Investigating the effects of bronchodilator induced cardiotoxicity

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Investigating the effects of Bronchodilator induced Cardiotoxicity

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Acknowledgements

"Whenever you feel the journey's too hard, remember He makes things happen for a reason. Every trial has a purpose…" Mufti Ismail ibn Musa Menk

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Abstract

Long acting muscarinic receptor antagonists (LAMAs) are utilised for the management of chronic obstructive pulmonary disease (COPD), to alleviate parasympathetic mediated bronchoconstriction. Amongst COPD patients, cardiovascular comorbidities are increasingly common, such as ischaemic heart disease, and account for the greatest proportion of deaths. Clinical studies have highlighted a possible link between the use of LAMAs such as Tiotropium bromide and adverse cardiovascular effects. Muscarinic receptors are present in the heart, and therefore all LAMAs possess potential cardiovascular risk. The current study investigated the effect of LAMA administration in whole heart Langendorff models of normoxic conditions and myocardial ischaemia/reperfusion injury using 3 month old Sprague Dawley rats. The Langendorff model was used to assess the effect of LAMAs on haemodynamic function; tissue from the models were assessed for infarct size analysis using triphenyl tetrazolium chloride (TTC) staining or were prepared for western blot analysis of Akt and CaMKII. Tissue from the models was also used for qPCR quantification of genes associated with myocardial infarction and oxidative stress. Cardiomyocytes were isolated from 3 month old rats using a modified Langendorff set up, with flow cytometric analysis of caspase-3, apoptotic cell death, oxidative stress and calcium release.

Aclidinium, Tiotropium and Umeclidinium bromide but not Glycopyyronium bromide exacerbated myocardial ischaemia/reperfusion injury, assessed by infarct size to risk ratio when administered at the onset of reperfusion without significantly affecting haemodynamic function. Tiotropium bromide has been on the market the longest and is the most widely prescribed long acting muscarinic receptor antagonist. This was further tested in normoxic cardiac models, and was also found to induce damage, assessed by infarct size analysis. The use of the natural muscarinic agonist, acetylcholine inhibited Tiotropium bromide induced infarct size in normoxic and ischaemia/reperfusion models, suggesting that direct muscarinic signalling was involved in Tiotropium bromide mediated cardiac damage. Inhibitors and adjunctive agents including cyclosporin A (CsA), wortmannin, KN-93, Z-DEVD-FMK, nifedipine and resveratrol were used to determine the mechanisms of cardiotoxicity following administration of Tiotropium bromide; as well as

determining the effect of Tiotropium bromide on Akt and CaMKII phosphorylation, reactive oxygen species generation and intracellular Ca^{2+} release and caspase-3 activation.

The study found that Tiotropium bromide mediated infarct size is attenuated following inhibition of pathways known to be involved in cardiotoxicity, such as mPTP opening, reactive oxygen species (ROS) generation, Ca^{2+} overload and caspase-3 activation. The study determined that Tiotropium bromide in cardiomyocytes exerts a greater effect on Ca^{2+} mediated signalling than on the generation of ROS. Additionally, caspase-3 is significantly activated, indicating a mechanism of cell death, however with no significant effect on apoptosis or necrosis as a whole. The study also showed that Tiotropium bromide affects the regulation of some genes such as caspase-3 involved in myocardial infarction through upregulation, and downregulates certain genes such as Akt1, peroxiredoxin 4 and superoxide dismutase 2 involved in cellular protection against oxidative stress.

In conclusion, the effect of LAMAs on myocardial ischaemia/reperfusion injury reflects the observation of increased cardiovascular complications amongst COPD patients, independently of other risk factors such as smoking. The effect of Tiotropium bromide in normoxic conditions suggests that muscarinic receptor antagonism in otherwise healthy heart exerts a Ca^{2+} driven mechanism of cardiotoxicity, involving abhorrent activation of Akt and CaMKII. These detrimental effects can be attenuated with the use of CsA, L-type Ca^{2+} channel blockers and antioxidants as well as acetylcholine. This study identifies the potential risks of LAMA use on the heart and urges for the development of robust preclinical tests which can identify compounds that may cause adverse cardiovascular complications. The observation that haemodynamic function was not adversely affected despite an increase in myocardial infarction, show that the dependence on functional parameters alone can not sufficiently identify cardiovascular risk.

Publications

Cassambai, S., Dean, S., Mee, C., and Hussain, A. (2018). *P17 Cyclosporin a Mediated Inhibition of the Mitochondrial Permeability Transition Pore (MPTP) Attenuates Tiotropium Bromide Mediated Cardiotoxicity* **Abstract**

Cassambai, S., Dean, S., Karvey, K., Mee, C., and Hussain, A. (2017) 'Long Acting Muscarinic Receptor Antagonists Exacerbate Myocardial Ischaemia Reperfusion Injury'. *Journal of Pharmacological and Toxicological Methods* 88, 217 **Abstract**

Cassambai, S., Dean, S., Mee, C., and Hussain, A. (2017) 'Role of Ca^{2+}/Calmodulin Kinase II in Tiotropium Induced Cardiotoxicity'. *Journal of Molecular and Cellular Cardiology* 109, 1-62 **Abstract and Oral presentation**

Cassambai, S., Dean, S., Mee, C., Harvey, K., and Hussain, A. (eds.) (2016) *Cardiovascular Research*. 'Cardiotoxicity of Long Acting Muscarinic Receptor Antagonists used for Chronic Obstructive Pulmonary Disease': Oxford Univ Press Great Clarendon St, Oxford OX2 6DP, England **Abstract**

Cassambai, S., Dean, S., Mee, C., Harvey, K., and Hussain, A. (2016) 'A Role for Antioxidants in Reversing Tiotropium Induced Cardiotoxicity'. *Journal of Molecular and Cellular Cardiology* 98, 1-85 **Abstract and Oral presentation**

Cassambai, S., Dean, S., Mee, C., Harvey, K., and Hussain, A. (2016) 'The Role of Ca2+/Calmodulin Kinase II in Tiotropium Bromide Mediated Cardiotoxicity'. *Proceedings of the British Pharmacological Society* 16 **Abstract and Oral Presentation**

Cassambai, S., Dean, S., Mee, C. J., and Hussain, A. (eds.) *Transforming Future*. 'Cardiac Safety Profiles of Long Acting Muscarinic Receptor Antagonists used in the Treatment of Chronic Obstructive Pulmonary Disease'. Held June 2015 at Coventry University **Abstract and Oral presentation**

List of Abbreviations

8-OHdG	8-hydroxydeoxyguanosine
AB	Aclidinium Bromide
ACCLAIM	Aclidinium bromide clinical trial assessing efficacy and safety in moderate to severe COPD patients
ACCORD	Aclidinium bromide in chronic obstructive respiratory disease
ACh	Acetylcholine
ADRs	Adverse drug reactions
AIF	Apoptosis inducing factor
Akt	Protein kinase B
ANOVA	Analysis of variance
ANT1	Adenine nucleotide translocase 1
Apaf-1	Apoptotic protease activating factor 1
ATP	Adenosine triphosphate
ATTAIN	Aclidinium bromide to treat airway obstruction in COPD patients
Bad	Bcl-2 family, BH-3 only domain, pro-apoptotic protein
Bax	Bcl-2 family, BH-1, BH-2 & BH-3 domain, pro-apoptotic protein
Bcl-2	B-cell lymphoma 2
BSA	Bovine serum albumin
CAD	Coronary artery disease
CaMKII	Ca^{2+}/Calmodulin kinase II
cA/GMP	Cyclic adenosine/guanosine monophosphate
CAP	Community acquired pneumonia
Caspase	Cysteine aspartate specific protease
CF	Coronary flow
CHD	Coronary heart disease
CICR	Ca^{2+} induced Ca^{2+} release
COPD	Chronic obstructive pulmonary disorder
CRP	C-reactive protein
CsA	Cyclosporin A
DAG	Diacylglycerol
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
ERK1/2	p42/44 extracellular signal-related kinase 1/2
FAB4	Fatty acid binding protein 4
FADD	Fas associated death domain
FDA	Food and drug administration
FEV_1	Forced expiratory volume in one second
GB	Glycopyrronium Bromide
GLOW1	Glycopyrronium bromide in COPD airways clinical study 1
GTP	Guanosine triphosphate
HCN	Hyperpolarisation-activated cation current

hERG	Human ether-a-go-go
HIF1	Hypoxia inducible factor
HMGB1	High mobility group box 1
HR	Heart rate
ICS	Inhaled corticosteroids
IHD	Ischaemic heart disease
I_h/I_f	Pacemaker depolarisation rate
IGF-1	Insuline like growth factor 1
IP_3	Inositol 1,4,5-trisphosphate
IPC	Ischaemic preconditioning
IR - I/R	Ischaemia/Reperfusion
JAK-STAT	Janus kinase-Signal transducer and activator of transcription
JNK	c-jun N-terminal kinase
KHB	Krebs-Heinsleit buffer
KN-93	Methoxybenzene-sulfonamide
L/SABA	Long/Short acting β_2 agonist
L/SAMA	Long/Short acting muscarinic receptor antagonist
LKB4	Leukotriene b_4
LVDP/EF	Left ventricular developed pressure/ejection fraction
MAO	Monoamine oxidase
MAPK	Mitogen-activated protein kinase
MCID	Minimal clinically important difference
MCP1	Monocyte chemoattractant protein 1
MI	Myocardial infarction
MiRs	Micro RNAs (ribonucleic acid)
MMP	Matrix metalloprotease/proteinase
mPTP	Mitochondrial permeability transition pore
MOMP	Mitochondrial Outer Membrane Permeabilisation
MUC5AC	Mucin 5AC
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NCX	Na^+/Ca^{2+} exchanger
NFAT	Nuclear factor of activated T cells
NF-κβ	Nuclear factor kappa light chain enchancer of activated B cells
NHE	Na^+/H^+ exchanger
NO	Nitric oxide
P/NPCD	Programmed/Non-programmed cell death
PCI	Percutaneous coronary intervention
PDGF	Platelet derived growth factor
PKA/C	Protein Kinase A/C
PI3K	Phosphotidyl inositol 3 kinase
PLC	Phospholipase C
PPAR	Peroxisome proliferator-activated receptor
PTCA	Percutaneous transluminal coronary angioplasty

PTEN	Phosphatase and tensin homology protein
RISK	Reperfusion injury salvage kinase
ROS	Reactive oxygen species
SEM	Standard error of the mean
SERCA	Sarcoplasmic/endoplasmic reticulum Ca^{2+}/ATPase
SOD	Superoxide dismutase
STEMI	ST elevated myocardial infarction
TB	Tiotropium bromide
TGF-β1	Transforming growth factor β1
TIOSPR®	Tiotropium bromide safety and performance in Respimat®
TNF-α/R	Tumour necrosis factor α /receptor
TORCH	Towards a revolution in COPD health
TTC	2,3,5-triphenyl-2H-tetrazolium-chloride
UB	Umeclidinium Bromide
UPLIFT	Understanding potemtial long-term impacts on function with Tiotropium
VCAM	Vascular adhesion molecule
VEGF	Vascular endothelial growth factor
Wort	Wortmannin
Z-DEVD-FMK	Benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-ValAsp(OMe)-fluoromethylketone

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Chapter 1: Introduction

Muscarinic receptor antagonists are prescribed to patients with chronic obstructive pulmonary disease (COPD); these block vagally mediated bronchoconstriction (Barnes 2013). Studies have observed a correlation with adverse cardiac effects in patients administered bronchodilators (Hilleman et al. 2009, Singh, Loke and Furberg 2008), particularly when long acting muscarinic antagonists (LAMAs) are newly administered (Wang et al. 2018, Liou et al. 2018). Ischaemic heart disease (IHD) is often clinically silent and a common comorbidity in COPD patients (Cazzola, Rogliani and Matera 2015). IHD is the leading cause of global mortality and presents a problem due to the paradoxical phenomenon of ischaemia/reperfusion injury. The restoration of blood flow to ischaemic

tissues can itself cause further damage through calcium overload and the production of reactive oxygen species (ROS) (Davidson et al. 2006, Hausenloy and Yellon 2015).

Previous studies have elucidated the cardiovascular risks associated with the use of β agonists such as salmetarol (Prevost et al. 1997, Salpeter, Ormiston and Salpeter 2004), but few have explored the effect of LAMAs in cardiovascular risk. Most studies where concerned, have focused on clinical data to consider the safety of LAMAs; however even fewer have considered the signalling pathways which may be associated. Singh et al. (2008) conducted a meta-analysis which found that anti-muscarinics such as Tiotropium bromide and Ipratropium bromide increase the risk of cardiovascular events in patients with COPD. This study was the first of several debated meta-analyses which began to challenge the safety of anti-muscarinics, which have classically been associated with fewer side effects and better safety profiles (Jara, Wentworth and Lanes 2012). Shaik et al. (2012) conducted a study in erythrocytes where Ipratropium bromide was found to result in suicidal cell death in a Ca^{2+} mechanism. This study was the first to suggest that a muscarinic antagonist could elicit a signalling cascade. Harvey et al. (2014) later showed that in an *ex vivo* model of ischaemia/reperfusion injury, Ipratropium bromide exacerbates myocardial damage. These studies perpetuated attention towards the cardiovascular safety of anti-muscarinics, however there are no studies which have observed the cellular mechanism of cardiotoxicity with LAMAs.

This study hypothesises that Tiotropium bromide and other LAMAs may exacerbate cardiovascular damage in the context of ischaemia/reperfusion injury. Additionally, it is hypothesised that Tiotropium bromide may orchestrate cell death in cardiomyocytes via Ca^{2+} overload or the production of reactive oxygen species. The aim of this study is to firstly understand and observe the effects of LAMAs on the heart in an ischaemia/reperfusion model, to mimic underlying ischaemia heart disease which is often a comorbidity associated with COPD. Secondly the study aims to elucidate the molecular mechanisms associated with cardiac damage, specifically with Tiotropium bromide in otherwise normal conditions. COPD patients are at greater risk of cardiovascular related mortality than respiratory consequences (Sin et al. 2006), therefore exacerbating pre-existing cardiac comorbidities may be fatal; this provides urgency in assessing the cardiac safety of drugs. Identifying signalling pathways involved in drug-induced cardiotoxicity may enable the

development of cardioprotective strategies, as well as enable the identification of novel biomarkers. Furthermore, evaluating the effect of LAMAs in cardiomyocyte signalling in addition to haemodynamic parameters can assess whether existing clinical assessments are sufficient to determine the cardiovascular safety of drugs.

1.1 Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is a progressive inflammatory condition with debilitating airflow limitations (Mannino et al. 2002). It is characterised by symptoms of breathlessness as a result of chronic irreversible obstruction and frequent chest infections (Barbu, Iordache and Man 2011, Cornwell et al. 2010, Daheshia 2005). COPD can not be cured; however, it can be managed pharmacologically; despite this, it is the third leading cause of mortality in the USA and the most common cause of hospitalisation in the UK, presenting a social and economic burden (Bhatt and Dransfield 2013, Qureshi, Sharafkhaneh and Hanania 2014). Deaths in COPD patients are often due to cardiovascular complications such as heart failure (Boudestein et al. 2009). Patients have increased risk of cardiovascular disease, and are accompanied by comorbidities, such as ischaemic heart disease (IHD). These disorders are often aggravated in patients with frequent COPD exacerbations (Patel et al. 2013). Co-existing cardiovascular disease and COPD exacerbations has significant clinical implications in terms of treatment regime (Qureshi, Sharafkhaneh and Hanania 2014), therefore identifying populations 'at risk' is essential.

