron and Doxorubicin in isolated rat hearts, rat ventricular myocytes and HL60 and HepG2 cancer cell lines.

In conclusion, the presented work demonstrates that Tiron exerted its protective mechanism against Doxorubicin induced cardiotoxicity via its antioxidative properties. Besides the beneficial effects on cardiac function, Tiron did not decrease the tumoricidal action of Doxorubicin in human cancerous cell lines. Therefore, this study highlights that Tiron could potentially be used as an adjunctive therapy that selectively alleviates the toxic side effects of Doxorubicin without affecting its anti-proliferative activity.

Chapter 5.Investigating the effect of Tiron against Doxorubicin induced cardiotoxicity in ischaemia/reperfusion conditions

5.1 Introduction

Methods for cancer diagnosis and treatment have progressed significantly in recent years by reducing the mortality and increasing life expectancy in cancer patients (Angsutararux, Luanpitpong and Issaragrisil 2015b, Florescu, Cinteza and Vinereanu 2013, Jain et al. 2017). Doxorubicin, an effective antineoplastic agent has been extensively used in the treatment of both solid and haematological cancers (Beretta and Zunino 2007, Green and Rose 2006, Kumar et al. 2014, Smith et al. 2006, Yaqub 2013). Its use has been limited due to negative effects on the functionality and contractility of the myocardium (De Angelis et al. 2016, Groarke and Nohria 2015, McGowan et al. 2017, Octavia et al. 2012, Salvatorelli et al. 2018, Zhao and Zhang 2017).

The mechanism of action of Doxorubicin is complex, but it has been shown that Doxorubicin induced cardiotoxicity can occur in a dose-dependent manner resulting in both acute and chronic cardiotoxic effects such as pericardiatis, arrhythmias, tachycardia, decline in the left ventricular function and cardiomyopathy (Barrett-Lee et al. 2009, Esteban et al. 2016, Rahman, Yusuf and Ewer 2007, Volkova and Russell 2011). The acute cardiotoxicity associated with Doxorubicin occurs during the treatment course, or immediately after cessation and it is reversible and manageable. Chronic cardiotoxicity may not be apparent until several years after the cessation of the treatment and can have serious and irreversible clinical implications such as cardiomyopathy, cardiomyocytes death and ultimately heart failure (Angsutararux, Luanpitpong

and Issaragrisil 2015a, Cardinale et al. 2015, Menna and Salvatorelli 2017, Minotti et al. 2004, Pecoraro et al. 2017).

The underlying mechanism of Doxorubicin induced myocardial injury has been linked with several adverse effects on cardiomyocytes including oxidative stress as a result of excessive production of ROS, changes in iron homeostasis, mitochondrial dysfunction leading to induction of apoptotic and necrotic cell death pathways (McGowan et al. 2017, Mistiaen 2016, Mitry and Edwards 2016, Pecoraro et al. 2017, Shaker et al. 2018, Zhao and Zhang 2017) (This has been described in detail in chapter 1).

Although the cardiotoxic effects induced by Doxorubicin have been previously reviewed and studied by several research groups (Chatterjee et al. 2010, Minotti et al. 2004, Octavia et al. 2012, Volkova and Russell 2011) the effects on the myocardium and cardiomyocytes viability in I/R setting are still elusive. Studies have shown the risk of cardiotoxic events associated with Doxorubicin administration increases in elderly patients with underlying diseases such as hypertension, diabetes mellitus, liver disease and underlying CVD, thus limiting its use in these patients (Barrett-Lee et al. 2009, Hershman et al. 2008).

As the prevalence of cancer rises in with age and the percentage of elderly population is increasing, there is a higher percentage of patients over 65 years with cancer (Aapro et al. 2010, Balducci and Extermann 2000). Due to strict inclusion and exclusion criteria, the elderly population are generally excluded or under-represented in clinical trials (Aapro et al. 2010, Hutchins et al. 1999, Lichtman et al. 2007). Mertens et al, (2008) have highlighted that a high mortality percentage of cancer patients subjected to Doxorubicin treatment is most likely

associated with the cardiac complications encountered during the chemotherapeutic treatment with this compound (Mertens et al. 2008). In a different investigation, Doyle et al, (2005) have highlighted that treatment with Doxorubicin resulted in three times increase in the cardiomyopathy adverse effects in the first year of cancer treatment in elderly population (Doyle et al. 2005). Several methods have been examined to extend the use of Doxorubicin, including dose optimisation, production and administration of similar agents or combined treatment (Geisberg and Sawyer 2010, Neilan et al. 2007, Spallarossa et al. 2004). Even though patients with underlying heart diseases are subjected to lower doses during cancer treatment, Doxorubicin administration could exacerbate the cardiac damage in patients with pre-existing heart conditions (Doyle et al. 2005). Therefore, it is crucial to investigate and elucidate the effects of Doxorubicin administration on cardiac dysfunction and especially in I/R injury conditions, with the aim to improve and find optimal therapeutic strategies for patients with ischaemic heart disease exposed to chemotherapy.

Significant evidence is present in the literature to highlight that reperfusion of the ischaemic myocardium may aggravate the myocardial injury (Frank et al. 2012, Hausenloy and Yellon 2013, Kalogeris et al. 2012). This phenomenon has been associated with disturbances in normal cellular metabolism (Frank et al. 2012, Hausenloy and Yellon 2013, Kalogeris et al. 2012). Under normal physiological conditions, the balance between ROS formation and ROS scavenging is highly regulated (Zorov, Juhaszova and Sollott 2014). However, uncontrolled oxidative and reductive stress caused by I/R, may result in exacerbated production of ROS, which in turn can produce severe damage to cellular function and integrity, induction of apoptotic cell death and heart

failure (Cadenas 2018, Granger and Kvietys 2015, Kalogeris et al. 2012, Zhou, Chuang and Zuo 2015) (As previously described in detail in Chapter 1)

Laboratory evidence suggests that elevated levels of oxidative stress with increased free radical generation and reduced myocardial endogenous antioxidants have an important role in the pathogenesis of Doxorubicin induced cardiotoxicity and heart damage (Ahmed et al. 2005, Mukherjee et al. 2003, Saleem, Chetty and Kavimani 2014, Singal et al. 1997). As previously stated, increasing attention has been focused on the use of antioxidants as a protective strategy with the aim to reduce Doxorubicin cardiotoxic side effects (Abushouk et al. 2017, Ananthanarayanan Ajith, Hema and Aswathi 2016, Erboga et al. 2016, Gao et al. 2016, Zhang, Yi and Huang 2017).

The investigation carried out in chapter 3 has demonstrated that Tiron offers myocardial protection by attenuating oxidative stress generated during I/R conditions (See chapter 3).

In chapter 4 the ability of Tiron to alleviate Doxorubicin induced cardiotoxicity was demonstrated in naïve conditions without affecting the tumoricidal activity of Doxorubicin. Since the majority of the elderly patients exposed to Doxorubicin chemotherapy have underlying I/R complications, it is essential to investigate the effects of combined treatment of Tiron and Doxorubicin in the setting of I/R conditions in the Sprague-Dawley rat heart model.

The aims of the current study are to assess and investigate the effect of Tiron (0.25,0.5 and 1mM) and Doxorubicin (1μM) during reperfusion in Langendorff perfused rat hearts and primary adult ventricular cardiomyocytes exposed to I/R and H/R conditions; the effect of combined treatment of Tiron and Doxorubicin on apoptosis, necrosis and cleaved-caspase-3 levels.

5.2 Methods

5.2.1 Chemicals

As previously mentioned in chapter 2 section 2.2, Tiron was purchased from Sigma Aldrich (Dorset,UK) and Doxorubicin hydrochloride was supplied from Tocris (Bristol, UK). Both drugs were prepared as detailed in Chapter 2 Section 2.2.

5.2.2 Animals

Sprague-Dawley rats with a body weight of 350±50g were purchased from Charles River, UK. Rats had unrestricted access to food and water, receiving human care assistance in concordance with the guidance in the operation of the animals (Scientific Procedure) Act 1986. Rats were sacrificed by cervical dislocation as outlined in Schedule 1 Home Office Procedure using the process of thoracotomy as previously described in Chapter 2 section 2.3.3

5.2.3 Isolated perfused rat heart model (Langendorff protocol)

Langendorff experimental protocol were carried out as described in Chapter 2, section 2.3. The experiment was carried out for 175 minutes. As previously described in chapter 2, section 2.3.3, hearts were allowed to stabilise for a period of 20 minutes, followed by 35 minutes of regional ischaemia and 120 minutes of reperfusion in the presence of Tiron (0.25-1mM) and/or Doxorubicin (1µM). Hearts were randomly allocated to the following treatment groups: either normoxic group: hearts perfused with Krebs-Hensleit (KH) buffer for 175 minutes; or ischaemia-reperfusion studies: where hearts were perfused with KH buffer for 20 minutes stabilisation period, followed by 35 minutes of regional ischaemia and 120 minutes of reperfusion; or hearts

were perfused with Tiron (0.25-1mM) and Doxorubicin (1µM) for 120 minutes during reperfusion period.

Throughout the duration of all experiments the stability of the heart was assessed by monitoring the haemodynamic parameters HR, CF and LVDP at regular intervals. Hearts that failed to meet the criteria outlined in Chapter 2 Section 2.3.2 were excluded from this study.

At the end of the reperfusion period, the infarct to risk ratio analysis was performed using Evans Blue solution and triphenyltetrazolium chloride (TTC) staining techniques. Viable, risk and infarct areas of the tissue were assessed and calculated in accordance with the protocol explained in detail in Chapter 2, Section 2.3.4-2.3.6.

5.2.4 Isolation of adult rat ventricular cardiomyocytes

Adult rat ventricular cardiomyocytes were isolated from Sprague Dawley rats using the process of enzymatic digestion as detailed in Chapter 2 Section 2.4 (Maddock et al, 2002, Gharanei et al, 2013).

5.2.5 Cell treatment protocol

Following the succesful isolation of adult rat ventricular cardiomyocytes as outlined in Chapter 2, Section 2.4, the freshly harvested cells were incubated for 1 hour with Esumi hypoxic buffer (described in Chapter 2, Section 2.2.1) and placed into a in New Brunswick hypoxic chamber at 37^{o}C, in 5%CO_2 and 0.01-1% O_2 to induce hypoxia (Details of the hypoxic buffer and preparation are detailed in Chapter 2, Section 2.5). Ventricular cardiomyocytes were then allocated to

different treatment groups and were incubated with either: Tiron (0.25mM, 0.5 and 1mM), Doxorubicin (1µM), Tiron (0.25, 0.5 and 1mM) and Doxorubicin (1µM) for 3 hours during the reoxygenation period. In addition, untreated cells were incubated for 4 hours in normoxic conditions at 37^{o}C, 5% CO_2 and 95% O_2.

Following the reoxygenation phase, treated cardiomyocytes were either assessed via MTT and Trypan Blue assay for cell viability or by flow cytometric analysis for apoptosis, necrosis or cleaved-caspase-3 activity as described below.

5.2.6 Assessment of cell viability using the colourimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)] assay

MTT (Sigma-Aldrich, UK) cell viability assay was performed in isolated rat ventricular cardiac myocytes exposed to drug treatment as previously stated in Chapter 2, Section 2.6.3, and the values obtained were calculated as a percentage change in MTT reductase activity of the mean absorbance of the control group.

5.2.7 Assessment of cell viability using Trypan blue staining

Trypan blue staining was performed in accordance with Chapter 2, Section 2.6.5. Cell viability was calculated as a percentage of the viable cells divided by total number of cells.

5.2.8 Asesement of apoptosis and necrosis in primary adult ventricular cardiomyocytes using Annexin V kit

Annexin V-FITC Assay (Abcam, UK) was used to assess the apoptotic and necrotic ratio of the cells. Following the incubation of adult rat cardiomyocytes in the presence and in the absence of Tiron (0.25mM) and Doxorubicin (1µM) throughout the reoxygenation period, $1x10^5$ cells were collected by centrifugation. Thereafter, cells were pelleted and re-suspended into 500µl of 1X Annexin V Binding buffer (provided by the manufacturer) and 5µl of Annexin V-FITC and 5µl of Propidium Iodide were added to each sample (in accordance with the manufacturer instructions). All samples were incubated at room temperature for 5 minutes in the dark, prior to flow cytometric analysis on BD Accuri C6 Plus. Annexin V-FITC was analysed using FL1 channel, whereas Propidium Iodide was measured using FL3 channel (according to manufacturer instructions).

5.2.9 Assessment of primary adult ventricular cardiomyocytes using cleaved caspase-3 staining

Caspase 3 staining was performed as described in Section 2.7.2. The samples were probed with the cleaved-caspase 3 antibody (Active/cleaved Caspase-3 Assay kit, Novus Biologicals/Bio-Techne, UK) and analysed in accordance with the manufacturer instructions on the BD Accuri C6 Plus flow cytometer.

5.2.10 Data analysis

All data is presented as mean± SEM. Data presented for the coronary flow parameter was adjusted by the heart weight and calculated as a percentage of mean stabilisation.

Haemodynamic parameters, heart rate, left ventricular developed pressure and coronary flow were analysed by one-way ANOVA for each time point. Significance was considered at $p<0.05$. All other data was analysed using one-way analysis of variance (ANOVA) with Tukey post-hoc tests to identify group differences.

5.3 Results

5.3.1 The effects of the co-administration of Tiron (0.25-1mM) and Doxorubicin (1µM) on the infarct size (IS) and area at risk (AAR) in the presence of ischaemia reperfusion injury in the Langendorff hearts.

As previously shown in Chapter 3, Figure 3.9 administration of Tiron alone (0.25mM-1mM) throughout reperfusion significantly reduced the infarct to risk ratio when compared to I/R control group (Tiron 0.25mM: 24.05±0.68% vs. 47.72±0.91%; Tiron 0.5mM: 23.39±0.84% vs. 47.72±0.91%; Tiron 1mM: 17.245±1.04% vs. 47.72±0.91%; $p<0.05$; Figure 3.9, Section 3.3.6, Chapter 3).

Furthermore, treatment with Doxorubicin (Dox) (1µM) resulted in a significant increase in the infarct to risk ratio when compared to I/R group (68.04±1.25% vs. 47.72±0.91%, respectively $p<0.0001$). Co-administration of Tiron (0.25-1mM) and Doxorubicin (1µM) during reperfusion significantly reduced the infarct size to risk ratio when compared to Doxorubicin group (Tiron 0.25mM+ Dox 1µM: 50.41±1.68% vs. 68.04±1.25%; Tiron 0.5mM+Dox 1µM: 47.12±0.67% vs. 68.04±1.25%; Tiron 1mM+Dox 1µM: 49.30±1.66 vs. 68.04±1.25%; $p<0.05$; Figure 5.1). In addition administration of Tiron in the presence of Doxorubicin throughout reperfusion reduced the

infarct size ratio to similar values observed in I/R control group (Tiron 0.25mM+ Dox 1µM: 50.41±1.68% vs. 47.72±0.91%; Tiron 0.5mM+Dox 1µM: 47.12±0.67% vs. 47.72±0.91%; Tiron 1mM+Dox 1µM: 49.30±1.66% vs. 47.72±0.91%) (Figure 5.1).

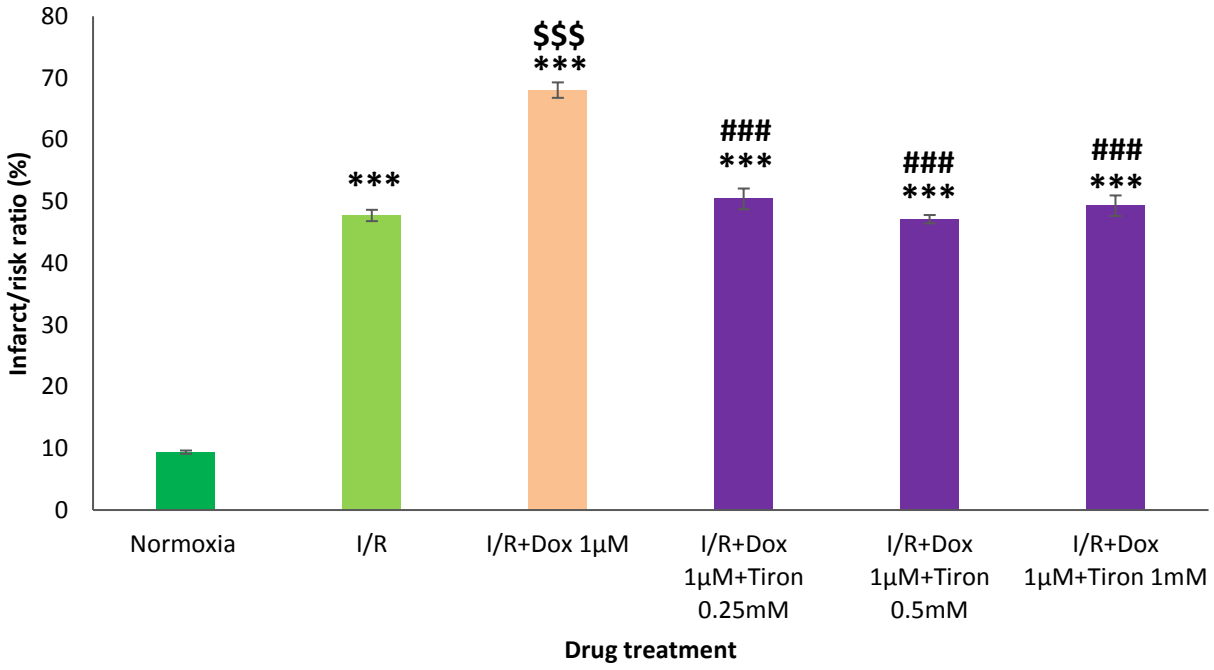


Figure 5.1 The effects of Tiron (0.25-1mM) and Doxorubicin (Dox) (1µM) on the infarct to risk ratio analysis on isolated Langendorff heart model of ischaemia reperfusion injury. The hearts were subjected to 20 minutes of stabilisation period, followed by 35 minutes of regional ischaemia and 155 minutes of reperfusion in the presence or absence of Doxorubicin (1µM) and Tiron (0.25-1mM). Data are presented as mean±SEM of 6 experiments; ***p<0.0001 vs. Normoxia; $^{###}$p<0.0001 vs. Doxorubicin group; $^{\$\$\$}$p<0.05 vs. I/R control group.

The AAR/left ventricle percentage was calculated in these experiments in order to confirm that there was no significant difference in the AAR between the experimental groups exposed to I/R. As observed in Figure 5.2, no significant difference was observed in the percentage area at risk (AAR)/left ventricular volume upon administration of Tiron (0.25-1mM) and Doxorubicin (1µM) when compared to I/R control group (I/R+Doxorubicin 1µM: 38.69±0.82% vs. 41.90±0.29%; I/R+Doxorubicin 1µM+ Tiron 0.25mM: 41.26±1.11% vs. 41.90±0.29%; I/R+Doxorubicin 1µM+ Tiron 0.5mM: 42.17±0.23% vs. 41.90±0.29%; I/R+Doxorubicin 1µM+ Tiron 1mM: 43.10±0.15% vs. 41.90±0.29%).

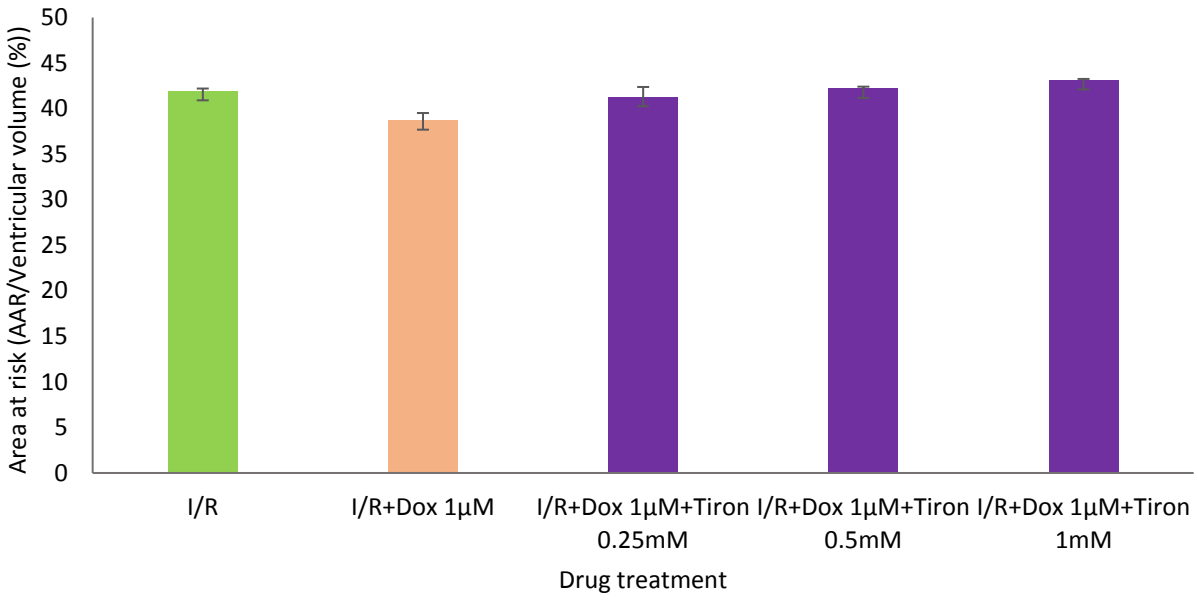


Figure 5.2 The effects of Tiron administration (0.25mM-1mM) and Doxorubicin (1µM) on the percentage of area at risk of the ventricular volume on isolated Langendorff heart model of ischaemia and reperfusion injury. Hearts were exposed to 20 minutes stabilisation, followed by 35 minutes of regional ischaemia and 120 minutes of reperfusion with KHB±Tiron (0.25-1mM) and Doxorubicin (1µM). Results were presented as mean±SEM, n=6 experiments.

5.3.2 The effects of co-administration of Tiron (0.25-1mM) and Doxorubicin (1µM) on the haemodynamic parameters of the heart in ischaemia-reperfusion studies

As previously shown in Chapter 3, Section 3.3.7, no significant difference was reported in the HR, CF and LVDP parameters when treated with Tiron (0.25-1mM) at reperfusion when compared to I/R control group (Figure 3.10A, B and C).

The effects of Tiron (0.25-1mM) and Doxorubicin (Dox) (1µM) on the coronary flow (CF) in the I/R model are illustrated in Figure 5.3. Treatment with Doxorubicin resulted in a significant decrease in CF after 45 minutes of treatment during reperfusion when compared to I/R group from (66.27±7.87% vs. 85.24±7.25% at 115 minutes into reperfusion; $p<0.05$). Co-administration of Doxorubicin with Tiron (0.25-1mM) significantly reversed the drop caused by Doxorubicin alone (Tiron 0.25mM+Doxorubicin 1µM: 76.87±1.67% vs. 66.27±7.87%; Tiron 0.5mM+Doxorubicin 1µM: 78.02±5.77% vs. 66.27±7.87%; Tiron 1mM+Doxorubicin 1µM: 81.78±6.18% vs. 66.27±7.87%; $p<0.05$ at 115 minutes reperfusion). No significant difference was reported in the co-treated groups when compared to I/R control group.

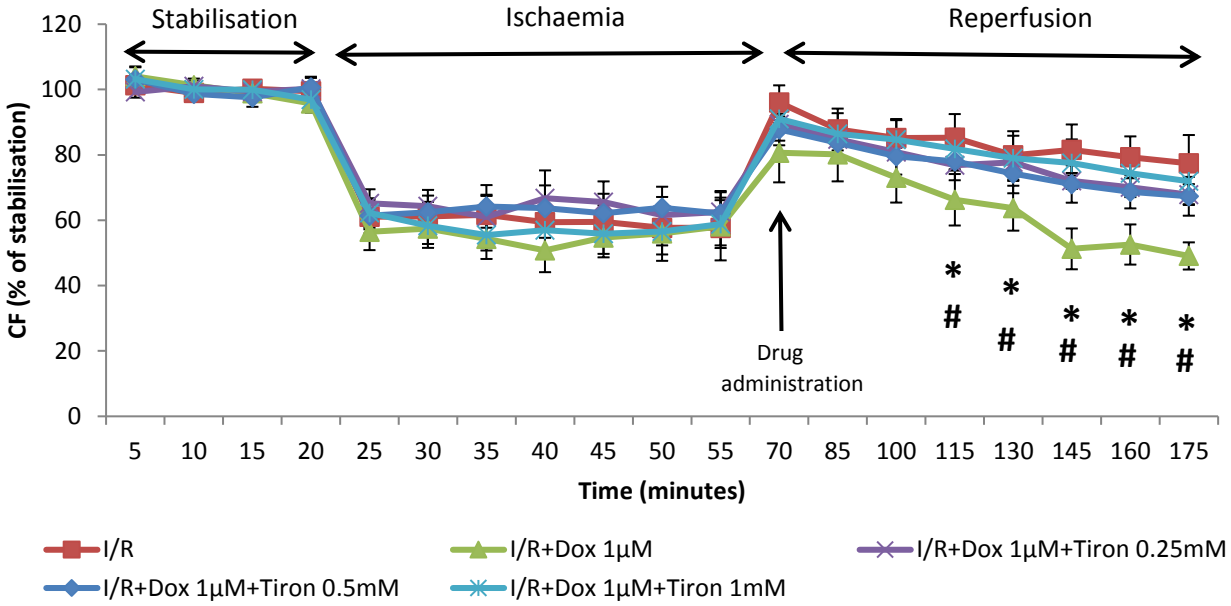


Figure 5.3 The effects of Tiron (0.25-1mM) and Doxorubicin (Dox) (1µM) on coronary flow. The hearts were subjected to 20 minutes of stabilisation period, 35 minutes of ischaemia and 120 minutes of reperfusion with drug treatment. Data are presented as a percentage of average stabilisation; n=6 experiments; *p<0.05 vs. I/R control; #p<0.05 vs. I/R+Dox (1µm) + Tiron (0.25-1mM).

The effects of Tiron and Doxorubicin on LVDP parameters in I/R model are shown in Figure 5.4. Treatment with Doxorubicin caused a significant reduction in in LVDP values after 75 minutes of treatment during reperfusion when compared to I/R control group (at 145 minutes: 59.38±3.37% vs. 75.04±0.79%; p<0.05). Co-treatment with Tiron (0.25-1mM) significantly reversed the reduction in LVDP observed with Doxorubicin group alone (at 145 minutes: Tiron 0.25mM+Doxorubicin 1µM: 71.43±8.75% vs. 59.38±3.37%; Tiron 0.5mM+Doxorubicin 1µM: 71.07±2.89% vs. 59.38±3.37%; Tiron 1mM+Doxorubicin 1µM: 69.78±4.75% vs. 59.38±3.37%; p<0.05).

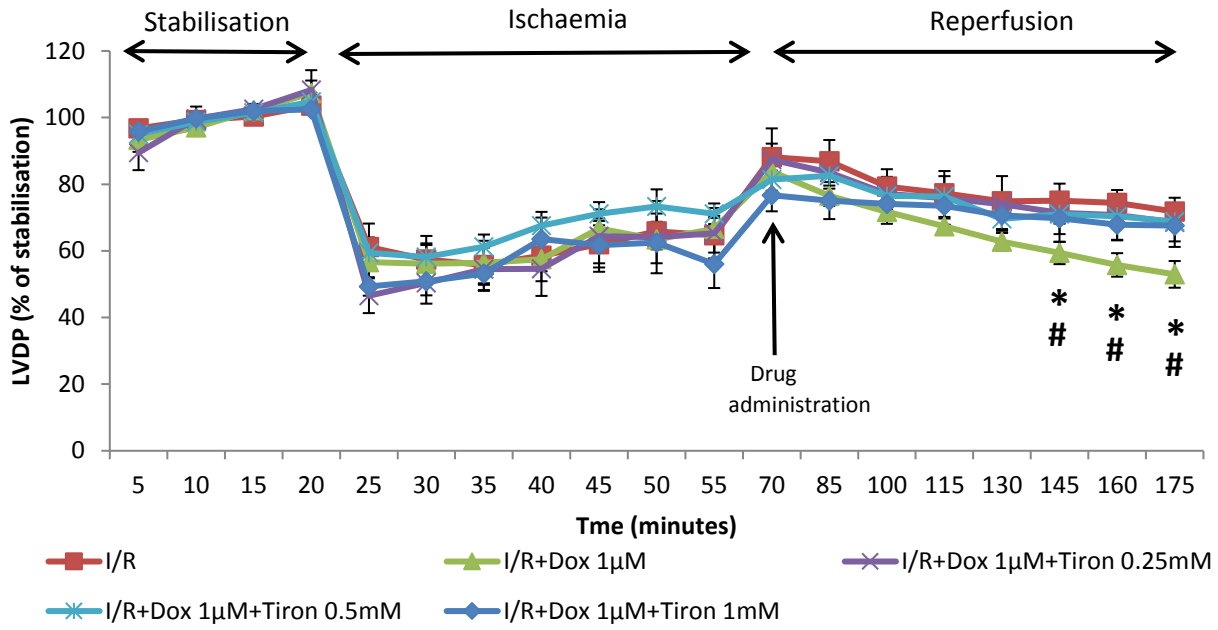


Figure 5.4 The effects of Tiron (0.25-1mM) and Doxorubicin (1µM) on left ventricular diastolic pressure. The hearts were subjected to 20 minutes of stabilization, 35 minutes of ischaemia and 120 minutes of reperfusion with drug treatment. Data are presented as mean±SEM of n=6 experiments; *$p<0.05$ vs. I/R control; $^{\#}p<0.05$ vs. I/R+Dox (1µm) + Tiron (0.25-1mM)

The effects of Tiron (0.25-1mM) and Doxorubicin (1µM) on the heart rate (HR) parameters in I/R model are shown in Figure 5.5. Administration of Doxorubicin (1µM) throughout reperfusion resulted in a significant decrease in the HR values when compared to I/R control group from 60 minutes into reperfusion stage (at 130 minutes: 78.59±5.10% vs. 98.61±4.58%; $p<0.05$). Interestingly, parallel administration of Tiron (0.25-1mM) and Doxorubicin (1µM) significantly mitigated the decrease in HR observed in Doxorubicin treated groups (at 130 minutes: Doxorubicin 1µM+Tiron 0.25mM: 93.30±4.58% vs. 78.59±5.10%; Doxorubicin 1µM+Tiron 0.5mM: 100.20±6.44% vs. 78.59±5.10%; Doxorubicin 1µM+Tiron 1mM: 109.84±6.60% vs.

78.59±5.10%; respectively p<0.05). No significant difference in the HR parameters was observed at any time point between co-treated groups and I/R control group.

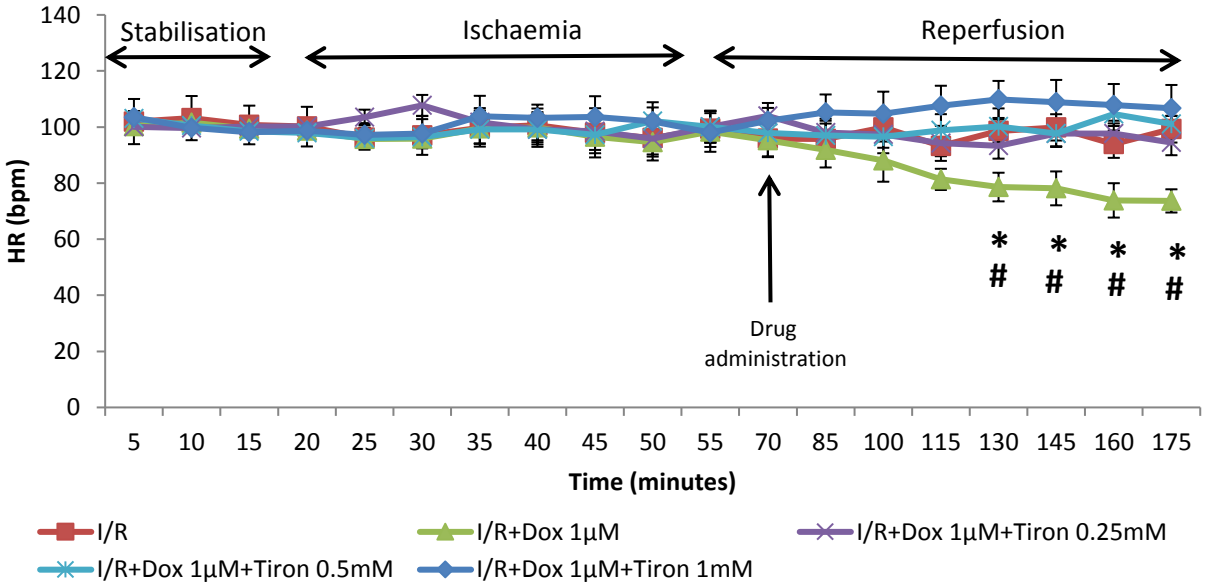


Figure 5.5 The effects of Tiron (0.25-1mM) and Doxorubicin (1µM) on heart rate. The hearts were subjected to 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion with drug treatment. Data are presented as average±SEM of n=6 experiments; *p<0.05 vs. I/R control; #p<0.05 vs. I/R+Dox (1µm) + Tiron (0.25-1mM)

5.3.3 The effect of Tiron (0.25mM) and Doxorubicin (1µM) on the viability of isolated rat ventricular cardiomyocytes subjected to H/R conditions

The MTT colorimetric assay (Figure 5.6) and Trypan Blue staining (Figure 5.7) showed that treatment with Doxorubicin at reoxygenation caused a significant decrease in the cell viability of isolated rat ventricular cardiomyocytes when compared to H/R group alone (MTT assay: 30.54±2.35% vs. 45.43±4.89%; Trypan Blue staining: 23.87±0.99% vs. 44.34±0.93%; respectively p<0.05). Co-administration of Tiron 0.25mM with Doxorubicin (1µM) significantly attenuated the

cellular damage observed in Doxorubicin treated group (MTT assay: 45.93±3.31% vs. 30.54±2.35%, respectively p<0.001 Trypan Blue assay: 44.34±0.93% vs. 23.87±0.99%; p<0.001). No significant difference in the cell viability was detected in the co-treated group when compared to H/R control group. Addition of Tiron (0.25mM) during reoxygenation significantly increase the cell viability when compared to H/R control group (MTT assay: 60.94±2.55% vs. 45.43±4.89%, respectively p<0.05, Figure 3.11, Section 3.3.8, Chapter 3; Trypan Blue: 57.73±1.15% vs. 44.34±0.93%; p<0.05; Figure 3.12, Section 3.3.8, Chapter 3).

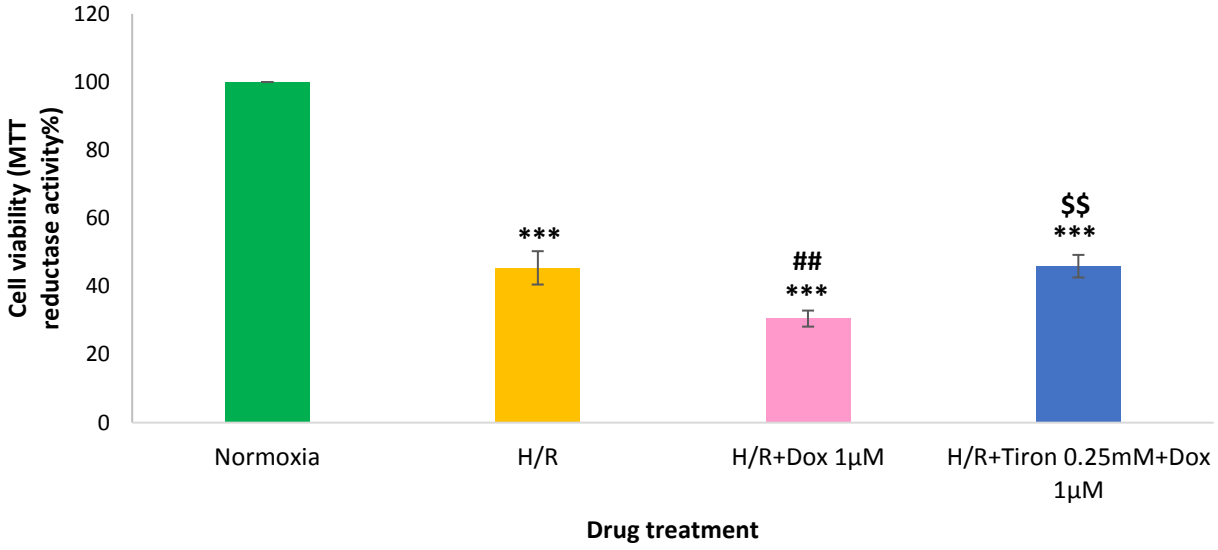


Figure 5.6 MTT analysis showing the viability of isolated adult rat ventricular cardiomyocytes subjected to 1 hour of hypoxia and 3 hours of reoxygenation in response to drug treatment with Tiron (0.25mM) and Doxorubicin (1µM). Data are presented as mean±SEM, with n=6 experiments, ***p<0.0001 vs. Normoxic control; $^{\#\#}$p<0.001 vs. H/R; $^{\$\$}$p<0.001 vs. Doxorubicin (1µM)

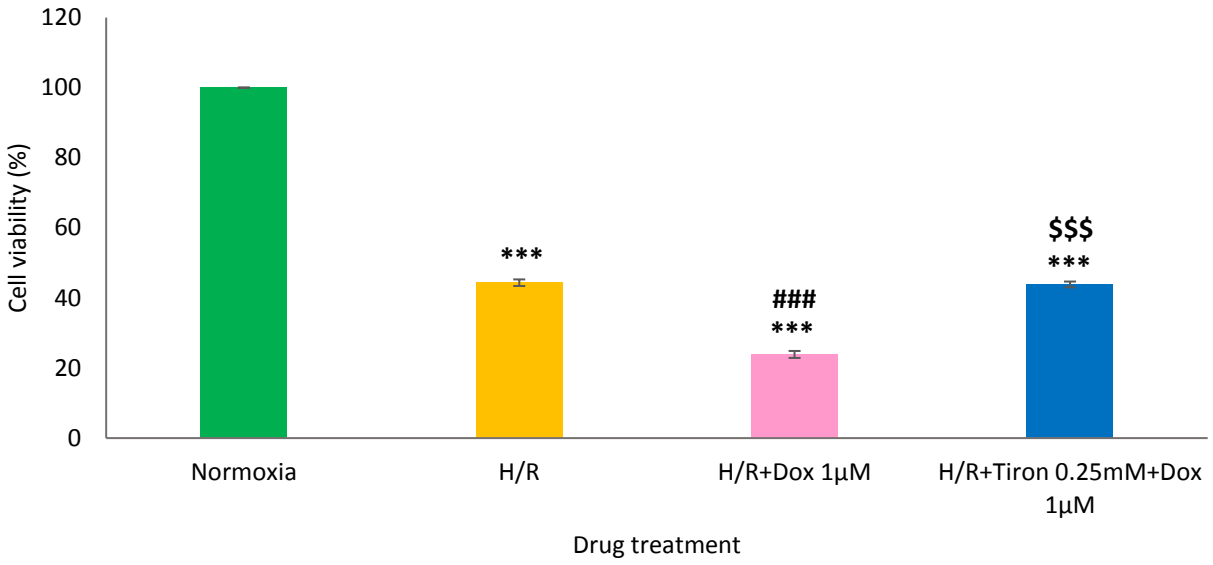


Figure 5.7 Trypan blue analysis showing the viability of isolated adult rat ventricular cardiomyocytes subjected to 1 hour of hypoxia and 3 hours of reoxygenation in response to drug treatment with Tiron (0.25mM) and Doxorubicin (1µM). Data are presented as mean±SEM, with n=6 experiments, ***p<0.0001 vs. Normoxic control; ##<0.001 vs. H/R; $^{\$\$}$p<0.001 vs. Doxorubicin (1µM)

5.3.4 The effects of Tiron (0.25mM) and Doxorubicin (1µM) on the apoptosis and necrosis in the primary ventricular myocytes exposed to H/R conditions

Due to potential non-specific binding of PI to the cardiac myocytes no data is available for the apoptosis and necrosis in these conditions. Technical guidance was sought from both BD and Abcam in order to solve this issue. The technical staff from BD advised to use a different kit with different fluorochromes that do not have overlapping spectra, however there was insufficient time to complete the experiments. The histogram plots are attached below (Figure 5.8). However, on reflection of the results obtained in chapters 3 and 4, caspase-3 activity was

assessed under the H/R conditions to better elucidate the potential protective pathway of Tiron against H/R and Doxorubicin in adult rat ventricular myocytes.

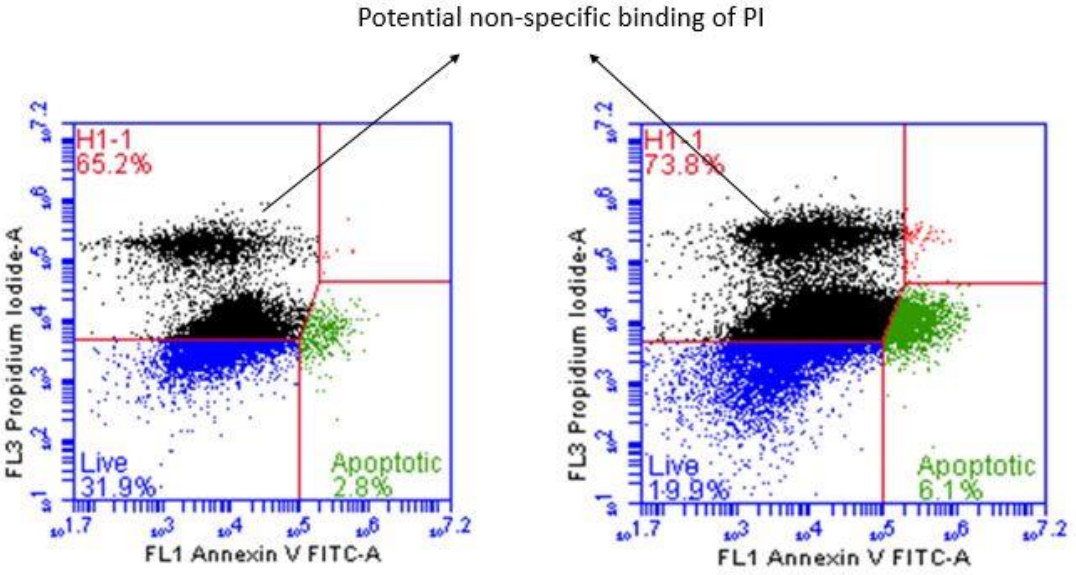


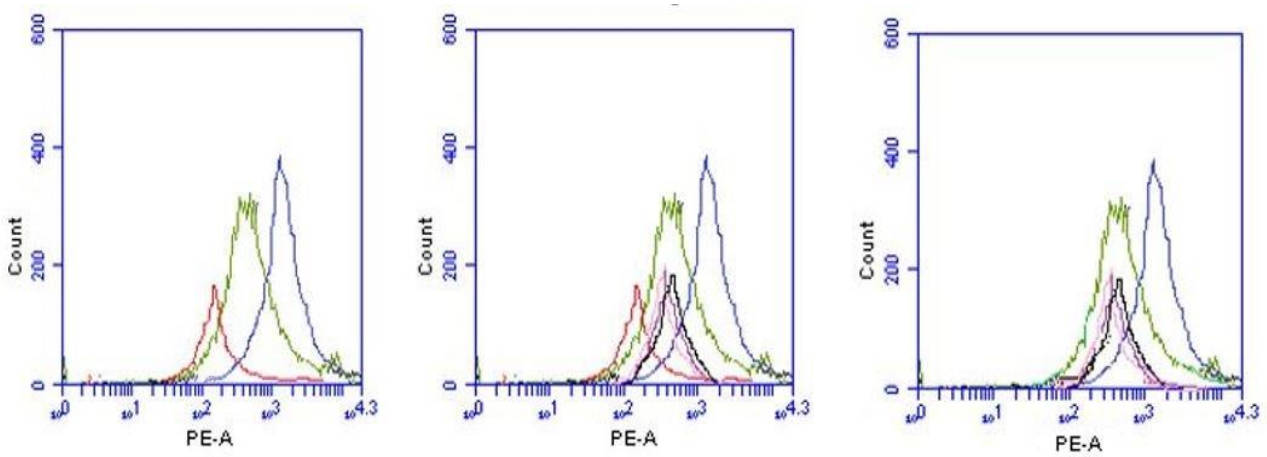
Figure 5.8 Representation of the histogram plots ilustrating the possibility of non-specific binding of propidium iodide (PI) observed in the samples treated with both Annexin V and PI.

5.3.5 The effects of combined treatment of Tiron (0.25-1mM) and Doxorubicin (1µM) on the cleaved caspase 3 activity.

The cleaved-caspase-3 flow cytometric analysis has shown a significant increase in the levels of cleaved-caspase-3 levels in H/R and Doxorubicin treated group when compared to H/R control group (742.02±6.48% vs. 454.31±8.21%, respectively $p<0.0001$) (as shown in Figure 5.9). Concomitant administration of Tiron (0.25-1mM) with Doxorubicin (1µM) throughout the reoxygenation phase significantly inhibited Doxorubicin increase in cleaved-caspase-3 activity

(Tiron 0.25mM+Dox 1µM: 262.81±9.55% vs. 742.02±6.48%; Tiron 0.5mM+Dox 1µM: 271.74±11.73% vs. 742.02±6.48%; Tiron 1mM+Dox 1µM: 322.59±11.70% vs. 742.02±6.48%; $p<0.0001$) (Figure 5.9). In addition, a significant decrease could be detected in the cleaved-caspase 3 levels in the co-treated samples and H/R control group (Tiron 0.25mM+Dox 1µM: 262.81±9.55% vs. 454.31±8.21%; Tiron 0.5mM+Dox 1µM: 271.74±11.73% vs. 454.31±8.21%; Tiron 1mM+Dox 1µM: 322.59±11.70% vs. 454.31±8.21%; $p<0.0001$). The significant decrease in the cleaved-caspase 3 levels in the co-treated samples did not achieve the values reported in the normoxic control group (Tiron 0.25mM+Dox 1µM: 262.81±9.55% vs. 100±0.0%; Tiron 0.5mM+Dox 1µM: 271.74±11.73% vs. 100±0.0%; Tiron 1mM+Dox 1µM: 322.59±11.70% vs. 100±0.0%; $p<0.0001$). The results obtained in chapter 3 showed that administration of Tiron (0.25mM-1mM) significantly decreased the caspase-3 activity when compared to H/R control group Tiron 0.25mM:98.69±7.14% vs. 454.31±8.21%; Tiron 0.5mM: 105.23±6.67% vs. 454.31±8.21%; Tiron 1mM: 109.86±4.76 vs. 454.31±8.21%, $p<0.0001$; Figure 3.13, Section 3.39, Chapter 3).

A



B

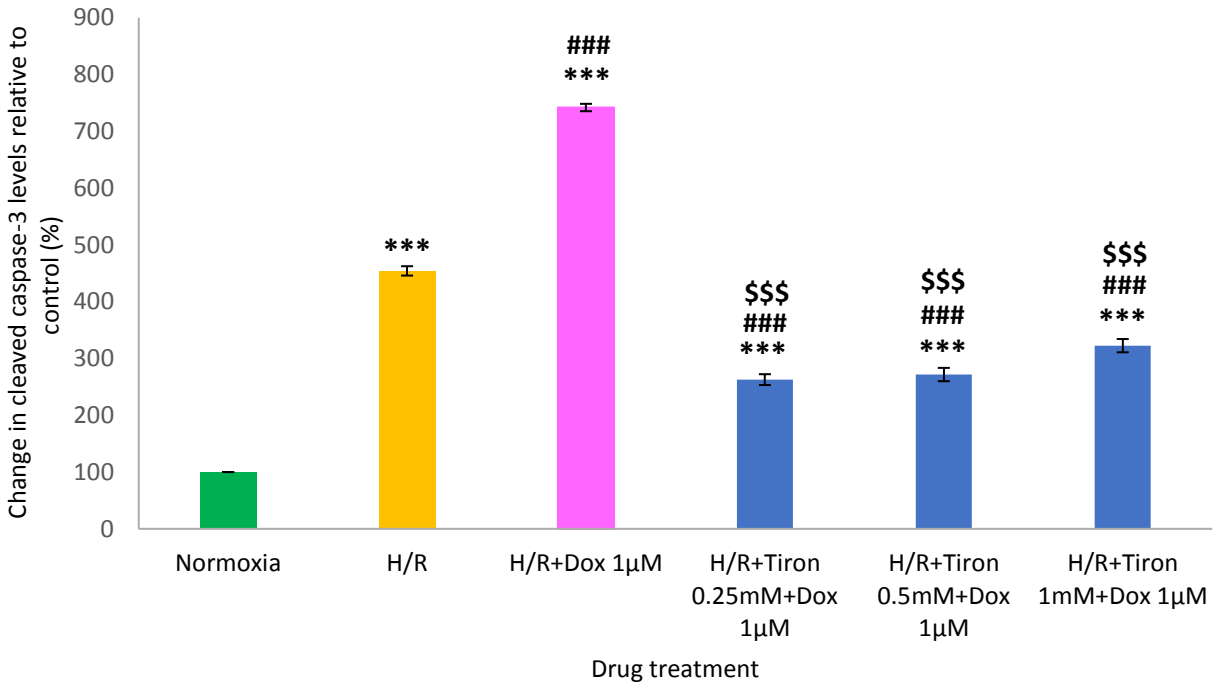


Figure 5.9 Cleaved-caspase-3 levels flow cytometric scatter plots (A) and analysis (B) in isolated rat ventricular myocytes subjected to 1 hour of hypoxia and 3 hours of reoxygenation with Tiron (0.25-1mM) and Dox (1µM). Data are presented as average±SEM, n=6 experiments, ***p<0.0001 vs. Normoxia; $^{###}$p<0.0001 vs. H/R; $^{\$\$\$}$p<0.0001 vs. Dox (Representation of the scatter plots Normoxic control (red); H/R control (green); Dox (blue); Tiron 0.25mM+Dox (pink); Tiron 0.5mM+Dox (purple); Tiron 1mM+Dox (black).

5.4 Discussion

In the present study, we investigated the effects of Tiron (0.25-1mM) on isolated Sprague Dawley perfused rats and primary ventricular myocytes subjected to Doxorubicin (1µM) treatment under I/R and respectively H/R conditions. This study demonstrates Tiron's ability to provide cardioprotection against Doxorubicin administration in I/R conditions and this effect was shown by a decrease in the infarct to risk ratio observed in the co-treated groups when compared to Doxorubicin group alone (Figure 5.1). In addition, we have also shown that Doxorubicin treatment throughout reperfusion of the ischaemic myocardium resulted in an increase in the myocardial damage and a delay in the heart recovery, findings that are consistent with previous published articles (Chahine et al. 2014, Gharanei et al. 2013). In a similar manner, using the Langendorff technique, Chahine et al, (2014) investigated the effects of Doxorubicin in the model of ex-vivo rabbit hearts subjected to 30 minutes of simulated global ischaemia and 40 minutes of drug reperfusion with Doxorubicin (5µg/ml) and showed that treatment with Doxorubicin resulted in significant alterations in the muscle striations such as disturbances in the orientation and space of the myocardial fibers and distinct areas of necrosis when compared to I/R control group (Chahine et al. 2014). In a different study, Gharanei et al, (2013) have shown that treatment with Doxorubicin administered at reperfusion for 120 minutes in the isolated Sprague-Dawley rat hearts was associated with an increase in the infarct to risk ratio in the left ventricle and a pronounced decrease in the cardiac function parameters CF, LVDP and HR (Gharanei, et al, 2013). In this regard, our findings highlighted in this study are consistent with the aforementioned published articles.

In addition, LVDP, CF and HR haemodynamic parameters were also monitored in order to assess the cardiac function. Our results have shown a marked decrease on the CF, LVDP and HR haemodynamic parameters in Doxorubicin treated hearts during reperfusion stage (Figure 5.3, 5.4 and 5.5). These outcomes have been previously confirmed in the Langendorff heart model, which revealed that administration of Doxorubicin at reperfusion resulted in a significant decline in the CF, LVDP and HR cardiac function (Cai et al, 2010; Chahine et al. 2014, Gharanei et al. 2013) As previously highlighted in chapter 3 and 4, our findings show that antioxidant Tiron protected the myocardium against I/R injury alone and against Doxorubicin induced toxicity in normoxic conditions. Interestingly, the haemodynamic and infarct to risk ratio data showed that co-treatment with Tiron (0.25-1mM) and Doxorubicin (1μM) in I/R injury model protected the myocardium by attenuating the damaging effects of Doxorubicin in the myocardium and reversed the decline observed in LVDP, CF and HR haemodynamic parameters thus improving contractility. In a similar manner, our results are in accordance with a previous study carried out in isolated rabbit hearts, in which it was shown that saffron extract (a powerful free radical scavenger) exerted significant protection against Doxorubicin and I/R injury by improving the heart recovery and contractility when administered at reperfusion (Chahine et al. 2014). Also, within the same study, the researchers have investigated the ratio of lipid peroxidase (LP), superoxide dismutase (SOD) and creatine kinase (CK) activity and showed that administration of saffron extract at reperfusion ameliorated the reduction in LP and SOD activity when compared to Doxorubicin and I/R group, whereas the CK activity was abrogated, reaching the control values (Chahine et al. 2014. The outcomes of this study suggest that the protective effects of saffron extract against Doxorubicin-induced cardiotoxicity might occur via its active scavenger properties against free

radicals induced by Doxorubicin. In addition, the results from previous chapters have shown the ability of Tiron to improve the cardiac function and myocardial damage in both I/R injury as well as Doxorubicin induced detrimental effects observed in naïve hearts. Therefore, it is reasonable to conclude that Tiron's cardioprotection against Doxorubicin induced damage in I/R is likely to be associated with its ability to scavenge the free radicals produced by both Doxorubicin and I/R injury.

In this study we have also examined the effects of concomitant administration of Tiron (0.25mM) and Doxorubicin (1µM) during reperfusion on primary isolated rat ventricular cardiomyocytes exposed to 1 hour of hypoxia and 3 hours of reoxygenation. The MTT assay and Trypan blue staining have shown that exposure of isolated rat myocyte cells to H/R and Doxorubicin treatment caused a significant injury to the cells which was shown by decreased cell viability (Figure 5.6 and 5.7). In addition, our results demonstrate that parallel treatment with Tiron (0.25mM) and Doxorubicin (1µM) at reperfusion protects the cardiomyocytes from H/R and Doxorubicin damage by increasing cell viability and decreasing the toxic effects of both Doxorubicin and H/R (Figure 5.6 and 5.7). An investigation carried out in the model of H9c2 cardiac cells have shown that exposure of these cells to 8 and 16 hours of hypoxia, following by 3 hours of reoxygenation with Doxorubicin caused a significant decrease in the cell viability, and concomitant treatment with Doxorubicin and saffron extract exhibited antioxidative properties by offering protection against I/R and Doxorubicin damage and increasing cell viability (Chahine et al. 2016). Therefore, in agreement with the previous studies and based on the findings highlighted in chapter 4, it is reasonable to hypothesise that the cardioprotective effects of Tiron against H/R and Doxorubicin injury are associated with the antioxidant properties of Tiron.

The effects of Tiron (0.25mM) and Doxorubicin (1µM) on the apoptosis and cleaved caspase-3 levels were also examined. Our results have shown that isolated rat ventricular myocytes subjected to 1 hour of hypoxia and 3 hours of reoxygenation showed a significant damage to the cells which was quantified by increased levels of cleaved caspase-3 activity when compared to control, untreated cells (Figure 5.9). Furthermore, treatment with Doxorubicin further enhanced the cell damage and showed a significant elevation in the cleaved-caspase 3, results that are confirmed by previous research papers (Al-Kuraishy and Hussein 2017, Green and Leeuwenburgh 2002b, Shi et al. 2012, Zhao and Zhang 2017). A possible explanation for the increase in cleaved-caspase-3 in H/R cells exposed to Doxorubicin treatment and a decrease in the cell viability supports the hypothesis of the apoptotic cell death mechanism associated with mitochondrial dysfunction and alterations in the mitochondrial membrane potential (Green and Leeuwenburgh 2002a, Marechal et al. 2011, Minotti et al. 2004, Montaigne, Hurt and Neviere 2012). This theory is supported by an investigation carried out by Gharanei et al, (2013) in which it was demonstrated that Doxorubicin induced cardiotoxicity disrupts the mitochondrial activity and integrity in I/R conditions, leading to mitochondrial dysfunction and cell death (Gharanei et al. 2013). A possible mechanism of Tiron's cardioprotection against I/R and Doxorubicin induced toxic effects might be related with its effects on the mitochondrial apoptotic signaling pathway, suggesting that possible cardioprotective mechanism might occur via the mitochondrial transition pore. However, this theory needs further elucidation due to the failure in assessment of apoptosis and necrosis in cardiomyocytes in this setting.

The mitochondrial permeability transition pore opening was suggested to be implicated in both Doxorubicin and I/R induced cell damage and death by disturbing the function and activity of a

number of factors and associated pathways such as intracellular levels of Ca^{2+}, free radical formation and generation, oxidative stress, Bcl-2 family proteins and ATP levels (Halestrap, Clarke and Javadov 2004, Hausenloy, Duchen and Yellon 2003, Murphy and Steenbergen 2008; (Childs et al. 2002, Khan et al. 2006). It has been shown that opening of the mitochondrial transition pore as a result of changes in the mitochondrial permeability leads to the release of apoptotic proteins from the mitochondria and induction of caspase-3 activity, leading to apoptotic cell death pathway (Baines 2009, Halestrap, Clarke and Javadov 2004, Hausenloy, Duchen and Yellon 2003, Weiss et al. 2003). In a previous study, Chahine et al, (2016) have demonstrated that the saffron extract treatment at reperfusion stage had cardioprotective effects against Doxorubicin and H/R injury in H9c2 cells by increasing the cell viability, reducing apoptosis, regulate the mitochondrial membrane potential and inhibit the activity of caspase-3 levels (Chahine et al. 2016). In addition, as previously mentioned in chapter 4, the protective effects of Tiron against chemotherapeutic copper-based drugs on the cardiac mitochondria metabolism and mitochondrial permeability transition pore were reported in the study carried out by Silva-Platas et al, (2016). Therefore, in agreement with these investigations, our results suggest that the cardioprotective effects of Tiron observed in this study could be associated with its mitochondrial targeted antioxidant activity, however the possible mechanism of Tiron on the mitochondrial permeability transition pore in these conditions requires further elucidation. Further investigations that aim to assess the mitochondrial function within the heart such as cytochrome C release and opening of the mitochondrial permeability transition pore could provide a better understanding of Tiron's cardioprotective effects against Doxorubicin induced cardiotoxicity in I/R setting. In addition, due to the potential non-specific binding of the PI staining, future work

should aim to assess the apoptosis and necrosis using different fluorochromes that do not possess overlapping spectra such as Metabolic Activity Dead Cell Apoptosis Kit (Molecular Probes, Thermo Fisher Scientific) containing C12 Resazurin, Annexin V APC and Sytox Green- a three colour fluorescence assay that has the ability to differentiate between the live cells and the cells that are undergoing the early and late phase of apoptosis. This assay could potentially confirm one of the mechanisms by which Tiron is offering protection in the heart.

5.5 Summary of the findings

In summary, the present study provides the first evidence that Tiron has cardioprotective properties against Doxorubicin-induced cardiotoxicity in ischaemia/reperfusion conditions in the Sragues-Dawley rat model. Our results show that treatment with Tiron exerts powerful antioxidant properties by suppressing caspase 3 activation and improving the cell viability in the model of H/R and Doxorubicin induced injury in primary adult rat ventricular myocytes. These outcomes highlight the potential use of Tiron as an adjunctive therapy in patients with pre-existing heart diseases exposed to Doxorubicin chemotherapy.

Chapter 6.General discussion

6.1 Summary of the findings and future work

6.1.1 The effects of Tiron in naïve and I/R conditions

IHD represents the leading cause of mortality worldwide and its incidence is increasing rapidly (Finegold et al. 2013). Clinical interventions carried out for the treatment of CVD such as thrombolysis, coronary artery bypass grafting, and percutaneous coronary angioplasty can effectively reduce the mortality rate (Hausenloy and Yellon, 2013); restoration of the blood flow through a previous ischaemic region can result in myocardial I/R injury (Piper et al, 1998; Hausenloy and Yellon, 2013; Frank et al, 2012). Recent studies carried out in animal models simulating the progression of human myocardial I/R injury have shown that oxidative stress, inflammatory response and apoptotic cell death are the main contributors in the incidence and progression of myocardial I/R injury (Yang, 2018; Granger et al, 2015; Rodrigo et al, 2013; Frangogiannis, 2014; Oyama et al, 2004; Zhao et al, 2000). Thus, research has focused on identifying cardioprotective strategies that could reduce the damaging effects caused by myocardial reperfusion injury. Previous studies have demonstrated that treatment with antioxidants and free radical scavengers could improve the cardiac function and reduce infarct size (Hung et al, 2002; Abe et al, 2008; Sagach et al, 2002). Therefore, in the light of this research and the promising results obtained with antioxidant Tiron in other models (Jiang et al. 2017; Oyewole et al. 2014; Arimura et al, 2001; Bosch et al, 2011. Fleming et al, 2001), the focus of this

thesis was to investigate the potential cardioprotective capacity of Tiron in the ex-vivo heart models using Sprague Dawley rats in naïve and stressed conditions.

The experiments carried out in this thesis found that treatment with increasing concentrations of Tiron (0.25-2.5mM) at reperfusion offered significant cardioprotection in both ex-vivo Langendorff heart model and isolated ventricular adult cardiomyocytes. The protective effects of Tiron were highlighted by a dose-dependent decrease in the myocardial I/R injury as shown by a reduction in the infarct size and an increase in the cell viability in isolated ventricular adult cardiomyocytes. Furthermore, the ability of Tiron to offer cardioprotection when administered at reoxygenation was correlated with its antioxidant and antiapoptotic properties, in which isolated rat ventricular myocytes showed a reduction in the exacerbated production of ROS and a decrease in the caspase-3 activity following treatment with Tiron. In a similar manner, an in vivo model of adult mice exposed to I/R showed that Tiron attenuated the increase in the caspase-3 levels in the myocardium and decreased the apoptotic cell death as detected by Tunel assay (Jiao, et al, 2009). Further investigations should be carried out to assess the apoptotic and necrotic cell death by measuring the concentration of enzymes such as lactate dehydrogenase and creatine kinase activity in the effluents collected during Langendorff experiments. This would potentially identify the mechanisms involved in Tiron's attenuation of infarct sizes in the model of I/R injury.

In addition to the beneficial effects of Tiron against ROS induced damage and apoptotic cell death, this study investigated the effects of Tiron on the myocardial Akt pathway with the purpose to potentially elucidate the intracellular mechanisms of Tiron induced cardioprotection.

Using Western blot, we have shown that the addition of Tiron in the first 20 minutes of reperfusion resulted in a significant increase in the levels of p-Akt. This effect was abrogated by the PI3K inhibitor Wortmannin; activation of PI3K/Akt pathway has been linked with a reduction in the apoptosis and its effectors as well as a decrease in the myocardial I/R injury (Hausenloy and Yellon, 2004; Jian et al, 2016). The results obtained in this study also suggest that Tiron induced activation of Akt pathway in I/R reperfusion could be linked with a reduction in the myocardial I/R injury. To the best of our knowledge, this is the first time Akt levels have been measured in response to Tiron treatment in myocardial I/R injury. Further studies are required to identify the involvement of other survival kinases such as Erk1/2 and JNK are involved in Tiron induced cardioprotection. It would be interesting to evaluate if Tiron exerts is cardioprotective mechanisms via the downstream effectors of Akt pathway such as eNOS and BAD, using techniques such as western blot and flow cytometry to assess the levels of these proteins in the heart tissue and isolated cardiomyocytes.

Our study results suggest that administration of Tiron in the heart reduces H/R induced apoptosis in cardiomyocytes, and this outcome is mediated by a dual effect of the phosphorylation of Akt in the cardiac tissue as well as the attenuation of caspase-3 activation. Several studies have indicated that treatment with cardioprotective agents such as eryhthropoietin (anti-apoptotic), PI3K- gamma/delta inhibitors (anti-inflammatory), and ischaemic post-conditioning (anti-apoptotic and anti-inflammatory) during reperfusion could reduce acute myocardial infarction (Gao et al, 2007; Doukas et al, 2006; Roubille et al, 2011; Hausenloy and Yellon, 2013). Based on these results, our findings suggest that administration of Tiron at reperfusion might be a practical choice in the clinical setting, having a potential in enhancing the benefits of clinical interventions

carried out for myocardial infarction, reducing cardiac tissue damage and improving the quality of life in patients.

In addition to the cardioprotective effects of Tiron in I/R and respectively H/R injury, this study has also investigated the effects of Tiron in naïve conditions in isolated rat heart and primary rat ventricular myocytes. Our results have shown that in normoxic conditions low concentrations of Tiron (0.25-1mM) did not have a negative impact on the heart, however the use of high concentrations of Tiron (2.5-10mM) resulted in cell toxicity, which may suggest Tiron is exhibiting a pro-oxidant effect. Previous investigations carried out with a number of antioxidants in different cell models have shown a similar pattern, which supports our hypothesis with the pro-oxidant properties of Tiron when administered in high concentrations (Azam et al, 2004; DeMarchi et al, 2009; Watjen et al, 2005; Ruiz et al, 2015). Although our results show Tiron at high concentrations has a harmful effect in healthy heart tissue, the use of low concentrations of Tiron could provide a beneficial role in cell protection and prevention against a variety of diseases as a result of oxidative stress.

6.1.2 The effects of Tiron against Doxorubicin induced cardiotoxicity in naïve and stressed conditions

Doxorubicin, an anticancer drug that belongs to anthracyclines family, is considered one of the most potent drug ever developed to treat several types of cancer. Unfortunately, its use has been limited due to cardiotoxic effects that limit its dosage (McGowan et al, 2017; Menna and Salvatorelli, 2017). The use of Doxorubicin based chemotherapy exposes the cancer patient to increased cardiovascular morbidity and mortality (Ewer et al, 2008). Studies have shown that

elderly cancer patients with underlying comorbidities and risk factors (history of heart failure, cardiac dysfunction, hypertension, CAD) exposed to Doxorubicin treatment have a higher risk of developing congestive heart failure (Aapro et al, 2010; Barrett-Lee et al, 2009). The fact that cardiotoxic effects of Doxorubicin are likely to develop in patients of all ages, but with a higher incidence in elderly population calls for development of optimal strategies and pharmacological therapies to prevent or limit Doxorubicin associated cardiotoxic side effects.

Numerous experimental investigations have highlighted that ROS play an important contributing role in the mechanism of cardiotoxicity induced by Doxorubicin suggesting that co-treatment with antioxidants might have promising outcomes in reducing and limiting cardiotoxicity (DeAtley, 1999; Abushouk et al, 2017; Sun et al, 2016). However, the use of antioxidants to prevent cardiotoxicity during cancer treatment with Doxorubicin resulted in a serious debate, as ROS is implicated in destroying cancer cells (Vincent et al. 2013).