1.1.1 Pathophysiology of COPD

COPD is characterised by a group of diseases, such as chronic bronchitis, emphysema and small airway disease. The disease is identified from spirometric tests measuring the volume of air expelled in a period of time, forced expiratory volume in one second (FEV_1) (MacNee 2005). The most common clinical manifestation is inflammation of the airways, resulting in obstructed airflow, lung remodelling and a loss of alveolar surface area (Tuder and Petrache 2012).

The inflammatory response underlying COPD pathophysiology is driven by various inflammatory mediators such as cytokines and chemokines triggered by inhalants and allergens commonly cigarette smoke, which triggers an adaptive immune response (Barnes

2008, MacNee 2005). COPD pathophysiology also involves pathological lesions such as fibrosis and alveolar wall destruction through proteinases and oxidative stress (Barnes 2008). Macrophages, neutrophils and TH_1 cells produce damaging cytotoxic effects; TH_1 cells mediate inflammation through the release of interferon γ (IFN γ); macrophages are activated by inhaled irritants such as cigarette smoke, causing the release of different chemical mediators such as TNFα and IL-6, and the release reactive oxygen species (ROS) by neutrophils. These mediators may also cause genotoxicity leading to the development of lung cancer (Houghton 2013).

Inflammatory cells initiate cytokine signalling following inhalant exposure, cigarette smoke is most commonly associated with COPD. However, only 15-20% of smokers develop clinically significant COPD, (Daheshia 2005, MacNee 2005). Genetic predisposition also underlies COPD, such as a deficiency of α1 anti-trypsin leads to emphysema often observed in younger patients (Daheshia 2005). Imbalance of proteinases/anti-proteinases is also associated with COPD; Min et al. (2011) found correlation between increased expression of proteostasis mediators and severity of emphysema, suggesting that proteostasis imbalance affects COPD severity via apoptosis, oxidative stress and chronic inflammation (Min et al. 2011). Tissue remodelling is also a key mediator of COPD pathology; apoptotic factors and metalloproteases underlie emphysema, whereas small airway fibrosis underlies chronic bronchitis (Chung and Adcock 2008).

COPD is a manageable condition; however, exacerbations present a major complication in treatment and on the healthcare system, imposing a substantial financial burden (Qureshi, Sharafkhaneh and Hanania 2014). Exacerbations can significantly deteriorate the existing condition; pulmonary infections are known to be the leading cause of COPD exacerbations (Sethi and Murphy 2008). Exacerbations can be complicated by the presence of other comorbid conditions such as congestive heart failure (Qureshi, Sharafkhaneh and Hanania 2014). In some cases, the treatment of these underlying conditions is essential in managing exacerbations (Qureshi, Sharafkhaneh and Hanania 2014).

Figure 1.1.1.1 highlights the relation of inhalants such as cigarette smoke, and bacterial or viral colonisation in COPD. Cellular signalling cascades amplify the response to the chronic exposure of inhalants such as cigarette smoke in COPD (Chung and Adcock 2008). Exposure

to cigarette smoke prompts the release of inflammatory mediators from the lung epithelium (Chung and Adcock 2008, Daheshia 2005). IL-8, TNFα and IL-1β are widely associated with exposure to cigarette smoke (Barbu, Iordache and Man 2011, Chung and Adcock 2008, Daheshia 2005). The release of these mediators is succeeded by neutrophil recruitment and macrophage activation in the alveoli and bronchioles (Barbu, Iordache and Man 2011) releasing matrix metalloproteinases (MMPs) and pro-inflammatory mediators such as IL-6 (Daheshia 2005). Inflammatory signalling pathways lead to progressive obstruction and destruction of bronchiolar and alveolar spaces, underlying the symptoms associated with COPD (Barnes, Shapiro and Pauwels 2003). Muscarinic signalling also plays an important role in the pathological consequences of COPD.

Figure 1.1.1.1: The complex cellular interactions involved in mediating the chronic inflammation associated with COPD pathology *(Chung and Adcock 2008). A range of different components are involved in mediating this response, with several different immunological cells as well as the epithelium. Ab – antibody, Th – T helper cell, MHC - major histocompatibility complex, TCR - T-cell receptor, CXCL - CXC chemokine ligand, IP -*

interferon (IFN)-c-inducible protein, CCL - CC chemokine ligand, RANTES - regulated on activation normal T-cell expressed and secreted, TSLP - thymic stromal lymphopoietin, IL – interleukin, TNF - tumour necrosis factor, MCP - monocyte chemotactic protein, LT – leukotriene, CRP - C-reactive protein, TGF - transforming growth factor, EGF - epidermal growth factor, VEGF - vascular endothelial growth factor, MMP - matrix metalloproteinase.

1.2 Muscarinic receptors

Muscarinic receptors are G-protein coupled receptors (GPCRs) (Katritch, Cherezov and Stevens 2012), G-proteins consist of an α, β and γ subunit; the α subunit exists in many forms, including $G_{\alpha s}$ and $G_{\alpha i}$, hydrolysing guanosine triphosphate (GTP) into guanosine diphosphate (GDP). The β and γ subunits also initiate responses through ion channels and proteins (Marinissen and Gutkind 2001). Second messenger signalling depends on the Gα; the α_s subunit is stimulatory via several effectors including, adenylyl cyclase (Liu, Erlichman and Weinstein 2003). $G\alpha_i$ inhibits adenylyl cyclase mediated cyclic AMP (cAMP), ion channels and other second messengers (Marinissen and Gutkind 2001). GPCRs share a generic signal transduction mechanism but are activated by various ligands including photons, which makes them a key target for pharmaceutical intervention (Katritch, Cherezov and Stevens 2013). Therapeutic targeting allows modulation of function in COPD, muscarinic antagonists block muscarinic bronchoconstriction, and dampen inflammation (Karakiulakis and Roth 2012).

1.2.1 Acetylcholine and Muscarinic receptors

Acetylcholine is an abundant neurotransmitter in the central and peripheral nervous systems, released from cholinergic neurons and non-neuronal sources such as endothelial and epithelial cells (Wessler and Kirkpatrick 2008). Acetylcholine modulates neuronal signalling; including synaptic transmission, synaptic plasticity, neuronal networks and responses to stimuli (Picciotto, Higley and Mineur 2012). Acetylcholine is released from parasympathetic nerves in the neuronal system and various cell types in the non-neuronal

system, binding to metabotropic muscarinic and ionotropic nicotinic receptors (Caulfield and Birdsall 1998, Coulson and Fryer 2003, Wess, Eglen and Gautam 2007). There are five muscarinic receptor subtypes, M_{1-5} (Caulfield and Birdsall 1998, Coulson and Fryer 2003). M_1, M_2 and M_3 exhibit a physiological function in the airways and the heart (Coulson and Fryer 2003, Harvey 2012). Muscarinic signalling activates downstream signalling molecules such as phospholipase C (PLC); the varying receptor expression patterns in different tissues leads to different physiological consequences. M_2 receptors mediate bradycardia in the heart, however expression of M_2 receptors in the brain contributes to anti-nociception (Wess, Eglen and Gautam 2007).

Airway smooth muscle control occurs via muscarinic and β_2-adrenergic GPCRs (Katritch, Cherezov and Stevens 2013). M_2 and M_3 receptors are involved in airway smooth muscle contractility; M_2 receptors inhibit β_2-adrenergic mediated smooth muscle relaxation, and M_3 receptors directly mediate contractility (Coulson and Fryer 2003). Muscarinic receptors are a common target in COPD due to increased parasympathetic tone (Coulson and Fryer 2003, Wess, Eglen and Gautam 2007). Hyperactivity of parasympathetic tone is characteristic of COPD, resulting in irritant mediated bronchoconstriction. Muscarinic antagonists counteract the increased vagal tone, and alleviate symptoms associated with bronchoconstriction (Wess, Eglen and Gautam 2007).

1.2.2 Muscarinic receptors in COPD and pharmacological intervention using muscarinic receptor antagonists

Muscarinic activity is involved in COPD due to increased vagal tone resulting in elevated bronchoconstriction, mucus secretion and hyper-responsiveness to allergenic agents (Buels and Fryer 2012). Increased cholinergic tone may be due to increased muscarinic receptor expression, or an enhanced sensitivity to cholinergic stimulation through elevated synthesis or reduced breakdown of acetylcholine (Pieper 2012). Muscarinic antagonists or anticholinergics were one of the earliest forms of treatment for COPD patients, with the short acting muscarinic antagonist, Ipratropium bromide and the long acting (LAMA), Tiotropium bromide (Hanania, Lareau and Yawn 2017, Karakiulakis and Roth 2012). They function as bronchodilators by reversing parasympathetic bronchoconstriction; however, they are not limited to antagonising cholinergic signalling (Gosens et al. 2006).

Cholinergic signalling is also involved in inflammation (Rosas-Ballina and Tracey 2009), attributed to airway remodelling in COPD (Karakiulakis and Roth 2012). Inflammation in COPD sees increased infiltrating neutrophils, lymphocytes and macrophages; these promote inflammation through leukotriene b_4 (LKB4) production (Profita et al. 2005). Acetylcholine may influence the release of leukotrienes from alveolar epithelial cells through increased eosinophil and neutrophil activity (Profita et al. 2005). The stimulation of muscarinic receptors promotes inflammation, this has been observed following the incubation of bronchial epithelial cell lines with acetylcholine, which results in increased inflammatory mediators from neutrophilic granulocytes, and is blocked with muscarinic antagonists, such as Tiotropium bromide (Pieper 2012), within the same dose range for bronchodilation.

Stable COPD shows neutrophil infiltration, $CD8^+$ cytotoxic lymphocytes, macrophages and monocytes (Gosens et al. 2006). These express muscarinic receptors, therefore suggesting that cholinergic signalling may also affect inflammation (Gosens et al. 2006, Pieper 2012). Both neuronal and non-neuronal acetylcholine and muscarinic receptors are involved in mediating inflammation in COPD. In COPD there is upregulation of mucin 5AC (MUC5AC) induced by cigarette smoke, which is inhibited by LAMAs such as Aclidinium bromide (Karakiulakis and Roth 2012). Muscarinic stimulation affects inflammation and airway remodelling, which are inhibited with LAMAs, such as Tiotropium bromide (Karakiulakis and Roth 2012, Pieper 2012). Fibroblasts also express muscarinic receptors, mainly $M_{1\text{-}3}$ and are influential in COPD airway remodelling, increased fibroblast proliferation occurs from muscarinic stimulation, leading to fibrosis and remodelling of the airways (Buels and Fryer 2012). Acetylcholine indirectly mediates smooth muscle proliferation via platelet derived growth factor (PDGF) and epidermal growth factor (EGF) (Karakiulakis and Roth 2012). Figure 1.2.2.1 summarises the effects of muscarinic signalling on inflammation and remodelling in the airway.

***Figure 1.2.2.1**: **Effects of acetylcholine and muscarinic signalling on airway inflammation and remodelling.** ACH – acetylcholine, PDGF-BB – platelet derived growth factor type B, EGF – epidermal growth factor, $M_{1,2,3}$ – muscarinic receptors 1,2,3, MMP – matrix metalloproteases, IL – interleukins, ERK1/2 – extracellular signal-regulated kinase, NFκB – nuclear factor κ-light-chain enhancer of activated B cells, TGF-β1 – transforming growth factor β 1 (Karakiulakis and Roth 2012).*

1.2.3 Muscarinic receptors in the heart

In the heart, muscarinic receptors function as part of the parasympathetic nervous system to regulate heart rate and contractility (Harvey 2012), with M_2 receptors the predominant subtype (Brodde and Michel 1999). Activation of M_2 receptors results in negative chronotropy and inotropy (Brodde and Michel 1999, Eglen 2012). M_2 receptors were previously thought to be the only functional muscarinic receptor in the heart, however M_1 and M_3 receptors are also present (Ockenga et al. 2013). M_2 receptors couple to the $G\alpha_i$, which inhibits cAMP through adenylyl cyclase (Marinissen and Gutkind 2001). M_2 receptors slow down the rate of action potentials fired from the sinoatrial node; this slows down

heart rate through hyperpolarisation of the maximum diastolic potential (Ang, Opel and Tinker 2012, Harvey 2012). M_2 receptor stimulation results in dissociation of the G-protein complex, the βγ subunit can activate the G-protein inward rectifying potassium channel (GIRK) leading to membrane hyperpolarsation (Harvey 2012, Olshansky et al. 2008). The α subunit inhibits adenylyl cyclase, leading to decreased cAMP and protein kinase A (PKA), which inhibits hyperpolarisation-activated cation currents (HCN) leading to decreased pacemaker depolarisation rate (I_h/I_f) (Ang, Opel and Tinker 2012).

Cardiac ion channels are regulated by cAMP largely through sympathetic innervations which increase heart rate, via β_1-adrenergic receptors (Rockman, Koch and Lefkowitz 2002). These activate the $G\alpha_s$ subunit promoting adenylyl cyclase mediated cAMP (Harvey and Belevych 2003). Increased cAMP can directly affect the pacemaker ion channel in the sinoatrial and atrioventricular nodes or through PKA, leading to PKA-dependent phosphorylation of ion channels, including the L-type Ca^{2+} channel (Ang, Opel and Tinker 2012). However, M_2 receptor stimulation can modulate the β-adrenergic stimulated cAMP-dependent response, through the L-type Ca^{2+} channel to affect heart rate and contractility (Harvey and Belevych 2003); acetylcholine has no direct effect on L-type Ca^{2+} channels, however it can antagonise the currents produced as a result of β-adrenergic stimulation (Ang, Opel and Tinker 2012, Belevych and Harvey 2000). Figure 1.2.3.1 summarises the M_2 muscarinic signalling pathways in supraventricular myocytes.

Figure 1.2.3.1: Acetylcholine binds to M_2 receptors and triggers the dissociation of the GPCR. *The separate subunit complexes (α and βγ) function by acting on adenylyl cyclase (AC) and K^+ channels, resulting in a decrease in cyclic AMP (cAMP) and hyperpolarisation. Noradrenaline-mediated (NEPI) activation of β_1-adrenergic receptors however, has a stimulatory effect on cAMP production and through protein kinase A (PKA) phosphorylation of L-type Ca^{2+} channels, results in an increase in heart rate (Harvey 2012). SR – sarcoplasmic reticiulum, PLn – phospholamban, RyR – ryanodine receptor, AKAP – A-kinase anchoring proteins.*

The roles of cardiac M_1 and M_3 receptors are not clear, due to the predominant expression of M_2 receptors, and due to poor ligand selectivity (Matsui et al. 2002a). However, these subtypes are thought to have a role in pathological conditions such as ischaemia/reperfusion injury (Borda et al. 1999, Hang et al. 2013). Cardiac M_3 receptors regulate heart rate and cardiac repolarisation; they modulate and regulate inotropic effects, confer cytoprotection against ischaemic attacks and are involved in atrial fibrillation (Wang, Lu and Wang 2007, Wang et al. 2012c, Wang, Shi and Wang 2004). M_3 receptors activate a delayed rectifying K^+ current, highly sensitive to M_3 antagonists,

involved in mediating physiological and pathological cardiac repolarisation (Wang, Lu and Wang 2007).