Considering this aspect and based on the results obtained in Chapter 3 in which the cardioprotective effects of Tiron were demonstrated, the work within Chapter 4 and 5 highlighted the potential use of Tiron as an adjunctive agent against Doxorubicin induced cardiotoxicity in naïve and simulated I/R conditions. Furthermore, our data showed that addition of Tiron in Doxorubicin treated human leukaemia HL60 and human liver carcinoma HepG2 cells did not interfere with the anticancer efficacy of Doxorubicin in these cell lines. In the clinical context, our findings suggest that combined treatment with Tiron and Doxorubicin could be a beneficial chemotherapeutic strategy that could selectively destroy malignant cells while simultaneously reducing and preventing cardiac damage.

Similar to numerous studies investigating Doxorubicin induced cardiotoxicity, we demonstrated that Doxorubicin treatment had significant detrimental effects on the isolated perfused rat hearts in both normoxic and I/R settings, which were marked by an increase in the infarct ratio and a decrease in the cardiac haemodynamic parameters LVDP, CF and HR (Gharanei et al. 2013; Liu et al. 2002; Chahine et al. 2013). Although the effects observed in this study were observed in rat heart model, similar clinical manifestations such as left ventricular dysfunction, hypertension and arrhythmias have been reported in cancer patients exposed to Doxorubicin treatment (Ewer et al, 2008; Swain et al, 2003). In this study administration of Tiron as an adjunctive therapy successfully reversed the increase in myocardial infarction and improved cardiac function parameters LVDP, CF and HR, implying the potential therapeutic benefits of Tiron when administered in combination with Doxorubicin.

In addition to these findings, our results have shown that concomitant treatment with Tiron and Doxorubicin in isolated rat ventricular myocytes in normoxic conditions completely abolished the increase in oxidative stress observed in Doxorubicin treated groups. The outcomes obtained from Western blot technique showed that administration of Doxorubicin resulted in a significant increase in the levels of p-Akt, which was reversed when co-treated with Tiron in normoxic conditions. The activation of intracellular Akt pathway in Doxorubicin treated hearts has been associated with cardiac hyperthrophy, which may lead to apoptotic cell death (Merten et al. 2006; Shioi et al. 2002). Co-treatment with the PI3K/Akt inhibitor Wortmannin, Tiron and Doxorubicin showed a significant decrease in the phosophorylation of Akt, suggesting that inhibition of Akt pathway could enhance the cardioprotective role of Tiron against Doxorubicin.

Furthermore, this project has demonstrated that treatment with Tiron a was able to successfully reverse the increase in caspase-3 levels that was noted with Doxorubicin treatment alone in naïve and conditions of simulated ischaemia, suggesting a significant potential for Tiron therapy to mediate cellular apoptosis and therefore alter the progression of Doxorubicin associated cell death.

Previous experimental studies have shown that Doxorubicin induced cardiotoxicity has been associated with an overload in the concentration of intracellular mitochondrial Ca^{2+}, which in turn activates the mitochondrial permeability transition pore, leading to damage of the mitochondrial membrane potential, mitochondrial swelling, fissure of the external membrane and subsequently cytochrome c and apoptosis inducing factor (AIF) release from the mitochondria (Varga et al, 2015; Gharanei et al, 2013). Thus, further investigations aiming to assess the mitochondrial function within the heart such as cytochrome C release and opening of the mitochondrial permeability transition pore could provide a better understanding of Tiron's cardioprotective effects against Doxorubicin induced cardiotoxicity in both naïve and I/R settings.

Based on the findings described by Kim et al, (2006) in which treatment with Tiron (1mM) for 72 hours exhibited pro-apoptotic properties in HL60 cells, our work aimed to identify the effects of Tiron (0.25-1mM) and Doxorubicin in HL60 and HepG2 cells. Our results showed that treatment with Tiron (0.25-1mM) for 24 hours in these cell lines did not impact the cell viability, cleaved caspase-3 or ROS levels. Similar findings were reported by Kumar et al, (2013), in which treatment with Tiron (1mM) for 12 and 24 hours in HL60 cells did not produce any significant changes in cell viability cleaved-caspase-3 and ROS levels (Kumar et al, 2013). Future work should aim to

investigate the effects of Tiron in HepG2 and HL60 cell lines to model the extended exposure at longer time points in order to identify the response of cancer cell lines to prolonged treatment with Tiron. However, the anti-cancer properties of Tiron were not the focus of this thesis.

We have also shown that administration of Tiron in Doxorubicin-treated HepG2 and HL60 cells did not interfere with its antineoplastic activity, marked by a decrease in cell viability and enhanced cleaved-caspase-3 levels. However, no significant changes were observed in the ROS activity in response to Doxorubicin alone and co-administered with Tiron, suggesting that Doxorubicin may exert its cytotoxic mechanism via autophagy and apoptosis mediated pathways. However, the effects of combined treatment of Tiron and Doxorubicin on autophagy and apoptosis in these cell lines requires further warrant. Future research should be carried out to identify the effects of Tiron alone and in combination with Doxorubicin on Bax protein, an important regulator of apoptosis and autophagy.

6.2 Study limitations

The purpose of this study was to assess and identify the potential cardioprotective properties of Tiron and its pharmacological effects as an adjunctive agent when used in myocardial I/R injury and combination with Doxorubicin in the Langendorff heart model and isolated adult ventricular myocytes. Within this study we were able to successfully show that Tiron attenuates myocardial damage in the model of simulated I/R injury and against Doxorubicin-induced cardiac damage using ex-vivo Langendorff heart preparations.

One of the main advantages of Langendorff heart preparation is that it allows a preliminary study of the effects of Tiron and Doxorubicin respectively on the heart, as well as enabling the

assessment of myocardial physiological parameters and function. Although this protocol has certain benefits, as with any ex-vivo technique there are a number of limitations implicated with its use. Excising the heart from the animal limits the clinical relevance of the study and it is difficult to predict if the same effects will be observed in an in vivo model. In addition, the lack of hormonal and neuronal control of myocardial function decreases the ability of this procedure to reproduce the physiological circumstances in clinical setting.

Another limitation of this study is the use of isolated ventricular cardiomyocytes to assess the effects of Tiron alone and in combination with Doxorubicin in this study. Although the use of isolated ventricular cardiomyocytes is considered the gold standard procedure in cardiovascular physiology, these cells do not divide (Louch et al. 2011). Therefore, future studies should investigate the effects of these drugs in different cellular models that show similarities to primary cardiomyocytes and have the ability to divide. For example, the H9C2 cells, an immortalised myoblast cell line derived from embryonic rat heart has been previously used as an *in vitro* cardiac model because it possesses several features that resembles the characteristics of primary cardiomyocytes (Parameswaran et al. 2013). Although this cell line does not have the contractile function, it has the ability to proliferate in culture while maintaining the biochemical and electrophysiological properties of primary cardiomyocytes, and therefore being utilised as an animal-free alternative (Parameswaran et al. 2013).

The results from this thesis have shown that addition of Tiron in the first 20 minutes of reperfusion induced the activation of Akt pathway, an effect that could be linked with a reduction in the myocardial injury and a protective mechanism elicited by Tiron. As previously mentioned

in chapter 3, the short-term activation of Akt pathway plays a crucial role in the survival of cardiomyocytes against I/R injury and apoptosis (Fujio et al. 2000, Mullonkal and Toledo-Pereyra, 2007), however sustained activation of Akt pathway has been associated with cardiac hypertrophy, leading to apoptotic cell death (Merten et al. 2006). Further studies are required to identify the effects of Akt pathway beyond the time course used in this project, because it will give a better understanding of the mechanism of action of Tiron on this pathway. Due to the fact that this thesis has only investigated the effects of Tiron via the PI3K/Akt pathway, additional investigations should be carried out to elucidate the involvement of other survival signaling kinases such as Erk ½ and JNK in Tiron induced cardioprotection.

In addition, in a previous investigation carried out in an *in vivo* canine model of tachycardia induced heart failure, it has been reported that treatment with Tiron significantly improved the endothelium vasodilation by enhancing the activity of nitric oxide (Arimura et al. 2001). Cumulative studies have investigated nitric oxide as a downstream target of PI3K/Akt pathway and have demonstrated that nitric oxide plays an important role in cardioprotection due to its antioxidant, vasodilator, and antiplatelet properties (Jugdutt, 2003; Smart et al. 2006). Therefore, it would be interesting to evaluate if Tiron exerts is cardioprotective mechanisms via the downstream targets of Akt pathway such as nitric oxide.

Furthermore, as previously mentioned in chapter 4, the study by Silva-Platas et al. (2016) highlighted the protective effects of Tiron against chemotherapeutic copper-based drugs on the cardiac mitochondria metabolism and mitochondrial permeability transition pore. Investigating

the mitochondrial function in the cardiomyocytes could provide a better insight in understanding Tiron's cardioprotective mechanism against both I/R and Doxorubicin induced cardiotoxicity.

Although the outcomes of this study demonstrated that Tiron has cardioprotective effects against both I/R and Doxorubicin-induced injury in the Langendorff heart model and isolated ventricular cardiomyocytes without impacting the Doxorubicin antineoplastic activity, these effects were not compared and assessed with a second antioxidant. Previous published literature has highlighted the ROS scavenger and anti-apoptotic properties of Tiron, this effect has not been compared with a different antioxidant. For instance, previous studies have assessed and compared the response of Tiron in comparison with a number of antioxidants including the vitamin E analogue Trolox, Tempol, NAC and Resveratrol and demonstrated that Tiron has shown greater protection against ROS in these studies (Vorobeja and Pinegin, 2016; Oyewole et al. 2014, Yang et al. 2007). Even though the results from published literature have shown the positive outcomes with Tiron, it is important that further studies to be carried out in order to identify if the same response will be observed in the heart and in combination with anti-cancer therapies.

As previously mentioned in the introduction Tiron is a vitamin E analogue, a SOD mimetic and a mitochondria localised antioxidant. Due to the fact that Tiron has the ability to penetrate and accumulate in the organelle and the availability of binding sites, the combination of this antioxidant with Doxorubicin should be further investigated. In regard to this, a previous study explored the mechanism of action of Tiron alone and in combination with a number of nutritional supplements including Vitamin E, selenium and zinc against vanadium intoxication in female albino rats (Shrivastava et al. 2007). It was demonstrated that administration of Vanadium in

female albino rats resulted in a significant injury which was marked by an increase in the activities of serum transaminases, serum alkaline phosphatases, lactate dehydrogenase and hepatic lipid peroxidation as well as a substantial reduction in glutathione levels and glucose-6-phosphatase in the liver. Interestingly, co-administration of Tiron alone reduced these effects, however significant recovery has been reported when Tiron was co-administered with vitamin E, zinc and selenium. The study has also revealed that upon administration with Tiron the concentration of vanadium significantly declined in the kidney and liver and the urinary excretion of vanadium was also enhanced (Shrivastava et al. 2007).

A possible explanation for Tiron's protective effects could be associated with its chelation properties as well as the availability of the binding sites within its structure and the stability constant of the metal chelator complex. The authors have also implied that Tiron could replace one of the hydrogen atoms and bind to vanadium with its oxygen atom, leading to the formation of a stable complex that reverses the toxic effects of vanadium (Shrivastava et al. 2007). Therefore, future studies should be carried out in order to elucidate the pharmacokinetic properties of Tiron in the presence and absence of Doxorubicin to investigate if a similar pattern is detected.

This study has also shown the potential range of concentrations of Tiron that are safe to use in the isolated perfused heart preparation is lower than the concentrations used previously in different physiological systems where the typical concentrations were ranging between 3-10mM. However, it is well known that the heart is more susceptible to cellular damage. Within the preclinical context, there is an earlier study published by Ortega et al, (1991) which assessed the

effects of Tiron in pregnant Swiss mice revealed that treatment with an average dose of Tiron did not exhibit any toxic effects, whereas high concentrations of Tiron (3000mg/kg/day) resulted in a significant increase in the death ratio, a decrease in the body weight and substantial alterations in the kidneys and liver weights as well as fetal toxicity (Ortega et al, 1991). Therefore, future studies should investigate the effects of Tiron within the in vivo models, and particularly in vivo tumour-bearing models as it will give a better preclinical assessment of the effects of Tiron in the heart and the interactions with the anti-cancer therapies. This will be an important step in the progression and translation into clinical trials. However, in order to advance to clinical investigations, the exact mechanism of Tiron induced-cardioprotection and its safe therapeutic dose needs to be fully elucidated.

6.3 Conclusion

The work carried out in this thesis successfully showed the cardioprotective effects of Tiron in both I/R and Doxorubicin induced-cardiotoxicity. To date, this is the first study that shows that Tiron cardioprotective effects occur via the Akt pathway in the Langendorff heart model exposed to I/R conditions. In addition, the work within this thesis has shown for the first time that Tiron successfully reversed the increase in ROS and cleaved-caspase-3 levels in both I/R and Doxorubicin induced damage in cardiomyocytes. The results from this thesis have also revealed that co-administration of Tiron with Doxorubicin did not affect the anti-cancer properties of Doxorubicin.

Importantly, the first results chapter (Chapter 3) has shown that administration of increasing concentrations of Tiron (0.25-2.5mM) during reperfusion had significant cardioprotective

responses in both isolated rat heart Langendorff perfusion model and primary ventricular cardiomyocytes. To our knowledge, no other study has investigated the cardioprotective effects of Tiron treatment in the heart. The majority of published investigations have used Tiron as a metal chelator and a ROS scavenger in different models (Jiao et al., 2009; Arimura et al. 2001). In addition, other experimental studies have only used one concentration of Tiron. For example, the study carried out by Prado et al. (2018) investigated the effects of Tiron at a concentration of 10mM in isolated Sprague-Dawley hearts exposed to acute episodes of ischaemia and showed that treatment with Tiron significantly increased the antioxidant power in the myocardial tissue when compared to the control group (Prado et al. 2018). However, no additional concentrations were tested in this study. The results in this thesis revealed for the first time the range of concentrations of Tiron that offer cardioprotective properties in the model of I/R injury. In addition to the cardioprotective properties of Tiron in I/R injury, this study has also investigated the effects of Tiron in naïve conditions. Our data has shown that low concentrations of Tiron (0.25-1mM) did not have an impact on the myocardium and primary ventricular myocytes, however the use of higher concentrations (2.5-10mM) resulted in cell toxicity, implying that Tiron exhibits a pro-oxidant effect in the heart. To date, there are no studies that have evaluated the effects of increasing concentrations of Tiron in normoxic conditions in the myocardium. However, in a similar manner, in an earlier *in vivo* investigation, Ortega et al. (1991) studies the effects of Tiron in pregnant Swizz mice and showed that treatment with low and average concentrations of Tiron did not reveal any toxic effects, whereas high concentrations of Tiron caused a significant increase in the death rates as well as a decline in the body weight, impairment in the kidney function and fetal toxicity. These findings suggest that treatment with Tiron is dose-dependent.

Although high concentrations of Tiron can have a harmful effect in the healthy heart tissue, the use of low concentrations of Tiron could be a valuable treatment in protecting against ROS-induced diseases.

Chapter 3 also showed the involvement of Akt pathway in Tiron induced cardioprotection in I/R conditions. Administration of Tiron stimulated the activation of Akt during the first 20 minutes of reperfusion, effect that was completely inhibited by Wortmannin. The results of this study also revealed that Tiron exhibited a potent antioxidative effect which was marked by a reduction in the H_2O_2 induced oxidative stress, a decrease in ROS production and caspase-3 activity. The involvement of Akt pathway in Tiron-induced cardioprotection has not been previously investigated, therefore further studies which investigate the other survival signalling proteins (Erk ½, JNK) could be important for establishing the cardioprotective mechanisms of Tiron.

The work carried out within chapter 4 and 5 emphasised the potential of Tiron as an adjunctive therapy against Doxorubicin induced cardiotoxicity in naïve and I/R conditions. Administration of Tiron (0.25-1mM) diminished Doxorubicin-induced myocardial infarction and significantly ameliorated the haemodynamic cardiac function. Our results have shown that Tiron completely abolished Doxorubicin-induced oxidative stress and decreased the levels of p-Akt observed in Doxorubicin treated hearts. As mentioned previously, the sustained stimulation of Akt pathway has been linked with cardiac hypertrophy and cell death (Merten et al. 2006; Shioi et al. 2002). Interestingly, co-administration of both Tiron and Wortmannin in Doxorubicin treated hearts significantly reduced Akt phosphorylation and reversed the decline in cardiac parameters by

Doxorubicin. These findings suggest that inhibition of Akt pathway could enhance the cardioprotective role of Tiron against Doxorubicin.

Furthermore, in this thesis Tiron demonstrated powerful antioxidative properties which were indicated by a decline in the elevated caspase-3 levels observed in Doxorubicin treated cardiomyocytes in both naïve H/R conditions. This suggest that Tiron possesses anti-apoptotic mechanism and its potential use as a therapy in mediating the off-target cardiotoxic effects exhibited by the treatment with the anti-cancer drug Doxorubicin.

Finally, the presented work demonstrates that besides that beneficial effects on cardiac function, Tiron did not inhibit the antineoplastic properties of Doxorubicin in both HL60 and HepG2 cancer cell lines. It is desirable and essential that a cardioprotective adjunctive therapy could be designed to maintain and preserve the anticancer properties of Doxorubicin. This study highlights the potential of Tiron as an adjunctive therapy that selectively reduces the off-target cardiotoxic effects of Doxorubicin without impacting the anti-cancer activity.

Chapter 7.References

Aapro, M., Bernard-Marty, C., Brain, E., Batist, G., Erdkamp, F., Krzemieniecki, K., Leonard, R., Lluch, A., Monfardini, S., and Ryberg, M. (2010) 'Anthracycline Cardiotoxicity in the Elderly Cancer Patient: A SIOG Expert Position Paper'. Annals of Oncology 22 (2), 257-267

Abdel-Sattar, E., El-Gayed, S., Shehata, I., Ashour, O. M., Nagy, A. A., and Mohamadin, A. M. (2012) 'Antioxidant and Cardioprotective Activity of Stachys Schimperi Vatke Against Doxorubicin-Induced Cardiotoxicity'. Bulletin of Faculty of Pharmacy, Cairo University 50 (1), 41-47

Abe, M., Takiguchi, Y., Ichimaru, S., Tsuchiya, K., and Wada, K. (2008) 'Comparison of the Protective Effect of N-Acetylcysteine by Different Treatments on Rat Myocardial Ischemia-Reperfusion Injury'. Journal of Pharmacological Sciences 106 (4), 571-577

Abel, E. D. and Doenst, T. (2011) 'Mitochondrial Adaptations to Physiological Vs. Pathological Cardiac Hypertrophy'. Cardiovascular Research 90 (2), 234-242

Abushouk, A. I., Ismail, A., Salem, A. M. A., Afifi, A. M., and Abdel-Daim, M. (2017) 'Cardioprotective Mechanisms of Phytochemicals Against Doxorubicin-Induced Cardiotoxicity'. Biomedicine & Pharmacotherapy 90, 935-946

Adameova, A., Goncalvesova, E., Szobi, A., and Dhalla, N. S. (2016) 'Necroptotic Cell Death in Failing Heart: Relevance and Proposed Mechanisms'. Heart Failure Reviews 21 (2), 213-221

Adlam, V. J., Harrison, J. C., Porteous, C. M., James, A. M., Smith, R. A., Murphy, M. P., and Sammut, I. A. (2005) 'Targeting an Antioxidant to Mitochondria Decreases Cardiac Ischemia-Reperfusion Injury'. FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology 19 (9), 1088-1095

Ahmed, H. H., Mannaa, F., Elmegeed, G. A., and Doss, S. H. (2005) 'Cardioprotective Activity of Melatonin and its Novel Synthesized Derivatives on Doxorubicin-Induced Cardiotoxicity'. Bioorganic & Medicinal Chemistry 13 (5), 1847-1857

Akhlaghi, M. and Bandy, B. (2009) 'Mechanisms of Flavonoid Protection Against Myocardial ischemia–reperfusion Injury'. Journal of Molecular and Cellular Cardiology 46 (3), 309-317

Alderton, W. K., Cooper, C. E., and Knowles, R. G. (2001) 'Nitric Oxide Synthases: Structure, Function and Inhibition'. The Biochemical Journal 357, 593-615

Aldieri, E., Bergandi, L., Riganti, C., Costamagna, C., Bosia, A., and Ghigo, D. (2002) 'Doxorubicin Induces an Increase of Nitric Oxide Synthesis in Rat Cardiac Cells that is Inhibited by Iron Supplementation'. Toxicology and Applied Pharmacology 185 (2), 85-90

Al-Harthi, S. E., Alarabi, O. M., Ramadan, W. S., Alaama, M. N., Al-Kreathy, H. M., Damanhouri, Z. A., Khan, L. M., and Osman, A. M. (2014) 'Amelioration of doxorubicin-induced Cardiotoxicity by Resveratrol'. Molecular Medicine Reports 10 (3), 1455-1460

Al-Kuraishy, H. and Hussein, R. G. (2017) 'Caspase-3 Levels (CASP-3) in Doxorubicin Induced-Cardiotoxicity: Role of Metformin Pretreatment'. Research Journal of Oncology 1 (1)

Almeida, D., Pinho, R., Correia, V., Soares, J., Bastos, M., Carvalho, F., Capela, J., and Costa, V. (2018) 'Mitoxantrone is More Toxic than Doxorubicin in SH-SY5Y Human Cells: A 'Chemobrain'In Vitro Study'. Pharmaceuticals 11 (2), 41

Alpsoy, S., Aktas, C., Uygur, R., Topcu, B., Kanter, M., Erboga, M., Karakaya, O., and Gedikbasi, A. (2013) 'Antioxidant and anti-apoptotic Effects of Onion (Allium Cepa) Extract on doxorubicin-induced Cardiotoxicity in Rats'. Journal of Applied Toxicology 33 (3), 202-208

Ambrosio, G., Zweier, J. L., and Flaherty, J. T. (1991) 'The Relationship between Oxygen Radical Generation and Impairment of Myocardial Energy Metabolism Following Post-Ischemic Reperfusion'. Journal of Molecular and Cellular Cardiology 23 (12), 1359-1374

Ananthanarayanan Ajith, T., Hema, U., and Aswathi, S. (2016) 'Zingiber Officinale Roscoe Ameliorates Anticancer Antibiotic Doxorubicin-Induced Acute Cardiotoxicity in Rat'. Journal of Experimental Therapeutics & Oncology 11 (3)

Andreadou, I., Mikros, E., Ioannidis, K., Sigala, F., Naka, K., Kostidis, S., Farmakis, D., Tenta, R., Kavantzas, N., Bibli, S. I., Gikas, E., Skaltsounis, L., Kremastinos, D. T., and Iliodromitis, E. K. (2014) 'Oleuropein Prevents Doxorubicin-Induced Cardiomyopathy Interfering with Signaling Molecules and Cardiomyocyte Metabolism'. Journal of Molecular and Cellular Cardiology 69, 4-16

Angsutararux, P., Luanpitpong, S., and Issaragrisil, S. (2015) 'Chemotherapy-Induced Cardiotoxicity: Overview of the Roles of Oxidative Stress'. Oxidative Medicine and Cellular Longevity 2015

Antunes, F., Erustes, A. G., Costa, A. J., Nascimento, A. C., Bincoletto, C., Ureshino, R. P., Pereira, G. J. S., and Smaili, S. S. (2018) 'Autophagy and Intermittent Fasting: The Connection for Cancer Therapy?'. Clinics 73

Anversa, P., Cheng, W., Liu, Y., Leri, A., Redaelli, G., and Kajstura, J. (1998) 'Apoptosis and Myocardial Infarction'. Basic Research in Cardiology 93 (3), s008-s012

Aoyagi, T. and Matsui, T. (2011) 'Phosphoinositide-3 Kinase Signaling in Cardiac Hypertrophy and Heart Failure'. Current Pharmaceutical Design 17 (18), 1818-1824

Arimura, K., Egashira, K., Nakamura, R., Ide, T., Tsutsui, H., Shimokawa, H., and Takeshita, A. (2001) 'Increased Inactivation of Nitric Oxide is Involved in Coronary Endothelial Dysfunction in Heart Failure'. American Journal of Physiology.Heart and Circulatory Physiology 280 (1), H68-75

Armstrong, G. T., Plana, J. C., Zhang, N., Srivastava, D., Green, D. M., Ness, K. K., Daniel Donovan, F., Metzger, M. L., Arevalo, A., and Durand, J. (2012) 'Screening Adult Survivors of Childhood Cancer for Cardiomyopathy: Comparison of Echocardiography and Cardiac Magnetic Resonance Imaging'. Journal of Clinical Oncology 30 (23), 2876-2884

Armstrong, S. C. (2004) 'Protein Kinase Activation and Myocardial ischemia/reperfusion Injury'. Cardiovascular Research 61 (3), 427-436

Aryal, B., Jeong, J., and Rao, V. A. (2014) 'Doxorubicin-Induced Carbonylation and Degradation of Cardiac Myosin Binding Protein C Promote Cardiotoxicity'. Proceedings of the National Academy of Sciences of the United States of America 111 (5), 2011-2016

Asensio-López, M. C., Soler, F., Pascual-Figal, D., Fernández-Belda, F., and Lax, A. (2017) 'Doxorubicin-Induced Oxidative Stress: The Protective Effect of Nicorandil on HL-1 Cardiomyocytes'. PloS One 12 (2), e0172803

Atkinson, A. and Winge, D. R. (2009) 'Metal Acquisition and Availability in the Mitochondria'. Chemical Reviews 109 (10), 4708-4721

Azam, S., Hadi, N., Khan, N. U., and Hadi, S. M. (2004) 'Prooxidant Property of Green Tea Polyphenols Epicatechin and Epigallocatechin-3-Gallate: Implications for Anticancer Properties'. Toxicology in Vitro 18 (5), 555-561

Babior, B. M. (2004) 'NADPH Oxidase'. Current Opinion in Immunology 16 (1), 42-47

Badalzadeh, R., Mokhtari, B., and Yavari, R. (2015) 'Contribution of Apoptosis in Myocardial Reperfusion Injury and Loss of Cardioprotection in Diabetes Mellitus'. The Journal of Physiological Sciences 65 (3), 201-215

Badavi, M., Sadeghi, N., Dianat, M., and Samarbafzadeh, A. (2017) 'Gallic Acid and Cyclosporine Mixture and their Effects on Cardiac Dysfunction Induced by Ischemia/Reperfusion and eNOS/iNOS Expression'. International Journal of Cardiovascular Sciences 30 (3), 207-218

Baines, C. P. (2009) 'The Mitochondrial Permeability Transition Pore and Ischemia-Reperfusion Injury'. Basic Research in Cardiology 104 (2), 181-188

Balaban, R. S. (2006) 'Maintenance of the Metabolic Homeostasis of the Heart'. Annals of the New York Academy of Sciences 1080 (1), 140-153

Balducci, L. and Extermann, M. (2000) 'Cancer and Aging. an Evolving Panorama'. Hematology/oncology Clinics of North America 14 (1), 1-16

Bandyopadhyay, D., Chattopadhyay, A., Ghosh, G., and Datta, A. G. (2004) 'Oxidative Stress-Induced Ischemic Heart Disease: Protection by Antioxidants'. Current Medicinal Chemistry 11 (3), 369-387

Bar-Joseph, H., Stemmer, S. M., Tsarfaty, I., Shalgi, R., and Ben-Aharon, I. (2015) 'Chemotherapy-Induced Vascular Toxicity--Real-Time in Vivo Imaging of Vessel Impairment'. Journal of Visualized Experiments : JoVE (95):e51650. doi (95), e51650

Barrett-Lee, P., Dixon, J. M., Farrell, C., Jones, A., Leonard, R., Murray, N., Palmieri, C., Plummer, C. J., Stanley, A., and Verrill, M. W. (2009) 'Expert Opinion on the use of Anthracyclines in Patients with Advanced Breast Cancer at Cardiac Risk'. Annals of Oncology : Official Journal of the European Society for Medical Oncology 20 (5), 816-827

Basinger, M. A. and Jones, M. M. (1981) 'Tiron (Sodium 4,5-Dihydroxybenzene-1,3-Disulfonate) as an Antidote for Acute Uranium Intoxication in Mice'. Research Communications in Chemical Pathology and Pharmacology 34 (2), 351-358

Becker, L. B. (2004) 'New Concepts in Reactive Oxygen Species and Cardiovascular Reperfusion Physiology'. Cardiovascular Research 61 (3), 461-470

Becker, L. B., vanden Hoek, T. L., Shao, Z., Li, C., and Schumacker, P. T. (1999) 'Generation of Superoxide in Cardiomyocytes during Ischemia before Reperfusion'. American Journal of Physiology-Heart and Circulatory Physiology 277 (6), H2240-H2246

Bedard, K. and Krause, K. (2007) 'The NOX Family of ROS-Generating NADPH Oxidases: Physiology and Pathophysiology'. Physiological Reviews 87 (1), 245-313

Bell, R. M., Mocanu, M. M., and Yellon, D. M. (2011) 'Retrograde Heart Perfusion: The Langendorff Technique of Isolated Heart Perfusion'. Journal of Molecular and Cellular Cardiology 50 (6), 940-950

Belmonte, F., Das, S., Sysa-Shah, P., Sivakumaran, V., Stanley, B., Guo, X., Paolocci, N., Aon, M. A., Nagane, M., Kuppusamy, P., Steenbergen, C., and Gabrielson, K. (2015) 'ErbB2 Overexpression Upregulates Antioxidant Enzymes, Reduces Basal Levels of Reactive Oxygen Species, and Protects Against Doxorubicin Cardiotoxicity'. American Journal of Physiology.Heart and Circulatory Physiology 309 (8), H1271-80

Benchekroun, M. N., Pourquier, P., Schott, B., and Robert, J. (1993) 'Doxorubicin-Induced Lipid Peroxidation and Glutathione Peroxidase Activity in Tumor Cell Lines Selected for Resistance to Doxorubicin'. European Journal of Biochemistry 211 (1-2), 141-146

Beretta, G. L. and Zunino, F. (2007) 'Molecular Mechanisms of Anthracycline Activity'. in . ed. by Anon: Springer, 1-19

Berg, J. M., Tymoczko, J. L., and Stryer, L. (2012) 'Oxidative Phosphorylation'. Biochemistry.WH Freeman, New York

Berthiaume, J. M. and Wallace, K. B. (2007) 'Adriamycin-Induced Oxidative Mitochondrial Cardiotoxicity'. Cell Biology and Toxicology 23 (1), 15-25

Bhaskar, P. T. and Hay, N. (2007) 'The Two TORCs and AKT'. Developmental Cell 12 (4), 487-502

Bien, S., Riad, A., Ritter, C. A., Gratz, M., Olshausen, F., Westermann, D., Grube, M., Krieg, T., Ciecholewski, S., Felix, S. B., Staudt, A., Schultheiss, H. P., Ewert, R., Volker, U., Tschope, C., and Kroemer, H. K. (2007) 'The Endothelin Receptor Blocker Bosentan Inhibits Doxorubicin-Induced Cardiomyopathy'. Cancer Research 67 (21), 10428-10435

Birnie, G. D. (1988) 'The HL60 Cell Line: A Model System for Studying Human Myeloid Cell Differentiation'. *The British Journal of Cancer.Supplement* 9, 41-45

Blaustein, A., Deneke, S. M., Stolz, R. I., Baxter, D., Healey, N., and Fanburg, B. L. (1989) 'Myocardial Glutathione Depletion Impairs Recovery After Short Periods of Ischemia'. Circulation 80 (5), 1449-1457

Bolli, R. and Marbán, E. (1999) 'Molecular and Cellular Mechanisms of Myocardial Stunning'. Physiological Reviews 79 (2), 609-634

Bolli, R. (1991) 'Oxygen-Derived Free Radicals and Myocardial Reperfusion Injury: An Overview'. Cardiovascular Drugs and Therapy 5 (2), 249-268

Bolooki, H. M. and Askari, A. (2010) 'Acute Myocardial Infarction'. Disease Manag Proj

Bonomini, F., Tengattini, S., Fabiano, A., Bianchi, R., and Rezzani, R. (2008) 'Atherosclerosis and Oxidative Stress'. Histology and Histopathology 23 (3), 381-390

Borchi, E., Bargelli, V., Stillitano, F., Giordano, C., Sebastiani, M., Nassi, P. A., d'Amati, G., Cerbai, E., and Nediani, C. (2010) Enhanced ROS Production by NADPH Oxidase is Correlated to Changes in Antioxidant Enzyme Activity in Human Heart Failure.