M_1 receptor knock out models have shown that there is a loss of response to the M_1 agonist McN-A-343, which facilitates an increase in heart rate and force of contraction (Hamilton et al. 2001, Hardouin et al. 2002). M_1 knockout mice show that these receptors are not involved in basal activity, but affect the heart via β_1-adrenergic receptors, through postganglionic sympathetic innervation (Hardouin et al. 2002). M_1 receptors are predominantly present in the brain and have well defined physiological and pathological roles such as memory impairment in dementia (Eglen 2012), but negligible expression in wild type mouse hearts. In rats, M_1 receptors are thought to increase intracellular Ca^{2+} and contractility, observed following the M_1 antagonist pirenzepine, which inhibits carbachol mediated Ca^{2+} transients (Sharma et al. 1997). In humans, M_1 receptors have been associated with episodic memory in cognitive dysfunction (Nathan et al. 2013).

M_1 and M_3 receptors couple to $G\alpha_{q/11}$, leading to mobilisation of inositol phospholipids stimulating increases in intracellular Ca^{2+} (Kruse et al. 2012, Wang, Shi and Wang 2004). M_1 and M_3 receptor stimulation activates membrane bound PLC, which results in the release of inositol 1,4,5-trisphosphate (IP_3) and diacyglycerol (DAG) mediating intracellular Ca^{2+} release and activating protein kinase C (PKC) (Brodde and Michel 1999, Ockenga et al. 2013). Figure 1.2.3.2 summarises the generalised muscarinic signalling pathways. However, the functional roles of these receptors are dependent on 'location', such that the role of M_2 receptors in ventricular myocytes will vary from those in atrial myocytes (Harvey 2012).

Figure 1.2.3.2: Signalling of muscarinic receptors. *M_2 and M_4 couple preferentially to $G_{i/0}$, whereas M_1, M_3 and M_5 couple to $G_{q/11}$. Upon stimulation, M_2 and M_4 inhibit adenylyl cyclase (AC), leading to decreased cAMP, they can also activate ion (K^+) channels. M_1, M_3 and M_5 activate PKC by PLC mediated increases in IP_3 and Ca^{2+}. PKC can activate Raf kinase, leading to activation of the MAPK cascade and ERK1/2. Activation of ERK1/2 via Src/PI3K is common to all muscarinic receptors (Ockenga et al. 2013).*

1.3 Cardiotoxicity and Bronchodilators

Cardiovascular toxicity is a major concern and an even urgent matter due to the growing number of COPD patients with comorbidities (Wood-Baker, Cochrane and Naughton 2010). Cardiotoxicity, hepatoxicity and respiratory depression account for 56% of all post marketing drug withdrawal, where the drugs resulted in death (Onakpoya, Heneghan and Aronson 2015). The definition for cardiotoxicity is vague and universally lacking, however 'cardiotoxicity' accounts for cardiomyopathy, heart failure and a reduction in left ventricular ejection fraction (LVEF) (Yeh et al. 2014). The National Cancer Institute (NCI) defines cardiotoxicity as a 'toxicity that affects the heart', including left ventricular dysfunction, rhythm disturbances and ischaemia (Brana and Tabernero 2010).

Attrition rates have increased considerably over the last few years; several reasons underlie withdrawal of drugs including, poor *in vitro* assays which fail to spot safety concerns at a preclinical level (Mordwinkin, Burridge and Wu 2013). Often, adverse drug reactions (ADRs) are only realised following long-term use, therefore the role of post marketing surveillance is very important (Coloma et al. 2013). However, any potential toxic effects of a drug compound should be established early, using screening assessments and *in vitro* assays (Bowes et al. 2012).

1.3.1 Bronchodilator induced cardiotoxicity

Drug induced cardiotoxicity is well established with many chemotherapeutic drugs, particularly anthracyclines such as doxorubicin (Octavia et al. 2012). Many anticancer drugs affect the cardiovascular system, including tyrosine kinase inhibitors, such as the monoclonal antibody, trastuzumab (Fiuza and Magalhães 2012). In contrast, little evidence suggests such toxicity with the use of bronchodilators however, this view is now being challenged.

β-adrenergic receptor agonists have been associated with cardiotoxicity particularly in a preclinical setting, but also long term cardiotoxic effects leading to fatal or near-death consequences (Spitzer et al. 1992). The cardiotoxicity of β_2 agonists has been well documented due to the presence of β_2-adrenergic receptors in cardiac muscle (Lipworth and McDevitt 1992), this is despite the introduction of newer drugs designed to be more specific (Sears and Lötvall 2005). In COPD, patients administered a β_2 agonist were more

likely to experience arrhythmias, and develop heart failure and other vascular diseases, leading to arrhythmia related death (Wilchesky et al. 2012). Although the use of many adrenergic bronchodilators alone, have been associated with cardiotoxicity, such as albuterol (salbutamol) (Coskun et al. 2001) and salmeterol (Sears 2002); anti-muscarinics with β_2 agonists have been shown to reduce adverse cardiovascular effects in patients, with the exception of Ipratropium bromide (Wood-Baker, Cochrane and Naughton 2010). However, few studies have looked at the potential mechanism of cardiotoxicity with anti-muscarinics alone (Harvey, Hussain and Maddock 2014).

The focus is on the various drug therapies patients are administered and the associated adverse effects, including short acting or long acting β_2 agonists (SABAs or LABAs), as well as short or long acting muscarinic receptor antagonists (SAMAs or LAMAs). With anti-muscarinics, the use of SAMAs increases the risk of arrhythmias, particularly in severe COPD (Wilchesky et al. 2012). This is particularly concerning in patients who may have underlying cardiovascular disease, an increasingly common comorbidity amongst COPD patients (Cazzola, Rogliani and Matera 2015). Comorbidities in patients mean that drugs may have off target effects which aggravate underlying conditions such as ischaemic heart disease.

Several large scale clinical studies have revealed a correlative relationship between the use of bronchodilators and adverse cardiovascular events; however, these studies do not show any mechanistic relationship (Hilleman et al. 2009, Oba, Zaza and Thameem 2008, Singh, Loke and Furberg 2008, Tashkin et al. 2008). Anti-muscarinics such as Tiotropium and Ipratropium bromide are widely prescribed amongst COPD patients, however the cardiovascular outcomes following the use of these drugs are largely unclear. Meta analyses of the use of these drugs highlight the relationship between the use of anti-muscarinics and adverse cardiotoxic effects amongst patient populations (Rodrigo et al. 2009, Singh, Loke and Furberg 2008).

1.3.2 Safety of Muscarinic antagonists

Long acting muscarinic receptor antagonists (LAMAs) and long acting β_2 agonists (LABAs) are first line therapy for patients, they are prescribed alone, or as a co-treatment with short acting agents, to treat moderate to severe exacerbations (Matera, Page and Cazzola 2011).

Several trials, namely the TORCH (towards a revolution in COPD health) and UPLIFT (understanding potential long-term impacts on function with Tiotropium bromide) studies have shown that in some patients, there is no reduction in lung function decline even after a period of treatment, suggesting that patients require assessment of Forced Expiratory Volume in 1 second (FEV_1) to decide which treatments are best (Cazzola and Page 2014). Most guidelines, recommend long acting bronchodilators, and in patients with breathlessness, short acting bronchodilators may also be prescribed (Cazzola and Page 2014). Inhaled corticosteroids show an improvement in the quality of life for patients, by alleviating inflammation associated with COPD (Kew, Dias and Cates 2014).

Anti-muscarinics have been used to alleviate symptoms of COPD for decades. These drugs are thought to have a better cardiac safety profile than other bronchodilator classes, due to their low gastrointestinal absorbance which limits the concentration of drug in the systemic circulation (Rabe 2010). Various trials have assessed the efficacy and safety of muscarinic antagonists; a five-year Lung Health Study showed safety concerns regarding Ipratropium bromide, with an increase in cardiovascular related hospitalisations (Wang et al. 2012b), particularly due to supraventricular tachycardia. Ipratropium bromide is a quaternary derivative of atropine, a non-selective muscarinic receptor antagonist with equal binding affinity for M_1, M_2 and M_3 receptors (Barnes 2000, Cazzola et al. 2012b), the short acting nature of the drug is thought to contribute to better safety tolerability. However, recent studies have shown a correlation between increased cardiovascular morbidity and mortality with Ipratropium bromide (Ogale et al. 2010, Singh, Loke and Furberg 2008, Wood-Baker, Cochrane and Naughton 2010).

The increased risk of cardiovascular events with Ipratropium bromide has shifted some focus towards other anti-muscarinics, such as Tiotropium bromide (Singh, Loke and Furberg 2008). Tiotropium bromide is a derivative of Ipratropium bromide; however, it has a slow dissociation affinity, making it more potent than its predecessor with greater selectivity over M_3 receptors (Barnes et al. 1995). The added benefit of Tiotropium bromide is the once daily dose, which makes it favourable amongst patients (Oba, Zaza and Thameem 2008, Rodrigo and Nannini 2007). Van Noord et al. (2000) compared the use of Ipratropium bromide with Tiotropium bromide in COPD. The study found that a single dose of Tiotropium bromide was more effective than multiple doses of Ipratropium bromide,

with similar safety profiles for both drugs. More recent studies assessing the cardiac safety of Tiotropium bromide have found conflicting information regarding the risk of cardiovascular events (Hilleman et al. 2009, Kesten et al. 2006, Singh, Loke and Furberg 2008, Wood-Baker, Cochrane and Naughton 2010); however, the possible implication of cardiac adverse effects has meant a greater emphasis on the development of new long acting muscarinic antagonists (LAMAs) (Suppli Ulrik 2012) with better cardiovascular safety profiles (Wise et al. 2018).

Aclidinium bromide, Glycopyrronium bromide and Umeclidinium bromide are LAMAs which have recently entered the market (Decramer et al. 2013, Prakash, Babu and Morjaria 2013). These newer LAMAs have potent activity at M_3 receptors, meaning that patients who are unable to tolerate Tiotropium bromide have other options (Prakash, Babu and Morjaria 2013). Due to the recent approval of these drugs, long term studies following the safety of these drugs have yet to be established; however, early trials such as ACCLAIM, ACCORD and ATTAIN for Aclidinium bromide, suggest that patients tolerated the drug well with rare cardiovascular events reported (Reid and Carlson 2014). Recently, a cohort study of over 100,000 new users of antimuscarinics to treat overactive bladder, found that the cardiovascular risk amongst the antimuscarinics used was variable; specifically, oxybutynin showed an elevated risk of cardiovascular events and mortality compared to the standard antimuscarinic, tolterodine (Arana et al. 2018). This suggests that antimuscarinics may have unknown cardiovascular risks associated with their use, and therefore urges investigation into the cardiovascular risk of antimuscarinics used for other conditions, such as COPD.

1.3.2.1 Glycopyrronium bromide

Glycopyrronium bromide is a once daily LAMA; phase III clinical trials have shown it to be effective for COPD patients with moderate to severe disease (Carter 2013, D'Urzo 2013). Glycopyrronium bromide has low oral bioavailability therefore low risk of systemic effects following oral administration (Buhl and Banerji 2012). Atropine and Glycopyrronium bromide are used to decrease oral and respiratory secretions however, atropine results in significant anticholinergic side effects such as dry mouth (Bennett et al. 2002, Gomez, Bellido and Sanchez de la Cuesta 1995). Glycopyrronium bromide is a competitive antagonist with high affinity and slow dissociation for M_3 receptors (Buhl and Banerji 2012).

All LAMAs carry cardiovascular risks associated due to non-selective receptor binding to M_2 receptors in the heart (Hanania, Lareau and Yawn 2017). Glycopyrronium bromide has 4- to 5-fold greater selectivity for M_3 receptors, therefore fewer cardiovascular events (Carter 2013). Glycopyrronium bromide has a rapid onset of action, assessed via inhibition of methacholine-induced calcium release, demonstrating a five times shorter half-life than Tiotropium bromide (Buhl and Banerji 2012).

The clinical effectiveness of Glycopyrronium bromide was observed during the GLOW1 trial (Glycopyrronium bromide in COPD airways clinical study 1, 2011); patients were administered 50µg of Glycopyrronium bromide or a placebo once daily via a dry powder inhaler with low internal resistance (D'Urzo 2013). Efficacy was measured as an improvement in FEV_1, at 24 hours following drug administration at 12 weeks; secondary parameters included breathlessness and health related quality of life. FEV_1 values showed considerable improvement with respect to placebo; this was maintained throughout the 26 weeks and observed from day 1. A 31% drop in COPD exacerbations was also observed vs. placebo (D'Urzo 2013, Ulrik 2012). Early studies have suggested a good safety profile for Glycopyrronium bromide, with adverse effects similar to placebo, apart from an increase in patients reporting dry mouth; and no considerable risk of cardiovascular or metabolic effects, however this may be as patients with comorbidities were excluded from trials (Ulrik 2012).

1.3.2.2 Aclidinium bromide

Aclidinium bromide is another novel LAMA for moderate to severe COPD, like Glycopyrronium bromide, it is also exhibits a quaternary ammonium salt structure, based on a (3R)-quinuclidinol ester (Sims and Panettieri Jr 2011). Aclidinium bromide was found as a potential anticholinergic following screening of compounds with high affinity M_3 receptor binding and low systemic availability (Joos et al. 2010). Aclidinium bromide features a long half-life at the M_3 receptor *in vitro* and *in vivo* (Gavalda et al. 2009). It is rapidly hydrolysed *in vivo;* resulting in low systemic availability and potentially decreased clinical side effects, with little effect on renal function in plasma clearance (Schmid et al. 2010, Sentellas et al. 2010). Aclidinium bromide was firstly tested as a once daily and then as a twice daily treatment (Cazzola, Page and Matera 2013).

The ACCLAIM (Aclidinium bromide clinical trial assessing efficacy and safety in moderate to severe COPD patients) trials evaluated the efficacy of once daily Aclidinium bromide 200 µg versus placebo (Jones et al. 2011). The studies showed maintained but non-significant bronchodilation with respect to minimal clinically important difference (MCID), indicating suboptimal therapy (Reid and Carlson 2014). The phase III ATTAIN (Aclidinium bromide to treat airway obstruction in COPD patients) trial evaluated twice daily Aclidinium bromide at 200 and 400 µg compared to placebo in a double-blind trial over 24 weeks. The study showed that patients with moderate to severe COPD showed significant improvement in FEV_1 with both doses; however, improvement with 400 µg was better and comparable to improvement seen with Tiotropium bromide in 6-12 month studies (Jones et al. 2012). The ACCORD (Aclidinium bromide in Chronic Obstructive Respiratory Disease) trial found only the 400 µg dose to show clinical significance. The St. George's Respiratory Questionnaire (SGRQ) was used to assess COPD symptoms (mornings and nights) and use of rescue medication; it revealed that frequency and severity of night-time symptoms were reduced with treatment (Reid and Carlson 2014). Twice daily Aclidinium bromide over 6-weeks has numerically greater improvement in COPD symptoms, compared to once daily Tiotropium bromide. This provides a use for twice daily LAMAs in the relief of symptomatic COPD (Cazzola and Page 2014).

1.3.2.3 Tiotropium bromide

Tiotropium bromide received FDA approval in 2004 as a single daily dose for patients with moderate to severe COPD (Michele, Pinheiro and Iyasu 2010, Yohannes, Connolly and Hanania 2013), and is the most widely prescribed LAMA (Jara, Wentworth and Lanes 2012, Rodrigo et al. 2009). [^{3}H] binding assays show a 10-fold greater affinity and 100 times slower dissociation for pulmonary M_3 receptors with Tiotropium bromide compared to Ipratropium bromide (Barnes et al. 1995, Barnes 2000), contributing to its potency. Previously, long acting β_2 agonists (LABA) and inhaled corticosteroids (ICS) were associated with increased pneumonia-related hospitalisations followed by death (Matera et al. 2018), however LAMAs have been associated with improved lung function and fewer hospitalisations in COPD (Oba, Zaza and Thameem 2008). Tiotropium bromide has limited systemic bioavailability and dissociates faster at M_2 receptors allowing for reduced acetylcholine release (Rodrigo et al. 2009), this suggests a lower risk of side effects.

Tiotropium bromide binding to its receptors involves interaction between hydroxy groups and asparagine residues within the receptor core (Popescu 2015), which may explain reduced M_2 receptor binding.

Studies looking at FEV_1 have found that Tiotropium bromide shows significant improvement compared to Ipratropium bromide (van Noord et al. 2000). The UPLIFT (Understanding Potential Long-term Impacts on Function with Tiotropium) trial was a randomised, double blinded trial examining COPD patients using placebo or Tiotropium bromide over four years, exclusion criteria for the study included any history of asthma, COPD exacerbations or pulmonary infections (Tashkin et al. 2008). Tiotropium bromide was shown to improve health related quality of life and lung function with decreased exacerbations; of 2987 patients randomised to the treatment group, there were 792 deaths, caused by cancer, respiratory and cardiac disorders (Celli et al. 2009) at a lower risk than the placebo group. Randomised clinical trials have shown fewer exacerbations and hospilisations, and improved lung function with Tiotropium bromide compared to placebo and Ipratropium bromide (Rodrigo and Nannini 2007).

Tiotropium bromide was approved in the HandiHaler® and the Respimat® Soft Mist™ formats, the latter being more favourable amongst patients (Ohno, Muraki and Tohda 2015). The HandiHaler delivers a dry formulation (18µg), whereas the Respimat inhaler delivers an aqueous solution (5µg) showing similar safety, efficacy and pharmacokinetic profiles (Halpin et al. 2015). Data from independent trials show reduced fatality with the HandiHaler® but a numerical increase with the Respimat®, however a causal relationship was unidentified (Wise et al. 2013a). The TIOSPIR® (Tiotropium bromide Safety and Performance in Respimat®) trial assessed all-cause mortality of HandiHaler® vs. Respimat® (Halpin et al. 2015, Mathioudakis et al. 2014, Wise et al. 2013b), involving over 17,000 patients across multiple countries with a 2-3 year follow up. The study showed no difference in mortality or exacerbations between the formats (Ohno, Muraki and Tohda 2015, Wise et al. 2013a, Wise et al. 2013b); however, the trial excluded patients with comorbidities (Mathioudakis et al. 2014). Trials reporting an increase in cardiovascular-related mortality with Respimat® included data with the higher dose of 10µg (Halpin et al. 2015, Karner, Chong and Poole 2014). However, the UPLIFT trial also used short acting anti-

muscarinics (SAMAs); this is the key difference to other trials which had shown increased risk with Tiotropium bromide use (Grosso et al. 2009).

1.3.2.4 Umeclidinium bromide

Umeclidinium bromide is a quaternary ammonium structure derived from quinuclidine (Segreti et al. 2014). Single and repeat doses of Umeclidinium bromide are well tolerated in patients and healthy volunteers (Decramer et al. 2013). *In vitro* studies have shown Umeclidinium bromide has a longer duration of action compared to Tiotropium bromide. Umeclidinium bromide received approval, both as a once-daily drug and combined with the long acting β_2 agonist, vilanterol (Cazzola and Page 2014). Umeclidinium bromide also has greater selectivity and dissociates slowly from M_3 receptors compared to M_2 (Cazzola et al. 2012a). In patients responsive to Ipratropium bromide, Umeclidinium bromide was rapidly absorbed, cleared and eliminated (Segreti et al. 2014).

Umeclidinium bromide (15.6, 31.25, 62.5 and 125 µg) was evaluated in a large-scale trial, administered twice daily over 7 days (Church et al. 2014), via the Ellipta™ dry formulation inhaler. Tiotropium bromide in the HandiHaler® format (18 µg) and placebo once daily were also used, FEV_1 determined primary efficacy. Umeclidinium bromide significantly improved lung function in patients with moderate to severe COPD compared to placebo (Church et al. 2014, Segreti et al. 2014) and was well tolerated at 62.5 and 125 µg, subsequently recommended at these doses for clinical assessment (Church et al. 2014). These doses showed improved lung function and good tolerance (Trivedi et al. 2014). Umeclidinium bromide received approval as a single dose; however, there is now focus on combinatorial therapy with long acting β_2 agonists (LABA) and inhaled corticosteroids (ICS).

Vilanterol (LABA) displays high receptor selectivity for β_2 over β_1, showing rapid onset of action in isolated small airways (Cazzola, Segreti and Matera 2013). Combinatorial therapy can potentially achieve a once-daily dose, encouraging patient compliance as multiple drugs would not need to be taken for the same effect. Moderate to severe COPD patients can be prescribed a LABA with a LAMA, (Malerba, Morjaria and Radaeli 2014). Umeclidinium bromide/vilanterol has been assessed at 62.5/25 µg and 125/25 µg respectively (Donohue et al. 2013, Malerba, Morjaria and Radaeli 2014), both show improved lung function, and quality of life (Cazzola and Page 2014), and improved FEV_1

with respect to placebo and monotherapies (Donohue et al. 2013, Malerba, Morjaria and Radaeli 2014). Recently, LAMA/LABA/ICS combinations such as Umeclidinium bromide/vilanterol/fluticasone have shown significant improvement in FEV_1 with respect to placebo/vilanterol/fluticasone; the frequency of adverse effects was equivalent amongst all groups, supporting triple therapy (Siler et al. 2015).

1.3.3 Clinical studies

In the last decade, there has been a surge in studies analysing therapeutic agents and adverse cardiovascular outcomes. Comorbidities amongst COPD patients elevate the need to identify drugs which may exacerbate underlying cardiac disease. Studies have shown an association with anti-muscarinic use, such as Ipratropium bromide and adverse cardiac events (Macie et al. 2008, Singh, Loke and Furberg 2008); however, multiple studies debate any increase in all-cause mortality (Sin and Tu 2000, Tashkin et al. 2008).

Singh et al. (2008) highlighted that exposure to Tiotropium bromide elevated the risk of adverse cardiac events and mortality. The risk of cardiovascular death, stroke or myocardial infarction was greater in those who had received Tiotropium bromide or Ipratropium bromide compared to controls (Singh, Loke and Furberg 2008). However, this study was criticised for methodological limitations, such as bias in study selection which specifically selected trials reporting cardiovascular events (Michele, Pinheiro and Iyasu 2010). A subsequent study by Singh et al. (2011) assessed the use of the Tiotropium bromide mist inhaler (Respimat ® Soft Mist inhaler) in COPD patients; the meta-analysis of five randomised controlled trials showed an increased risk of mortality with the mist inhaler. Another study observing the impact of adherence to Tiotropium bromide found that the device used can significantly alter patient adherence. Poor adherence and underuse of the Respimat® format were associated with a significant increase in mortality, and overuse was associated with increased morbidity such as community acquired pneumonia (CAP) (Koehorst-ter Huurne et al. 2013). Additionally, it was shown that use of the Handihaler® format was at much less risk of overuse (Koehorst-ter Huurne et al. 2018). This highlights the fact that adherence and format of LAMA may substantially increase the risk of adverse events.

Certain patient populations showed increased susceptibility, indicating the role of comorbidities in exacerbating cardiac events. Although anti-muscarinics have a better safety profile in COPD patients, patients with cardiac complications are often excluded from trials, limiting the support to use these bronchodilators within these populations (Hawkins et al. 2010, Mentz et al. 2012). COPD is a strong risk factor for cardiovascular events (Sin and Man 2005); a large-scale study analysing mortality and hospitalisations amongst COPD patients found that cardiovascular disease is more frequent and may represent an increased burden compared to primary disease (Huiart, Ernst and Suissa 2005). There is a strong association of COPD and cardiac disease, which is independent of other risk factors such as smoking status (Bhatt and Dransfield 2013, Cazzola, Rogliani and Matera 2015).

1.3.4 Patients with underlying morbid heart conditions

A large-scale study reviewing the associations of cardiovascular disease and COPD found significant correlation, indicating a five-fold greater risk of major cardiac diseases including ischaemic heart disease (IHD) in COPD patients (Cazzola et al. 2015). Finkelstein et al. (2009) assessed whether COPD was an independent risk factor of cardiovascular disease; the study showed that COPD patients were at a greater risk of cardiovascular events with respect to matched controls, matched for various sociodemographic, health and co-morbidity factors, concluding that COPD is an independent risk factor of cardiac disease. Increased cardiac complications amongst COPD patients have seen a rise in hospitalisations associated with cardiac related mortality (Sidney et al. 2005). This is not restricted to COPD patients but is also observed amongst patients with asthma and other respiratory diseases (Guite, Dundas and Burney 1999).

This co-existent relationship is also present in the inverse, where patients with existing cardiovascular disease also present with COPD or other airflow restricting respiratory conditions. A study conducted amongst Japanese patients with diagnosed cardiovascular disease assessed patients for COPD, a quarter of patients had COPD which was largely undiagnosed (Onishi et al. 2014).

The mechanism for increased cardiovascular risk in COPD is poorly defined. Several potential mechanisms are attributed to the increased risk amongst COPD patients,

including arterial stiffness and blood pressure (Mills et al. 2008). Increased arterial stiffness and systemic inflammation are attributed to COPD, as patients without comorbidities also presented the same. Arterial stiffness is a validated marker of cardiovascular risk, especially atherosclerosis (McAllister et al. 2007, Patel et al. 2013, Sabit et al. 2007). However, multiple mechanisms may be involved with the increased cardiovascular risk, including airway infection and inflammation. The low grade chronic inflammation underlying COPD is also an important risk factor for cardiovascular disease, independent of smoking (Sin et al. 2006). It is hypothesised that markers such as C-reactive protein (CRP) are directly involved in the pathogenesis of atherosclerosis (Sin and Man 2003). CRP is thought to deposit into the arterial wall and interact with other inflammatory mediators to form foam cells which are the prerequisite of atherosclerotic plaques (Calverley and Scott 2006, Sin and Man 2003).

1.4 Ischaemic Heart Disease

Ischaemic heart disease (IHD) also known as coronary artery disease (CAD) is the leading cause of death worldwide according to the World Health Organisation (Finegold, Asaria and Francis 2013, Kim and Johnston 2011, Wong 2014). IHD is defined as reduced blood flow to the coronary artery, which reduces blood supply to the heart (Hansson 2005). IHD involves atherosclerotic lesions in the arterial wall narrowing the lumen, leading to myocardial infarction (Hansson 2005, Marzilli et al. 2012, Polonsky et al. 2010). The risk of cardiac complications is significantly greater with age, with an elevated risk of atherosclerosis and arterial stiffening (Lakatta and Levy 2003).

Understanding the mechanisms underlying atherosclerotic plaques is important in prevention. However, defining atherosclerosis in a patient is necessary to manage disease with modification of life style factors to invasive percutaneous transluminal coronary angioplasty (PTCA) (Libby and Theroux 2005).

1.4.1 Atherosclerosis

Atherosclerosis involves local immune mediators such as T cells and macrophages (Danesh et al. 2004, Epstein and Ross 1999, Lind 2003), it is not fatal itself; however, thrombosis along with plaque formation leads to stroke and acute coronary syndromes (Falk 2006).

Figure 1.4.1.1 depicts the stages of plaque build-up and thrombosis. Arterial endothelial cells express receptors enabling interaction with immune cells (Pamukcu, Lip and Shantsila 2011, Rudijanto 2007) such as macrophages, monocytes and T cells (Falk 2006, Fordjour et al. 2015, Lind 2003, Moore and Tabas 2011). Immune involvement is supported by elevated levels of auto-antibodies and cytokines (Hansson et al. 2002). The expression of pro-attachment molecules such as vascular adhesion molecule (VCAM) and monocyte chemoattractant protein 1 (MCP1) follows exposure to hypertension, diabetes mellitus, smoking or blood flow induced laminar shear stress (Cunningham and Gotlieb 2005, Libby, Ridker and Hansson 2011).

Apolipoprotein B containing lipoproteins (apoB-LPs) accumulate within the arterial wall, which are then converted into atherogenic remnant lipoproteins known as the fatty streak (Moore and Tabas 2011). Macrophages are predominantly involved in the formation of foam cells; foam cells are common in infants and young children and may resolve untreated (Epstein and Ross 1999, Falk 2006). Macrophage derived foam cells are important in atherosclerosis and are the primary source of atherogenic plaques (Shashkin, Dragulev and Ley 2005, Yu et al. 2013). The release and production of reactive oxygen species (ROS) by macrophages aid the progressive oxidation of LDL. This oxidised LDL is taken up by macrophages via scavenger receptors such as CD36 and SR class-A (Yu et al. 2013), leading to internalisation and storage as lipid droplets (Rudijanto 2007).

Once foam cells and lipid droplets accumulate, an irreversible atheroma develops (Hansson 2005). A fibrous cap forms as a protective scar over tha atheroma, however when ruptured this leads to exposure of the thrombogenic core to the blood. This core is composed of apoptotic and necrotic immune cells; necrosis occurs through the defective clearance of apoptotic macrophages, leading to lesional macrophages. Apoptosis in macrophages is triggered by oxidative stress and the activation of death receptors (Moore and Tabas 2011). Dendritic cells also accumulate in atherosclerotic lesions contributing to early foam cell development; the functional role of dendritic cells and macrophages has significant overlap in this context (Zernecke 2015).