Bouayed, J. and Bohn, T. (2010) 'Exogenous antioxidants—double-Edged Swords in Cellular Redox State: Health Beneficial Effects at Physiologic Doses Versus Deleterious Effects at High Doses'. Oxidative Medicine and Cellular Longevity 3 (4), 228-237

Bozcali, E., Dedeoglu, D. B., Karpuz, V., Suzer, O., and Karpuz, H. (2012) 'Cardioprotective Effects of Zofenopril, Enalapril and Valsartan Against ischaemia/reperfusion Injury as Well as Doxorubicin Cardiotoxicity'. Acta Cardiologica 67 (1), 87-96

Bozulic, L. and Hemmings, B. A. (2009) 'PIKKing on PKB: Regulation of PKB Activity by Phosphorylation'. Current Opinion in Cell Biology 21 (2), 256-261

Brand, M. D. (2010) 'The Sites and Topology of Mitochondrial Superoxide Production'. Experimental Gerontology 45 (7-8), 466-472

Braunersreuther, V. and Jaquet, V. (2012) 'Reactive Oxygen Species in Myocardial Reperfusion Injury: From Physiopathology to Therapeutic Approaches'. Current Pharmaceutical Biotechnology 13 (1), 97-114

Braunwald, E. and Kloner, R. A. (1985) 'Myocardial Reperfusion: A Double-Edged Sword?'. The Journal of Clinical Investigation 76 (5), 1713-1719

British, H. F. (2015) 'Cardiovascular Disease Statistics 2015'

Brown, J. M. (1993) 'SR 4233 (Tirapazamine): A New Anticancer Drug Exploiting Hypoxia in Solid Tumours'. British Journal of Cancer 67 (6), 1163

Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999) 'Akt Promotes Cell Survival by Phosphorylating and Inhibiting a Forkhead Transcription Factor'. Cell 96 (6), 857-868

Bryan, R. M.,J, Steenberg, M. L., and Marrelli, S. P. (2001) 'Role of Endothelium in Shear Stress-Induced Constrictions in Rat Middle Cerebral Artery'. Stroke 32 (6), 1394-1400

Buja, L. M. and Weerasinghe, P. (2010) 'Unresolved Issues in Myocardial Reperfusion Injury'. Cardiovascular Pathology 19 (1), 29-35

Buja, L. M. and Vela, D. (2008) 'Cardiomyocyte Death and Renewal in the Normal and Diseased Heart'. Cardiovascular Pathology 17 (6), 349-374

Buja, L. M. (2005) 'Myocardial Ischemia and Reperfusion Injury'. Cardiovascular Pathology 14 (4), 170-175

Buja, L. M. and Willerson, J. T. (1981) 'Abnormalities of Volume Regulation and Membrane Integrity in Myocardial Tissue Slices After Early Ischemic Injury in the Dog: Effects of Mannitol, Polyethylene Glycol, and Propranolol'. The American Journal of Pathology 103 (1), 79-95

Burstein, B., Maguy, A., Clement, R., Gosselin, H., Poulin, F., Ethier, N., Tardif, J. C., Hebert, T. E., Calderone, A., and Nattel, S. (2007) 'Effects of Resveratrol (Trans-3,5,4'-Trihydroxystilbene) Treatment on Cardiac Remodeling Following Myocardial Infarction'. The Journal of Pharmacology and Experimental Therapeutics 323 (3), 916-923

C Bompotis, ,Georgios, Deftereos, S., Angelidis, C., Choidis, E., Panagopoulou, V., Kaoukis, A., P Vassilikos, ,Vassilios, W Cleman, ,Michael, and Giannopoulos, G. (2016) 'Altered Calcium Handling in Reperfusion Injury'. Medicinal Chemistry 12 (2), 114-130

Cadenas, S. (2018) ROS and Redox Signaling in Myocardial Ischemia-Reperfusion Injury and Cardioprotection.

Cai, C., Lothstein, L., Morrison, R. R., and Hofmann, P. A. (2010) 'Protection from Doxorubicin-Induced Cardiomyopathy using the Modified Anthracycline N-Benzyladriamycin-14-Valerate (AD 198)'. The Journal of Pharmacology and Experimental Therapeutics 335 (1), 223-230

Cai, J., Jing, D., Shi, M., Liu, Y., Lin, T., Xie, Z., Zhu, Y., Zhao, H., Shi, X., and Du, F. (2014) 'Epigallocatechin Gallate (EGCG) Attenuates Infrasound-Induced Neuronal Impairment by Inhibiting Microglia-Mediated Inflammation'. The Journal of Nutritional Biochemistry 25 (7), 716-725

Camacho, P., Fan, H., Liu, Z., and He, J. Q. (2016) 'Small Mammalian Animal Models of Heart Disease'. American Journal of Cardiovascular Disease 6 (3), 70-80

Camaggi, C. M., Comparsi, R., Strocchi, E., Testoni, F., Angelelli, B., and Pannuti, F. (1988) 'Epirubicin and Doxorubicin Comparative Metabolism and Pharmacokinetics. A Cross-Over Study'. Cancer Chemotherapy and Pharmacology 21 (3), 221-228

Cancer Research, U. (2014) 'Cancer Statistics for the UK'

Cantley, L. C. (2002) 'The Phosphoinositide 3-Kinase Pathway'. Science (New York, N.Y.) 296 (5573), 1655-1657

Cao, J., Jiang, L., Liu, Y., Yang, G., Yao, X., and Zhong, L. (2007) 'Curcumin-Induced Genotoxicity and Antigenotoxicity in HepG2 Cells'. *Toxicon* 49 (8), 1219-1222

Cao, Y., Ruan, Y., Shen, T., Huang, X., Li, M., Yu, W., Zhu, Y., Man, Y., Wang, S., and Li, J. (2014) 'Astragalus Polysaccharide Suppresses Doxorubicin-Induced Cardiotoxicity by Regulating the PI3k/Akt and p38MAPK Pathways'. Oxidative Medicine and Cellular Longevity 2014, 674219

Carden, D. L. and Granger, D. N. (2000) 'Pathophysiology of Ischaemia-Reperfusion Injury'. The Journal of Pathology 190 (3), 255-266

Cardinale, D., Colombo, A., Bacchiani, G., Tedeschi, I., Meroni, C. A., Veglia, F., Civelli, M., Lamantia, G., Colombo, N., Curigliano, G., Fiorentini, C., and Cipolla, C. M. (2015) 'Early Detection of Anthracycline Cardiotoxicity and Improvement with Heart Failure Therapy'. Circulation 131 (22), 1981-1988

Cardinale, D., Colombo, A., Lamantia, G., Colombo, N., Civelli, M., De Giacomi, G., Rubino, M., Veglia, F., Fiorentini, C., and Cipolla, C. M. (2010) 'Anthracycline-Induced Cardiomyopathy: Clinical Relevance and Response to Pharmacologic Therapy'. Journal of the American College of Cardiology 55 (3), 213-220

Carnero, A., Blanco-Aparicio, C., Renner, O., Link, W., and Leal, J. F. M. (2008) 'The PTEN/PI3K/AKT Signalling Pathway in Cancer, Therapeutic Implications'. Current Cancer Drug Targets 8 (3), 187-198

Carvalho, R. A., Sousa, R. P. B., Cadete, V. J. J., Lopaschuk, G. D., Palmeira, C. M. M., Bjork, J. A., and Wallace, K. B. (2010) 'Metabolic Remodeling Associated with Subchronic Doxorubicin Cardiomyopathy'. Toxicology 270 (2), 92-98

Catalucci, D. and Condorelli, G. (2006) . Effects of Akt on Cardiac Myocytes: Location Counts

Chahine, N., Nader, M., Duca, L., Martiny, L., and Chahine, R. (2016) 'Saffron Extracts Alleviate Cardiomyocytes Injury Induced by Doxorubicin and Ischemia-Reperfusion in Vitro'. Drug and Chemical Toxicology 39 (1), 87-96

Chahine, N., Makhlouf, H., Duca, L., Martiny, L., and Chahine, R. (2014) 'Cardioprotective Effect of Saffron Extracts Against Acute Doxorubicin Toxicity in Isolated Rabbit Hearts Submitted to Ischemia-Reperfusion Injury'. Zeitschrift f ├╚ r Naturforschung C 69 (11-12), 459-470

Chan, D. W., Liu, V. W., Tsao, G. S., Yao, K. M., Furukawa, T., Chan, K. K., and Ngan, H. Y. (2008) 'Loss of MKP3 Mediated by Oxidative Stress Enhances Tumorigenicity and Chemoresistance of Ovarian Cancer Cells'. Carcinogenesis 29 (9), 1742-1750

Chang, W., Li, J., Haung, H., Liu, H., Han, M., Ramachandran, S., Li, C., Sharp, W. W., Hamann, K. J., and Yuan, C. (2011) 'Baicalein Protects Against doxorubicin-induced Cardiotoxicity by Attenuation of Mitochondrial Oxidant Injury and JNK Activation'. Journal of Cellular Biochemistry 112 (10), 2873-2881

Chang, H., Li, C., Wang, Q., Lu, L., Zhang, Q., Zhang, Y., Zhang, N., Wang, Y., and Wang, W. (2017) 'QSKL Protects Against Myocardial Apoptosis on Heart Failure Via PI3K/Akt-p53 Signaling Pathway'. Scientific Reports 7 (1), 16986

Chang, W., Wu, Q., Xiao, Y., Jiang, X., Yuan, Y., Zeng, X., and Tang, Q. (2017) 'Acacetin Protects Against Cardiac Remodeling After Myocardial Infarction by Mediating MAPK and PI3K/Akt Signal Pathway'. Journal of Pharmacological Sciences 135 (4), 156-163

Chatterjee, K., Zhang, J., Honbo, N., and Karliner, J. S. (2010) 'Doxorubicin Cardiomyopathy'. Cardiology 115 (2), 155-162

Chen, Z., Siu, B., Ho, Y., Vincent, R., Chua, C. C., Hamdy, R. C., and Chua, B. H. (1998) 'Overexpression of MnSOD Protects Against Myocardial ischemia/reperfusion Injury in Transgenic Mice'. Journal of Molecular and Cellular Cardiology 30 (11), 2281-2289

Chen, B., Peng, X., Pentassuglia, L., Lim, C. C., and Sawyer, D. B. (2007) 'Molecular and Cellular Mechanisms of Anthracycline Cardiotoxicity'. Cardiovascular Toxicology 7 (2), 114-121

Chen, K., Craige, S. E., and Keaney Jr, J. F. (2009) 'Downstream Targets and Intracellular Compartmentalization in Nox Signaling'. Antioxidants & Redox Signaling 11 (10), 2467-2480

Chen, Q., Xu, T., Li, D., Pan, D., Wu, P., Luo, Y., Ma, Y., and Liu, Y. (2016) 'JNK/PI3K/Akt Signaling Pathway is Involved in Myocardial ischemia/reperfusion Injury in Diabetic Rats: Effects of Salvianolic Acid A Intervention'. American Journal of Translational Research 8 (6), 2534-2548

Chen, Y. R. and Zweier, J. L. (2014) 'Cardiac Mitochondria and Reactive Oxygen Species Generation'. Circulation Research 114 (3), 524-537

Chen, Z., Siu, B., Ho, Y., Vincent, R., Chua, C. C., Hamdy, R. C., and Chua, B. H. L. (1998) 'Overexpression of MnSOD Protects Against Myocardial ischemia/reperfusion Injury in Transgenic Mice'. Journal of Molecular and Cellular Cardiology 30 (11), 2281-2289

Cheng, E. H., Sheiko, T. V., Fisher, J. K., Craigen, W. J., and Korsmeyer, S. J. (2003) 'VDAC2 Inhibits BAK Activation and Mitochondrial Apoptosis'. Science (New York, N.Y.) 301 (5632), 513-517

Childs, A. C., Phaneuf, S. L., Dirks, A. J., Phillips, T., and Leeuwenburgh, C. (2002) 'Doxorubicin Treatment in Vivo Causes Cytochrome C Release and Cardiomyocyte Apoptosis, as Well as Increased Mitochondrial Efficiency, Superoxide Dismutase Activity, and Bcl-2:Bax Ratio'. Cancer Research 62 (16), 4592-4598

Chiong, M., Wang, Z. V., Pedrozo, Z., Cao, D. J., Troncoso, R., Ibacache, M., Criollo, A., Nemchenko, A., Hill, J. A., and Lavandero, S. (2011) 'Cardiomyocyte Death: Mechanisms and Translational Implications'. Cell Death & Disease 2 (12), e244

Chipuk, J. E., Moldoveanu, T., Llambi, F., Parsons, M. J., and Green, D. R. (2010) 'The BCL-2 Family Reunion'. Molecular Cell 37 (3), 299-310

Chularojmontri, L., Gerdprasert, O., and Wattanapitayakul, S. K. (2013) 'Pummelo Protects Doxorubicin-Induced Cardiac Cell Death by Reducing Oxidative Stress, Modifying Glutathione Transferase Expression, and Preventing Cellular Senescence'. Evidence-Based Complementary and Alternative Medicine : ECAM 2013, 254835

Chun, O. K., Kim, D., and Lee, C. Y. (2003) 'Superoxide Radical Scavenging Activity of the Major Polyphenols in Fresh Plums'. Journal of Agricultural and Food Chemistry 51 (27), 8067-8072

Civitarese, A. E. and Ravussin, E. (2008) 'Mitochondrial Energetics and Insulin Resistance'. Endocrinology 149 (3), 950-954

Cole, M. P., Chaiswing, L., Oberley, T. D., Edelmann, S. E., Piascik, M. T., Lin, S., Kiningham, K. K., and St. Clair, D. K. (2006) 'The Protective Roles of Nitric Oxide and Superoxide Dismutase in Adriamycin-Induced Cardiotoxicity'. Cardiovascular Research 69 (1), 186-197

Costa, L. G., Garrick, J. M., Roqu ├¿, P. J., and Pellacani, C. (2016) 'Mechanisms of Neuroprotection by Quercetin: Counteracting Oxidative Stress and More'. Oxidative Medicine and Cellular Longevity 2016

Crow, M. T., Mani, K., Nam, Y. J., and Kitsis, R. N. (2004) 'The Mitochondrial Death Pathway and Cardiac Myocyte Apoptosis'. Circulation Research 95 (10), 957-970

Cullen, J. J., Weydert, C., Hinkhouse, M. M., Ritchie, J., Domann, F. E., Spitz, D., and Oberley, L. W. (2003) 'The Role of Manganese Superoxide Dismutase in the Growth of Pancreatic Adenocarcinoma'. Cancer Research 63 (6), 1297-1303

Danaei, G., Ding, E. L., Mozaffarian, D., Taylor, B., Rehm, J., Murray, C. J., and Ezzati, M. (2009) 'The Preventable Causes of Death in the United States: Comparative Risk Assessment of Dietary, Lifestyle, and Metabolic Risk Factors'. PLoS Medicine 6 (4), e1000058

Danial, N. N. and Korsmeyer, S. J. (2004) 'Cell Death: Critical Control Points'. Cell 116 (2), 205-219

Das, D. K., Sato, M., Ray, P. S., Maulik, G., Engelman, R. M., Bertelli, A. A., and Bertelli, A. (1999) 'Cardioprotection of Red Wine: Role of Polyphenolic Antioxidants'. Drugs Under Experimental and Clinical Research 25 (2-3), 115-120

Das, D. K., Mukherjee, S., and Ray, D. (2011) 'Erratum to: Resveratrol and Red Wine, Healthy Heart and Longevity'. Heart Failure Reviews 16 (4), 425-435

Das, S., Santani, D. D., and Dhalla, N. S. (2007) 'Experimental Evidence for the Cardioprotective Effects of Red Wine'. Experimental and Clinical Cardiology 12 (1), 5-10

Davidson, S. M. and Duchen, M. R. (2007) 'Endothelial Mitochondria: Contributing to Vascular Function and Disease'. Circulation Research 100 (8), 1128-1141

Davis, C. W., Hawkins, B. J., Ramasamy, S., Irrinki, K. M., Cameron, B. A., Islam, K., Daswani, V. P., Doonan, P. J., Manevich, Y., and Madesh, M. (2010) 'Nitration of the Mitochondrial Complex I Subunit NDUFB8 Elicits RIP1-and RIP3-Mediated Necrosis'. Free Radical Biology and Medicine 48 (2), 306-317

Dayton, A., Selvendiran, K., Meduru, S., Khan, M., Kuppusamy, M. L., Naidu, S., Kalai, T., Hideg, K., and Kuppusamy, P. (2011) 'Amelioration of Doxorubicin-Induced Cardiotoxicity by an Anticancer-Antioxidant Dual-Function Compound, HO-3867'. The Journal of Pharmacology and Experimental Therapeutics 339 (2), 350-357

De Angelis, A., Urbanek, K., Cappetta, D., Piegari, E., Ciuffreda, L. P., Rivellino, A., Russo, R., Esposito, G., Rossi, F., and Berrino, L. (2016) 'Doxorubicin Cardiotoxicity and Target Cells: A Broader Perspective'. Cardio-Oncology 2 (1), 2

de Britto, R. M., da Silva-Neto, J. A., Mesquita, T. R. R., de Vasconcelos, Carla Maria Lins, de Almeida, Grace Kelly Melo, de Jesus, Itamar Couto Guedes, dos Santos, P. H., Souza, D. S., Miguel-dos-Santos, R., and de Sá, L. A. (2018) 'Myrtenol Protects Against Myocardial Ischemia-Reperfusion Injury through Antioxidant and Anti-Apoptotic Dependent Mechanisms'. Food and Chemical Toxicology 111, 557-566

De Marchi, U., Biasutto, L., Garbisa, S., Toninello, A., and Zoratti, M. (2009) 'Quercetin can Act either as an Inhibitor Or an Inducer of the Mitochondrial Permeability Transition Pore: A Demonstration of the Ambivalent Redox Character of Polyphenols'. Biochimica Et Biophysica Acta (BBA)-Bioenergetics 1787 (12), 1425-1432

DeAtley, S. M., Aksenov, M. Y., Aksenova, M. V., Harris, B., Hadley, R., Harper, P. C., Carney, J. M., and Butterfield, D. A. (1999) 'Antioxidants Protect Against Reactive Oxygen Species Associated with Adriamycin-Treated Cardiomyocytes'. Cancer Letters 136 (1), 41-46

Della Torre, P., Mazue, G., Podesta, A., Moneta, D., Sammartini, U., and Imondi, A. R. (1999) 'Protection Against Doxorubicin-Induced Cardiotoxicity in Weanling Rats by Dexrazoxane'. Cancer Chemotherapy and Pharmacology 43 (2), 151-156

Delmas, D., Jannin, B., and Latruffe, N. (2005) 'Resveratrol: Preventing Properties Against Vascular Alterations and Ageing'. Molecular Nutrition & Food Research 49 (5), 377-395

Deng, S., Kruger, A., Kleschyov, A. L., Kalinowski, L., Daiber, A., and Wojnowski, L. (2007) 'Gp91phox-Containing NAD (P) H Oxidase Increases Superoxide Formation by Doxorubicin and NADPH'. Free Radical Biology and Medicine 42 (4), 466-473

Dent, P. (2013) 'The Flip Side of Doxorubicin: Inflammatory and Tumor Promoting Cytokines'. Cancer Biology & Therapy 14 (9), 774-775

Deres, P., Halmosi, R., Toth, A., Kovacs, K., Palfi, A., Habon, T., Czopf, L., Kalai, T., Hideg, K., Sumegi, B., and Toth, K. (2005) 'Prevention of Doxorubicin-Induced Acute Cardiotoxicity by an Experimental Antioxidant Compound'. Journal of Cardiovascular Pharmacology 45 (1), 36-43

Dhalla, N. S., Temsah, R. M., and Netticadan, T. (2000) 'Role of Oxidative Stress in Cardiovascular Diseases'. Journal of Hypertension 18 (6), 655-673

Di Lisa, F., Canton, M., Carpi, A., Kaludercic, N., Menabo, R., Menazza, S., and Semenzato, M. (2011) 'Mitochondrial Injury and Protection in Ischemic Pre- and Postconditioning'. Antioxidants & Redox Signaling 14 (5), 881-891

Diplock, A. T., Charleux, J. L., Crozier-Willi, G., Kok, F. J., Rice-Evans, C., Roberfroid, M., Stahl, W., and Vina-Ribes, J. (1998) 'Functional Food Science and Defence Against Reactive Oxidative Species'. The British Journal of Nutrition 80 Suppl 1, S77-112

Domingo, J. (1994) 'Metal-induced Developmental Toxicity in Mammals: A Review'. Journal of Toxicology and Environmental Health, Part A Current Issues 42 (2), 123-141

Doroshow, J. H., Locker, G. Y., and Myers, C. E. (1980) 'Enzymatic Defenses of the Mouse Heart Against Reactive Oxygen Metabolites: Alterations Produced by Doxorubicin'. The Journal of Clinical Investigation 65 (1), 128-135

dos Santos, D. S. and dos Santos Goldenberg, Regina Coeli (2018) 'Doxorubicin-Induced Cardiotoxicity: From Mechanisms to Development of Efficient Therapy'. in *Cardiotoxicity*. ed. by Anon: IntechOpen

Doukas, J., Wrasidlo, W., Noronha, G., Dneprovskaia, E., Fine, R., Weis, S., Hood, J., Demaria, A., Soll, R., and Cheresh, D. (2006) 'Phosphoinositide 3-Kinase gamma/delta Inhibition Limits Infarct Size After Myocardial ischemia/reperfusion Injury'. Proceedings of the National Academy of Sciences of the United States of America 103 (52), 19866-19871

Doyle, J. J., Neugut, A. I., Jacobson, J. S., Grann, V. R., and Hershman, D. L. (2005) 'Chemotherapy and Cardiotoxicity in Older Breast Cancer Patients: A Population-Based Study'. Journal of Clinical Oncology 23 (34), 8597-8605

Drafts, B. C., Twomley, K. M., D'Agostino, R., Lawrence, J., Avis, N., Ellis, L. R., Thohan, V., Jordan, J., Melin, S. A., Torti, F. M., Little, W. C., Hamilton, C. A., and Hundley, W. G. (2013) Low to Moderate Dose Anthracycline-Based Chemotherapy is Associated with Early Noninvasive Imaging Evidence of Subclinical Cardiovascular Disease.

Dumont, E. A., Hofstra, L., van Heerde, W. L., van, d. E., Doevendans, P. A., DeMuinck, E., Daemen, M. A., Smits, J. F., Frederik, P., Wellens, H. J., Daemen, M. J., and Reutelingsperger, C. P. (2000) 'Cardiomyocyte Death Induced by Myocardial Ischemia and Reperfusion: Measurement with Recombinant Human Annexin-V in a Mouse Model'. Circulation 102 (13), 1564-1568

Dusting, G. J. and Triggle, C. (2005) 'Are we Over Oxidized? Oxidative Stress, Cardiovascular Disease, and the Future of Intervention Studies with Antioxidants'. Vascular Health and Risk Management 1 (2), 93-97

Eefting, F., Rensing, B., Wigman, J., Pannekoek, W. J., Liu, W. M., Cramer, M. J., Lips, D. J., and Doevendans, P. A. (2004) 'Role of Apoptosis in Reperfusion Injury'. Cardiovascular Research 61 (3), 414-426

Eisenberg-Lerner, A., Bialik, S., Simon, H., and Kimchi, A. (2009) 'Life and Death Partners: Apoptosis, Autophagy and the Cross-Talk between them'. Cell Death and Differentiation 16 (7), 966

Eisenreich, A. and Rauch, U. (2011) 'PI3K Inhibitors in Cardiovascular Disease'. Cardiovascular Therapeutics 29 (1), 29-36

Elahi, M. M., Kong, Y. X., and Matata, B. M. (2009) 'Oxidative Stress as a Mediator of Cardiovascular Disease'. Oxidative Medicine and Cellular Longevity 2 (5), 259-269

Elmore, S. (2007) 'Apoptosis: A Review of Programmed Cell Death'. Toxicologic Pathology 35 (4), 495-516

El-Sherbeeny, N., Hassan, Z. A., and Ateyya, H. (2016) 'Tiron Ameliorates Oxidative Stress and Inflammation in a Murine Model of Airway Remodeling'. International Immunopharmacology 39, 172-180

Eltzschig, H. K. and Collard, C. D. (2004) 'Vascular Ischaemia and Reperfusion Injury'. British Medical Bulletin 70 (1), 71-86

Erboga, M., Donmez, Y. B., Sener, U., Erboga, Z. F., Aktas, C., and Kanter, M. (2016) 'Effect of Urtica Dioica Against Doxorubicin-Induced Cardiotoxicity in Rats through Suppression of Histological Damage, Oxidative Stress and Lipid Peroxidation'. European Journal of General Medicine 13 (2)

Esteban, L. L., Medina, A. L., Fernández, R. D., and García, T. M. (2016) . CP-179 Patients Exceeding Doxorubicin Recommended Cumulative Dose

Ewer, M. S. and Lenihan, D. J. (2008) 'Left Ventricular Ejection Fraction and Cardiotoxicity: Is our Ear really to the Ground?'. Journal of Clinical Oncology 26 (8), 1201-1203

Falk, E. (2006) 'Pathogenesis of Atherosclerosis'. Journal of the American College of Cardiology 47 (8), C7-C12

Farber, J. L., Chien, K. R., and Mittnacht, S.,J. (1981) 'Myocardial Ischemia: The Pathogenesis of Irreversible Cell Injury in Ischemia'. The American Journal of Pathology 102 (2), 271-281

Ferretti, S., Allegrini, P. R., Becquet, M. M., and McSheehy, P. M. (2009) 'Tumor Interstitial Fluid Pressure as an Early-Response Marker for Anticancer Therapeutics'. Neoplasia (New York, N.Y.) 11 (9), 874-881

Ferro, C. J. and Webb, D. J. (1997) 'Endothelial Dysfunction and Hypertension'. Drugs 53 (1), 30-41

Finegold, J. A., Asaria, P., and Francis, D. P. (2013) 'Mortality from Ischaemic Heart Disease by Country, Region, and Age: Statistics from World Health Organisation and United Nations'. International Journal of Cardiology 168 (2), 934-945

Fink, S. L. and Cookson, B. T. (2005) 'Apoptosis, Pyroptosis, and Necrosis: Mechanistic Description of Dead and Dying Eukaryotic Cells'. Infection and Immunity 73 (4), 1907-1916

Fleming, I., Michaelis, U. R., Bredenkotter, D., Fisslthaler, B., Dehghani, F., Brandes, R. P., and Busse, R. (2001) 'Endothelium-Derived Hyperpolarizing Factor Synthase (Cytochrome P450 2C9) is a Functionally Significant Source of Reactive Oxygen Species in Coronary Arteries'. Circulation Research 88 (1), 44-51

Fliss, H. and Gattinger, D. (1996) 'Apoptosis in Ischemic and Reperfused Rat Myocardium'. Circulation Research 79 (5), 949-956

Florescu, M., Cinteza, M., and Vinereanu, D. (2013) 'Chemotherapy-Induced Cardiotoxicity'. Maedica 8 (1), 59-67

Fogli, S., Nieri, P., and Breschi, M. C. (2004) 'The Role of Nitric Oxide in Anthracycline Toxicity and Prospects for Pharmacologic Prevention of Cardiac Damage'. FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology 18 (6), 664-675

Forini, F., Nicolini, G., and Iervasi, G. (2015) 'Mitochondria as Key Targets of Cardioprotection in Cardiac Ischemic Disease: Role of Thyroid Hormone Triiodothyronine'. International Journal of Molecular Sciences 16 (3), 6312-6336

Forman, H. J., Maiorino, M., and Ursini, F. (2010) 'Signaling Functions of Reactive Oxygen Species'. Biochemistry 49 (5), 835-842

Frangogiannis, N. G. (2014) 'The Inflammatory Response in Myocardial Injury, Repair, and Remodelling'. Nature Reviews Cardiology 11 (5), 255

Frangogiannis, N. G. (2015) 'Pathophysiology of Myocardial Infarction'. Comprehensive Physiology

Frank, A., Bonney, M., Bonney, S., Weitzel, L., Koeppen, M., and Eckle, T. (eds.) (2012) . 'Myocardial Ischemia Reperfusion Injury: From Basic Science to Clinical Bedside': SAGE Publications Sage CA: Los Angeles, CA

Frei, B. (1995) 'Cardiovascular Disease and Nutrient Antioxidants: Role of lowΓÇÉdensity Lipoprotein Oxidation'. Critical Reviews in Food Science & Nutrition 35 (1-2), 83-98

Fu, J., Yamamoto, K., Guan, Z., Kimura, S., and Iyanagi, T. (2004) 'Human Neuronal Nitric Oxide Synthase can Catalyze One-Electron Reduction of Adriamycin: Role of Flavin Domain'. Archives of Biochemistry and Biophysics 427 (2), 180-187

Fujio, Y., Nguyen, T., Wencker, D., Kitsis, R. N., and Walsh, K. (2000) 'Akt Promotes Survival of Cardiomyocytes in Vitro and Protects Against Ischemia-Reperfusion Injury in Mouse Heart'. Circulation 101 (6), 660-667

Fukuda, S., Kaga, S., Zhan, L., Bagchi, D., Das, D. K., Bertelli, A., and Maulik, N. (2006) 'Resveratrol Ameliorates Myocardial Damage by Inducing Vascular Endothelial Growth Factor-Angiogenesis and Tyrosine Kinase Receptor Flk-1'. Cell Biochemistry and Biophysics 44 (1), 43-49

Fulbright, J. M., Egas-Bejar, D., Huh, W. W., and Chandra, J. (2015) 'Analysis of Redox and Apoptotic Effects of Anthracyclines to Delineate a Cardioprotective Strategy'. Cancer Chemotherapy and Pharmacology 76 (6), 1297-1307

Fulda, S. (2010) 'Evasion of Apoptosis as a Cellular Stress Response in Cancer'. International Journal of Cell Biology 2010, 370835

Fulton, D., Gratton, J., McCabe, T. J., Fontana, J., Fujio, Y., Walsh, K., Franke, T. F., Papapetropoulos, A., and Sessa, W. C. (1999) 'Regulation of Endothelium-Derived Nitric Oxide Production by the Protein Kinase Akt'. Nature 399 (6736), 597

Galluzzi, L., Kepp, O., Krautwald, S., Kroemer, G., and Linkermann, A. (eds.) (2014) . 'Molecular Mechanisms of Regulated Necrosis': Elsevier

Galluzzi, L., Kepp, O., and Kroemer, G. (2014) '5 Pathophysiology of Cancer Cell Death'. in . ed. by Anon, 69-77. e3

Gammella, E., Recalcati, S., Rybinska, I., Buratti, P., and Cairo, G. (2015) 'Iron-Induced Damage in Cardiomyopathy: Oxidative-Dependent and Independent Mechanisms'. Oxidative Medicine and Cellular Longevity 2015, 230182

Gammella, E., Maccarinelli, F., Buratti, P., Recalcati, S., and Cairo, G. (2014) 'The Role of Iron in Anthracycline Cardiotoxicity'. Frontiers in Pharmacology 5, 25

Ganz, P. A., Hussey, M. A., Moinpour, C. M., Unger, J. M., Hutchins, L. F., Dakhil, S. R., Giguere, J. K., Goodwin, J. W., Martino, S., and Albain, K. S. (2008) 'Late Cardiac Effects of Adjuvant Chemotherapy in Breast Cancer Survivors Treated on Southwest Oncology Group Protocol s8897'. Journal of Clinical Oncology 26 (8), 1223-1230

Gao, E., Boucher, M., Chuprun, J. K., Zhou, R., Eckhart, A. D., and Koch, W. J. (2007) 'Darbepoetin Alfa, a Long-Acting Erythropoietin Analog, Offers Novel and Delayed Cardioprotection for the Ischemic Heart'. American Journal of Physiology-Heart and Circulatory Physiology 62 (1), H60

Gao, J., Chen, T., Zhao, D., Zheng, J., and Liu, Z. (2016) 'Ginkgolide B Exerts Cardioprotective Properties Against Doxorubicin-Induced Cardiotoxicity by Regulating Reactive Oxygen Species, Akt and Calcium Signaling Pathways in Vitro and in Vivo'. PloS One 11 (12), e0168219

Garcia-Dorado, D., Ruiz-Meana, M., Inserte, J., Rodriguez-Sinovas, A., and Piper, H. M. (2012) 'Calcium-Mediated Cell Death during Myocardial Reperfusion'. Cardiovascular Research 94 (2), 168-180

Geisberg, C. A. and Sawyer, D. B. (2010) 'Mechanisms of Anthracycline Cardiotoxicity and Strategies to Decrease Cardiac Damage'. Current Hypertension Reports 12 (6), 404-410

Genestra, M. (2007) 'Oxyl Radicals, Redox-Sensitive Signalling Cascades and Antioxidants'. Cellular Signalling 19 (9), 1807-1819

Gerczuk, P. Z. and Kloner, R. A. (2012) 'An Update on Cardioprotection: A Review of the Latest Adjunctive Therapies to Limit Myocardial Infarction Size in Clinical Trials'. Journal of the American College of Cardiology 59 (11), 969-978

Gewirtz, D. (1999) 'A Critical Evaluation of the Mechanisms of Action Proposed for the Antitumor Effects of the Anthracycline Antibiotics Adriamycin and Daunorubicin'. Biochemical Pharmacology 57 (7), 727-741

Gharanei, M., Hussain, A., Janneh, O., and Maddock, H. (2013) 'Attenuation of Doxorubicin-Induced Cardiotoxicity by Mdivi-1: A Mitochondrial division/mitophagy Inhibitor'. PloS One 8 (10), e77713

Gharanei, M., Hussain, A., Janneh, O., and Maddock, H. L. (2013) 'Doxorubicin Induced Myocardial Injury is Exacerbated Following Ischaemic Stress Via Opening of the Mitochondrial Permeability Transition Pore'. Toxicology and Applied Pharmacology 268 (2), 149-156

Ghosh, M., Wang, H. D., and McNeill, J. R. (2002) 'Tiron Exerts Effects Unrelated to its Role as a Scavenger of Superoxide Anion: Effects on Calcium Binding and Vascular Responses'. Canadian Journal of Physiology and Pharmacology 80 (8), 755-760

Giacco, F. and Brownlee, M. (2010) 'Oxidative Stress and Diabetic Complications'. Circulation Research 107 (9), 1058-1070

Gilleron, M., Marechal, X., Montaigne, D., Franczak, J., Neviere, R., and Lancel, S. (2009) 'NADPH Oxidases Participate to Doxorubicin-Induced Cardiac Myocyte Apoptosis'. Biochemical and Biophysical Research Communications 388 (4), 727-731

Giorgio, M. (2015) 'Oxidative Stress and the Unfulfilled Promises of Antioxidant Agents'. Ecancermedicalscience 9, 556

Glass, C. K. and Witztum, J. L. (2001) 'Atherosclerosis. the Road Ahead'. Cell 104 (4), 503-516

Gomes, A. P. and Blenis, J. (2015) 'A Nexus for Cellular Homeostasis: The Interplay between Metabolic and Signal Transduction Pathways'. Current Opinion in Biotechnology 34, 110-117

Goormaghtigh, E., Chatelain, P., Caspers, J., and Ruysschaert, J. M. (1980) 'Evidence of a Complex between Adriamycin Derivatives and Cardiolipin: Possible Role in Cardiotoxicity'. Biochemical Pharmacology 29 (21), 3003-3010

Gottlieb, R. A., Burleson, K. O., Kloner, R. A., Babior, B. M., and Engler, R. L. (1994) 'Reperfusion Injury Induces Apoptosis in Rabbit Cardiomyocytes'. The Journal of Clinical Investigation 94 (4), 1621-1628

Gottwald, E. M., Duss, M., Bugarski, M., Haenni, D., Schuh, C. D., Landau, E. M., and Hall, A. M. (2018) 'The Targeted anti-oxidant MitoQ Causes Mitochondrial Swelling and Depolarization in Kidney Tissue'. *Physiological Reports* 6 (7), e13667.

Graham, R. M., Frazier, D. P., Thompson, J. W., Haliko, S., Li, H., Wasserlauf, B. J., Spiga, M. G., Bishopric, N. H., and Webster, K. A. (2004) 'A Unique Pathway of Cardiac Myocyte Death Caused by Hypoxia-Acidosis'. The Journal of Experimental Biology 207, 3189-3200

Granger, D. N. and Kvietys, P. R. (2015) Reperfusion Injury and Reactive Oxygen Species: The Evolution of a Concept.

Grann, V. R., Hershman, D., Jacobson, J. S., Tsai, W., Wang, J., McBride, R., Mitra, N., Grossbard, M. L., and Neugut, A. I. (2006) 'Outcomes and Diffusion of doxorubicin-based Chemotherapy among Elderly Patients with Aggressive non-Hodgkin Lymphoma'. Cancer 107 (7), 1530-1541

Green, P. S. and Leeuwenburgh, C. (2002) 'Mitochondrial Dysfunction is an Early Indicator of Doxorubicin-Induced Apoptosis'. Biochimica Et Biophysica Acta (BBA)-Molecular Basis of Disease 1588 (1), 94-101

Grein, A. (1987) 'Antitumor Anthracyclines Produced by Streptomyces Peucetius'. Advances in Applied Microbiology 32, 203-214

Groarke, J. D. and Nohria, A. (2015) 'Anthracycline Cardiotoxicity: A New Paradigm for an Old Classic'. Circulation 131 (22), 1946-1949

Gu, J., Hu, W., and Zhang, D. (2015) 'Resveratrol, a Polyphenol Phytoalexin, Protects Against doxorubicin-induced Cardiotoxicity'. Journal of Cellular and Molecular Medicine 19 (10), 2324-2328

Guaricci, A. I., Bulzis, G., Pontone, G., Scicchitano, P., Carbonara, R., Rabbat, M., De Santis, D., and Ciccone, M. M. (2018) Current Interpretation of Myocardial Stunning [online] . available from <http://www.sciencedirect.com/science/article/pii/S105017381730169X>

Guarnieri, C., Flamigni, F., and Caldarera, C. M. (1980) Role of Oxygen in the Cellular Damage Induced by Re-Oxygenation of Hypoxic Heart.

Guha, M. and Mackman, N. (2002) 'The PI3K-Akt Pathway Limits LPS Activation of Signaling Pathways and Expression of Inflammatory Mediators in Human Monocytic Cells'. Journal of Biological Chemistry

Guin, P. S., Das, S., and Mandal, P. C. (2011) 'Electrochemical Reduction of Quinones in Different Media: A Review'. International Journal of Electrochemistry 2011

Gustafsson, Å. B. and Gottlieb, R. A. (2007) 'Heart Mitochondria: Gates of Life and Death'. Cardiovascular Research 77 (2), 334-343

Habener, A., Chowdhury, A., Echtermeyer, F., Lichtinghagen, R., Theilmeier, G., and Herzog, C. (2016) 'MitoNEET Protects HL-1 Cardiomyocytes from Oxidative Stress Mediated Apoptosis in an in Vitro Model of Hypoxia and Reoxygenation'. PloS One 11 (5), e0156054

Hahn, V. S., Lenihan, D. J., and Ky, B. (2014) 'Cancer Therapy-Induced Cardiotoxicity: Basic Mechanisms and Potential Cardioprotective Therapies'. Journal of the American Heart Association 3 (2), e000665

Halestrap, A. P., Clarke, S. J., and Javadov, S. A. (2004) 'Mitochondrial Permeability Transition Pore Opening during Myocardial reperfusion—a Target for Cardioprotection'. Cardiovascular Research 61 (3), 372-385

Halestrap, A. P., Clarke, S. J., and Khaliulin, I. (2007) The Role of Mitochondria in Protection of the Heart by Preconditioning.

Halestrap, A. P., McStay, G. P., and Clarke, S. J. (2002) 'The Permeability Transition Pore Complex: Another View'. Biochimie 84 (2-3), 153-166

Halliwell, B. (2007) 'Biochemistry of Oxidative Stress'. Biochemical Society Transactions 35 (Pt 5), 1147-1150

Halliwell, B. (2007) 'Oxidative Stress and Cancer: Have we Moved Forward?'. The Biochemical Journal 401 (1), 1-11

Halliwell, B. and Gutteridge, J. (1989) 'Protection Against Oxidants in Biological Systems: The Superoxide Theory of Oxygen Toxicity'. Free Radicals in Biology and Medicine 86

Hamilton, K. L. (2007) 'Antioxidants and Cardioprotection'. Medicine and Science in Sports and Exercise 39 (9), 1544-1553

Hamza, A. A., Ahmed, M. M., Elwey, H. M., and Amin, A. (2016) 'Melissa Officinalis Protects Against Doxorubicin-Induced Cardiotoxicity in Rats and Potentiates its Anticancer Activity on MCF-7 Cells'. PloS One 11 (11), e0167049

Han, W., Li, L., Qiu, S., Lu, Q., Pan, Q., Gu, Y., Luo, J., and Hu, X. (2007) 'Shikonin Circumvents Cancer Drug Resistance by Induction of a Necroptotic Death'. Molecular Cancer Therapeutics 6 (5), 1641-1649

Handy, D. E. and Loscalzo, J. (2012) 'Redox Regulation of Mitochondrial Function'. Antioxidants & Redox Signaling 16 (11), 1323-1367

Hanna, A. D., Lam, A., Tham, S., Dulhunty, A. F., and Beard, N. A. (2014) 'Adverse Effects of Doxorubicin and its Metabolic Product on Cardiac RyR2 and SERCA2A'. Molecular Pharmacology 86 (4), 438-449

Hansson, G. K. (2005) 'Inflammation, Atherosclerosis, and Coronary Artery Disease'. New England Journal of Medicine 352 (16), 1685-1695

Haramaki, N., Stewart, D. B., Aggarwal, S., Ikeda, H., Reznick, A. Z., and Packer, L. (1998) 'Networking Antioxidants in the Isolated Rat Heart are Selectively Depleted by Ischemia-Reperfusion'. Free Radical Biology and Medicine 25 (3), 329-339

Hausenloy, D. J. and Yellon, D. M. (2004) 'New Directions for Protecting the Heart Against ischaemia–reperfusion Injury: Targeting the Reperfusion Injury Salvage Kinase (RISK)-Pathway'. Cardiovascular Research 61 (3), 448-460

Hausenloy, D. J., Maddock, H. L., Baxter, G. F., and Yellon, D. M. (2002) 'Inhibiting Mitochondrial Permeability Transition Pore Opening: A New Paradigm for Myocardial Preconditioning?'. Cardiovascular Research 55 (3), 534-543

Hausenloy, D. J. and Yellon, D. M. (2013) 'Myocardial Ischemia-Reperfusion Injury: A Neglected Therapeutic Target'. The Journal of Clinical Investigation 123 (1), 92-100

Hausenloy, D. J. (2012) 'Conditioning the Heart to Prevent Myocardial Reperfusion Injury during PPCI'. European Heart Journal.Acute Cardiovascular Care 1 (1), 13-32

Hausenloy, D. J., Tsang, A., Mocanu, M. M., and Yellon, D. M. (2005) 'Ischemic Preconditioning Protects by Activating Prosurvival Kinases at Reperfusion'. American Journal of Physiology.Heart and Circulatory Physiology 288 (2), H971-6

Hausenloy, D. J. and Yellon, D. M. (2007) 'Reperfusion Injury Salvage Kinase Signalling: Taking a RISK for Cardioprotection'. Heart Failure Reviews 12 (3-4), 217-234

Hayes, J. D., Flanagan, J. U., and Jowsey, I. R. (2005) 'Glutathione Transferases'. Annu.Rev.Pharmacol.Toxicol. 45, 51-88

Hedayati, M., Niazmand, S., Hosseini, M., Baghcheghi, Y., and Beheshti, F. (2017) 'Vitamin E Improved Redox Homeostasis in Heart and Aorta of Hypothyroid Rats'. Endocrine Regulations 51 (4), 205-212

Hennekens, C. H., Buring, J. E., Manson, J. E., Stampfer, M., Rosner, B., Cook, N. R., Belanger, C., LaMotte, F., Gaziano, J. M., and Ridker, P. M. (1996) 'Lack of Effect of Long-Term Supplementation with Beta Carotene on the Incidence of Malignant Neoplasms and Cardiovascular Disease'. New England Journal of Medicine 334 (18), 1145-1149

Hercberg, S., Preziosi, P., Briançon, S., Galan, P., Triol, I., Malvy, D., Roussel, A., and Favier, A. (1998) 'A Primary Prevention Trial using Nutritional Doses of Antioxidant Vitamins and Minerals in Cardiovascular Diseases and Cancers in a General Population: The SU. VI. MAX study—design, Methods, and Participant Characteristics'. Controlled Clinical Trials 19 (4), 336-351

Hers, I., Vincent, E. E., and Tavaré, J. M. (2011) 'Akt Signalling in Health and Disease'. Cellular Signalling 23 (10), 1515-1527

Herscher, L. L., Krishna, M. C., Cook, J. A., Norman Coleman, C., Biaglow, J. E., Tuttle, S. W., Gonzalez, F. J., and Mitchell, J. B. (1994) Protection Against Sr 4233 (Tirapazamine) Aerobic Cytotoxicity by the Metal Chelators Desferrioxamine and Tiron.

Hershman, D. L., McBride, R. B., Eisenberger, A., Tsai, W. Y., Grann, V. R., and Jacobson, J. S. (2008) 'Doxorubicin, Cardiac Risk Factors, and Cardiac Toxicity in Elderly Patients with Diffuse B-Cell Non-Hodgkin's Lymphoma'. Journal of Clinical Oncology 26 (19), 3159-3165

Holmes-McNary, M. and Baldwin, A. S.,J (2000) 'Chemopreventive Properties of Trans-Resveratrol are Associated with Inhibition of Activation of the IkappaB Kinase'. Cancer Research 60 (13), 3477-3483

Hooning, M. J., Botma, A., Aleman, B. M. P., Baaijens, M. H. A., Bartelink, H., Klijn, J. G. M., Taylor, C. W., and Van Leeuwen, F. E. (2007) 'Long-Term Risk of Cardiovascular Disease in 10-Year Survivors of Breast Cancer'. Journal of the National Cancer Institute 99 (5), 365-375

Hortobagyi, G. N. (1997) 'Anthracyclines in the Treatment of Cancer'. Drugs 54 (4), 1-7

Hoshino, S., Kikuchi, Y., Nakajima, M., Kimura, H., Tsuyama, S., Uemura, K., and Yoshida, K. (2005) 'Endothelial NO Synthase (eNOS) Phosphorylation Regulates Coronary Diameter during Ischemia-Reperfusion in Association with Oxidative Stress'. Free Radical Research 39 (5), 481-489

Hosseinzadeh, H., Modaghegh, M. H., and Saffari, Z. (2009) 'Crocus Sativus L. (Saffron) Extract and its Active Constituents (Crocin and Safranal) on Ischemia-Reperfusion in Rat Skeletal Muscle'. Evidence-Based Complementary and Alternative Medicine : ECAM 6 (3), 343-350

Hua, F., Ha, T., Ma, J., Li, Y., Kelley, J., Gao, X., Browder, I. W., Kao, R. L., Williams, D. L., and Li, C. (2007) 'Protection Against Myocardial ischemia/reperfusion Injury in TLR4-Deficient Mice is Mediated through a Phosphoinositide 3-Kinase-Dependent Mechanism'. Journal of Immunology (Baltimore, Md.: 1950) 178 (11), 7317-7324

Huk, I., Brovkovych, V., Nanobash Vili, J., Weigel, G., Neumayer, C., Partyka, L., Patton, S., and Malinski, T. (1998) 'Bioflavonoid Quercetin Scavenges Superoxide and Increases Nitric Oxide Concentration in ischaemiaΓÇôreperfusion Injury: An Experimental Study'. Bjs 85 (8), 1080-1085

Hung, L., Su, M., and Chen, J. (2004) 'Resveratrol Protects Myocardial ischemia–reperfusion Injury through both NO-Dependent and NO-Independent Mechanisms'. Free Radical Biology and Medicine 36 (6), 774-781

Hung, L., Su, M., Chu, W., Chiao, C., Chan, W., and Chen, J. (2002) 'The Protective Effect of Resveratrols on ischaemia-reperfusion Injuries of Rat Hearts is Correlated with Antioxidant Efficacy'. British Journal of Pharmacology 135 (7), 1627-1633

Hung, L., Chen, J., Huang, S., Lee, R., and Su, M. (2000) 'Cardioprotective Effect of Resveratrol, a Natural Antioxidant Derived from Grapes'. Cardiovascular Research 47 (3), 549-555

Huo, Y., Li, G., Duan, R., Gou, Q., Fu, C., Hu, Y., Song, B., Yang, Z., Wu, D., and Zhou, P. (2008) 'PTEN Deletion Leads to Deregulation of Antioxidants and Increased Oxidative Damage in Mouse Embryonic Fibroblasts'. Free Radical Biology and Medicine 44 (8), 1578-1591

Hutchins, L. F., Unger, J. M., Crowley, J. J., Coltman Jr, C. A., and Albain, K. S. (1999) 'Underrepresentation of Patients 65 Years of Age Or Older in Cancer-Treatment Trials'. New England Journal of Medicine 341 (27), 2061-2067

Hüttemann, M., Lee, I., Samavati, L., Yu, H., and Doan, J. W. (2007) 'Regulation of Mitochondrial Oxidative Phosphorylation through Cell Signaling'. Biochimica Et Biophysica Acta (BBA)-Molecular Cell Research 1773 (12), 1701-1720

Ichihara, S., Yamada, Y., Kawai, Y., Osawa, T., Furuhashi, K., Duan, Z., and Ichihara, G. (2007) 'Roles of Oxidative Stress and Akt Signaling in Doxorubicin Cardiotoxicity'. Biochemical and Biophysical Research Communications 359 (1), 27-33

Ichikawa, Y., Ghanefar, M., Bayeva, M., Wu, R., Khechaduri, A., Prasad, S. V. N., Mutharasan, R. K., Naik, T. J., and Ardehali, H. (2014) 'Cardiotoxicity of Doxorubicin is Mediated through Mitochondrial Iron Accumulation'. The Journal of Clinical Investigation 124 (2), 617-630

Irani, K., Xia, Y., Zweier, J. L., Sollott, S. J., Der, C. J., Fearon, E. R., Sundaresan, M., Finkel, T., and Goldschmidt-Clermont, P. (1997) 'Mitogenic Signaling Mediated by Oxidants in Ras-Transformed Fibroblasts'. Science (New York, N.Y.) 275 (5306), 1649-1652

Isner, J. M. and Asahara, T. (1999) 'Angiogenesis and Vasculogenesis as Therapeutic Strategies for Postnatal Neovascularization'. The Journal of Clinical Investigation 103 (9), 1231-1236

Iwata, K., Matsuno, K., and Yabe-Nishimura, C. (2016) 'Reactive Oxygen Species Derived from the NOX1 Isoform of NADPH Oxidase Exacerbate Doxorubicin-Induced Cardiotoxicity by Promoting Cardiac Remodeling'. Free Radical Biology and Medicine 100, S144

Jackson, T. S., Xu, A., Vita, J. A., and Keaney, J. F.,J (1998) 'Ascorbate Prevents the Interaction of Superoxide and Nitric Oxide Only at very High Physiological Concentrations'. Circulation Research 83 (9), 916-922

Jadeski, L., Mataraza, J. M., Jeong, H. W., Li, Z., and Sacks, D. B. (2008) 'IQGAP1 Stimulates Proliferation and Enhances Tumorigenesis of Human Breast Epithelial Cells'. The Journal of Biological Chemistry 283 (2), 1008-1017

Jain, D., Ahmad, T., Cairo, M., and Aronow, W. (2017) 'Cardiotoxicity of Cancer Chemotherapy: Identification, Prevention and Treatment'. Annals of Translational Medicine 5 (17)

Jennings, R. B. and Reimer, K. A. (1981) 'Lethal Myocardial Ischemic Injury'. The American Journal of Pathology 102 (2), 241-255

Jiang, P., Zhang, D., Qiu, H., Yi, X., Zhang, Y., Cao, Y., Zhao, B., Xia, Z., and Wang, C. (2017) 'Tiron Ameliorates High glucose-induced Cardiac Myocyte Apoptosis by PKC δ-dependent Inhibition of Osteopontin'. Clinical and Experimental Pharmacology and Physiology 44 (7), 760-770

Jiang, F., Zhang, Y., and Dusting, G. J. (2011) 'NADPH Oxidase-Mediated Redox Signaling: Roles in Cellular Stress Response, Stress Tolerance, and Tissue Repair'. Pharmacological Reviews 63 (1), 218-242

Jiang, Y. Q., Chang, G. L., Wang, Y., Zhang, D. Y., Cao, L., and Liu, J. (2016) 'Geniposide Prevents Hypoxia/Reoxygenation-Induced Apoptosis in H9c2 Cells: Improvement of Mitochondrial

Dysfunction and Activation of GLP-1R and the PI3K/AKT Signaling Pathway'. Cellular Physiology and Biochemistry : International Journal of Experimental Cellular Physiology, Biochemistry, and Pharmacology 39 (1), 407-421

Jiani, L., Yanxia, L., Mingzhi, S., Meng, Z., Yali, Z., and Mingge, D. (2011) 'The Regulation of DIDS on PI3K/Akt and ROS Signal Transduction in the Ischemia-Reperfusion Injury Myocardium'. Heart 97 (Suppl 3), A55-A55

Jiao, X. Y., Gao, E., Yuan, Y., Wang, Y., Lau, W. B., Koch, W., Ma, X. L., and Tao, L. (2009) 'INO-4885 5,10,15,20-tetraN-(Benzyl-4'-Carboxylate)-2-Pyridinium]-21H,23H-Porphine Iron(III) Chloride], a Peroxynitrite Decomposition Catalyst, Protects the Heart Against Reperfusion Injury in Mice'. The Journal of Pharmacology and Experimental Therapeutics 328 (3), 777-784

Jin, Z. and El-Deiry, W. (2005) 'Overview of Cell Death Signaling Pathways'. Cancer Biology & Therapy 4 (2), 147-171

Jones, S. P., Hoffmeyer, M. R., Sharp, B. R., Ho, Y., and Lefer, D. J. (2003) 'Role of Intracellular Antioxidant Enzymes After in Vivo Myocardial Ischemia and Reperfusion'. American Journal of Physiology-Heart and Circulatory Physiology 284 (1), H277-H282

Jones, K., Saxon, L., Cunningham, W., Adams, P., and Guideline, D. G. (2013) 'Secondary Prevention for Patients After a Myocardial Infarction: Summary of Updated NICE Guidance'. BMJ (Clinical Research Ed.) 347, f6544

Jugdutt, B. I. (2003) 'Nitric Oxide and Cardiovascular Protection'. Heart Failure Reviews 8 (1), 29-34

Kaga, S., Zhan, L., Matsumoto, M., and Maulik, N. (2005) 'Resveratrol Enhances Neovascularization in the Infarcted Rat Myocardium through the Induction of Thioredoxin-1, Heme Oxygenase-1 and Vascular Endothelial Growth Factor'. Journal of Molecular and Cellular Cardiology 39 (5), 813-822

Kalanuria, A. A., Nyquist, P., and Ling, G. (2012) 'The Prevention and Regression of Atherosclerotic Plaques: Emerging Treatments'. Vascular Health and Risk Management 8, 549-561

Kalivendi, S. V., Kotamraju, S., Zhao, H., Joseph, J., and Kalyanaraman, B. (2001) 'Doxorubicin-Induced Apoptosis is Associated with Increased Transcription of Endothelial Nitric-Oxide Synthase. Effect of Antiapoptotic Antioxidants and Calcium'. The Journal of Biological Chemistry 276 (50), 47266-47276

Kalogeris, T., Baines, C. P., Krenz, M., and Korthuis, R. J. (2012) 'Cell Biology of ischemia/reperfusion Injury'. International Review of Cell and Molecular Biology 298, 229-317

Kalyanaraman, B., Joseph, J., Kalivendi, S., Wang, S., Konorev, E., and Kotamraju, S. (2002) 'Doxorubicin-Induced Apoptosis: Implications in Cardiotoxicity'. Molecular and Cellular Biochemistry 234 (1), 119-124

Kang, Y. J. (1999) 'The Antioxidant Function of Metallothionein in the Heart'. Proceedings of the Society for Experimental Biology and Medicine 222 (3), 263-273

Kanmura, Y., Raeymaekers, L., and Casteels, R. (1989) 'Effects of Doxorubicin and Ruthenium Red on Intracellular Ca2 Stores in Skinned Rabbit Mesenteric Smooth-Muscle Fibres'. Cell Calcium 10 (6), 433-439

Kashfi, K., Israel, M., Sweatman, T. W., Seshadri, R., and Cook, G. A. (1990) 'Inhibition of Mitochondrial Carnitine Palmitoyltransferases by Adriamycin and Adriamycin Analogues'. Biochemical Pharmacology 40 (7), 1441-1448

Kattoor, A. J., Pothineni, N. V. K., Palagiri, D., and Mehta, J. L. (2017) 'Oxidative Stress in Atherosclerosis'. Current Atherosclerosis Reports 19 (11), 42

Kavazis, A. N., Morton, A. B., Hall, S. E., and Smuder, A. J. (2017) 'Effects of Doxorubicin on Cardiac Muscle Subsarcolemmal and Intermyofibrillar Mitochondria'. Mitochondrion 34, 9-19

Kedzierski, R. M. and Yanagisawa, M. (2001) 'Endothelin System: The Double-Edged Sword in Health and Disease'. Annual Review of Pharmacology and Toxicology 41 (1), 851-876

Keizer, H. G., FAU, P. H. M., FAU, S. G. J., and Joenje, H. Doxorubicin (Adriamycin): A Critical Review of Free Radical-Dependent Mechanisms of Cytotoxicity.

Keoni, C. L. I. and Brown, T. L. (2015) 'Inhibition of Apoptosis and Efficacy of Pan Caspase Inhibitor, Q-VD-OPh, in Models of Human Disease'. Journal of Cell Death 8, JCD. S23844

Ketelhuth, D. F. J. and Hansson, G. (2011) 'Cellular Immunity, Low-Density Lipoprotein and Atherosclerosis: Break of Tolerance in the Artery Wall'. Thrombosis and Haemostasis 105 (05), 779-786

Khakoo, A. Y., Liu, P. P., Force, T., Lopez-Berestein, G., Jones, L. W., Schneider, J., and Hill, J. (2011) 'Cardiotoxicity due to Cancer Therapy'. Texas Heart Institute Journal 38 (3), 253-256

Khan, G., Haque, S. E., Anwer, T., Ahsan, M. N., Safhi, M. M., and Alam, M. F. (2014) 'Cardioprotective Effect of Green Tea Extract on Doxorubicin-Induced Cardiotoxicity in Rats'. Acta Pol Pharm 71 (5), 861-868

Khan, M., Varadharaj, S., Shobha, J. C., Naidu, M. U., Parinandi, N. L., Kutala, V. K., and Kuppusamy, P. (2006) 'C-Phycocyanin Ameliorates Doxorubicin-Induced Oxidative Stress and

Apoptosis in Adult Rat Cardiomyocytes'. Journal of Cardiovascular Pharmacology 47 (1), 9-20

Khavari, T. A. and Rinn, J. L. (2007) 'Ras/Erk MAPK Signaling in Epidermal Homeostasis and Neoplasia'. Cell Cycle 6 (23), 2928-2931

Kiechle, F. L. and Zhang, X. (2002) Apoptosis: Biochemical Aspects and Clinical Implications.

Kilickap, S., Akgul, E., Aksoy, S., Aytemir, K., and Barista, I. (2005) 'Doxorubicin-Induced Second Degree and Complete Atrioventricular Block'. EP Europace 7 (3), 227-230

Kim, S., Kim, S., Kim, B., Rah, S., Chung, S. M., Im, M., and Kim, U. (2006) 'Doxorubicin-Induced Reactive Oxygen Species Generation and Intracellular Ca2 Increase are Reciprocally Modulated in Rat Cardiomyocytes'. Experimental & Molecular Medicine 38 (5), 535

Kim, B. (2017) 'Western Blot Techniques'. Methods in Molecular Biology (Clifton, N.J.) 1606, 133-139

Kim, C., Hao, J., Ahn, H., and Kim, S. W. (2012) 'Activation of Akt/protein Kinase B Mediates the Protective Effects of Mechanical Stretching Against Myocardial Ischemia-Reperfusion Injury'. Journal of Veterinary Science 13 (3), 235-244

Kim, D., Kim, H., Woo, E., Kwon, D., Kim, M., Chae, S., and Chae, H. (2006) 'Protective Effect of Calceolarioside on Adriamycin-Induced Cardiomyocyte Toxicity'. European Journal of Pharmacology 541 (1), 24-32

Kim, J. S., Cho, E. W., Chung, H. W., and Kim, I. G. (2006) 'Effects of Tiron, 4, 5-Dihydroxy-1, 3-Benzene Disulfonic Acid, on Human Promyelotic HL-60 Leukemia Cell Differentiation and Death'. Toxicology 223 (1-2), 36-45

Kim, N. and Kang, P. M. (2010) 'Apoptosis in Cardiovascular Diseases: Mechanism and Clinical Implications'. Korean Circulation Journal 40 (7), 299-305

Kim, S., Kim, S., Kim, B., Rah, S., Chung, S. M., Im, M., and Kim, U. (2006) 'Doxorubicin-Induced Reactive Oxygen Species Generation and Intracellular Ca2 Increase are Reciprocally Modulated in Rat Cardiomyocytes'. Experimental & Molecular Medicine 38 (5), 535

Kim, Y. A., Kim, M. Y., and Jung, Y. S. (2013) 'Glutathione Depletion by L-Buthionine-S,R-Sulfoximine Induces Apoptosis of Cardiomyocytes through Activation of PKC-Delta'. Biomolecules & Therapeutics 21 (5), 358-363

Kim, Y., Lee, D., Jeong, J., Guo, Z. S., and Lee, Y. J. (2008) 'Quercetin Augments TRAIL-Induced Apoptotic Death: Involvement of the ERK Signal Transduction Pathway'. Biochemical Pharmacology 75 (10), 1946-1958

Kitakaze, M., Asakura, M., Kim, J., Shintani, Y., Asanuma, H., Hamasaki, T., Seguchi, O., Myoishi, M., Minamino, T., and Ohara, T. (2007) 'Human Atrial Natriuretic Peptide and Nicorandil as Adjuncts to Reperfusion Treatment for Acute Myocardial Infarction (J-WIND): Two Randomised Trials'. The Lancet 370 (9597), 1483-1493

Klawitter, P. F., Murray, H. N., Clanton, T. L., and Angelos, M. G. (2002) 'Reactive Oxygen Species Generated during Myocardial Ischemia Enable Energetic Recovery during Reperfusion'. American Journal of Physiology-Heart and Circulatory Physiology 283 (4), H1656-H1661

Kleinbongard, P., Schulz, R., and Heusch, G. (2011) 'TNFα in Myocardial ischemia/reperfusion, Remodeling and Heart Failure'. Heart Failure Reviews 16 (1), 49-69

Kloner, R. A. and Rezkalla, S. H. (2004) Cardiac Protection during Acute Myocardial Infarction: Where do we Stand in 2004?.

Kluza, J., Lansiaux, A., Wattez, N., Mahieu, C., Osheroff, N., and Bailly, C. (2000) 'Apoptotic Response of HL-60 Human Leukemia Cells to the Antitumor Drug TAS-103'. *Cancer Research* 60 (15), 4077-4084

Ko, C. H., Shen, S., Hsu, C., and Chen, Y. (2005) Mitochondrial-Dependent, Reactive Oxygen Species-Independent Apoptosis by Myricetin: Roles of Protein Kinase C, Cytochrome c, and Caspase Cascade [online] available from <http://www.sciencedirect.com/science/article/pii/S0006295204008214>

Koene, R. J., Prizment, A. E., Blaes, A., and Konety, S. H. (2016) 'Shared Risk Factors in Cardiovascular Disease and Cancer'. Circulation 133 (11), 1104-1114

Koka, S. and Kukreja, R. C. (2011) 'Phosphodiesterase-5 Inhibitors in Protection Against Doxorubicin-Induced Cardiomyopathy'. in . ed. by Anon: Springer, 243-255

Konstantinidis, K., Whelan, R. S., and Kitsis, R. N. (2012) 'Mechanisms of Cell Death in Heart Disease'. Arteriosclerosis, Thrombosis, and Vascular Biology 32 (7), 1552-1562

Korkmaz, S., Barnucz, E., Loganathan, S., Li, S., Radovits, T., Hegedus, P., Zubarevich, A., Hirschberg, K., Weymann, A., and Puskás, L. G. (2013) 'Q50, an Iron-Chelating and Zinc-Complexing Agent, Improves Cardiac Function in Rat Models of ischemia/reperfusion-Induced Myocardial Injury'. Circulation Journal 77 (7), 1817-1826

Koshinuma, S., Miyamae, M., Kaneda, K., Kotani, J., and Figueredo, V. M. (2014) 'Combination of Necroptosis and Apoptosis Inhibition Enhances Cardioprotection Against Myocardial ischemia–reperfusion Injury'. Journal of Anesthesia 28 (2), 235-241

Kostin, S., Pool, L., Elsasser, A., Hein, S., Drexler, H. C., Arnon, E., Hayakawa, Y., Zimmermann, R., Bauer, E., Klovekorn, W. P., and Schaper, J. (2003) 'Myocytes Die by Multiple Mechanisms in Failing Human Hearts'. Circulation Research 92 (7), 715-724

Kotamraju, S., Konorev, E. A., Joseph, J., and Kalyanaraman, B. (2000) 'Doxorubicin-Induced Apoptosis in Endothelial Cells and Cardiomyocytes is Ameliorated by Nitrone Spin Traps and Ebselen. Role of Reactive Oxygen and Nitrogen Species'. The Journal of Biological Chemistry 275 (43), 33585-33592

Koti, B. C., Vishwanathswamy, A., Wagawade, J., and Thippeswamy, A. (2009) 'Cardioprotective Effect of Lipistat Against Doxorubicin Induced Myocardial Toxicity in Albino Rats'

Krijnen, P. A., Nijmeijer, R., Meijer, C. J., Visser, C. A., Hack, C. E., and Niessen, H. W. (2002) 'Apoptosis in Myocardial Ischaemia and Infarction'. Journal of Clinical Pathology 55 (11), 801-811

Kris-Etherton, P., Lichtenstein, A. H., Howard, B. V., Steinberg, D., Witztum, J. L., and Nutrition Committee of the American Heart Association Council,on Nutrition (2004) 'Antioxidant Vitamin Supplements and Cardiovascular Disease'. Circulation 110 (5), 637-641

Krishna, C. M., Liebmann, J. E., Kaufman, D., DeGraff, W., Hahn, S. M., McMurry, T., Mitchell, J. B., and Russo, A. (1992) 'The Catecholic Metal Sequestering Agent 1,2-Dihydroxybenzene-3,5-Disulfonate Confers Protection Against Oxidative Cell Damage'. Archives of Biochemistry and Biophysics 294 (1), 98-106

Kumar, S., Kumar, A., Pathania, A. S., Guru, S. K., Jada, S., Sharma, P. R., Bhushan, S., Saxena, A. K., Kumar, H. S., and Malik, F. (2013) 'Tiron and Trolox Potentiate the Autophagic Cell Death Induced by Magnolol Analog Ery5 by Activation of Bax in HL-60 Cells'. Apoptosis 18 (5), 605-617

Kumar, A., Gautam, B., Dubey, C., and Tripathi, P. K. (2014) 'A Review: Role of Doxorubicin in Treatment of Cancer'. International Journal of Pharmaceutical Sciences and Research 5 (10), 4105

Kumar, D., Kirshenbaum, L. A., Li, T., Danelisen, I., and Singal, P. K. (2001) 'Apoptosis in Adriamycin Cardiomyopathy and its Modulation by Probucol'. Antioxidants and Redox Signaling 3 (1), 135-145

Kurian, G. A., Rajagopal, R., Vedantham, S., and Rajesh, M. (2016) 'The Role of Oxidative Stress in Myocardial Ischemia and Reperfusion Injury and Remodeling: Revisited'. Oxidative Medicine and Cellular Longevity 2016, 1656450

Lander, H. M., Hajjar, D. P., Hempstead, B. L., Mirza, U. A., Chait, B. T., Campbell, S., and Quilliam, L. A. (1997) 'A Molecular Redox Switch on p21ras Structural Basis for the Nitric Oxide-p21ras Interaction'. Journal of Biological Chemistry 272 (7), 4323-4326

Langer, S. W. (2014) 'Dexrazoxane for the Treatment of Chemotherapy-Related Side Effects'. Cancer Management and Research 6, 357-363

Lavandero, S., Chiong, M., Rothermel, B. A., and Hill, J. A. (2015) 'Autophagy in Cardiovascular Biology'. The Journal of Clinical Investigation 125 (1), 55-64