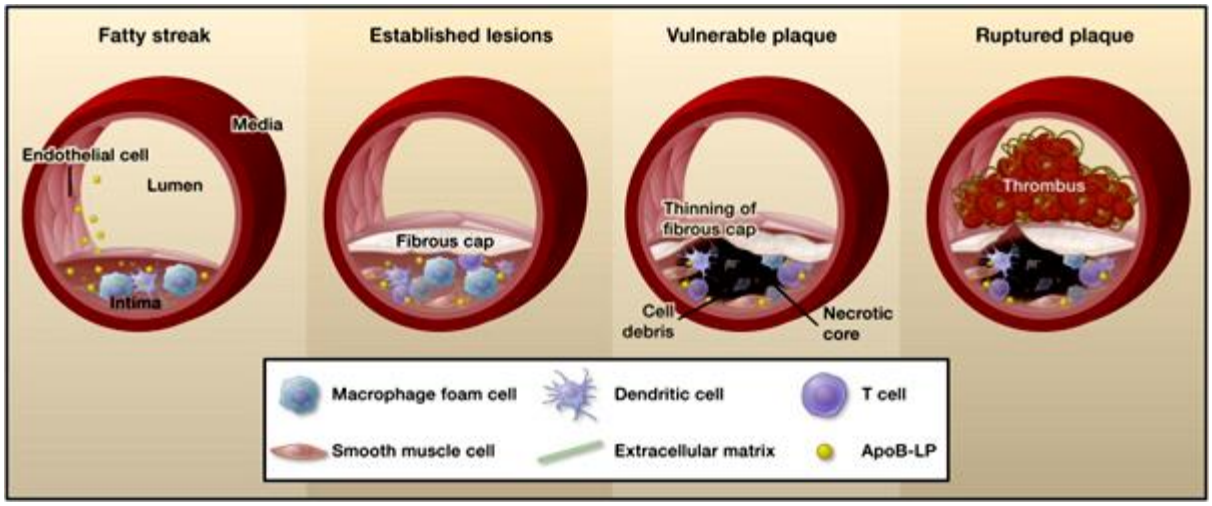

Figure 1.4.1.1: The formation of atherosclerotic plaque, from the fatty streak to the problematic ruptured plaque. *All stages involve the inter-play of several cell types including immune cells and smooth muscle cells. Fatty streaks may be reversed before the atheroma stage is reached, before macrophages become foam cells upon interaction with lipids. ApoB-LP – apolipoprotein B containing lipoproteins (Moore and Tabas 2011).*

The thinning and rupture of the fibrous cap leads to thrombosis; it is this step which leads to myocardial infarction, unstable angina and thromboemoblic stroke (Lee et al. 2013, Moore and Tabas 2011). The integrity of the cap is compromised due to proteolytic enzymes such as matrix metalloproteinases (MMPs) (Rudijanto 2007). MMP-2 and MMP-9 are strongly correlated with plaque instability and are over expressed in atherosclerosis (Heo et al. 2011). Plaque stability is also associated with microcalcifications; which increase plaque stability, and reduce rupture; for this reason, coronary calcification is used as a prognostic marker (Maldonado et al. 2012). However, calcification at the fibrous cap increases the probability of rupture therefore, the location of microcalcifications is important in determining plaque stability. Although plaque stability is crucial in determining the fate of rupture, certain genes are closely linked to ruptured plaques. Fatty acid binding protein 4 (FABP4) and leptin were expressed highly in samples of ruptured plaques from patients undergoing carotid stenosis surgery. These genes are linked to lipid metabolism and inflammation, showing a correlation between these pathways and plaque instability (Lee et al. 2013).

1.4.2. Ischaemia and Reperfusion

Ischaemia occurs when the demand for oxygen consumption exceeds the supply; therefore, obstructions in blood vessels are critical (Crossman 2004). Prolonged ischaemia results in altered cellular metabolism and structure, and irreversible damage. Myocardial ischaemia underlies the pathological conditions of angina, arrhythmias and myocardial infarction (Eltzschig and Collard 2004).

Tissue acidosis occurs during ischaemia due to increased H^+ (Turer and Hill 2010). Deprivation of oxygen and metabolites lead to suspension of oxidative phosphorylation, which depolarises the mitochondrial membrane and results in ATP depletion (Hausenloy and Yellon 2013). The Na^+-H^+ exchanger (NHE) becomes activated and results in the accumulation of Na^+ whilst expelling protons; accumulation of Na^+ reverses the Na^+/Ca^{2+} exchanger (NCX), leading to calcium overload (Hausenloy and Yellon 2013). Increased Ca^{2+} entry compensates for the expulsion of accumulated Na^+ and is exacerbated via L-type calcium channels and the Ca^{2+}-ATPase, which promote calcium overload and leads to ATP depletion. In cardiomyocytes this means hypercontracture, mitochondrial damage and myocardial stunning (Dorn and Maack 2013, Murphy and Steenbergen 2008).

To curb the lethal consequences of ischaemia, restoration of blood flow is critical. In a clinical context, this is achieved by percutaneous coronary intervention (PCI) (Hausenloy and Yellon 2013). PCI is standard therapy for patients who have experienced ST elevated myocardial infarction (STEMI), whereby blood flow is restored by thrombolysis, which has been shown to reduce infarct size (Sardella et al. 2009). However, the restoration of blood flow to ischaemic tissues itself results in paradoxical injury. This restoration of blood flow is necessary to protect the myocardium from the damaging consequences of ischaemia alone (Yellon and Hausenloy 2007). Figure 1.4.2.1 depicts the cellular processes underlying myocardial ischaemia/reperfusion injury. It is well established that the mitochondrial permeability transition pore (mPTP) has an important role in reperfusion injury, particularly with regards to cell death (Dhalla and Duhamel 2007, Halestrap and Richardson 2015, Halestrap 2006, Yellon and Hausenloy 2007). During acute ischaemia, the drop in pH prevents the opening of the mPTP, however during reperfusion the reactivation of the electron transport chain generates ROS, leading to opening of the mPTP. This stage is the gateway to cardiomyocyte cell death (Hausenloy and Yellon 2013).

Figure 1.4.2.1: Cardiomyocyte damage occurs during both ischaemia and reperfusion*. This is due to the initial deprivation of oxygenated blood flow leading to anaerobic respiration; however, following re-establishment of blood flow, the production of reactive oxygen species (ROS) cause opening of the mitochondrial permeability transition pore (mPTP) and* Ca^{2+} *efflux from the sarcoplasmic reticulum (SR) leads to further injury (Hausenloy and Yellon 2013).*

Clinically, aside from cell death there are reversible and permanent injuries sustained with myocardial ischaemia/reperfusion including arrhythmias, contractile dysfunction, ultrastructural damage, vasoconstriction and endothelial dysfunction (Dhalla and Duhamel 2007). The 'no-reflow' phenomenon also known as microvascular obstruction is a lethal consequence of ischaemia/reperfusion injury (Jaffe et al. 2008) from incomplete reperfusion (Reffelmann and Kloner 2002), a common clinical occurrence where 39% of cases may show unsuccessful reperfusion via angiography following successful PCI intervention (Galiuto, Rebuzzi and Crea 2009). Microvascular obstruction involves neutrophil accumulation which release inflammatory mediators, proteases and ROS (Buja 2005); de novo thrombosis also contributes to the no-reflow phenomenon (Moens et al. 2005).

Myocardial stunning describes depressed contractility following reperfusion of previously ischaemic regions (Buja 2005). Stunning is due to oxidative stress and calcium overload; repeated periods of myocardial stunning may lead to the development of heart failure and cardiomyopathy (Hausenloy and Yellon 2013, Moens et al. 2005). Myocardial stunning persists for long periods, despite cells being viable. Several mechanisms including depleted ATP due to ischaemia, microvascular obstruction and cellular damage from ROS may contribute to stunning (Maxwell and Lip 1997).

Reperfusion induced ventricular arrhythmias are potentially lethal. These remain for long periods of time; however arrhythmias are usually self-terminating and are reversible (Hausenloy and Yellon 2013). The critical mechanisms responsible for these manifestations are oxidative stress and calcium overload (Dhalla and Duhamel 2007). A key component of these cascading pathways is the mitochondrial permeability transition pore (mPTP); during ischaemia the mPTP remains unopened however, in reperfusion this pore opens due to ROS generation and thus leads to mitochondrial mediated cell death (Hausenloy and Yellon 2013).

Several genes are linked to cardiovascular dysfunction, the expression of which are altered in certain disease phenotypes. The peroxisome proliferator-activated receptor gamma (PPARγ) is involved in heart failure and myocardial infarction, but also possesses contrasting cardioprotective properties when activated (Chandra, Miriyala and Panchatcharam 2017), particularly in the context of ischaemic preconditioning (Han et al. 2010). Coactivators enhance the activity of transcriptional factors; in the case of PPARγ, the most well profile coactivator is PGC-1α, which has the capacity to affect many transcription factors (Rowe, Jiang and Arany 2010). Circulating levels of PGC-1α have been used to predict myocardial salvage after acute myocardial infarction (Fabregat-Andrés et al. 2011), where higher baseline levels have correlated with increased myocardial salvage; however excessive levels of PGC-1α lead to damaging ventricular remodelling through the upregulation of adenine nucleotide translocase (ANT1) (Fabregat-Andrés et al. 2015, Lynn et al. 2010).

Another potential biomarker of acute myocardial infarction is VEGF-A (Zhao et al. 2010b), during hypoxic conditions, there is an increase in VEGF-A to promote angiogenesis, this

increase in expression can be used as a biomarker, with prognostic value in predicting adverse outcome in patients with acute coronary syndrome (Heeschen et al. 2003). However, a case-controlled study of acute myocardial infarction patients, found VEGF-A to have a lower power as a biomarker when adjusted for traditional risk factors and C-reactive protein (CRP) (Iribarren et al. 2011). Nonetheless, VEGF-A is a lucrative therapeutic target in many cancers such as renal cell carcinoma (Ciamporcero et al. 2015), gastric cancer cells (Chen et al. 2014) and myeloid cells (Klose et al. 2016). Greater emphasis is now based upon targeting VEGF-A mRNA using microRNAs (miRs) and silencing microRNAs (siRNA), for the treatment of breast cancer (Chen et al. 2017a) and oesophageal cancer (Li et al. 2017). These suggest that VEGF-A may be targeted at a transcript level and may therefore have prognostic value in acute myocardial infarction, particularly when paired together with another biomarker.

1.4.3 Mitochondrial Permeability Transition Pore (mPTP)

Apoptosis and necrosis are implicated in ischaemia/reperfusion injury. Opening of the mPTP leads to the initiation of both apoptosis and necrosis and leads to a cessation of ATP production (Halestrap 2006). The pore is a high conductance, non-specific channel which is influenced by calcium overload, leading to mPTP opening and enabling the loss of membrane potential due to ionic disturbance. This leads to a collapse in oxidative phosphorylation and initiates cell death signalling (Mott et al. 2004). The mPTP is important in ischaemia/reperfusion injury; the use of a pore inhibitor such as cyclosporin A (CsA) can significantly attenuate infarct development in the heart (Piot et al. 2008). The cardioprotective effects of CsA have been demonstrated both experimentally and in humans, confirming the role of the mPTP in cell death signalling (Wong, Steenbergen and Murphy 2012). CsA binds to mitochondrial cyclophilin D, a protein found in the mitochondrial matrix (Hausenloy et al. 2010, Mott et al. 2004), which is involved in the generation of reactive oxygen species (ROS). Cardiomyocytes without cyclophilin D do not generate ROS in hypoxic conditions (Hausenloy et al. 2010). CsA also has inhibitory effects on the calcium dependent phosphatase, calcineurin, via binding of cyclophilin A, which may lead to undesirable consequences on the heart (Mott et al. 2004). Figure 1.4.3.1 shows a schematic demonstrating the steps that occur in deciding cell fate.

Figure 1.4.3.1: The steps occurring to decide cellular fate, in response to stress factors *(Halestrap 2006). The schematic highlights the role of calcium overload (Ca^{2+}- overload) in affecting the mPTP (or MPT), which ultimately decides the mode of cell death.*

Mitochondria support cell survival, through oxidative phosphorylation and modulate cell death via the mPTP. Several factors underlie mPTP opening such as matrix pH, [Ca^{2+}] and the mitochondrial membrane potential difference (Di Lisa et al. 2001). The opening of the permeability pore is known to affect several metabolic pathways, including NAD^+ catabolism. This pathway is important due to the role of mitochondrial NAD^+ in cardioprotection (Di Lisa et al. 2001).

Calcium overload is the key driver of mPTP dysfunction. Release of Ca^{2+} from the endoplasmic reticulum (ER) causes mitochondrial calcium influx via calcium-selective uniporters, however this influx is rapidly followed by efflux (Baumgartner et al. 2009). In

physiological circumstances, increased mitochondrial Ca^{2+} is met with increased ATP synthesis (Tarasov, Griffiths and Rutter 2012); however, in pathological conditions calcium overload results in mPTP opening. The rate at which the Ca^{2+} concentrations change, is the key difference; in pathological circumstances the increase in Ca^{2+} is initially slow and progressive, allowing management of Ca^{2+} across the inner mitochondrial membrane, however once the cytosolic concentration rises proportionally greater than 1-3µM, calcium overload occurs; this concentration is referred to as a 'set point' (Crompton 1999, Wong, Steenbergen and Murphy 2012). mPTP opening allows free movement of small (<1.5 KDa) ions, which disturbs colloidal osmotic pressure of the inner mitochondrial membrane. The outer membrane of the mitochondria ruptures to release cytochrome *c* and key apoptotic proteins such as apoptosis inducing factor (AIF). The rupture of the outer membrane exposes the inner membrane and thus allows free movement of protons, resulting in a breakdown of oxidative phosphorylation and a cessation in ATP production, which quickly reverses to ATP hydrolysis (Halestrap, Clarke and Javadov 2004).

The duration of mPTP opening has significance in whether apoptosis or necrosis is adopted (Halestrap 2006). Brief opening of the pore leads to transient depolarisation of the mitochondria and ROS generation occurring as oxygen is re-introduced to the inhibited respiratory chain and can further trigger mPTP opening and ROS production in neighbouring mitochondria, causing sustained mPTP opening leading to cell death (Halestrap, Clarke and Javadov 2004, Kinnally et al. 2011, Wong, Steenbergen and Murphy 2012). An important pathway highly exploited to curb the damaging consequences of ischaemia/reperfusion injury, is known as the reperfusion injury salvage kinase (RISK) pathway, involving Akt and ERK1/2 which mediate ischaemic preconditioning via non-pathological mPTP opening and small quantities of ROS.

1.4.4 RISK pathway

The reperfusion injury salvage kinase (RISK) pathway is a cardioprotective pathway, involving the rapid activation of pro-survival kinase pathways such as phosphatidylinositol-3-kinase (PI3K)-Akt and p42/44 extracellular signal-regulated kinase 1/2 (ERK1/2) (Davidson et al. 2006, Hausenloy and Yellon 2004, Zhu et al. 2006). Several other kinases are also known to confer cytoprotection such as p38, JNK, MAPK and the Jak-Stat pathway, (Hausenloy and Yellon 2004). The Jak-Stat pathway is an evolutionary conserved pathway;

Stat3 (Signal transducer and activator of transcription 3) in particular is a transcription factor which localises to the mitochondria and is involved in oxidative phosphorylation and membrane permeability, and has been shown to exert protective effects via modulation of mitochondrial and transcriptional responses as well as anti-apoptotic proteins (Harada et al. 2005, O'Sullivan et al. 2016).

The protective effects of Stat3 are particularly apparent in myocardial infarction (MI); a cardiac specific knockout of Stat3 in mice showed an increase in detrimental cardiac remodelling following MI with exaggerated fibrosis, therefore the induction of Stat3 in MI confers resistance to cardiac remodelling (Enomoto et al. 2015). In contrast, the RISK pathway elicits a cardioprotective state through inhibiting mPTP opening, via phosphorylation of pro-apoptotic proteins by Akt and ERK1/2 (Hausenloy, Tsang and Yellon 2005, Hausenloy and Yellon 2004). This pathway is important in ischaemia/reperfusion injury, which is a consequential phenomenon occurring during the restoration of blood flow to ischaemic areas. Myocardial tissue can cope with brief periods of ischaemia, up to 15 minutes without significant damage. However, once damage occurs from myocyte death, it is irreversible and can only be subsided by reperfusion to prevent further cardiomyocyte death (Verma et al. 2002).