Lebrecht, D., Geist, A., Ketelsen, U., Haberstroh, J., Setzer, B., and Walker, U. (2007) 'Dexrazoxane Prevents doxorubicin-induced long-term Cardiotoxicity and Protects Myocardial Mitochondria from Genetic and Functional Lesions in Rats'. British Journal of Pharmacology 151 (6), 771-778

L'Ecuyer, T., Allebban, Z., Thomas, R., and Vander Heide, R. (2004) 'Glutathione S-Transferase Overexpression Protects Against Anthracycline-Induced H9C2 Cell Death'. American Journal of Physiology-Heart and Circulatory Physiology 286 (6), H2057-H2064

LeDoux, S. P., Driggers, W. J., Hollensworth, B. S., and Wilson, G. L. (1999) 'Repair of Alkylation and Oxidative Damage in Mitochondrial DNA'. Mutation Research 434 (3), 149-159

Lee, I., Cook, N. R., Manson, J. E., Buring, J. E., and Hennekens, C. H. (1999) 'B-Carotene Supplementation and Incidence of Cancer and Cardiovascular Disease: The Women's Health Study'. Journal of the National Cancer Institute 91 (24), 2102-2106

Lee, S. R., Yang, K. S., Kwon, J., Lee, C., Jeong, W., and Rhee, S. G. (2002) 'Reversible Inactivation of the Tumor Suppressor PTEN by H2O2'. The Journal of Biological Chemistry 277 (23), 20336-20342

Lehenbauer Ludke, A. R., Al-Shudiefat, A., Dhingra, S., Jassal, D. S., and Singal, P. K. (2009) 'A Concise Description of Cardioprotective Strategies in Doxorubicin-Induced Cardiotoxicity'. Canadian Journal of Physiology and Pharmacology 87 (10), 756-763

Leichtweis, S. and Ji, L. (2001) 'Glutathione Deficiency Intensifies ischaemia-reperfusion Induced Cardiac Dysfunction and Oxidative Stress'. Acta Physiologica Scandinavica 172 (1), 1-10

Leist, M., Single, B., Castoldi, A. F., Kuhnle, S., and Nicotera, P. (1997) 'Intracellular Adenosine Triphosphate (ATP) Concentration: A Switch in the Decision between Apoptosis and Necrosis'. The Journal of Experimental Medicine 185 (8), 1481-1486

Leong, X., Ng, C., and Jaarin, K. (2015) 'Animal Models in Cardiovascular Research: Hypertension and Atherosclerosis'. BioMed Research International 2015

Levraut, J., Iwase, H., Shao, Z., Vanden Hoek, T. L., and Schumacker, P. T. (2003) 'Cell Death during Ischemia: Relationship to Mitochondrial Depolarization and ROS Generation'. American Journal of Physiology-Heart and Circulatory Physiology 284 (2), H549-H558

Li, J., McQuade, T., Siemer, A. B., Napetschnig, J., Moriwaki, K., Hsiao, Y., Damko, E., Moquin, D., Walz, T., and McDermott, A. (2012) 'The RIP1/RIP3 Necrosome Forms a Functional Amyloid Signaling Complex Required for Programmed Necrosis'. Cell 150 (2), 339-350

Li, Z., Hao, C., Wu, Y., Wang, T., Yang, L., and Wang, W. (2018) 'Protective Effect of Apigenin Against Myocardium Injury in Ischemia Reperfusion Rats by Stimulating JAK2/STAT3 Signal Pathway'. International Journal of Clinical and Experimental Medicine 11 (1), 118-124

Li, J., Liu, H., Ramachandran, S., Waypa, G. B., Yin, J., Li, C., Han, M., Huang, H., Sharp, W. W., and Vanden Hoek, T. L. (2010) 'Grape Seed Proanthocyanidins Ameliorate Doxorubicin-Induced Cardiotoxicity'. The American Journal of Chinese Medicine 38 (03), 569-584

Libby, P., Ridker, P. M., and Maseri, A. (2002) 'Inflammation and Atherosclerosis'. Circulation 105 (9), 1135-1143

Lichtman, S. M., Wildiers, H., Chatelut, E., Steer, C., Budman, D., Morrison, V. A., Tranchand, B., Shapira, I., and Aapro, M. (2007) 'International Society of Geriatric Oncology Chemotherapy Taskforce: Evaluation of Chemotherapy in Older patients—an Analysis of the Medical Literature'. Journal of Clinical Oncology 25 (14), 1832-1843

Lieberthal, W., Menza, S. A., and Levine, J. S. (1998) 'Graded ATP Depletion can Cause Necrosis Or Apoptosis of Cultured Mouse Proximal Tubular Cells'. American Journal of Physiology-Renal Physiology 274 (2), F315-F327

Liebgott, T., Miollan, M., Berchadsky, Y., Drieu, K., Culcasi, M., and Pietri, S. (2000) 'Complementary Cardioprotective Effects of Flavonoid Metabolites and Terpenoid Constituents of Ginkgo Biloba Extract (EGb 761) during Ischemia and Reperfusion'. Basic Research in Cardiology 95 (5), 368-377

Limaye, V., Li, X., Hahn, C., Xia, P., Berndt, M. C., Vadas, M. A., and Gamble, J. R. (2005) 'Sphingosine Kinase-1 Enhances Endothelial Cell Survival through a PECAM-1-Dependent Activation of PI-3K/Akt and Regulation of Bcl-2 Family Members'. Blood 105 (8), 3169-3177

Lin, S., Fujii, M., and Hou, D. (2003) Rhein Induces Apoptosis in HL-60 Cells Via Reactive Oxygen Species-Independent Mitochondrial Death Pathway [online] . available from <http://www.sciencedirect.com/science/article/pii/S0003986103004223>

Lin, Y., Choksi, S., Shen, H. M., Yang, Q. F., Hur, G. M., Kim, Y. S., Tran, J. H., Nedospasov, S. A., and Liu, Z. G. (2004) 'Tumor Necrosis Factor-Induced Nonapoptotic Cell Death Requires

Receptor-Interacting Protein-Mediated Cellular Reactive Oxygen Species Accumulation'. The Journal of Biological Chemistry 279 (11), 10822-10828;

Lin, C., Lee, C., Shih, Y., Lin, T., Wang, S., Lin, Y., and Shih, C. (2012) 'Resveratrol Enhances the Therapeutic Effect of Temozolomide Against Malignant Glioma in Vitro and in Vivo by Inhibiting Autophagy'. *Free Radical Biology and Medicine* 52 (2), 377-391;

Ling, Y. H., Liebes, L., Zou, Y., and Perez-Soler, R. (2003) 'Reactive Oxygen Species Generation and Mitochondrial Dysfunction in the Apoptotic Response to Bortezomib, a Novel Proteasome Inhibitor, in Human H460 Non-Small Cell Lung Cancer Cells'. The Journal of Biological Chemistry 278 (36), 33714-33723

Liou, G. Y. and Storz, P. (2010) 'Reactive Oxygen Species in Cancer'. Free Radical Research 44 (5), 479-496

Lipshultz, S. E., Rifai, N., Dalton, V. M., Levy, D. E., Silverman, L. B., Lipsitz, S. R., Colan, S. D., Asselin, B. L., Barr, R. D., and Clavell, L. A. (2004) 'The Effect of Dexrazoxane on Myocardial Injury in Doxorubicin-Treated Children with Acute Lymphoblastic Leukemia'. New England Journal of Medicine 351 (2), 145-153

Lipshultz, S. E., Lipsitz, S. R., Mone, S. M., Goorin, A. M., Sallan, S. E., Sanders, S. P., Orav, E. J., Gelber, R. D., and Colan, S. D. (1995) 'Female Sex and Higher Drug Dose as Risk Factors for Late Cardiotoxic Effects of Doxorubicin Therapy for Childhood Cancer'. New England Journal of Medicine 332 (26), 1738-1744

Liu, C., Yang, F., Cheng, S., Liu, Y., Wan, L., and Cong, H. (2017) 'Rosuvastatin Postconditioning Protects Isolated Hearts Against ischemia–reperfusion Injury: The Role of Radical Oxygen Species, PI 3K–Akt–GSK-3β Pathway, and Mitochondrial Permeability Transition Pore'. Cardiovascular Therapeutics 35 (1), 3-9

Liu, L., Hu, X., Xia, C., He, J., Zhou, Q., Shi, X., Fang, J., and Jiang, B. (2006) 'Reactive Oxygen Species Regulate Epidermal Growth Factor-Induced Vascular Endothelial Growth Factor and Hypoxia-Inducible Factor-1α Expression through Activation of AKT and P70S6K1 in Human Ovarian Cancer Cells'. Free Radical Biology and Medicine 41 (10), 1521-1533

Liu, B., Li, H., Qu, H., and Sun, B. (2006) 'Nitric Oxide Synthase Expressions in ADR-Induced Cardiomyopathy in Rats'. Journal of Biochemistry and Molecular Biology 39 (6), 759-765

Liu, J., Mao, W., Ding, B., and Liang, C. (2008) 'ERKs/p53 Signal Transduction Pathway is Involved in Doxorubicin-Induced Apoptosis in H9c2 Cells and Cardiomyocytes'. American Journal of Physiology-Heart and Circulatory Physiology 295 (5), H1956-H1965

Liu, X., Chen, Z., Chua, C. C., Ma, Y., Youngberg, G. A., Hamdy, R., and Chua, B. H. L. (2002) 'Melatonin as an Effective Protector Against Doxorubicin-Induced Cardiotoxicity'. American Journal of Physiology-Heart and Circulatory Physiology 283 (1), H254-H263

Lochner, A., Genade, S., and Moolman, J. (2003) 'Ischemic Preconditioning: Infarct Size is a More Reliable Endpoint than Functional Recovery'. Basic Research in Cardiology 98 (5), 337-346

Longhi, A., Ferrari, S., Bacci, G., and Specchia, S. (2007) 'Long-Term Follow-Up of Patients with Doxorubicin-Induced Cardiac Toxicity After Chemotherapy for Osteosarcoma'. Anti-Cancer Drugs 18 (6), 737-744

Loreto, C., La Rocca, G., Anzalone, R., Caltabiano, R., Vespasiani, G., Castorina, S., Ralph, D. J., Cellek, S., Musumeci, G., Giunta, S., Djinovic, R., Basic, D., and Sansalone, S. (2014) 'The Role of Intrinsic Pathway in Apoptosis Activation and Progression in Peyronie's Disease'. BioMed Research International 2014, 616149

Lou, Y. U., Wang, Z., Xu, Y. I., Zhou, P., Cao, J., Li, Y., Chen, Y., Sun, J., and Fu, L. U. (2015) 'Resveratrol Prevents Doxorubicin-Induced Cardiotoxicity in H9c2 Cells through the Inhibition of Endoplasmic Reticulum Stress and the Activation of the Sirt1 Pathway'. International Journal of Molecular Medicine 36 (3), 873-880

Lu, L., Liu, M., Sun, R., Zheng, Y., and Zhang, P. (2015) 'Myocardial Infarction: Symptoms and Treatments'. Cell Biochemistry and Biophysics 72 (3), 865-867

Lu, M. and Wang, W. (1998) 'Reaction of Nitric Oxide with Superoxide Inhibits Basolateral K Channels in the Rat CCD'. American Journal of Physiology-Cell Physiology 275 (1), C309-C316

Lucero, A. A., Lambrick, D. M., Faulkner, J. A., Fryer, S., Tarrant, M. A., Poudevigne, M., Williams, M. A., and Stoner, L. (2014) 'Modifiable Cardiovascular Disease Risk Factors among Indigenous Populations'. Advances in Preventive Medicine 2014, 547018

Ludke, A., Akolkar, G., Ayyappan, P., Sharma, A. K., and Singal, P. K. (2017) 'Time Course of Changes in Oxidative Stress and Stress-Induced Proteins in Cardiomyocytes Exposed to Doxorubicin and Prevention by Vitamin C'. PloS One 12 (7), e0179452

Luedde, M., Lutz, M., Carter, N., Sosna, J., Jacoby, C., Vucur, M., Gautheron, J., Roderburg, C., Borg, N., and Reisinger, F. (2014) 'RIP3, a Kinase Promoting Necroptotic Cell Death, Mediates Adverse Remodelling After Myocardial Infarction'. Cardiovascular Research 103 (2), 206-216

Luo, J., Xuan, Y., Gu, Y., and Prabhu, S. D. (2006) 'Prolonged Oxidative Stress Inverts the Cardiac force–frequency Relation: Role of Altered Calcium Handling and Myofilament Calcium Responsiveness'. Journal of Molecular and Cellular Cardiology 40 (1), 64-75

Luo, J., Xuan, Y., Gu, Y., and Prabhu, S. D. (2006) 'Prolonged Oxidative Stress Inverts the Cardiac force–frequency Relation: Role of Altered Calcium Handling and Myofilament Calcium Responsiveness'. Journal of Molecular and Cellular Cardiology 40 (1), 64-75

Lv, L., Meng, Q., Xu, J., Gong, J., Cheng, Y., and Jiang, S. (2012) 'Ligustrazine Attenuates Myocardial Ischemia Reperfusion Injury in Rats by Activating the Phosphatidylinositol 3-kinase/Akt Pathway'. Annals of Clinical and Laboratory Science 42 (2), 198-202

Lyu, Y. L., Kerrigan, J. E., Lin, C., Azarova, A. M., Tsai, Y., Ban, Y., and Liu, L. F. (2007) 'Topoisomerase IIβ–Mediated DNA Double-Strand Breaks: Implications in Doxorubicin Cardiotoxicity and Prevention by Dexrazoxane'. Cancer Research [online] 67 (18), 8839-8846. available from <http://cancerres.aacrjournals.org/content/67/18/8839.abstract>

Lyu, Y. L., Lin, C., Azarova, A. M., Cai, L., Wang, J. C., and Liu, L. F. (2006) 'Role of Topoisomerase IIβ in the Expression of Developmentally Regulated Genes'. Molecular and Cellular Biology [online] 26 (21), 7929-7941. Available from http://mcb.asm.org/content/26/21/7929.abstract

Ma, S., Wang, Y., Chen, Y., and Cao, F. (2015) 'The Role of the Autophagy in Myocardial ischemia/reperfusion Injury'. Biochimica Et Biophysica Acta (BBA)-Molecular Basis of Disease 1852 (2), 271-276

MacKenzie, A. and Martin, W. (1998) 'Loss of endothelium-derived Nitric Oxide in Rabbit Aorta by Oxidant Stress: Restoration by Superoxide Dismutase Mimetics'. British Journal of Pharmacology 124 (4), 719-728

Madamanchi, N. R., Vendrov, A., and Runge, M. S. (2005) 'Oxidative Stress and Vascular Disease'. Arteriosclerosis, Thrombosis, and Vascular Biology 25 (1), 29-38

Maddaford, T. G. and Pierce, G. N. (1997) 'Myocardial Dysfunction is Associated with Activation of Na /H Exchange Immediately during Reperfusion'. American Journal of Physiology-Heart and Circulatory Physiology 273 (5), H2232-H2239

Magyar, K., Halmosi, R., Palfi, A., Feher, G., Czopf, L., Fulop, A., Battyany, I., Sumegi, B., Toth, K., and Szabados, E. (2012) 'Cardioprotection by Resveratrol: A Human Clinical Trial in Patients with Stable Coronary Artery Disease'. Clinical Hemorheology and Microcirculation 50 (3), 179-187

Mahmood, T. and Yang, P. C. (2012) 'Western Blot: Technique, Theory, and Trouble Shooting'. North American Journal of Medical Sciences 4 (9), 429-434

Maillet, A., Tan, K., Chai, X., Sadananda, S. N., Mehta, A., Ooi, J., Hayden, M. R., Pouladi, M. A., Ghosh, S., and Shim, W. (2016) 'Modeling Doxorubicin-Induced Cardiotoxicity in Human Pluripotent Stem Cell Derived-Cardiomyocytes'. Scientific Reports 6, 25333

Majno, G. and Joris, I. (1995) 'Apoptosis, Oncosis, and Necrosis. an Overview of Cell Death'. The American Journal of Pathology 146 (1), 3-15

Mani, H., Tanaka, H., Adachi, T., Ikegawa, M., Dai, P., Fujita, N., and Takamatsu, T. (2015) 'How does the Ca2 -Paradox Injury Induce Contracture in the Heart?—A Combined Study of the Intracellular Ca2 Dynamics and Cell Structures in Perfused Rat Hearts—'. Acta Histochemica Et Cytochemica 48 (1), 1-8

Marchioli, R., Levantesi, G., Macchia, A., Marfisi, R. M., Nicolosi, G. L., Tavazzi, L., Tognoni, G., Valagussa, F., and GISSI-Prevenzione Investigators (2006) 'Vitamin E Increases the Risk of Developing Heart Failure After Myocardial Infarction: Results from the GISSI-Prevenzione Trial'. Journal of Cardiovascular Medicine (Hagerstown, Md.) 7 (5), 347-350

Marechal, X., Montaigne, D., Marciniak, C., Marchetti, P., Hassoun, S. M., Beauvillain, J. C., Lancel, S., and Neviere, R. (2011) 'Doxorubicin-Induced Cardiac Dysfunction is Attenuated by Ciclosporin Treatment in Mice through Improvements in Mitochondrial Bioenergetics'. Clinical Science (London, England : 1979) 121 (9), 405-413

Mari, M., Morales, A., Colell, A., García-Ruiz, C., and Fernández-Checa, J. C. (2009) 'Mitochondrial Glutathione, a Key Survival Antioxidant'. Antioxidants & Redox Signaling 11 (11), 2685-2700

Marín-García, J. and Goldenthal, M. J. (2002) 'The Mitochondrial Organelle and the Heart'. Revista Espanola De Cardiologia 55 (12), 1293-1310

Masini, E., Cuzzocrea, S., Mazzon, E., Marzocca, C., Mannaioni, P. F., and Salvemini, D. (2002) 'Protective Effects of M40403, a Selective Superoxide Dismutase Mimetic, in Myocardial Ischaemia and Reperfusion Injury in Vivo'. British Journal of Pharmacology 136 (6), 905-917

Matsui, T., Tao, J., del Monte, F., Lee, K. H., Li, L., Picard, M., Force, T. L., Franke, T. F., Hajjar, R. J., and Rosenzweig, A. (2001) 'Akt Activation Preserves Cardiac Function and Prevents Injury After Transient Cardiac Ischemia in Vivo'. Circulation 104 (3), 330-335

Matsumoto, S., Koshiishi, I., Inoguchi, T., Nawata, H., and Utsumi, H. (2003) 'Confirmation of Superoxide Generation Via Xanthine Oxidase in Streptozotocin-Induced Diabetic Mice'. Free Radical Research 37 (7), 767-772

Maulik, A., Davidson, S. M., Piotrowska, I., Walker, M., and Yellon, D. M. (2018) 'Ischaemic Preconditioning Protects Cardiomyocytes from Anthracycline-Induced Toxicity Via the PI3K Pathway'. Cardiovascular Drugs and Therapy

Maxwell, S. R. J. (1997) 'Anti-Oxidant Therapy: Does it have a Role in the Treatment of Human Disease?'. Expert Opinion on Investigational Drugs 6 (3), 211-236

McCubrey, J. A., Steelman, L. S., Chappell, W. H., Abrams, S. L., Wong, E. W. T., Chang, F., Lehmann, B., Terrian, D. M., Milella, M., and Tafuri, A. (2007) 'Roles of the Raf/MEK/ERK Pathway in Cell Growth, Malignant Transformation and Drug Resistance'. Biochimica Et Biophysica Acta (BBA)-Molecular Cell Research 1773 (8), 1263-1284

McCully, J. D., Wakiyama, H., Hsieh, Y., Jones, M., and Levitsky, S. (2004) 'Differential Contribution of Necrosis and Apoptosis in Myocardial Ischemia-Reperfusion Injury'. American Journal of Physiology-Heart and Circulatory Physiology 286 (5), H1923-H1935

McGowan, J. V., Chung, R., Maulik, A., Piotrowska, I., Walker, J. M., and Yellon, D. M. (2017) 'Anthracycline Chemotherapy and Cardiotoxicity'. Cardiovascular Drugs and Therapy 31 (1), 63-75

McNamara, C. R. and Degterev, A. (2011) 'Small-Molecule Inhibitors of the PI3K Signaling Network'. Future Medicinal Chemistry 3 (5), 549-565

McQueen, M. J., Lonn, E., Gerstein, H. C., Bosch, J., and Yusuf, S. (2005) 'The Hope (Heart Outcomes Prevention Evaluation) Study and its Consequences'. Scandinavian Journal of Clinical and Laboratory Investigation 65, 143-156

Melov, S. (2000) 'Mitochondrial Oxidative Stress. Physiologic Consequences and Potential for a Role in Aging'. Annals of the New York Academy of Sciences 908, 219-225

Menna, P. and Salvatorelli, E. (2017) 'Primary Prevention Strategies for Anthracycline Cardiotoxicity: A Brief Overview'. Chemotherapy 62 (3), 159-168

Mertens, A. C., Liu, Q., Neglia, J. P., Wasilewski, K., Leisenring, W., Armstrong, G. T., Robison, L. L., and Yasui, Y. (2008) 'Cause-Specific Late Mortality among 5-Year Survivors of Childhood Cancer: The Childhood Cancer Survivor Study'. Journal of the National Cancer Institute 100 (19), 1368-1379

Mewton, N., Croisille, P., Gahide, G., Rioufol, G., Bonnefoy, E., Sanchez, I., Cung, T. T., Sportouch, C., Angoulvant, D., and Finet, G. (2010) 'Effect of Cyclosporine on Left Ventricular Remodeling After Reperfused Myocardial Infarction'. Journal of the American College of Cardiology 55 (12), 1200-1205

Miller, A. (2012) 'Superoxide Dismutases: Ancient Enzymes and New Insights'. FEBS Letters 586 (5), 585-595

Minotti, G., Menna, P., Salvatorelli, E., Cairo, G., and Gianni, L. (2004) 'Anthracyclines: Molecular Advances and Pharmacologic Developments in Antitumor Activity and Cardiotoxicity'. Pharmacological Reviews 56 (2), 185-229

Minotti, G., Cairo, G., and Monti, E. (1999) 'Role of Iron in Anthracycline Cardiotoxicity: New Tunes for an Old Song?'. FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology 13 (2), 199-212

Minotti, G., Recalcati, S., Mordente, A., Liberi, G., Calafiore, A. M., Mancuso, C., Preziosi, P., and Cairo, G. (1998) 'The Secondary Alcohol Metabolite of Doxorubicin Irreversibly Inactivates aconitase/iron Regulatory Protein-1 in Cytosolic Fractions from Human Myocardium'. FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology 12 (7), 541-552

Miranda, C. J., Makui, H., Soares, R. J., Bilodeau, M., Mui, J., Vali, H., Bertrand, R., Andrews, N. C., and Santos, M. M. (2003) 'Hfe Deficiency Increases Susceptibility to Cardiotoxicity and Exacerbates Changes in Iron Metabolism Induced by Doxorubicin'. Blood 102 (7), 2574-2580

Mistiaen, W. P. (2016) 'Effect of Doxorubicin on Cardiac Myocytes: Role of Apoptosis, Autophagy and Other Proteolytic Pathways'

Mitry, M. A. and Edwards, J. G. (2016) 'Doxorubicin Induced Heart Failure: Phenotype and Molecular Mechanisms'. IJC Heart & Vasculature 10, 17-24

Mocanu, M. M., Baxter, G. F., and Yellon, D. M. (2000) 'Caspase Inhibition and Limitation of Myocardial Infarct Size: Protection Against Lethal Reperfusion Injury'. British Journal of Pharmacology 130 (2), 197-200

Mochizuki, T., Furuta, S., Mitsushita, J., Shang, W. H., Ito, M., Yokoo, Y., Yamaura, M., Ishizone, S., Nakayama, J., and Konagai, A. (2006) 'Inhibition of NADPH Oxidase 4 Activates Apoptosis Via the AKT/apoptosis Signal-Regulating Kinase 1 Pathway in Pancreatic Cancer PANC-1 Cells'. Oncogene 25 (26), 3699-3707

Mockridge, J. W., Marber, M. S., and Heads, R. J. (2000) 'Activation of Akt during Simulated ischemia/reperfusion in Cardiac Myocytes'. Biochemical and Biophysical Research Communications 270 (3), 947-952

Monassier, J. P. (2008) 'Reperfusion Injury in Acute Myocardial Infarction. from Bench to Cath Lab. Part I: Basic Considerations'. Archives of Cardiovascular Diseases 101 (7-8), 491-500

Montaigne, D., Marechal, X., Preau, S., Baccouch, R., Modine, T., Fayad, G., Lancel, S., and Neviere, R. (2011) 'Doxorubicin Induces Mitochondrial Permeability Transition and Contractile Dysfunction in the Human Myocardium'. Mitochondrion 11 (1), 22-26

Montaigne, D., Hurt, C., and Neviere, R. (2012) 'Mitochondria death/survival Signaling Pathways in Cardiotoxicity Induced by Anthracyclines and Anticancer-Targeted Therapies'. Biochemistry Research International 2012

Monticone, M., Taherian, R., Stigliani, S., Carra, E., Monteghirfo, S., Longo, L., Daga, A., Dono, M., Zupo, S., and Giaretti, W. (2014) 'NAC, Tiron and Trolox Impair Survival of Cell Cultures Containing Glioblastoma Tumorigenic Initiating Cells by Inhibition of Cell Cycle Progression'. PloS One 9 (2), e90085

Morry, J., Ngamcherdtrakul, W., and Yantasee, W. (2017) 'Oxidative Stress in Cancer and Fibrosis: Opportunity for Therapeutic Intervention with Antioxidant Compounds, Enzymes, and Nanoparticles'. Redox Biology 11, 240-253

Mozaffarian, D., Benjamin, E. J., Go, A. S., Arnett, D. K., Blaha, M. J., Cushman, M., de Ferranti, S., Despres, J. P., Fullerton, H. J., Howard, V. J., Huffman, M. D., Judd, S. E., Kissela, B. M., Lackland, D. T., Lichtman, J. H., Lisabeth, L. D., Liu, S., Mackey, R. H., Matchar, D. B., McGuire, D. K., Mohler, E. R.,3, Moy, C. S., Muntner, P., Mussolino, M. E., Nasir, K., Neumar, R. W., Nichol, G., Palaniappan, L., Pandey, D. K., Reeves, M. J., Rodriguez, C. J., Sorlie, P. D., Stein, J., Towfighi, A., Turan, T. N., Virani, S. S., Willey, J. Z., Woo, D., Yeh, R. W., Turner, M. B., and American Heart Association Statistics Committee and Stroke,Statistics Subcommittee (2015) 'Heart Disease and Stroke Statistics--2015 Update: A Report from the American Heart Association'. Circulation 131 (4), e29-322

Mukherjee, S., Banerjee, S. K., Maulik, M., Dinda, A. K., Talwar, K. K., and Maulik, S. K. (2003) 'Protection Against Acute Adriamycin-Induced Cardiotoxicity by Garlic: Role of Endogenous Antioxidants and Inhibition of TNF-α Expression'. BMC Pharmacology 3 (1), 16

Mukhopadhyay, P., Rajesh, M., Batkai, S., Kashiwaya, Y., Hasko, G., Liaudet, L., Szabo, C., and Pacher, P. (2009) 'Role of Superoxide, Nitric Oxide, and Peroxynitrite in Doxorubicin-Induced Cell Death in Vivo and in Vitro'. American Journal of Physiology.Heart and Circulatory Physiology 296 (5), H1466-83

Muller, F. L., Liu, Y., and Van Remmen, H. (2004) 'Complex III Releases Superoxide to both Sides of the Inner Mitochondrial Membrane'. The Journal of Biological Chemistry 279 (47), 49064-49073

Mullonkal, C. J. and Toledo-Pereyra, L. (2007) 'Akt in Ischemia and Reperfusion'. Journal of Investigative Surgery 20 (3), 195-203

Münzel, T., Hink, U., Yigit, H., Macharzina, R., Harrison, D. G., and Mülsch, A. (1999) 'Role of Superoxide Dismutase in in Vivo and in Vitro Nitrate Tolerance'. British Journal of Pharmacology 127 (5), 1224-1230

Murata, T., Yamawaki, H., Yoshimoto, R., Hori, M., Sato, K., Ozaki, H., and Karaki, H. (2001) 'Chronic Effect of Doxorubicin on Vascular Endothelium Assessed by Organ Culture Study'. Life Sciences 69 (22), 2685-2695

Murphy, E. and Steenbergen, C. (2008) 'Mechanisms Underlying Acute Protection from Cardiac Ischemia-Reperfusion Injury'. Physiological Reviews 88 (2), 581-609

Murphy, E. and Steenbergen, C. (2007) 'Preconditioning: The Mitochondrial Connection'. Annu.Rev.Physiol. 69, 51-67

Murphy, M. P. (2009) 'How Mitochondria Produce Reactive Oxygen Species'. The Biochemical Journal 417 (1), 1-13

Myers, C. (1998) 'The Role of Iron in Doxorubicin-Induced Cardiomyopathy'. Seminars in Oncology 25 (4), 10-14

Nakao, M., Takio, S., and Ono, K. (1998) 'Alkyl Peroxyl Radical-Scavenging Activity of Catechins'. Phytochemistry 49 (8), 2379-2382

Nazeyrollas, P., Pr ├⌐vost, A., Baccard, N., Manot, L., Devillier, P., and Millart, H. (1999) 'Effects of Amifostine on Perfused Isolated Rat Heart and on Acute Doxorubicin-Induced Cardiotoxicity'. Cancer Chemotherapy and Pharmacology 43 (3), 227-232

Neilan, T. G., Blake, S. L., Ichinose, F., Raher, M. J., Buys, E. S., Jassal, D. S., Furutani, E., Perez-Sanz, T., Graveline, A., Janssens, S. P., Picard, M. H., Scherrer-Crosbie, M., and Bloch, K. D. (2007) 'Disruption of Nitric Oxide Synthase 3 Protects Against the Cardiac Injury, Dysfunction, and Mortality Induced by Doxorubicin'. Circulation 116 (5), 506-514

Ng, C. S., Wan, S., and Yim, A. P. (2005) 'Pulmonary Ischaemia-Reperfusion Injury: Role of Apoptosis'. The European Respiratory Journal 25 (2), 356-363

NIH,National Center for Biotechnology Information. (2005) 'Compound Summary for Tiron'

Nishida, K., Kyoi, S., Yamaguchi, O., Sadoshima, J., and Otsu, K. (2009) 'The Role of Autophagy in the Heart'. Cell Death and Differentiation 16 (1), 31

Nojiri, S., Daida, H., and Inaba, Y. (2004) 'Antioxidants and Cardiovascular Disease: Still a Topic of Interest'. Environmental Health and Preventive Medicine 9 (5), 200-213

Nozaki, N., Shishido, T., Takeishi, Y., and Kubota, I. (2004) 'Modulation of Doxorubicin-Induced Cardiac Dysfunction in Toll-Like Receptor-2-Knockout Mice'. Circulation 110 (18), 2869-2874

Octavia, Y., Tocchetti, C. G., Gabrielson, K. L., Janssens, S., Crijns, H. J., and Moens, A. L. (2012) Doxorubicin-Induced Cardiomyopathy: From Molecular Mechanisms to Therapeutic Strategies.