The RISK pathway is also involved in ischaemic preconditioning (IPC) through GPCR activation and nitric oxide (NO) mediated mPTP inhibition (Hausenloy, Tsang and Yellon 2005). ERK1/2 and protein kinase C (PKC) facilitate the cardioprotective response of the RISK pathway (Hausenloy, Tsang and Yellon 2005). Figure 1.4.4.1 depicts the mediators of the RISK pathway involved in promoting cardiomyocyte survival following ischaemia/reperfusion injury. Other signalling molecules also utilise the PI3K/Akt and ERK1/2 signalling pathways to confer cardioprotection, such as insulin-like growth factor-1 (IGF-1) and transforming growth factor β1 (TGF- β1) (Hausenloy and Yellon 2004).

Figure 1.4.4.1: The components of the RISK pathway. *The components in green are pro-survival whereas those in red are pro-apoptotic. Components in yellow are the key components of the RISK pathway (Hausenloy and Yellon 2004).*

1.5 Cell death pathways

Programmed cell death (PCD) and non-programmed cell death (NPCD) are distinct mechanisms, however aspects of these signalling pathways often overlap; therefore, determining the activation of one pathway over another can prove difficult. The most well-known form of PCD is apoptosis, an organised and sequential form of death resulting in characteristic features. Necrosis on the other hand is perceived as a disorganised NPCD process leading to damaging inflammatory consequences; however, a 'programmed' form of necrosis is also thought to exists (Jin and El-Deiry 2005).

Apoptosis is commonly associated with cell death in physiological conditions, notably in the regulation of bone physiology (Clarke 2008); whereas necrosis is often associated with pathological conditions. However programmed necrosis has been implicated in microbial infections; necrosis often takes over when apoptosis is inhibited, thus implying a physiological role for necrotic cell death (Edinger and Thompson 2004). Apoptosis is also

involved in pathological conditions, such as ischaemia/reperfusion injury, where both necrosis and apoptosis are implicated (Ibáñez et al. 2015, McCully et al. 2004). Cell death signalling is interwoven with many essential pathways including calcium signalling, where calcium dependent processes are interconnected with apoptotic caspases (Orrenius, Zhivotovsky and Nicotera 2003).

1.5.1 Apoptosis

Caspases are also involved in ischaemia/reperfusion injury. Both apoptosis and necrosis mediate cardiomyocyte death following ischaemia/reperfusion injury. Apoptosis is a controlled and sequential process of events which does not involve inflammation and is an energy dependent process. Caspases, death receptors and effectors are instrumental in apoptosis; other key players are the tumour necrosis factor/receptor family (TNF/R) and the Bcl-2 protein family members which regulate apoptosis (Strasser, O'Connor and Dixit 2000).

Caspases are cysteine-aspartate proteases, existing as zymogens and require cleavage for activation (Brentnall et al. 2013, Salvesen and Dixit 1997); caspase-8, caspase-9, and caspase-3 are critically important in apoptosis (Mocanu, Baxter and Yellon 2000). Apoptosis and necrosis are thought to be on going processes during ischaemia; however, apoptosis is thought to be boosted during reperfusion (Eefting et al. 2004). Figure 1.5.1.1 illustrates the apoptotic signalling pathway involved in cardiomyocyte death.

Figure 1.5.1.1: The key components in the apoptotic pathway in a cardiomyocyte *(Eefting et al. 2004). Caspases have an important role as the mediators of apoptosis, with caspase-3 as an effector caspase, directly affecting DNA. The activation of caspase-3 relies on the release of cytochrome c from the mitochondria but also, as a result of the activation of death receptors. AIF – apoptosis inducible factor, Apaf 1 – apoptotic protease activating factor 1, Bcl-2 – B cell lymphoma 2, Iκ-B – inhibitor of Kappa B, FADD – Fas associated protein with death domain, FLICE – FADD like interleukin 1 β converting enzyme, FLIP – FLICE like inhibitory protein , RSK90 – 90KDa ribosomal s6 kinase (Eefting et al. 2004).*

Apoptosis sees cell shrinkage, disintegration of the nuclear envelope, DNA fragmentation and extracellular presentation of epitopes to promote phagocytosis (Jin and El-Deiry 2005, Strasser, O'Connor and Dixit 2000). Apoptosis may be triggered intrinsically or extrinsically (Brentnall et al. 2013). The extrinsic pathway can be stimulated via death receptor ligands such as tumour necrosis factor α (TNF-α) and Fas ligand, but also by chemical or chemotherapeutic agents, such as cisplatin and doxorubicin (Han, Kim and Kim 2008). These interact with death receptors, in contrast to the intrinsic pathway which involves permeabilsation of the mitochondrial outer membrane leading to cytochrome *c* release and caspase activation (Harr and Distelhorst 2010). Apoptotic protease activating factor 1 (Apaf-1) interacts with cytochrome *c* causing oligomerisation of Apaf-1 which recruits dimerised caspase-9, leading to its activation (Green 2005). Both strands of the apoptotic signalling pathway converge to involve the activation of caspases (Strasser, O'Connor and Dixit 2000).

Caspases are the driving force behind apoptosis, therefore targeting of caspases in myocardial infarction may have therapeutic significance (Mocanu, Baxter and Yellon 2000). Caspase-3 is known as an effector caspase in apoptosis (Brentnall et al. 2013, Lakhani et al. 2006, Porter and Jänicke 1999), figure 1.5.1.1 shows how caspase-3 is one of the final caspases to be activated during an apoptotic cascade, resulting in DNA fragmentation and condensation of chromatin, both hallmarks of apoptosis (Eefting et al. 2004). For these reasons, caspase-3 may be targeted for therapeutic benefit. In cardiac post-conditioning studies conducted on guinea pig whole heart models, the post-conditioning effect conferred with the use of an inhalational anaesthetic sevoflurane, shows inhibition of caspase-3 activity; this protective effect was abolished with an Akt and ERK inhibitor respectively (Inamura et al. 2010). This study confirmed the role of caspase-3 in mediating the damaging consequences of myocardial ischaemia/reperfusion injury, and that Akt and ERK are involved in eliciting cardioprotection during postconditioning. Interestingly in cancers, caspase-3 activation is correlated with improved survival; a study conducted in patients with gastric cancer found that increased caspase-3 detection in tumour samples was associated with improved clinical outcome such as 5-year survival (Huang et al. 2018). This may also be of benefit to cardiac disease, where caspase-3 activation is increased in

heart failure and myocardial infarction (Scheubel et al. 2002), however the mechanism of improved survival may be specific to cancers

Another protein with a crucial role in apoptosis is the Bcl-2 associated X (Bax) protein. Bcl-2 is an anti-apoptotic protein whereas Bax is a pro-apoptotic protein, which interacts with the mitochondrial outer membrane leading to the formation of a pore, these pores leak out pro-apoptotic proteins and enhance apoptosis (Ola, Nawaz and Ahsan 2011). Cells lacking Bax and another pro-apoptotic Bcl-2 protein Bak, are resistant to apoptotic cell death (Karch et al. 2017). Bax is also involved in necrotic cell death and autophagy; a study using mouse embryonic fibroblasts and $Bax^{-/-}$ mice, found that Bax mediates mPTP opening observed in necrotic cell death, which is absent in Bax knock out mice (Karch et al. 2013). The same group also found that mice embryonic fibroblasts deficient in Bax, were also resistant to autophagic cell death, associated with lysosomal permeability; restoring Bax to the lysosomes was sufficient to enable autophagy in null mice (Karch et al. 2017). The ratio of Bcl-2 to Bax is a measure of survival following apoptotic stimuli; in autopsied hearts from myocardial infarction (MI), there was an overexpression of Bax in patients who had died >1 month after MI, however patients who had died following an acute MI showed increased Bcl-2 in salvaged areas (Misao et al. 1996). This highlights the role of apoptotic proteins with a clinical significance and the potential use of Bax as a clinical marker following MI. These studies show that Bax may mediate multiple forms of cell death aside from apoptosis, therefore it is an important regulator of cell death and shows the crossover between various cell death pathways (Biala and Kirshenbaum 2014).

1.5.2 Necrosis

Necrosis triggers an inflammatory response via swelling and subsequent rupture of affected cells (Jin and El-Deiry 2005). Breakdown of the plasma membrane releases cellular contents, affecting surrounding cells. Necrosis is associated with severe cell swelling and rupture, breakdown down of organelles and coagulation of proteins in the cardiomyocyte cytoplasm leading to an inflammatory response (Zhao et al. 2000). Ischaemia is often associated with necrosis, where oxygen and metabolite deprived cells undergo necrosis on an enormous scale (Jin and El-Deiry 2005). High mobility group box 1 (HMGB1) is a nuclear protein released extracellularly following necrosis, triggering a necrotic inflammatory response (Han, Kim and Kim 2008, Krysko et al. 2008). Preventing HMGB1 release in lung

cancer prevents excessive ROS formation (Han, Kim and Kim 2008). Necrosis is the consequence of extreme stress including heat, chemical and osmotic (Krysko et al. 2008) and is known to be a cause of tumour promotion and invasion due to excessive inflammation (Han, Kim and Kim 2008).

Necrosis is seen as an assortment of random processes (Lopez-Neblina, Toledo and Toledo-Pereyra 2005) triggering an inflammatory response as the cell membrane is ruptured, and cellular contents exit to elicit an immune response (Hausenloy and Yellon 2004). Apoptosis and necrosis can be distinguished based on morphological changes; however, as both mechanisms of cell death interact, it can be difficult to distinguish the pathways (Eefting et al. 2004). In cardiomyocyte ischaemia/reperfusion injury, necrosis also mediates cell death through the mitochondrial permeability transition pore (mPTP) like apoptosis (Green 2005, Lopez-Neblina, Toledo and Toledo-Pereyra 2005), therefore mPTP opening alone cannot distinguish the cell death pathway. Whilst necrosis and apoptosis are thought of as a set of programmed actions versus random actions, there is no biochemical distinction and morphological differences can also overlap; in the absence of phagocytosis in apoptosis, cells appear morphologically similar to primary necrosis (Krysko et al. 2008, Kung, Konstantinidis and Kitsis 2011). Necrosis is involved in physiological processes and may also be programmed and can be regulated via cyclophilin D in the mPTP and in a caspase-dependent manner (Edinger and Thompson 2004, Han, Kim and Kim 2008). Where apoptosis is pharmacologically or genetically blocked, necrosis occurs following death receptor activation; there is evidence to suggest that necrosis may be preferred in physiological conditions where an immune reaction to a dying cell may be desirable such as with a bacterial infection (Edinger and Thompson 2004).

1.6 Role of Calcium in cell death signalling

Calcium overload and reactive oxygen species (ROS) drive ischaemia/reperfusion injury and initiate cardiomyocyte death, however both have important functions in healthy cardiomyocytes. External consequences such as ischaemia result in dysfunctional production of these mediators, therefore it important to understand the associated signalling pathways (Moens et al. 2005). In cardiomyocytes, systole occurs by L-type Ca^{2+}

channel mediated Ca^{2+} entry, triggering Ca^{2+} induced Ca^{2+} release (CICR) via the sarcoplasmic reticulum (Bootman 2012); relaxation follows Ca^{2+} removal via the Na^{+}/ Ca^{2+} exchanger (NCX), and the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Fearnley, Roderick and Bootman 2011, Murphy and Steenbergen 2008). Physiological cytosolic [Ca^{2+}] is maintained at 100-200 nM (Farber 1990), an electrochemical force maintains Ca^{2+} homeostasis consisting of the intracellular-extracellular difference in [Ca^{2+}] and the electrical potential of the plasma membrane (Kristian and Siesjo 1998).

1.6.1 Changes in calcium handling and involvement in cardiomyocyte death

Calcium is implicated in several cellular processes such as excitability, cell motility and gene transcription; there are several sensor and adaptor proteins which respond to changes in calcium concentrations and initiate a cellular response (Clapham 2007). Disturbance in calcium balance is best exemplified with compartmental dependence on calcium homeostasis, and its role in initiating cell death signalling (Orrenius, Zhivotovsky and Nicotera 2003). Calcium homeostasis is essential for mitochondrial function via the Na^{+}/Ca^{2+} ion antiporter which maintains the ionic gradient across the inner mitochondrial membrane, required for oxidative phosphorylation (Halestrap 2006). An overload in mitochondrial calcium causes the transporter to cease function. Figure 1.6.1.1 depicts compartmental calcium regulation. Calcium overload is often accompanied by oxidative stress and depleting levels of ATP, forcing opening of the mPTP (Baumgartner et al. 2009, Halestrap 2006). mPTP opening is one of the initiating factors of cell death signalling, particularly apoptosis; however, calcium signalling has also been associated with initiating non-apoptotic cell death and promoting phagocytosis of dying cells (Halestrap 2006, Orrenius, Zhivotovsky and Nicotera 2003).

Figure 1.6.1.1: The compartmental regulation of calcium*. Several channels and transporters are involved in order to maintain calcium homeostasis (Orrenius, Zhivotovsky and Nicotera 2003). PTP refers to the mitochondrial permeability transition pore (mPTP). NCX – Na^+/Ca^{2+} exchanger, PLCγ – phospholipase C γ, $Ins(1,4,5)P_3/R$ – inositol 1,4,5 triphosphate receptor, RYR – ryanodine receptor, ER – endoplasmic reticulum, SERCA – sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase, DAG – diacylglycerol, $PtdIns(4,5)P_2$ – phosphatidylinositol bisphosphate, PMCA – plasma membrane Ca^{2+} ATPase.*

The calcium hypothesis suggests that the cellular ability to regulate calcium is impaired following ischaemia; leading to the accumulation of toxic intracellular concentrations of calcium (Moens et al. 2005). Calcium influx can be linked to the dysfunction of several key channels; the sarcolemmal L-type calcium channel and SERCA dysfunction result in hypercontracture and mitochondrial damage (Turer and Hill 2010). The calpain family of calcium dependent serine proteases are also involved in apoptosis; therefore, tight control of calcium concentrations has significant importance in cell death (Matsumura et al. 2001). Much like the paradoxical damage sustained with restoration of blood flow to ischaemic areas, the introduction of Ca^{2+} into previously Ca^{2+}- free environments also has damaging consequences, known as the 'Calcium Paradox' (Piper 2000). Preconditioning studies have

shown that signalling pathways involved in reducing Ca^{2+} overload are activated for cardioprotection (Murphy and Steenbergen 2008).

1.6.2 Calcium in Ischaemia/Reperfusion injury

Despite the significant role of calcium involvement in ischaemia/reperfusion injury, there is no clinical evidence to suggest that blocking calcium channels at the onset of reperfusion is cardioprotective (Hausenloy and Yellon 2013), suggesting that other calcium pathways may underlie the observed consequences. One such target for calcium signalling in apoptosis is the activation of the Ca^{2+}/calmodulin dependent phosphatase; calcineurin, as well as the activation of calpains (Garcia-Dorado et al. 2012). Figure 1.6.2.1 depicts the interactions involved in Ca^{2+} overload mediated cell death in the context of reperfusion injury.

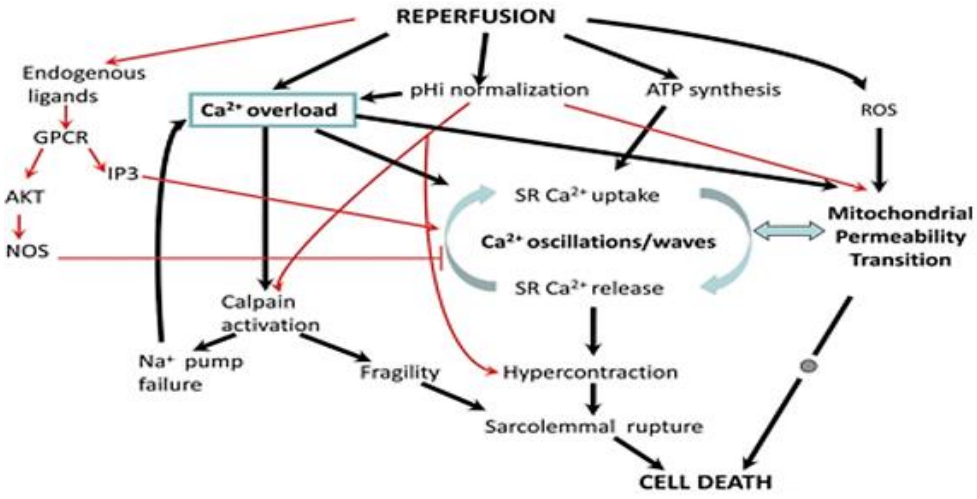

Figure 1.6.2.1: Alterations in calcium handling during reperfusion in cardiomyocytes leads to calcium mediated cardiomyocyte death*. There is involvement of reactive oxygen species (ROS), mitochondrial permeability transition pore (mPTP), SR mediated Ca^{2+} release and Ca^{2+} overload in cell death (Garcia-Dorado et al. 2012). NOS – nitric oxide synthase, GPCR – G-protein coupled receptor, IP3- inositol triphosphate.*

Activation of the Na^{+}/Ca^{2+} exchanger (NCX), particularly during ischaemia sheds light on the role of Ca^{2+} in ischaemia/reperfusion. NCX increases cytosolic calcium, correlating with

hypercontracture and cell death (Moens et al. 2005). Figure 1.6.2.2 summarises the roles of the Na^+ and Ca^{2+} in ischaemia/reperfusion. Calcium also plays a role in cardiomyocyte death during reperfusion, mediated via the opening of the mPTP and influx of mitochondrial calcium.

Figure 1.6.2.2: The roles of sodium and calcium ions in ischaemia/reperfusion induced hypercontracture in cardiomyocytes *(Moens et al. 2005), and the potential roles of inhibitors for the transporters.*

Ca^{2+} overload in cell death specifically in cardiac ischaemia/reperfusion injury, has been elucidated through the examination of the mPTP and the disturbance of mitochondrial calcium homeostasis. However, few studies have examined the role of calcium signalling in the context of drug exacerbated ischaemia/reperfusion injury. Drugs such as the short acting muscarinic antagonist Ipratropium bromide, are prescribed to patients with COPD; however, these patients often have underlying comorbidities such as ischaemic heart disease. Harvey et al. (2014) showed that the use of Ipratropium bromide in myocardial ischaemia/reperfusion injury can exacerbate ischaemia/reperfusion injury via necrosis and apoptosis. Although the study revealed that Ipratropium bromide exacerbated myocardial ischaemia/reperfusion injury, the specific mediators involved were not described.

However, Shaik et al. (2012) showed that Ipratropium bromide initiated Ca^{2+} mediated suicidal death in erythrocytes (Shaik et al. 2012), showing cell shrinkage a hallmark of eryptosis and membrane scrambling, a mechanism of identifying suicidal erythrocytes, by exposing phosphatidylserine residues on the cell surface (Shaik et al. 2012). This study highlights the role of calcium in mediating anti-muscarinic dependent cell death. Ipratropium bromide and similar drugs may also initiate cardiomyocyte death in a calcium dependent manner.

1.7 Role of Reactive Oxygen Species in cardiomyocyte cell death

Classically, reactive oxygen species (ROS) in cells were thought to be detrimental, however they also have protective effects (Becker 2004) observed in preconditioning, where a stress response leads to survival. Conditioning responses are associated with decreased oxidative stress during ischaemia/reperfusion injury therefore providing cytoprotection (Becker 2004, Pasdois et al. 2011). This is also observed with drug-induced cardioprotection in ischaemia/reperfusion injury; acetylcholine mediated cardioprotection correlates with an increase in intracellular ROS prior to the onset of ischaemia, the use of an anti-oxidant abolishes acetylcholine mediated cardioprotection and also blocks ROS increase (Yao et al. 1999). ROS are generated from oxidative phosphorylation in the mitochondria, or via interaction with xenobiotics (Ray, Huang and Tsuji 2012). Oxidation of cellular metabolites can affect the functional properties of these entities, influence the "Ca^{2+} code" and modify essential pathways (Ermak and Davies 2002). In the context of T cell activation, ROS influence calcium channel opening via oxidation of IP_3R and ryanodine receptors (Harr and Distelhorst 2010).

1.7.1 Types of Reactive Oxygen Species

Highly toxic ROS include superoxide anions (O^{-}_2), hydrogen peroxide (H_2O_2) and the hydroxyl radical (^{-}OH), produced from the universal reduction of freely available oxygen (Bandyopadhyay, Das and Banerjee 1999, Ray, Huang and Tsuji 2012). Other radicals include nitric oxide ($NO^{\cdot}$) and peroxynitrite ($ONOO^{-}$) (Griendling and FitzGerald 2003). Superoxides and H_2O_2 are involved in signalling at low concentrations, however at higher concentrations they form hydroxyl radicals that can lead to cell death (Pasdois et al. 2011).

1-5% of the oxygen consumed by the mitochondria is involved in ROS formation through the respiratory chain (Becker 2004, Circu and Aw 2010). In this process, oxygen accepts an electron and becomes the superoxide anion, along with the protonated $\cdot HO_2$; during ischaemia where the pH drops, the protonated form is more favourable and can cause oxidative damage to lipids and the cell membrane (Becker 2004). This can be circumvented by dismutation producing hydrogen peroxide, which is only toxic at high concentrations; cells have adapted mechanisms which enable the conversion of H_2O_2 to H_2O through the catalase enzyme or the glutathione system (Becker 2004).

ROS generation was first observed in phagocytic cells' NADPH oxidase; ROS were produced as a result of respiratory burst, utilising electrons from intracellular NAPDH to form superoxide anions which are processed to H_2O_2, used as a defence against microbial invasion (Orient et al. 2007). Phagocyte-type NAPDH oxidase have homologs in other cell types including vascular smooth muscle cells, known as Nox; these produce ROS in vascular smooth muscle, enhanced with stimuli such as angiotensin II or TNF-α (Li et al. 2002). Sources of mitochondrial ROS also include α-ketoglutarate dehydrogenase, $p66^{shc}$ and monoamine oxidase (MAO); generation of ROS may also occur in the cytosol. Peroxisomes, endoplasmic reticular monooxygenases, cytochrome P450 and membrane associated NADPH oxidase also generate ROS, the latter results in the generation of ROS implicated in cellular signalling (Circu and Aw 2010, Griendling and FitzGerald 2003). Damage is not restricted to ROS generated cells; damaged cells also release ROS resulting in injury to surrounding cells. Tissue injury can also cause oxidative stress via ischaemia/reperfusion, trauma or infection (Bandyopadhyay, Das and Banerjee 1999).

1.7.2 Role of ROS in Physiological and Pathological conditions

Reactive oxygen species (ROS) can be detrimental; in physiological conditions, molecular oxygen is taken up by the mitochondria, forming the site of ROS generation in aerobic conditions (Orrenius, Gogvadze and Zhivotovsky 2015). ROS and nitrogen species also function as signalling molecules in physiological conditions (Ermak and Davies 2002). The phosphatase and tensin homology (PTEN) protein negatively regulates the synthesis of membrane bound phosphatidylinositol 1, 4, 5 trisphosphate (PIP3), which recruits Akt by dephosphorylating to PIP2; PTEN can be inactivated via H_2O_2 oxidation which modulates this pathway (Griendling et al. 2000, Ray, Huang and Tsuji 2012).

In pathological conditions, ROS promote the oxidation of mitochondrial membrane phospholipids and proteins, resulting in a compromised membrane (Bandyopadhyay, Das and Banerjee 1999, Orrenius, Zhivotovsky and Nicotera 2003, Orrenius, Gogvadze and Zhivotovsky 2015). ROS can also damage DNA through base modifications, deoxyribose oxidation, strand breakage or cross-linking DNA proteins, resulting in mutagenesis or carcinogenesis (Bandyopadhyay, Das and Banerjee 1999, Devasagayam et al. 2004) leading to apoptosis (Griendling and FitzGerald 2003). Attack from $^{\cdot}OH$ on pyrimidines results in thymine glycol, uracil glycol, urea residue, 5-hydroxydeoxyuradine, hydantoin or 5-hydroxydeoxycytidine. Similarly, $^{\cdot}OH$ attack of purine bases results in the formation of 8-hydroxydeoxyguanosine (8-OHdG), formamidopyrimidines and 8-hydroxydeoxyadenosine (Devasagayam et al. 2004). 8-OHdG has been implicated in cancer and is a biomarker for oxidative damage of DNA. Figure 1.7.2.1 shows a schematic summarising the involvement of ROS and antioxidants in pathological conditions.

Figure 1.7.2.1: The involvement of reactive oxygen species in pathological conditions. *Conditions such as ischaemia/reperfusion injury, acidosis and tissue damage are described, along with endogenous scavengers, the sources and consequences of ROS. SOD - superoxide dismutase, GSH – glutathione, adapted from (Bandyopadhyay, Das and Banerjee 1999).*

Mitochondria are the major contributor to reperfusion-induced ROS (Becker 2004, Chouchani et al. 2014), produced from the reduction of molecular oxygen when re-

introduced into previously ischaemic tissue (Murphy and Steenbergen 2008, Verma et al. 2002). ROS are also generated from enzymes such as mitochondrial cytochrome oxidase, and activated neutrophils (Pasdois et al. 2011, Verma et al. 2002). Free radicals induce the release of platelet activating factor from endothelial cells, attracting more neutrophils and amplifying ROS production. ROS causes reperfusion injury by reacting with membrane-bound polyunsaturated fatty acids (Verma et al. 2002). Whilst ROS generation is attributed to reperfusion following mPTP opening (Hausenloy et al. 2002, Murphy 2009), there is evidence to suggest ROS may also be produced during ischaemia; however, failure to provide a completely anoxic simulated environment leaves differences in studies regarding ROS production (Murphy 2009). Ischaemic preconditioning relies on short periods of ischaemia induced sequentially before an ischaemic attack; the PI3K pathway leads to the involvement of the mitochondrial K_{ATP} channel which activates survival kinases such as Akt, p38, ERK1/2, JNK and PKC following ROS generation (Hausenloy and Yellon 2006). Conversely, postconditioning is based on attenuation of ROS generation early on during reperfusion, through the inhibition of neutrophil related events (Sun et al. 2005).

1.7.3 ROS Scavengers and Antioxidants

Several redox systems neutralise ROS, including the gluthathione redox system, thioredoxin system, pyridine nucleotide system, NAPDH, NAD^+ and sirtuins (Circu and Aw 2010). Antioxidant enzymes responsible for primary defence against ROS include superoxide dismutase, gluthathione peroxidise, horseradish peroxidise and lactoperoxidase as well as catalase (Bandyopadhyay, Das and Banerjee 1999, Birben et al. 2012). Exogenous or dietary substances can also detoxify ROS or free radicals; these are collectively known as antioxidants. Antioxidants also exist in non-enzymatic forms, such as vitamin C, vitamin E and glutathione (Birben et al. 2012). Free radical scavengers are antioxidants which scavenge and detoxify free radical species (Carocho and Ferreira 2013, Hamilton 2007). ROS can activate redox sensitive transcription factors, such as nuclear factor κβ (NF-κβ), nuclear factor of activated T cells (NFAT) and hypoxia inducible factor 1 (HIF1) (Birben et al. 2012). Figure 1.7.3.1 describes the effect of ROS on pathways affecting transcription factors.

Figure 1.7.3.1: The signalling cascades occurring within a cell in response to mitochondrial and non-mitochondrial ROS*. ROS signalling results in the activation of proinflammatory and antioxidant transcription genes. NFAT – nuclear factor of activated T cells, NF-κB/IκB - nuclear factor kappa light chain enhancer of activated B-cells/ inhibitor, NADPH – nicotinamide adenine dinucleotide phosphate, MEK – mitogen activated protein kinase kinase, ERK – extracellular signal regulated kinase, JNK, c-Jun n-terminal kinase, ROS – reactive oxygen species, AP1 – activator protein 1 (Birben et al. 2012).*

Resveratrol is a polyphenol compound found in red wine, implicated in conferring cardioprotection (Hung, Su and Chen 2004, Ray et al. 1999). Low to moderate consumers of red wine have decreased incidence of coronary heart disease, compared to excessive drinking or no alcohol consumption, this protective effect has been attributed to polyphenols (Magyar et al. 2012, Wu and Hsieh 2011). Polyphenols react with superoxides, hydroxyl or lipid hydroxyl radicals, reducing lipid oxidation and atherosclerosis (Hung et al. 2002). Resveratrol acts as free radical scavenger but does not function as a potent scavenger *in vitro* (Das and Maulik 2006); instead, the protective effects of resveratrol are likely to be from the upregulation of endogenous anti-oxidant systems such as Fe/Mn-superoxide dismutase and increased peroxidase activity (Mokni et al. 2013, Wang et al.

2012a). Resveratrol improves left ventricular function and lowers low density lipoproteins in post-infarction patients (Magyar et al. 2012).

Resveratrol is cardioprotective via nitric oxide (NO) (Das and Maulik 2006, Thirunavukkarasu et al. 2007) and adenosine, leading to NO mediated coronary flow increase in Langendorff models (Du et al. 2014, Magyar et al. 2012, Mokni et al. 2013, Thuc et al. 2012). Adenosine also acts through NO by activating NO signalling, which is abolished with adenosine inhibitors (Bradamante et al. 2003, Das and Maulik 2006). Resveratrol pretreatment in models of ischaemia/reperfusion injury shows significant upregulation of endothelial and neuronal NO synthase (eNOS and nNOS), this results in a significant reduction in infarct size (Dernek et al. 2004, Hung, Su and Chen 2004). Resveratrol induces relaxation in rat aortic rings mediated via inhibition of NADH/NADPH oxidase activity and activation of the L-arginine/NO/cGMP pathway; this activation is from an increased synthesis of NO and due to a decrease in NO inactivation. Diffusion of NO from endothelial cells to smooth muscle cells leads to the stimulation of guanylate cyclase resulting in cGMP mediated vascular relaxation (Orallo et al. 2002).