Oerlemans, M. I., Liu, J., Arslan, F., den Ouden, K., van Middelaar, B. J., Doevendans, P. A., and Sluijter, J. P. (2012) 'Inhibition of RIP1-Dependent Necrosis Prevents Adverse Cardiac

Remodeling After Myocardial ischemia–reperfusion in Vivo'. Basic Research in Cardiology 107 (4), 270

Oikawa, M., Wu, M., Lim, S., Knight, W. E., Miller, C. L., Cai, Y., Lu, Y., Blaxall, B. C., Takeishi, Y., Abe, J., and Yan, C. (2013) 'Cyclic Nucleotide Phosphodiesterase 3A1 Protects the Heart Against Ischemia-Reperfusion Injury'. Journal of Molecular and Cellular Cardiology 64, 11-19

Olson, B. J. and Markwell, J. (2007) 'Assays for Determination of Protein Concentration'. Current Protocols in Pharmacology Appendix 3, 3A

O'Neill, B. T. and Abel, E. D. (2005) 'Akt1 in the Cardiovascular System: Friend Or Foe?'. The Journal of Clinical Investigation 115 (8), 2059-2064

Organization, W. H. (2015) 'WHO Model List of Essential Medicines'. Ed 15

Osman, A. M., Al-Harthi, S., AlArabi, O. M., Elshal, M. F., Ramadan, W. S., Alaama, M. N., Al-Kreathy, H., Damanhouri, Z. A., and Osman, O. H. (2013) 'Chemosensetizing and Cardioprotective Effects of Resveratrol in Doxorubicin-Treated Animals'. Cancer Cell International 13 (1), 52

Ostrakhovitch, E. A. and Cherian, M. G. (2005) 'Inhibition of Extracellular Signal Regulated Kinase (ERK) Leads to Apoptosis Inducing Factor (AIF) Mediated Apoptosis in Epithelial Breast Cancer Cells: The Lack of Effect of ERK in p53 Mediated Copper Induced Apoptosis'. Journal of Cellular Biochemistry 95 (6), 1120-1134

Oyama, J., Blais Jr, C., Liu, X., Pu, M., Kobzik, L., Kelly, R. A., and Bourcier, T. (2004) 'Reduced Myocardial Ischemia-Reperfusion Injury in Toll-Like Receptor 4-Deficient Mice'. Circulation 109 (6), 784-789

Oyewole, A. O., Wilmot, M. C., Fowler, M., and Birch-Machin, M. (2014) 'Comparing the Effects of Mitochondrial Targeted and Localized Antioxidants with Cellular Antioxidants in Human Skin Cells Exposed to UVA and Hydrogen Peroxide'. FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology 28 (1), 485-494

Oyewole, A. O. and Birch-Machin, M. A. (2015) 'Mitochondria-Targeted Antioxidants'. *The FASEB Journal* 29 (12), 4766-4771

Panchuk, R. R., Skorokhyd, N. R., S Kozak, Yuliya, Lehka, L. V., Chumak, V. V., Omelyanchik, S. N., Gurinovich, V. A., Moiseenok, A. G., and Stoika, R. S. (2016) 'Antioxidants Selenomethionine and D-Pantethine Decrease the Negative Side Effects of Doxorubicin in NL/Ly Lymphoma-Bearing Mice'. Croatian Medical Journal 57 (2), 180-192

Pani, G., Bedogni, B., Anzevino, R., Colavitti, R., Palazzotti, B., Borrello, S., and Galeotti, T. (2000) 'Deregulated Manganese Superoxide Dismutase Expression and Resistance to Oxidative Injury in p53-Deficient Cells'. Cancer Research 60 (16), 4654-4660

Panjrath, G. S., Patel, V., Valdiviezo, C. I., Narula, N., Narula, J., and Jain, D. (2007) 'Potentiation of Doxorubicin Cardiotoxicity by Iron Loading in a Rodent Model'. Journal of the American College of Cardiology 49 (25), 2457-2464

Papadopoulou, L. C., Theophilidis, G., Thomopoulos, G. N., and Tsiftsoglou, A. S. (1999) 'Structural and Functional Impairment of Mitochondria in Adriamycin-Induced Cardiomyopathy in Mice: Suppression of Cytochrome c Oxidase II Gene Expression'. Biochemical Pharmacology 57 (5), 481-489

Park, S. A., Na, H. K., Kim, E. H., Cha, Y. N., and Surh, Y. J. (2009) '4-Hydroxyestradiol Induces Anchorage-Independent Growth of Human Mammary Epithelial Cells Via Activation of IkappaB Kinase: Potential Role of Reactive Oxygen Species'. Cancer Research 69 (6), 2416-2424

Patel, A. G. and Kaufmann, S. H. (2012) 'Cancer: How does Doxorubicin Work?'. Elife 1, e00387

Paulsen, C. E. and Carroll, K. S. (2009) 'Orchestrating Redox Signaling Networks through Regulatory Cysteine Switches'. ACS Chemical Biology 5 (1), 47-62

Pecoraro, M., Rodríguez-Sinovas, A., Marzocco, S., Ciccarelli, M., Iaccarino, G., Pinto, A., and Popolo, A. (2017) 'Cardiotoxic Effects of Short-Term Doxorubicin Administration: Involvement of Connexin 43 in Calcium Impairment'. International Journal of Molecular Sciences 18 (10), 2121

Perrelli, M. G., Pagliaro, P., and Penna, C. (2011) 'Ischemia/reperfusion Injury and Cardioprotective Mechanisms: Role of Mitochondria and Reactive Oxygen Species'. World Journal of Cardiology 3 (6), 186-200

Petrovski, G., Gurusamy, N., and Das, D. K. (2011) 'Resveratrol in Cardiovascular Health and Disease'. Annals of the New York Academy of Sciences 1215 (1), 22-33

Pham-Huy, L., He, H., and Pham-Huy, C. (2008) 'Free Radicals, Antioxidants in Disease and Health'. International Journal of Biomedical Science : IJBS 4 (2), 89-96

Piot, C., Croisille, P., Staat, P., Thibault, H., Rioufol, G., Mewton, N., Elbelghiti, R., Cung, T. T., Bonnefoy, E., and Angoulvant, D. (2008) 'Effect of Cyclosporine on Reperfusion Injury in Acute Myocardial Infarction'. New England Journal of Medicine 359 (5), 473-481

Piper, H., Garcña-Dorado, D., and Ovize, M. (1998) 'A Fresh Look at Reperfusion Injury'. Cardiovascular Research 38 (2), 291-300

Pocock, G. and Simons, T. (1987) 'Effects of Lead Ions on Events Associated with Exocytosis in Isolated Bovine Adrenal Medullary Cells'. Journal of Neurochemistry 48 (2), 376-382

Poeggeler, B., Thuermann, S., Dose, A., Schoenke, M., Burkhardt, S., and Hardeland, R. (2002) 'Melatonin's Unique Radical Scavenging properties–roles of its Functional Substituents as Revealed by a Comparison with its Structural Analogs'. Journal of Pineal Research 33 (1), 20-30

Pollard, S. E., Kuhnle, G. G. C., Vauzour, D., Vafeiadou, K., Tzounis, X., Whiteman, M., Rice-Evans, C., and Spencer, J. P. E. (2006) 'The Reaction of Flavonoid Metabolites with Peroxynitrite'. Biochemical and Biophysical Research Communications 350 (4), 960-968

Pomblum, V. J., Korbmacher, B., Cleveland, S., Sunderdiek, U., Klocke, R. C., and Schipke, J. D. (2010) 'Cardiac Stunning in the Clinic: The Full Picture'. Interactive Cardiovascular and Thoracic Surgery 10 (1), 86-91

Pouna, P., Bonoron-Adèle, S., Gouverneur, G., Tariosse, L., Besse, P., and Robert, J. (1995) 'Evaluation of Anthracycline Cardiotoxicity with the Model of Isolated, Perfused Rat Heart: Comparison of New Analogues Versus Doxorubicin'. Cancer Chemotherapy and Pharmacology 35 (3), 257-261

Powell, S. R., Hall, D., Aiuto, L., Wapnir, R. A., Teichberg, S., and Tortolani, A. J. (1994) 'Zinc Improves Postischemic Recovery of Isolated Rat Hearts through Inhibition of Oxidative Stress'. The American Journal of Physiology 266 (6), H2497-507

Prasad, N., Topping, R. S., Zhou, D., and Decker, S. J. (2000) 'Oxidative Stress and Vanadate Induce Tyrosine Phosphorylation of Phosphoinositide-Dependent Kinase 1 (PDK1)'. Biochemistry 39 (23), 6929-6935

Przyklenk, K., Dong, Y., Undyala, V. V., and Whittaker, P. (2012) 'Autophagy as a Therapeutic Target for ischaemia/reperfusion Injury? Concepts, Controversies, and Challenges'. Cardiovascular Research 94 (2), 197-205

Qi, X. J., Wildey, G. M., and Howe, P. H. (2006) 'Evidence that Ser87 of BimEL is Phosphorylated by Akt and Regulates BimEL Apoptotic Function'. The Journal of Biological Chemistry 281 (2), 813-823

Qin, F., Yan, C., Patel, R., Liu, W., and Dong, E. (2006) 'Vitamins C and E Attenuate Apoptosis, β-Adrenergic Receptor Desensitization, and Sarcoplasmic Reticular Ca2 ATPase Downregulation After Myocardial Infarction'. Free Radical Biology and Medicine 40 (10), 1827-1842

Qu, D., Han, J., Ren, H., Yang, W., Zhang, X., Zheng, Q., and Wang, D. (2016) 'Cardioprotective Effects of Astragalin Against Myocardial ischemia/reperfusion Injury in Isolated Rat Heart'. Oxidative Medicine and Cellular Longevity 2016

QuanJun, Y., GenJin, Y., LiLi, W., YongLong, H., Yan, H., Jie, L., JinLu, H., Jin, L., Run, G., and Cheng, G. (2017) 'Protective Effects of Dexrazoxane Against Doxorubicin-Induced Cardiotoxicity: A Metabolomic Study'. PloS One 12 (1), e0169567

Raedschelders, K., Ansley, D. M., and Chen, D. D. Y. (2012) 'The Cellular and Molecular Origin of Reactive Oxygen Species Generation during Myocardial Ischemia and Reperfusion'. Pharmacology & Therapeutics 133 (2), 230-255

Raghuvanshi, R., Kaul, A., Bhakuni, P., Mishra, A., and Misra, M. K. (2007) 'Xanthine Oxidase as a Marker of Myocardial Infarction'. Indian Journal of Clinical Biochemistry 22 (2), 90-92

Rahman, A. M., Yusuf, S. W., and Ewer, M. S. (2007) 'Anthracycline-Induced Cardiotoxicity and the Cardiac-Sparing Effect of Liposomal Formulation'. International Journal of Nanomedicine 2 (4), 567-583

Rai, G., Mishra, S., Suman, S., and Shukla, Y. (2016) 'Resveratrol Improves the Anticancer Effects of Doxorubicin in Vitro and in Vivo Models: A Mechanistic Insight'. Phytomedicine 23 (3), 233-242

Ramachandran, C., Samy, T. A., Huang, X. L., Yuan, Z. K., and Krishan, A. (1993) 'Doxorubicin-Induced DNA Breaks, Topoisomerase II Activity and Gene Expression in Human Melanoma Cells'. Biochemical Pharmacology 45 (6), 1367-1371

Ramos, J. W. (2008) 'The Regulation of Extracellular Signal-Regulated Kinase (ERK) in Mammalian Cells'. The International Journal of Biochemistry & Cell Biology 40 (12), 2707-2719

Ramu, E., Korach, A., Houminer, E., Schneider, A., Elami, A., and Schwalb, H. (2006) 'Dexrazoxane Prevents Myocardial ischemia/reperfusion-Induced Oxidative Stress in the Rat Heart'. Cardiovascular Drugs and Therapy 20 (5), 343-348

Rao, A. V. and Agarwal, S. (1999) 'Role of Lycopene as Antioxidant Carotenoid in the Prevention of Chronic Diseases: A Review'. Nutrition Research 19 (2), 305-323

Raphael, J., Abedat, S., Rivo, J., Meir, K., Beeri, R., Pugatsch, T., Zuo, Z., and Gozal, Y. (2006) 'Volatile Anesthetic Preconditioning Attenuates Myocardial Apoptosis in Rabbits After Regional Ischemia and Reperfusion Via Akt Signaling and Modulation of Bcl-2 Family Proteins'. The Journal of Pharmacology and Experimental Therapeutics 318 (1), 186-194

Ratnam, D. V., Ankola, D. D., Bhardwaj, V., Sahana, D. K., and Kumar, M. R. (2006) 'Role of Antioxidants in Prophylaxis and Therapy: A Pharmaceutical Perspective'. Journal of Controlled Release 113 (3), 189-207

Ray, D., Mukherjee, S., Falchi, M., Bertelli, A., Carlo Braga, P., and K Das, ,Dipak (2010) 'Amelioration of Myocardial Ischemic Reperfusion Injury with Calendula Officinalis'. Current Pharmaceutical Biotechnology 11 (8), 849-854

Razavi-Azarkhiavi, K., Iranshahy, M., Sahebkar, A., Shirani, K., and Karimi, G. (2016) 'The Protective Role of Phenolic Compounds Against Doxorubicin-Induced Cardiotoxicity: A Comprehensive Review'. Nutrition and Cancer 68 (6), 892-917

Reddy, K., Khaliq, A., and Henning, R. J. (2015) 'Recent Advances in the Diagnosis and Treatment of Acute Myocardial Infarction'. World Journal of Cardiology 7 (5), 243-276

Redfors, B., Shao, Y., and Omerovic, E. (2012) 'Myocardial Infarct Size and Area at Risk Assessment in Mice'. Experimental and Clinical Cardiology 17 (4), 268-272

Reed, J. C. (2000) 'Mechanisms of Apoptosis'. The American Journal of Pathology 157 (5), 1415-1430

Renu, K., Abilash, V., PB, T. P., and Arunachalam, S. (2018) 'Molecular Mechanism of Doxorubicin-Induced cardiomyopathy–An Update'. European Journal of Pharmacology 818, 241-253

Reynolds, A., Laurie, C., Mosley, R. L., and Gendelman, H. E. (2007) 'Oxidative Stress and the Pathogenesis of Neurodegenerative Disorders'. International Review of Neurobiology 82, 297-325

Rhee, S. G., Bae, Y. S., Lee, S., and Kwon, J. (2000) 'Hydrogen Peroxide: A Key Messenger that Modulates Protein Phosphorylation through Cysteine Oxidation'. Sci STKE 2000 (53), PE1

Riccioni, G., De Santis, A., Cerasa, V., Menna, V., Di Ilio, C., Schiavone, C., Ballone, E., and D'Orazio, N. (2003) 'Atherosclerotic Plaque Formation and Risk Factors'. International Journal of Immunopathology and Pharmacology 16 (1), 25-31

Rimal, H., Lee, S., Lee, J., and Oh, T. (2015) 'Understanding of Real Alternative Redox Partner of Streptomyces Peucetius DoxA: Prediction and Validation using in Silico and in Vitro Analyses'. Archives of Biochemistry and Biophysics 585, 64-74

Rodrigo, R., Libuy, M., Feliu, F., and Hasson, D. (2013) 'Oxidative Stress-Related Biomarkers in Essential Hypertension and Ischemia-Reperfusion Myocardial Damage'. Disease Markers 35 (6), 773-790

Rodrigo, R., Libuy, M., Feliu, F., and Hasson, D. (2013) 'Molecular Basis of Cardioprotective Effect of Antioxidant Vitamins in Myocardial Infarction'. BioMed Research International 2013, 437613

Rodrigo, R., Prieto, J. C., and Castillo, R. (2013) 'Cardioprotection Against ischaemia/reperfusion by Vitamins C and E Plus n-3 Fatty Acids: Molecular Mechanisms and Potential Clinical Applications'. Clinical Science (London, England : 1979) 124 (1), 1-15

Rosano, G., Fini, M., Caminiti, G., and Barbaro, G. (2008) 'Cardiac Metabolism in Myocardial Ischemia'. Current Pharmaceutical Design 14 (25), 2551-2562

Rossello, X. and Yellon, D. M. (2018) 'The RISK Pathway and Beyond'. Basic Research in Cardiology 113 (1), 2

Roubille, F., Franck-Miclo, A., Covinhes, A., Lafont, C., Cransac, F., Combes, S., Vincent, A., Fontanaud, P., Sportouch-Dukhan, C., Redt-Clouet, C., Nargeot, J., Piot, C., and Barrere-Lemaire, S. (2011) 'Delayed Postconditioning in the Mouse Heart in Vivo'. Circulation 124 (12), 1330-1336

Ruan, Y., Dong, C., Patel, J., Duan, C., Wang, X., Wu, X., Cao, Y., Pu, L., Lu, D., Shen, T., and Li, J. (2015) 'SIRT1 Suppresses Doxorubicin-Induced Cardiotoxicity by Regulating the Oxidative Stress and p38MAPK Pathways'. Cellular Physiology and Biochemistry : International Journal of Experimental Cellular Physiology, Biochemistry, and Pharmacology 35 (3), 1116-1124

Rubbo, H., Radi, R., Trujillo, M., Telleri, R., Kalyanaraman, B., Barnes, S., Kirk, M., and Freeman, B. A. (1994) 'Nitric Oxide Regulation of Superoxide and Peroxynitrite-Dependent Lipid Peroxidation. Formation of Novel Nitrogen-Containing Oxidized Lipid Derivatives'. The Journal of Biological Chemistry 269 (42), 26066-26075

Ruiz, L. M., Salazar, C., Jensen, E., Ruiz, P. A., Tiznado, W., Quintanilla, R. A., Barreto, M., and Elorza, A. A. (2015) 'Quercetin Affects Erythropoiesis and Heart Mitochondrial Function in Mice'. Oxidative Medicine and Cellular Longevity 2015, 836301

Rydén, L., Standl, E., Bartnik, M., Van den Berghe, G., Betteridge, J., De Boer, M., Cosentino, F., Jönsson, B., Laakso, M., and Malmberg, K. (2007) 'Guidelines on Diabetes, Pre-Diabetes, and Cardiovascular Diseases: Executive Summary: The Task Force on Diabetes and Cardiovascular Diseases of the European Society of Cardiology (ESC) and of the European Association for the Study of Diabetes (EASD)'. European Heart Journal 28 (1), 88-136

Ryter, S. W., Kim, H. P., Hoetzel, A., Park, J. W., Nakahira, K., Wang, X., and Choi, A. M. K. (2007) 'Mechanisms of Cell Death in Oxidative Stress'. Antioxidants & Redox Signaling 9 (1), 49-89

S Singh, ,Sylvia and M Kang, ,Peter (2011) 'Mechanisms and Inhibitors of Apoptosis in Cardiovascular Diseases'. Current Pharmaceutical Design 17 (18), 1783-1793

Saeki, K., Obi, I., Ogiku, N., Shigekawa, M., Imagawa, T., and Matsumoto, T. (2002) 'Doxorubicin Directly Binds to the Cardiac-Type Ryanodine Receptor'. Life Sciences 70 (20), 2377-2389

Sag, C. M., Köhler, A. C., Anderson, M. E., Backs, J., and Maier, L. S. (2011) 'CaMKII-Dependent SR Ca Leak Contributes to Doxorubicin-Induced Impaired Ca Handling in Isolated Cardiac Myocytes'. Journal of Molecular and Cellular Cardiology 51 (5), 749-759

Sagach, V. F., Scrosati, M., Fielding, J., Rossoni, G., Galli, C., and Visioli, F. (2002) 'The Water-Soluble Vitamin E Analogue Trolox Protects Against ischaemia/reperfusion Damage in Vitro and Ex Vivo. A Comparison with Vitamin E'. Pharmacological Research 45 (6), 435-439

Saleem, M. T. S., Chetty, M. C., and Kavimani, S. (2014) 'Antioxidants and Tumor Necrosis Factor Alpha-Inhibiting Activity of Sesame Oil Against Doxorubicin-Induced Cardiotoxicity'. Therapeutic Advances in Cardiovascular Disease 8 (1), 4-11

Salvatorelli, E., Menna, P., Chello, M., Covino, E., and Minotti, G. (2018) 'Low-Dose Anthracycline and Risk of Heart Failure in a Pharmacokinetic Model of Human Myocardium Exposure: Analog Specificity and Role of Secondary Alcohol Metabolites'. The Journal of Pharmacology and Experimental Therapeutics 364 (2), 323-331

Salvayre, R., Negre-Salvayre, A., and Camaré, C. (2016) 'Oxidative Theory of Atherosclerosis and Antioxidants'. Biochimie 125, 281-296

Salzer, W. L., Devidas, M., Carroll, W. L., Winick, N., Pullen, J., Hunger, S. P., and Camitta, B. A. (2010) 'Long-Term Results of the Pediatric Oncology Group Studies for Childhood Acute Lymphoblastic Leukemia 1984–2001: A Report from the Children's Oncology Group'. Leukemia 24 (2), 355

Sanada, S., Komuro, I., and Kitakaze, M. (2011) 'Pathophysiology of Myocardial Reperfusion Injury: Preconditioning, Postconditioning, and Translational Aspects of Protective Measures'. American Journal of Physiology.Heart and Circulatory Physiology 301 (5), H1723-41

Santhakumar, A. B., Kundur, A. R., Fanning, K., Netzel, M., Stanley, R., and Singh, I. (2015) 'Consumption of Anthocyanin-Rich Queen Garnet Plum Juice Reduces Platelet Activation Related Thrombogenesis in Healthy Volunteers'. Journal of Functional Foods 12, 11-22

Santhakumar, A. B., Bulmer, A. C., and Singh, I. (2014) 'A Review of the Mechanisms and Effectiveness of Dietary Polyphenols in Reducing Oxidative Stress and Thrombotic Risk'. Journal of Human Nutrition and Dietetics 27 (1), 1-21

Santos, C. X., Raza, S., and Shah, A. M. (2016) 'Redox Signaling in the Cardiomyocyte: From Physiology to Failure'. The International Journal of Biochemistry & Cell Biology 74, 145-151

Santos, C. X. C., Anilkumar, N., Zhang, M., Brewer, A. C., and Shah, A. M. (2011) 'Redox Signaling in Cardiac Myocytes'. Free Radical Biology and Medicine 50 (7), 777-793

Santulli, G., Pagano, G., Sardu, C., Xie, W., Reiken, S., D'Ascia, S. L., Cannone, M., Marziliano, N., Trimarco, B., and Guise, T. A. (2015) 'Calcium Release Channel RyR2 Regulates Insulin Release and Glucose Homeostasis'. The Journal of Clinical Investigation 125 (5), 1968-1978

Sardão, V. A., Oliveira, P. J., Holy, J., Oliveira, C. R., and Wallace, K. B. (2009) 'Doxorubicin-Induced Mitochondrial Dysfunction is Secondary to Nuclear p53 Activation in H9c2 Cardiomyoblasts'. Cancer Chemotherapy and Pharmacology 64 (4), 811-827

Sarvothaman, S., Undi, R. B., Pasupuleti, S. R., Gutti, U., and Gutti, R. K. (2015) 'Apoptosis: Role in Myeloid Cell Development'. Blood Research 50 (2), 73-79

Sawyer, D. B., Fukazawa, R., Arstall, M. A., and Kelly, R. A. (1999) 'Daunorubicin-Induced Apoptosis in Rat Cardiac Myocytes is Inhibited by Dexrazoxane'. Circulation Research 84 (3), 257-265

Sawyer, D. B., Peng, X., Chen, B., Pentassuglia, L., and Lim, C. C. (2010) 'Mechanisms of Anthracycline Cardiac Injury: Can we Identify Strategies for Cardioprotection?'. Progress in Cardiovascular Diseases 53 (2), 105-113

Sayed-Ahmed, M. M., Khattab, M. M., Gad, M. Z., and Osman, A. M. (2001) 'Increased Plasma endothelin-1 and Cardiac Nitric Oxide during doxorubicin-induced Cardiomyopathy'. Pharmacology & Toxicology 89 (3), 140-144

Scherrer-Crosbie, M., Ullrich, R., Bloch, K. D., Nakajima, H., Nasseri, B., Aretz, H. T., Lindsey, M. L., Vancon, A. C., Huang, P. L., Lee, R. T., Zapol, W. M., and Picard, M. H. (2001) 'Endothelial Nitric Oxide Synthase Limits Left Ventricular Remodeling After Myocardial Infarction in Mice'. Circulation 104 (11), 1286-1291

Schiffrin, E. L. (2001) 'Role of Endothelin-1 in Hypertension and Vascular Disease'. American Journal of Hypertension 14 (S3), 83S-89S

Schlame, M., Rua, D., and Greenberg, M. L. (2000) 'The Biosynthesis and Functional Role of Cardiolipin'. Progress in Lipid Research 39 (3), 257-288

Schott, B. and Robert, J. (1989) 'Comparative Cytotoxicity, DNA Synthesis Inhibition and Drug Incorporation of Eight Anthracyclines in a Model of Doxorubicin-Sensitive and -Resistant Rat Glioblastoma Cells'. Biochemical Pharmacology 38 (1), 167-172

Scorrano, L., Oakes, S. A., Opferman, J. T., Cheng, E. H., Sorcinelli, M. D., Pozzan, T., and Korsmeyer, S. J. (2003) 'BAX and BAK Regulation of Endoplasmic Reticulum Ca2+: A Control Point for Apoptosis'. Science (New York, N.Y.) 300 (5616), 135-139

Seki, S., Flavahan, N. A., Smedira, N. G., and Murray, P. A. (1999) 'Superoxide Anion Scavengers Restore NO-Mediated Pulmonary Vasodilation After Lung Transplantation'. American Journal of Physiology-Heart and Circulatory Physiology 276 (1), H42-H46

Sena, L. A. and Chandel, N. S. (2012) 'Physiological Roles of Mitochondrial Reactive Oxygen Species'. Molecular Cell 48 (2), 158-167

Sepe, D. M., Ginsberg, J. P., and Balis, F. M. (2010) 'Dexrazoxane as a Cardioprotectant in Children Receiving Anthracyclines'. The Oncologist 15 (11), 1220-1226

Shaker, R. A., Abboud, S. H., Assad, H. C., and Hadi, N. (2018) 'Enoxaparin Attenuates Doxorubicin Induced Cardiotoxicity in Rats Via Interfering with Oxidative Stress, Inflammation and Apoptosis'. BMC Pharmacology and Toxicology 19 (1), 3

Shakir, D. K. and Rasul, K. I. (2009) 'Chemotherapy Induced Cardiomyopathy: Pathogenesis, Monitoring and Management'. Journal of Clinical Medicine Research 1 (1), 8-12

Shan, K., Lincoff, A. M., and Young, J. B. (1996) 'Anthracycline-Induced Cardiotoxicity'. Annals of Internal Medicine 125 (1), 47-58

Shanmugam, K., Ravindran, S., Kurian, G. A., and Rajesh, M. (2018) 'Fisetin Confers Cardioprotection Against Myocardial Ischemia Reperfusion Injury by Suppressing Mitochondrial Oxidative Stress and Mitochondrial Dysfunction and Inhibiting Glycogen Synthase Kinase 3β Activity'. Oxidative Medicine and Cellular Longevity 2018

Sharma, P., Johri, S., and Shukla, S. (2000) 'Beryllium-induced Toxicity and its Prevention by Treatment with Chelating Agents'. Journal of Applied Toxicology: An International Journal 20 (4), 313-318

Shen, M., Jia, G., Wang, Y., and Ma, H. (2006) 'Cardioprotective Effect of Resvaratrol Pretreatment on Myocardial ischemia–reperfusion Induced Injury in Rats'. Vascular Pharmacology 45 (2), 122-126

Shi, J., Zhang, L., Zhang, Y., Surma, M., Mark Payne, R., and Wei, L. (2012) 'Downregulation of Doxorubicin-Induced Myocardial Apoptosis Accompanies Postnatal Heart Maturation'. American Journal of Physiology-Heart and Circulatory Physiology 302 (8), H1603-H1613

Shi, J., Abdelwahid, E., and Wei, L. (2011) 'Apoptosis in Anthracycline Cardiomyopathy'. Current Pediatric Reviews 7 (4), 329-336

Shinzawa, K. and Tsujimoto, Y. (2003) 'PLA2 Activity is Required for Nuclear Shrinkage in Caspase-Independent Cell Death'. The Journal of Cell Biology 163 (6), 1219-1230

Shiojima, I. and Walsh, K. (2002) 'Role of Akt Signaling in Vascular Homeostasis and Angiogenesis'. Circulation Research 90 (12), 1243-1250

Sishi, B. J., Loos, B., van Rooyen, J., and Engelbrecht, A. (2013) 'Autophagy Upregulation Promotes Survival and Attenuates Doxorubicin-Induced Cardiotoxicity'. Biochemical Pharmacology 85 (1), 124-134

Shizukuda, Y., Matoba, S., Mian, O. Y., Nguyen, T., and Hwang, P. M. (2005) 'Targeted Disruption of p53 Attenuates Doxorubicin-Induced Cardiac Toxicity in Mice'. Molecular and Cellular Biochemistry 273 (1-2), 25-32

Silva-Platas, C., Guerrero-Beltrán, C. E., Carrancá, M., Castillo, E. C., Bernal-Ramírez, J., Oropeza-Almazán, Y., González, L. N., Rojo, R., Martínez, L. E., and Valiente-Banuet, J. (2016) 'Antineoplastic Copper Coordinated Complexes (Casiopeinas) Uncouple Oxidative Phosphorylation and Induce Mitochondrial Permeability Transition in Cardiac Mitochondria and Cardiomyocytes'. Journal of Bioenergetics and Biomembranes 48 (1), 43-54

Šimůnek, T., Štěrba, M., Popelová, O., Adamcová, M., Hrdina, R., and Geršl, V. (2009) 'Anthracycline-Induced Cardiotoxicity: Overview of Studies Examining the Roles of Oxidative Stress and Free Cellular Iron'. Pharmacological Reports 61 (1), 154-171

Singal, P. K., Iliskovic, N., Li, T., and Kumar, D. (1997) 'Adriamycin Cardiomyopathy: Pathophysiology and Prevention'. FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology 11 (12), 931-936

Singh, G., Singh, A. T., Abraham, A., Bhat, B., Mukherjee, A., Verma, R., Agarwal, S. K., Jha, S., Mukherjee, R., and Burman, A. C. (2008) 'Protective Effects of Terminalia Arjuna Against Doxorubicin-Induced Cardiotoxicity'. Journal of Ethnopharmacology 117 (1), 123-129

Singh, U. and Jialal, I. (2006) 'Oxidative Stress and Atherosclerosis'. Pathophysiology : The Official Journal of the International Society for Pathophysiology 13 (3), 129-142

Skrzypiec-Spring, M., Grotthus, B., Szelag, A., and Schulz, R. (2007) 'Isolated Heart Perfusion According to Langendorff---Still Viable in the New Millennium'. Journal of Pharmacological and Toxicological Methods 55 (2), 113-126

Slee, E. A., Harte, M. T., Kluck, R. M., Wolf, B. B., Casiano, C. A., Newmeyer, D. D., Wang, H. G., Reed, J. C., Nicholson, D. W., Alnemri, E. S., Green, D. R., and Martin, S. J. (1999) 'Ordering the Cytochrome c-Initiated Caspase Cascade: Hierarchical Activation of Caspases-2, -3, -6, -7, -8, and -10 in a Caspase-9-Dependent Manner'. The Journal of Cell Biology 144 (2), 281-292

Smart, N., Mojet, M. H., Latchman, D. S., Marber, M. S., Duchen, M. R., and Heads, R. J. (2006) 'IL-6 Induces PI 3-Kinase and Nitric Oxide-Dependent Protection and Preserves Mitochondrial Function in Cardiomyocytes'. *Cardiovascular Research* 69 (1), 164-177

Smith, C. C., Davidson, S. M., Lim, S. Y., Simpkin, J. C., Hothersall, J. S., and Yellon, D. M. (2007) 'Necrostatin: A Potentially Novel Cardioprotective Agent?'. Cardiovascular Drugs and Therapy 21 (4), 227-233

Smith, L., Watson, M. B., O'Kane, S. L., Drew, P. J., Lind, M. J., and Cawkwell, L. (2006) 'The Analysis of Doxorubicin Resistance in Human Breast Cancer Cells using Antibody Microarrays'. Molecular Cancer Therapeutics 5 (8), 2115-2120

Smith, L. A., Cornelius, V. R., Plummer, C. J., Levitt, G., Verrill, M., Canney, P., and Jones, A. (2010) 'Cardiotoxicity of Anthracycline Agents for the Treatment of Cancer: Systematic Review and Meta-Analysis of Randomised Controlled Trials'. BMC Cancer 10 (1), 337

Song, D., Jang, Y., Kim, J. H., Chun, K., Lee, D., and Xu, Z. (2010) 'Polyphenol (-)-Epigallocatechin Gallate during Ischemia Limits Infarct Size Via Mitochondrial KATP Channel Activation in Isolated Rat Hearts'. Journal of Korean Medical Science 25 (3), 380-386

Spallarossa, P., Garibaldi, S., Altieri, P., Fabbi, P., Manca, V., Nasti, S., Rossettin, P., Ghigliotti, G., Ballestrero, A., Patrone, F., Barsotti, A., and Brunelli, C. (2004) 'Carvedilol Prevents Doxorubicin-Induced Free Radical Release and Apoptosis in Cardiomyocytes in Vitro'. Journal of Molecular and Cellular Cardiology 37 (4), 837-846

Spath, N. B., Mills, N. L., and Cruden, N. L. (2016) 'Novel Cardioprotective and Regenerative Therapies in Acute Myocardial Infarction: A Review of Recent and Ongoing Clinical Trials'. Future Cardiology 12 (6), 655-672

Spieker, L. E., Noll, G., Ruschitzka, F. T., and Lüscher, T. F. (2001) 'Endothelin Receptor Antagonists in Congestive Heart Failure: A New Therapeutic Principle for the Future?'. Journal of the American College of Cardiology 37 (6), 1493-1505

Stadtman, E. R. and Levine, R. L. (2000) 'Protein Oxidation'. Annals of the New York Academy of Sciences 899, 191-208

Steare, S. and Yellon, D. (1993) 'The Protective Effect of Heat Stress Against Reperfusion Arrhythmias in the Rat'. Journal of Molecular and Cellular Cardiology 25 (12), 1471-1481

Steinberg, D. and Witztum, J. L. (2010) 'Oxidized Low-Density Lipoprotein and Atherosclerosis'. Arteriosclerosis, Thrombosis, and Vascular Biology 30 (12), 2311-2316

Stephens, N. G., Parsons, A., Brown, M. J., Schofield, P. M., Kelly, F., Cheeseman, K., and Mitchinson, M. (1996) Randomised Controlled Trial of Vitamin E in Patients with Coronary Disease: Cambridge Heart Antioxidant Study (CHAOS).

Štěrba, M., Popelová, O., Vávrová, A., Jirkovský, E., Kovaříková, P., Geršl, V., and Šimůnek, T. (2013) 'Oxidative Stress, Redox Signaling, and Metal Chelation in Anthracycline Cardiotoxicity and Pharmacological Cardioprotection'. Antioxidants & Redox Signaling 18 (8), 899-929

Stewart, J., Manmathan, G., and Wilkinson, P. (2017) 'Primary Prevention of Cardiovascular Disease: A Review of Contemporary Guidance and Literature'. JRSM Cardiovascular Disease 6, 2048004016687211

Stocker, R. and Keaney Jr, J. F. (2004) 'Role of Oxidative Modifications in Atherosclerosis'. Physiological Reviews 84 (4), 1381-1478

Storz, P. and Toker, A. (2002) '3'-Phosphoinositide-Dependent Kinase-1 (PDK-1) in PI 3-Kinase Signaling'. Front Biosci 7, d886-902

Storz, P. (2005) 'Reactive Oxygen Species in Tumor Progression'. Front Biosci 10 (1-3), 1881-1896

Su, C., Yang, J., Leu, H., Chen, Y., and Wang, P. H. (2011) 'Mitochondrial Akt-Regulated Mitochondrial Apoptosis Signaling in Cardiac Muscle Cells'. American Journal of Physiology-Heart and Circulatory Physiology 302 (3), H716-H723

Sugamura, K. and Keaney Jr, J. F. (2011) 'Reactive Oxygen Species in Cardiovascular Disease'. Free Radical Biology and Medicine 51 (5), 978-992

Sullivan, L. B. and Chandel, N. S. (2014) 'Mitochondrial Reactive Oxygen Species and Cancer'. Cancer & Metabolism 2 (1), 1

Sun, H., Lesche, R., Li, D. M., Liliental, J., Zhang, H., Gao, J., Gavrilova, N., Mueller, B., Liu, X., and Wu, H. (1999) 'PTEN Modulates Cell Cycle Progression and Cell Survival by Regulating Phosphatidylinositol 3,4,5,-Trisphosphate and Akt/protein Kinase B Signaling Pathway'. Proceedings of the National Academy of Sciences of the United States of America 96 (11), 6199-6204

Sun, J., Sun, G., Cui, X., Meng, X., Qin, M., and Sun, X. (2016) 'Myricitrin Protects Against Doxorubicin-Induced Cardiotoxicity by Counteracting Oxidative Stress and Inhibiting Mitochondrial Apoptosis Via ERK/P53 Pathway'. Evidence-Based Complementary and Alternative Medicine 2016

Sun, J., Sun, G., Meng, X., Wang, H., Luo, Y., Qin, M., Ma, B., Wang, M., Cai, D., and Guo, P. (2013) 'Isorhamnetin Protects Against Doxorubicin-Induced Cardiotoxicity in Vivo and in Vitro'. PloS One 8 (5), e64526

Sundaresan, M., Yu, Z., Ferrans, V. J., Irani, K., and Finkel, T. (1995) 'Requirement for Generation of H2O2 for Platelet-Derived Growth Factor Signal Transduction'. Science 270 (5234), 296

Supinski, G., Nethery, D., Stofan, D., and DiMarco, A. (1999) 'Extracellular Calcium Modulates Generation of Reactive Oxygen Species by the Contracting Diaphragm'. Journal of Applied Physiology 87 (6), 2177-2185

Suter, T. M. and Ewer, M. S. (2012) 'Cancer Drugs and the Heart: Importance and Management'. European Heart Journal 34 (15), 1102-1111

Sutherland, F. J. and Hearse, D. J. (2000) 'The Isolated Blood and Perfusion Fluid Perfused Heart'. Pharmacological Research 41 (6), 613-627

Swain, S. M., Whaley, F. S., Gerber, M. C., Weisberg, S., York, M., Spicer, D., Jones, S. E., Wadler, S., Desai, A., Vogel, C., Speyer, J., Mittelman, A., Reddy, S., Pendergrass, K., Velez-Garcia, E., Ewer, M. S., Bianchine, J. R., and Gams, R. A. (1997) 'Cardioprotection with Dexrazoxane for Doxorubicin-Containing Therapy in Advanced Breast Cancer'. Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology 15 (4), 1318-1332

Swain, S. M., Whaley, F. S., and Ewer, M. S. (2003) 'Congestive Heart Failure in Patients Treated with Doxorubicin'. Cancer 97 (11), 2869-2879

Tacar, O., Sriamornsak, P., and Dass, C. R. (2013) 'Doxorubicin: An Update on Anticancer Molecular Action, Toxicity and Novel Drug Delivery Systems'. Journal of Pharmacy and Pharmacology 65 (2), 157-170

Tait, S. W., Ichim, G., and Green, D. R. (2014) 'Die another Way--Non-Apoptotic Mechanisms of Cell Death'. Journal of Cell Science 127, 2135-2144

Tait, S. W. G. and Green, D. R. (2010) 'Mitochondria and Cell Death: Outer Membrane Permeabilization and Beyond'. Nature Reviews Molecular Cell Biology 11 (9), 621

Takahashi, N., Duprez, L., Grootjans, S., Cauwels, A., Nerinckx, W., DuHadaway, J., Goossens, V., Roelandt, R., Van Hauwermeiren, F., and Libert, C. (2012) 'Necrostatin-1 Analogues: Critical Issues on the Specificity, Activity and in Vivo use in Experimental Disease Models'. Cell Death & Disease 3 (11), e437

Takemura, G. and Fujiwara, H. (2007) 'Doxorubicin-Induced Cardiomyopathy from the Cardiotoxic Mechanisms to Management'. Progress in Cardiovascular Diseases 49 (5), 330-352

Tam, K. (2013) 'The Roles of Doxorubicin in Hepatocellular Carcinoma'. ADMET and DMPK 1 (3), 29-44

Tang, Z., Yang, L., and Zhang, X. (2017) 'Vitexin Mitigates Myocardial Ischemia Reperfusion-Induced Damage by Inhibiting Excessive Autophagy to Suppress Apoptosis Via the PI3K/Akt/mTOR Signaling Cascade'. RSC Advances 7 (89), 56406-56416

Tebbi, C. K., London, W. B., Friedman, D., Villaluna, D., De Alarcon, P. A., Constine, L. S., Mendenhall, N. P., Sposto, R., Chauvenet, A., and Schwartz, C. L. (2007) 'Dexrazoxane-Associated Risk for Acute Myeloid leukemia/myelodysplastic Syndrome and Other Secondary Malignancies in Pediatric Hodgkin's Disease'. Journal of Clinical Oncology 25 (5), 493-500

Teresa, M. S. and Lunawati, L. B. (2011) 'The Role of Antioxidant Vitamins in Cardiovascular Disease'. Journal of Pharmacy Technology 27 (1), 19-26

Testai, L., Martelli, A., Cristofaro, M., Breschi, M. C., and Calderone, V. (2013) 'Cardioprotective Effects of Different Flavonoids Against Myocardial ischaemia/reperfusion Injury in L angendorff-perfused Rat Hearts'. Journal of Pharmacy and Pharmacology 65 (5), 750-756

Thannickal, V. J. and Fanburg, B. L. (2000) 'Reactive Oxygen Species in Cell Signaling'. American Journal of Physiology.Lung Cellular and Molecular Physiology 279 (6), L1005-28

Thomas, C. E. and Aust, S. D. (1986) 'Release of Iron from Ferritin by Cardiotoxic Anthracycline Antibiotics'. *Archives of Biochemistry and Biophysics* 248 (2), 684-689

Thorn, C. F., Oshiro, C., Marsh, S., Hernandez-Boussard, T., McLeod, H., Klein, T. E., and Altman, R. B. (2011) 'Doxorubicin Pathways: Pharmacodynamics and Adverse Effects'. Pharmacogenetics and Genomics 21 (7), 440-446

Tokarska-Schlattner, M., Zaugg, M., Zuppinger, C., Wallimann, T., and Schlattner, U. (2006) 'New Insights into Doxorubicin-Induced Cardiotoxicity: The Critical Role of Cellular Energetics'. Journal of Molecular and Cellular Cardiology 41 (3), 389-405

Toldo, S., Goehe, R. W., Lotrionte, M., Mezzaroma, E., Sumner, E. T., Biondi-Zoccai, G., Seropian, I. M., Van Tassell, B. W., Loperfido, F., and Palazzoni, G. (2013) 'Comparative Cardiac Toxicity of Anthracyclines in Vitro and in Vivo in the Mouse'. PloS One 8 (3), e58421

Tompkins, A. J., Burwell, L. S., Digerness, S. B., Zaragoza, C., Holman, W. L., and Brookes, P. S. (2006) 'Mitochondrial Dysfunction in Cardiac ischemia–reperfusion Injury: ROS from Complex I, without Inhibition'. Biochimica Et Biophysica Acta (BBA)-Molecular Basis of Disease 1762 (2), 223-231

Torres, V. M. and Simic, V. D. (2012) 'Doxorubicin-Induced Oxidative Injury of Cardiomyocytes-do we have Right Strategies for Prevention?'. in . ed. by Anon: InTech

Toth, A., Halmosi, R., Kovacs, K., Deres, P., Kalai, T., Hideg, K., Toth, K., and Sumegi, B. (2003) 'Akt Activation Induced by an Antioxidant Compound during Ischemia-Reperfusion'. Free Radical Biology & Medicine 35 (9), 1051-1063

Tribble, D. L. (1999) 'AHA Science Advisory. Antioxidant Consumption and Risk of Coronary Heart Disease: Emphasison Vitamin C, Vitamin E, and Beta-Carotene: A Statement for Healthcare Professionals from the American Heart Association'. Circulation 99 (4), 591-595

Tsang, A., Hausenloy, D. J., Mocanu, M. M., and Yellon, D. M. (2004) 'Postconditioning: A Form of "Modified Reperfusion" Protects the Myocardium by Activating the Phosphatidylinositol 3-Kinase-Akt Pathway'. Circulation Research 95 (3), 230-232

Turrens, J. F. (2003) 'Mitochondrial Formation of Reactive Oxygen Species'. The Journal of Physiology 552, 335-344

Uchiyama, T., Engelman, R. M., Maulik, N., and Das, D. K. (2004) 'Role of Akt Signaling in Mitochondrial Survival Pathway Triggered by Hypoxic Preconditioning'. Circulation 109 (24), 3042-3049

Upston, J. M., Witting, P. K., Brown, A. J., Stocker, R., and Keaney Jr, J. F. (2001) 'Effect of Vitamin E on Aortic Lipid Oxidation and Intimal Proliferation After Arterial Injury in Cholesterol-Fed Rabbits'. Free Radical Biology and Medicine 31 (10), 1245-1253

Ushio-Fukai, M., Alexander, R. W., Akers, M., Yin, Q., Fujio, Y., Walsh, K., and Griendling, K. K. (1999) 'Reactive Oxygen Species Mediate the Activation of Akt/protein Kinase B by Angiotensin II in Vascular Smooth Muscle Cells'. The Journal of Biological Chemistry 274 (32), 22699-22704

Valko, M., Rhodes, C., Moncol, J., Izakovic, M., and Mazur, M. (2006) 'Free Radicals, Metals and Antioxidants in Oxidative Stress-Induced Cancer'. Chemico-Biological Interactions 160 (1), 1-40

Valko, M., Izakovic, M., Mazur, M., Rhodes, C. J., and Telser, J. (2004) 'Role of Oxygen Radicals in DNA Damage and Cancer Incidence'. Molecular and Cellular Biochemistry 266 (1-2), 37-56

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., and Telser, J. (2007) 'Free Radicals and Antioxidants in Normal Physiological Functions and Human Disease'. The International Journal of Biochemistry & Cell Biology 39 (1), 44-84

van Empel, V.,P.M., Bertrand, A. T. A., Hofstra, L., Crijns, H. J., Doevendans, P. A., and De Windt, L. J. (2005) 'Myocyte Apoptosis in Heart Failure'. Cardiovascular Research 67 (1), 21-29

Vanden Hoek, T. L., Shao, Z., Li, C., Zak, R., Schumacker, P. T., and Becker, L. B. (1996) 'Reperfusion Injury on Cardiac Myocytes After Simulated Ischemia'. The American Journal of Physiology 270 (4), H1334-41

Vandenabeele, P., Galluzzi, L., Berghe, T. V., and Kroemer, G. (2010) 'Molecular Mechanisms of Necroptosis: An Ordered Cellular Explosion'. Nature Reviews Molecular Cell Biology 11 (10), 700

Vanlangenakker, N., Berghe, T. V., Krysko, D. V., Festjens, N., and Vandenabeele, P. (2008) 'Molecular Mechanisms and Pathophysiology of Necrotic Cell Death'. Current Molecular Medicine 8 (3), 207-220

Varga, Z. V., Ferdinandy, P., Liaudet, L., and Pacher, P. (2015) 'Drug-Induced Mitochondrial Dysfunction and Cardiotoxicity'. American Journal of Physiology-Heart and Circulatory Physiology 309 (9), H1453-H1467

Varga, Z. V., Ferdinandy, P., Liaudet, L., and Pacher, P. (2015) 'Drug-Induced Mitochondrial Dysfunction and Cardiotoxicity'. American Journal of Physiology.Heart and Circulatory Physiology 309 (9), H1453-67

Vásquez-Vivar, J., Martasek, P., Hogg, N., Masters, B. S. S., Pritchard, K. A., and Kalyanaraman, B. (1997) 'Endothelial Nitric Oxide Synthase-Dependent Superoxide Generation from Adriamycin'. Biochemistry 36 (38), 11293-11297

Vejpongsa, P. and Yeh, E. T. H. (2014) 'Prevention of Anthracycline-Induced Cardiotoxicity: Challenges and Opportunities'. Journal of the American College of Cardiology 64 (9), 938-945

Venardos, K. M. and Kaye, D. M. (2007) 'Myocardial Ischemia-Reperfusion Injury, Antioxidant Enzyme Systems, and Selenium: A Review'. Current Medicinal Chemistry 14 (14), 1539-1549

Ventura-Clapier, R., Garnier, A., and Veksler, V. (2004) 'Energy Metabolism in Heart Failure'. The Journal of Physiology 555 (1), 1-13

Venturini, M., Michelotti, A., Del Mastro, L., Gallo, L., Carnino, F., Garrone, O., Tibaldi, C., Molea, N., Bellina, R. C., Pronzato, P., Cyrus, P., Vinke, J., Testore, F., Guelfi, M., Lionetto, R., Bruzzi, P., Conte, P. F., and Rosso, R. (1996) 'Multicenter Randomized Controlled Clinical Trial to Evaluate Cardioprotection of Dexrazoxane Versus no Cardioprotection in Women Receiving Epirubicin Chemotherapy for Advanced Breast Cancer'. Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology 14 (12), 3112-3120

Verma, S., Lovren, F., Dumont, A. S., Mather, K. J., Maitland, A., Kieser, T. M., Kidd, W., McNeill, J. H., Stewart, D. J., and Triggle, C. R. (2001) 'Endothelin Receptor Blockade Improves

Endothelial Function in Human Internal Mammary Arteries'. Cardiovascular Research 49 (1), 146-151

Verma, S., Fedak, P. W., Weisel, R. D., Butany, J., Rao, V., Maitland, A., Li, R. K., Dhillon, B., and Yau, T. M. (2002) 'Fundamentals of Reperfusion Injury for the Clinical Cardiologist'. Circulation 105 (20), 2332-2336

Virtamo, J., Rapola, J. M., Ripatti, S., Heinonen, O. P., Taylor, P. R., Albanes, D., and Huttunen, J. K. (1998) 'Effect of Vitamin E and Beta Carotene on the Incidence of Primary Nonfatal Myocardial Infarction and Fatal Coronary Heart Disease'. Archives of Internal Medicine 158 (6), 668-675

Voest, E. E., van Acker, Saskia A. B. E., van, d. V., van Asbeck, B. S., and Bast, A. (1994) Comparison of Different Iron Chelators as Protective Agents Against Acute Doxorubicin-Induced Cardiotoxicity.

Volkova, M. and Russell, R.,3 (2011) 'Anthracycline Cardiotoxicity: Prevalence, Pathogenesis and Treatment'. Current Cardiology Reviews 7 (4), 214-220

Von Hoff, D. D., Layard, M. W., Basa, P., Davis, H. L.,J, Von Hoff, A. L., Rozencweig, M., and Muggia, F. M. (1979) 'Risk Factors for Doxorubicin-Induced Congestive Heart Failure'. Annals of Internal Medicine 91 (5), 710-717;

Vorobjeva, N. V. and Pinegin, B. V. (2016) 'Effects of the Antioxidants Trolox, Tiron and Tempol on Neutrophil Extracellular Trap Formation'. *Immunobiology* 221 (2), 208-219.

Walker, J. M. (1994) 'The Bicinchoninic Acid (BCA) Assay for Protein Quantitation'. Methods in Molecular Biology (Clifton, N.J.) 32, 5-8

Wallace, K. B. (2003) 'Doxorubicin-induced Cardiac Mitochondrionopathy'. Pharmacology & Toxicology 93 (3), 105-115

Wallace, D. C. (1999) 'Mitochondrial Diseases in Man and Mouse'. Science (New York, N.Y.) 283 (5407), 1482-1488

Wallace, D. C. (2005) 'A Mitochondrial Paradigm of Metabolic and Degenerative Diseases, Aging, and Cancer: A Dawn for Evolutionary Medicine'. Annu.Rev.Genet. 39, 359-407

Walsh, K. (2006) 'Akt Signaling and Growth of the Heart'. Circulation 113 (17), 2032-2034

Wambi-Kiéssé, C. O. and Katusic, Z. S. (1999) 'Inhibition of copper/zinc Superoxide Dismutase Impairs NO·-Mediated Endothelium-Dependent Relaxations'. American Journal of Physiology-Heart and Circulatory Physiology 276 (3), H1043-H1048

Wang, D., Zhang, X., Li, D., Hao, W., Meng, F., Wang, B., Han, J., and Zheng, Q. (2017) 'Kaempferide Protects Against Myocardial Ischemia/Reperfusion Injury through Activation of the PI3K/Akt/GSK-3β Pathway'. Mediators of Inflammation 2017

Wang, J., Pareja, K. A., Kaiser, C. A., and Sevier, C. S. (2014) 'Redox Signaling Via the Molecular Chaperone BiP Protects Cells Against Endoplasmic Reticulum-Derived Oxidative Stress'. Elife 3, e03496

Warpe, V. S., Mali, V. R., Arulmozhi, S., Bodhankar, S. L., and Mahadik, K. R. (2015) 'Cardioprotective Effect of Ellagic Acid on Doxorubicin Induced Cardiotoxicity in Wistar Rats'. Journal of Acute Medicine 5 (1), 1-8

Watjen, W., Michels, G., Steffan, B., Niering, P., Chovolou, Y., Kampkotter, A., Tran-Thi, Q., Proksch, P., and Kahl, R. (2005) 'Low Concentrations of Flavonoids are Protective in Rat H4IIE Cells Whereas High Concentrations Cause DNA Damage and Apoptosis'. The Journal of Nutrition 135 (3), 525-531

Wei, Y., Lu, M., and Wang, W. (2001) 'Ca2 Mediates the Effect of Inhibition of Na -K -ATPase on the Basolateral K Channels in the Rat CCD'. American Journal of Physiology-Cell Physiology 280 (4), C920-C928

Weinstein, D. M., Mihm, M. J., and Bauer, J. A. (2000) 'Cardiac Peroxynitrite Formation and Left Ventricular Dysfunction Following Doxorubicin Treatment in Mice'. The Journal of Pharmacology and Experimental Therapeutics 294 (1), 396-401

Weise-Cross, L., Sands, M. A., Sheak, J. R., Broughton, B. R., Snow, J. B., Gonzalez Bosc, L. V., Jernigan, N. L., Walker, B. R., and Resta, T. C. (2018) 'Actin Polymerization Contributes to Enhanced Pulmonary Vasoconstrictor Reactivity After Chronic Hypoxia'. American Journal of Physiology-Heart and Circulatory Physiology 314 (5), H1011-H1021

Weiss, J. N., Korge, P., Honda, H. M., and Ping, P. (2003) 'Role of the Mitochondrial Permeability Transition in Myocardial Disease'. Circulation Research 93 (4), 292-301

Weiss, R. B. (1992) 'The Anthracyclines: Will we Ever Find a Better Doxorubicin?'. Seminars in Oncology 19 (6), 670-686

Wende, A. R., O'Neill, B. T., Bugger, H., Riehle, C., Tuinei, J., Buchanan, J., Tsushima, K., Wang, L., Caro, P., Guo, A., Sloan, C., Kim, B. J., Wang, X., Pereira, R. O., McCrory, M. A., Nye, B. G., Benavides, G. A., Darley-Usmar, V., Shioi, T., Weimer, B. C., and Abel, E. D. (2015) 'Enhanced Cardiac Akt/protein Kinase B Signaling Contributes to Pathological Cardiac Hypertrophy in Part by Impairing Mitochondrial Function Via Transcriptional Repression of Mitochondrion-Targeted Nuclear Genes'. Molecular and Cellular Biology 35 (5), 831-846

Winter, E., Chiaradia, L. D., Silva, A. H., Nunes, R. J., Yunes, R. A., and Creczynski-Pasa, T. B. (2014) 'Involvement of Extrinsic and Intrinsic Apoptotic Pathways Together with Endoplasmic Reticulum Stress in Cell Death Induced by Naphthylchalcones in a Leukemic Cell Line: Advantages of Multi-Target Action'. Toxicology in Vitro 28 (5), 769-777

Winterbourn, C. C. (1995) 'Toxicity of Iron and Hydrogen Peroxide: The Fenton Reaction'. Toxicology Letters 82, 969-974

Witztum, J. L. and Steinberg, D. (2001) 'The Oxidative Modification Hypothesis of Atherosclerosis: Does it Hold for Humans?'. Trends in Cardiovascular Medicine 11 (3-4), 93-102

Wouters, K. A., Kremer, L., Miller, T. L., Herman, E. H., and Lipshultz, S. E. (2005) 'Protecting Against anthracyclineΓÇÉinduced Myocardial Damage: A Review of the most Promising Strategies'. British Journal of Haematology 131 (5), 561-578

Wright, V. P., Klawitter, P. F., Iscru, D. F., Merola, A. J., and Clanton, T. L. (2005) 'Superoxide Scavengers Augment Contractile but Not Energetic Responses to Hypoxia in Rat Diaphragm'. Journal of Applied Physiology 98 (5), 1753-1760

Wu, J. M. and Hsieh, T. (2011) 'Resveratrol: A Cardioprotective Substance'. Annals of the New York Academy of Sciences 1215 (1), 16-21

Wu, W., Lee, W. L., Wu, Y. Y., Chen, D., Liu, T. J., Jang, A., Sharma, P. M., and Wang, P. H. (2000) 'Expression of Constitutively Active Phosphatidylinositol 3-Kinase Inhibits Activation of Caspase 3 and Apoptosis of Cardiac Muscle Cells'. The Journal of Biological Chemistry 275 (51), 40113-40119

Xiao, J., Sun, G., Sun, B., Wu, Y., He, L., Wang, X., Chen, R., Cao, L., Ren, X., and Sun, X. (2012) 'Kaempferol Protects Against Doxorubicin-Induced Cardiotoxicity in Vivo and in Vitro'. Toxicology 292 (1), 53-62

Xu, M., Shi, H., Gao, X., and Wang, H. (2013) 'Salidroside Attenuates Myocardial ischemiaΓÇôreperfusion Injury Via PI3K/Akt Signaling Pathway'. Journal of Asian Natural Products Research 15 (3), 244-252

Xu, M. F., Tang, P. L., Qian, Z. M., and Ashraf, M. (2001) 'Effects by Doxorubicin on the Myocardium are Mediated by Oxygen Free Radicals'. Life Sciences 68 (8), 889-901

Xu, W., Barrientos, T., and Andrews, N. C. (2013) 'Iron and Copper in Mitochondrial Diseases'. Cell Metabolism 17 (3), 319-328

Xu, X., Persson, H. L., and Richardson, D. R. (2005) 'Molecular Pharmacology of the Interaction of Anthracyclines with Iron'. Molecular Pharmacology 68 (2), 261-271

Yamashita, J., Ogawa, M., and Shirakusa, T. (1995) 'Plasma endothelin-1 as a Marker for Doxorubicin Cardiotoxicity'. International Journal of Cancer 62 (5), 542-547

Yang, C. (2018) 'Clinical Manifestations and Basic Mechanisms of Myocardial ischemia/reperfusion Injury'. Tzu-Chi Medical Journal 30 (4), 209

Yang, F., Teves, S. S., Kemp, C. J., and Henikoff, S. (2014) 'Doxorubicin, DNA Torsion, and Chromatin Dynamics'. Biochimica Et Biophysica Acta (BBA)-Reviews on Cancer 1845 (1), 84-89

Yang, H., Wang, Y., and Peng, S. (2010) 'Metallothionein-I/II Null Cardiomyocytes are Sensitive to Fusarium Mycotoxin Butenolide-Induced Cytotoxicity and Oxidative DNA Damage'. Toxicon 55 (7), 1291-1296

Yaqub, F. (2013) 'Mechanism of Action of Anthracycline Drugs'. The Lancet Oncology 14 (8), e296

Yellon, D. M. and Hausenloy, D. J. (2007) 'Myocardial Reperfusion Injury'. The New England Journal of Medicine 357 (11), 1121-1135

Yen, H., Oberley, T. D., Gairola, C. G., Szweda, L. I., and Clair, D. K. S. (1999) 'Manganese Superoxide Dismutase Protects Mitochondrial Complex I Against Adriamycin-Induced Cardiomyopathy in Transgenic Mice'. Archives of Biochemistry and Biophysics 362 (1), 59-66

Yim, T. K. and Ko, K. M. (1999) 'Schisandrin B Protects Against Myocardial Ischemia-Reperfusion Injury by Enhancing Myocardial Glutathione Antioxidant Status'. in . ed. by Anon: Springer, 151-156

Yoshida, Y., Shioi, T., and Izumi, T. (2007) 'Resveratrol Ameliorates Experimental Autoimmune Myocarditis'. Circulation Journal 71 (3), 397-404

Yu, W., Sun, H., Zha, W., Cui, W., Xu, L., Min, Q., and Wu, J. (2017) 'Apigenin Attenuates Adriamycin-Induced Cardiomyocyte Apoptosis Via the PI3K/AKT/mTOR Pathway'. Evidence-Based Complementary and Alternative Medicine 2017

Yusuf, S., Lessem, J., Jha, P., and Lonn, E. (1993) 'Primary and Secondary Prevention of Myocardial Infarction and Strokes: An Update of Randomly Allocated, Controlled Trials'. Journal of Hypertension.Supplement : Official Journal of the International Society of Hypertension 11 (4), S61-73

Zare Mirakabadi, A., Shahramyar, Z., Morovvati, H., Lotfi, M., and Nouri, A. (2012) 'Induction of Apoptosis in Human Leukemia Cell Line (HL60) by Animal's Venom Derived Peptides (ICD-85)'. *Iranian Journal of Pharmaceutical Research : IJPR* 11 (3), 931-938

Zeng, C., Jiang, W., Zheng, R., He, C., Li, J., and Xing, J. (2018) 'Cardioprotection of Tilianin Ameliorates Myocardial Ischemia-Reperfusion Injury: Role of the Apoptotic Signaling Pathway'. PloS One 13 (3), e0193845

Zhang, H., Xiong, Z., Wang, J., Zhang, S., Lei, L., Yang, L., and Zhang, Z. (2016) 'Glucagon-like peptide-1 Protects Cardiomyocytes from Advanced Oxidation Protein product-induced Apoptosis Via the PI3K/Akt/Bad Signaling Pathway'. Molecular Medicine Reports 13 (2), 1593-1601

Zhang, J., Wang, X., Vikash, V., Ye, Q., Wu, D., Liu, Y., and Dong, W. (2016) 'ROS and ROS-Mediated Cellular Signaling'. Oxidative Medicine and Cellular Longevity 2016, 4350965

Zhang, P., Lu, Y., Yu, D., Zhang, D., and Hu, W. (2015) 'TRAP1 Provides Protection Against Myocardial Ischemia-Reperfusion Injury by Ameliorating Mitochondrial Dysfunction'. Cellular Physiology and Biochemistry : International Journal of Experimental Cellular Physiology, Biochemistry, and Pharmacology 36 (5), 2072-2082

Zhang, Q., Shang, M., Zhang, M., Wang, Y., Chen, Y., Wu, Y., Liu, M., Song, J., and Liu, Y. (2016) 'Microvesicles Derived from hypoxia/reoxygenation-Treated Human Umbilical Vein Endothelial Cells Promote Apoptosis and Oxidative Stress in H9c2 Cardiomyocytes'. BMC Cell Biology 17 (1), 25

Zhang, S., Liu, Y., Wang, F., Qiang, J., Liu, P., Zhang, J., and Xu, J. (2017) 'Ilexsaponin A Attenuates Ischemia-Reperfusion-Induced Myocardial Injury through Anti-Apoptotic Pathway'. PloS One 12 (2), e0170984

Zhang, S., Liu, X., Bawa-Khalfe, T., Lu, L., Lyu, Y. L., Liu, L. F., and Yeh, E. T. H. (2012) 'Identification of the Molecular Basis of Doxorubicin-Induced Cardiotoxicity'. Nature Medicine 18 (11), 1639

Zhang, X., Liang, X., Lin, X., Zhang, S., Huang, Z., Chen, C., Guo, Y., Xuan, F., Xu, X., and Huang, R. (2014) 'Mechanism of the Protective Effect of Yulangsan Flavonoid on Myocardial ischemia/reperfusion Injury in Rats'. Cellular Physiology and Biochemistry : International Journal of Experimental Cellular Physiology, Biochemistry, and Pharmacology 34 (4), 1050-1062

Zhang, Y., Zhao, W., Zhang, H. J., Domann, F. E., and Oberley, L. W. (2002) 'Overexpression of Copper Zinc Superoxide Dismutase Suppresses Human Glioma Cell Growth'. Cancer Research 62 (4), 1205-1212

Zhang, Y., Yi, M., and Huang, Y. (2017) 'Oxymatrine Ameliorates Doxorubicin-Induced Cardiotoxicity in Rats'. Cellular Physiology and Biochemistry 43 (2), 626-635

Zhang, Y., Shi, J., Li, Y., and Wei, L. (2009) 'Cardiomyocyte Death in Doxorubicin-Induced Cardiotoxicity'. Archivum Immunologiae Et Therapiae Experimentalis 57 (6), 435-445

Zhao, Z., Nakamura, M., Wang, N., Wilcox, J. N., Shearer, S., Ronson, R. S., Guyton, R. A., and Vinten-Johansen, J. (2000) 'Reperfusion Induces Myocardial Apoptotic Cell Death'. Cardiovascular Research 45 (3), 651-660

Zhao, L. and Zhang, B. (2017) 'Doxorubicin Induces Cardiotoxicity through Upregulation of Death Receptors Mediated Apoptosis in Cardiomyocytes'. Scientific Reports 7, 44735

Zhao, L. and Zhang, B. (2017) 'Doxorubicin Induces Cardiotoxicity through Upregulation of Death Receptors Mediated Apoptosis in Cardiomyocytes'. Scientific Reports 7, 44735

Zhao, M., Ding, X., Shen, J., Zhang, X., Ding, X., and Xu, B. (2017) 'Use of Liposomal Doxorubicin for Adjuvant Chemotherapy of Breast Cancer in Clinical Practice'. Journal of Zhejiang University-SCIENCE B 18 (1), 15-26

Zhao, Z., Nakamura, M., Wang, N., Wilcox, J. N., Shearer, S., Ronson, R. S., Guyton, R. A., and Vinten-Johansen, J. (2000) 'Reperfusion Induces Myocardial Apoptotic Cell Death'. Cardiovascular Research 45 (3), 651-660

Zhe-Wei, S., Li-Sha, G., and Yue-Chun, L. (2018) 'The Role of Necroptosis in Cardiovascular Disease'. Frontiers in Pharmacology 9

Zhou, S., Starkov, A., Froberg, M. K., Leino, R. L., and Wallace, K. B. (2001) 'Cumulative and Irreversible Cardiac Mitochondrial Dysfunction Induced by Doxorubicin'. Cancer Research 61 (2), 771-777

Zhou, S., Palmeira, C. M., and Wallace, K. B. (2001) 'Doxorubicin-Induced Persistent Oxidative Stress to Cardiac Myocytes'. Toxicology Letters 121 (3), 151-157

Zhou, T., Prather, E. R., Garrison, D. E., and Zuo, L. (2018) 'Interplay between ROS and Antioxidants during Ischemia-Reperfusion Injuries in Cardiac and Skeletal Muscle'. International Journal of Molecular Sciences 19 (2), 417

Zhou, T., Chuang, C., and Zuo, L. (2015) 'Molecular Characterization of Reactive Oxygen Species in Myocardial Ischemia-Reperfusion Injury'. BioMed Research International 2015

Zhu, W., Soonpaa, M. H., Chen, H., Shen, W., Payne, R. M., Liechty, E. A., Caldwell, R. L., Shou, W., and Field, L. J. (2009) 'Acute Doxorubicin Cardiotoxicity is Associated with p53-Induced Inhibition of the Mammalian Target of Rapamycin Pathway'. Circulation 119 (1), 99-106

Zhu, X. and Zuo, L. (2013) 'Characterization of Oxygen Radical Formation Mechanism at Early Cardiac Ischemia'. Cell Death & Disease 4 (9), e787

Zong, W. X., Li, C., Hatzivassiliou, G., Lindsten, T., Yu, Q. C., Yuan, J., and Thompson, C. B. (2003) 'Bax and Bak can Localize to the Endoplasmic Reticulum to Initiate Apoptosis'. The Journal of Cell Biology 162 (1), 59-69

Zorov, D. B., Juhaszova, M., and Sollott, S. J. (2014) 'Mitochondrial Reactive Oxygen Species (ROS) and ROS-Induced ROS Release'. Physiological Reviews 94 (3), 909-950

Zucchi, R. and Danesi, R. (2003) 'Cardiac Toxicity of Antineoplastic Anthracyclines'. Current Medicinal Chemistry.Anti-Cancer Agents 3 (2), 151-171

Zuo, L., Youtz, D. J., and Wold, L. E. (2011) 'Particulate Matter Exposure Exacerbates High Glucose-Induced Cardiomyocyte Dysfunction through ROS Generation'. PloS One 6 (8), e23116

Zweier, J. L. (1988) 'Measurement of Superoxide-Derived Free Radicals in the Reperfused Heart. Evidence for a Free Radical Mechanism of Reperfusion Injury'. The Journal of Biological Chemistry 263 (3), 1353-1357

Zweier, J. L., Flaherty, J. T., and Weisfeldt, M. L. (1987) 'Direct Measurement of Free Radical Generation Following Reperfusion of Ischemic Myocardium'. Proceedings of the National Academy of Sciences of the United States of America 84 (5), 1404-1407

Zweier, J. L. and Talukder, M. H. (2006) 'The Role of Oxidants and Free Radicals in Reperfusion Injury'. Cardiovascular Research 70 (2), 181-190