Resveratrol effects several pathways related to inflammation and gene expression, as seen in figure 1.7.3.2 (Das and Maulik 2006), with anti-apoptotic and anti-inflammatory effects (Wang et al. 2012a). At low concentrations, resveratrol induces autophagy following ischaemia/reperfusion via AMPK mediated activation of mammalian target of rapamycin 2 (mTOR2) and inhibition of mTOR1 (Gurusamy et al. 2009). However, AMPK activation by resveratrol inhibits cardiac hypertrophy by suppressing protein synthesis and gene transcription by NFAT and inhibition of Akt (Chan et al. 2008). Resveratrol also has genomic mechanisms to confer cardioprotection (Wu and Hsieh 2011), with differential expression observed with some MicroRNAs (miRs); ischaemia/reperfusion hearts pre-treated with resveratrol were shown to have miR patterns similar to basal levels with significant increases in ERK1/2 phosphorylation regulated by miR-21 (Mukhopadhyay et al. 2010). These studies show the diverse effect of resveratrol in cardiomyocytes to confer protection.

Figure 1.7.3.2: Resveratrol interacts with a wide range of different proteins and pathways*. These account for the anti-oxidant properties which can be manipulated to confer protection in a vast number of disease phenotypes (Das and Maulik 2006).*

1.8 Aims and Objectives

This study hypothesises that LAMAs may exacerbate cardiovascular damage in ischaemia/reperfusion injury. Additionally, it is hypothesised that Tiotropium bromide results in cardiomyocyte death via Ca^{2+} overload or reactive oxygen species.

The primary aim of this study was to elucidate the cardiotoxicity of long acting muscarinic receptor antagonists, notably how Tiotropium bromide induces myocardial damage in *ex vivo* and *in vitro* models of normoxic conditions and ischaemia/reperfusion injury. The objectives of this study were:

(a) Determining the cardiotoxicity of other long acting muscarinic receptor antagonists in *ex vivo* models of myocardial ischaemia/reperfusion injury
(b) Understanding the effect of Tiotropium bromide on calcium signalling and oxidative stress in *ex vivo* and *in vitro* normoxic conditions via Ca^{2+}/calmodulin kinase II (CaMKII) and the antioxidant, resveratrol
(c) Elucidating the function of muscarinic, PI3K-Akt, caspase-3 and the mPTP in Tiotropium bromide mediated myocardial damage
(d) To observe the effect of Tiotropium bromide on gene expression profiles associated with myocardial infarction and oxidative stress.

Chapter 2: Methods and Materials

2.1 Drugs and Materials

Aclidinium bromide, Tiotropium bromide, Glycopyrronium bromide and Umeclidinium bromide (Sequoia Research Products Ltd, UK) were dissolved in DMSO at 10 mM or 1 mM (Sigma Aldrich, UK) before use and diluted in Krebs Henseleit buffer for subsequent concentrations. The final dilution of DMSO has been shown to have no significant effect on infarct size (Joyeux et al. 2002), due to negligible final concentrations. All stock solutions were stored at -20^{o}C. The 2,3,5-Triphenyltetrazolium Chloride (TTC) and Evans blue dye were purchased from Sigma Aldrich, UK. All other reagents used were purchased from Fisher Scientific, UK. Haemodynamic function was recorded using a physiological pressure transducer connected to a latex balloon and PowerLab (ADI, UK), using LabChart® software v7.

2.1.1 Antibodies used for Flow cytometry

Cleaved caspase-3 $_{(Asp175)}$ Alexa Fluor® 488 antibody (Cell Signalling), Annexin-V FITC apoptosis detection kit (Abcam, UK), CellROX® green flow cytometry assay kit and Fluo 3-AM (Invitrogen, ThermoFisher, UK) were used with the BD FACSCalibur (BD Biosciences, UK).

2.1.2 Antibodies and reagents used for Western blotting

Antibodies from Cell Signalling (UK) include; anti-Akt antibody and phospho-Akt $_{(Ser473)}$, anti-CaMKII antibody and p-CaMKII $_{(Thr286)}$ antibody, biotinylated protein ladder detection pack; secondary antibodies for anti-biotin and HRP-linked anti-rabbit IgG. Any KDa Mini-PROTEAN® TGX™ pre-cast gels, the Mini-PROTEAN tetra cell, Trans-blot® Turbo™,PVDF membranes and Precision Plus Protein ™ Kaleidoscope prestained ladder were purchased from Bio-Rad Ltd. (UK).

2.1.3 Reagents used for quantitative PCR (qPCR)

Reagents for sample preparation, including RNase Zap® and RNA Later® were purchased from Ambion®, ThermoFisher, UK. SYBR Green, dNTPs and PrimePCR pathway plates were obtained from Bio-Rad Ltd. (UK), whilst the GAPDH primer was purchased from Qiagen, UK; all other reagents were from Invitrogen®, ThermoFisher, UK.

2.2. Animals

Adult male Sprague-Dawley rats (300g ± 50g body weight – Charles River, UK) were kept in humane conditions and fed a standard laboratory diet. Procedures were in accordance with the Guidelines on the Operation of the Animals (Scientific Procedures) Act 1986. Whole hearts were used for Langendorff models to record haemodynamic parameters and evaluate infarct development, tissue collection (Western blotting/qPCR) and cardiomyocyte cell isolations. All studies were approved by the Coventry University Ethics department (UK).

2.3 Langendorff Perfused Heart Model

2.3.1 Krebs-Henseleit solution and mounted Langendorff

For the whole heart perfusion models, animals were sacrificed via cervical dislocation in accordance to the Schedule I Home Office procedure, and the intact hearts were excised. The excised hearts were immediately placed in ice cold Krebs-Henseleit (KH - 118.5 mM NaCl, 25 mM $NaHCO_3$, 12 mM Glucose, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4.7H_2O$ and 1.7 mM $CaCl_2.2H_2O$) buffer before being cannulated securely onto the Langendorff apparatus (figure 2.3.1.1), and retrogradely perfused with KH buffer (37°C, pH 7.4) saturated with 95% O_2 and 5% CO_2. Figure 2.3.1.1 depicts Langendorff mounted hearts.

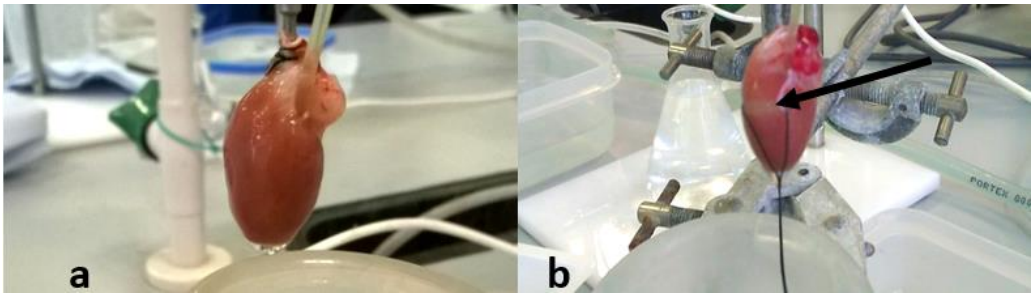

Figure 2.3.1.1: Hearts mounted on a Langendorff. *a) A normoxic heart cannulated with a latex balloon inflated in the left ventricle, through the excised left atrium. b) A heart following 35 minutes of regional ischaemia and 120 minutes of reperfusion. There is a clear ischaemic band formed horizontally across the heart (arrow).*

2.3.2 Haemodynamics Data Collection

Haemodynamics and experimental protocols were as described by Gharanei et al. (2013b). Figure 2.3.2.1 shows left ventricular developed pressure (LVDP) and heart rate (Beats per Minute – BPM). The coronary flow (ml/min) was recorded by collecting the coronary perfusate at timed intervals. Haemodynamic parameters were recorded at 5 minute intervals during stabilisation and ischaemia, and 15 minute intervals during reperfusion. Appendix 1 depicts the primary data recorded from a single experiment.

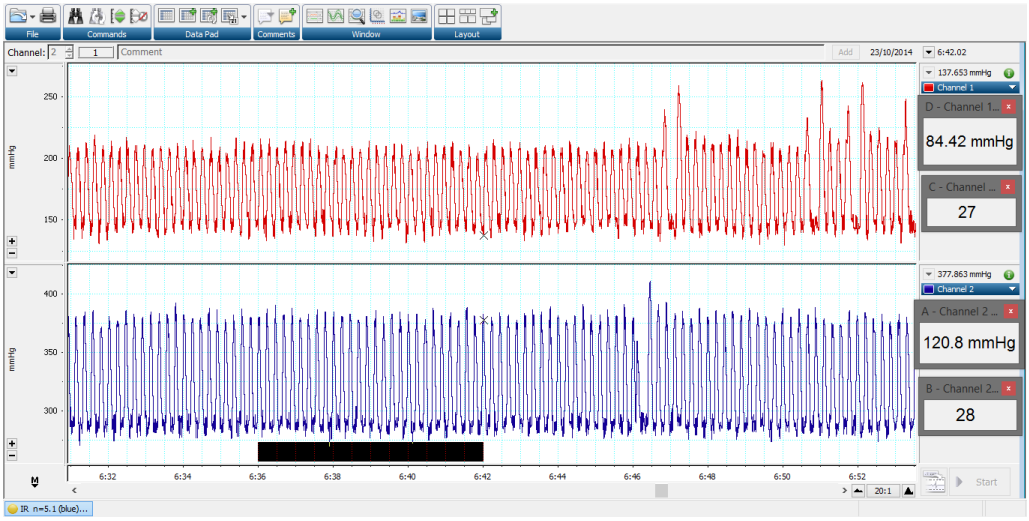

Figure 2.3.2.1: Haemodynamic trace for LVDP and heart rate*. The parameters were recorded at timed intervals as mentioned in section 2.3.2.*

2.3.3 Experimental Design – Concentration Response and Adjunctive therapies

2.3.3.1 Normoxia Langendorff Models – Adjunctive Therapies and Tiotropium bromide

Tiotropium bromide (10 nM – 0.1 nM) was used in the concentration response model; 1 nM was subsequently used for all other studies. For the adjunctive drug groups: acetylcholine (100 nM), resveratrol (10 µM), KN-93 (400 nM), wortmannin (100 nM), cyclosporin A (200 nM), Z-DEVD-FMK (140 nM) and nifedipine (1 nM) were used. The data from these drug groups were compared to normoxic controls (n = 4-6).

Normoxic controls received Krebs-Henseleit (KH) buffer for the duration of the experiment (175-mins) with no intervention. For all drug groups, following 20 minutes of stabilisation,

drug administration was initiated and remained for the duration of the experimental protocol (155 minutes) as demonstrated in figure 2.3.3.1.1. At the end of the experimental protocol, the weight of the hearts were recorded and stored at -20°C, before further analysis using TTC staining.

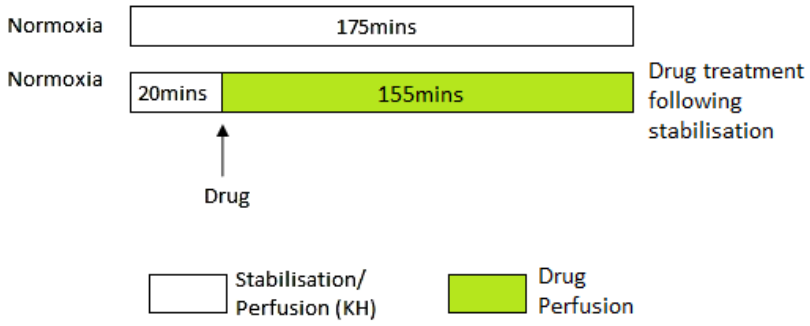

Figure 2.3.3.1.1: Normoxic Langendorff experimental model. *The drug treated groups accounts for Tiotropium bromide (10 – 0.1 nM), and the adjunctive drugs following stabilisation. The normoxic control group did not receive any drug treatment.*

2.3.3.2 Ischaemia/Reperfusion Langendorff Model

Tiotropium bromide (10 µM – 0.1 nM), Aclidinium and Glycopyrronium bromides (10 µM – 1 nM) and Umeclidinium bromide (1 µM – 1 nM) were used for the concentration response in ischaemia/reperfusion (n = 4-6), to include a range of concentrations including those administered in humans. Figure 2.3.3.2.1 depicts the experimental protocol. The normoxic controls were the same as described in 2.3.3.1. For ischaemia/reperfusion (I/R), following 20 minutes of stabilisation, regional ischaemia was induced for 35 minutes. Ischaemia was induced through the insertion of surgical sutures to occlude the left descending coronary arteries; the sutures were tightened and secured in place using plastic tubes forming a snare. Following the end of the ischaemia period, the snare around the coronary arteries was released and reperfusion commenced for 120 minutes. Drug administration began at the onset of reperfusion and lasted throughout.

Following reperfusion, the coronary arteries were re-ligated and hearts were perfused with 0.25% Evans blue dye (EBD). This perfusion with Evans blue solution allows discrimination

between the ischaemic tissue (unstained), and non-ischaemic 'risk' tissue, comprising the areas which are not subjected to regional ischaemia (stained blue) (Bell, Mocanu and Yellon 2011). The weight of the hearts were recorded and stored at -20°C, before further analysis. TTC staining was undertaken for all Langendorff groups, including normoxic groups.

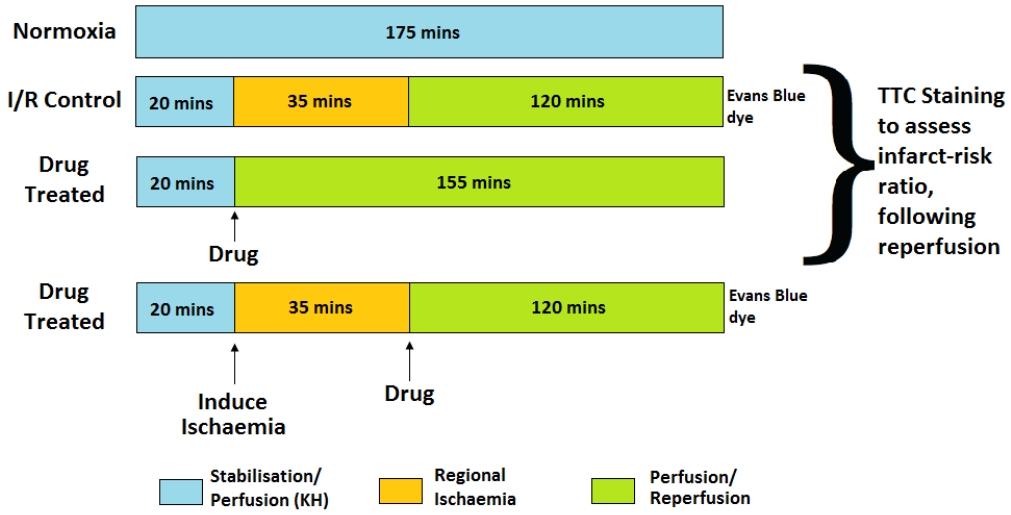

Figure 2.3.3.2.1: The standard experimental protocols for ischaemia/reperfusion (I/R) and normoxic conditions. *Following the end of the experimental protocol, all hearts were weighed and prepared for TTC staining.*

2.3.3.3 Staining procedure using 2, 3, 5-Triphenyl Tetrazolium Chloride (TTC)

The TTC staining procedure was as previously described by Bell et al (2011). Viable tissue appeared red (tetrazolium positive) whilst infarct areas appeared pale (tetrazolium negative). For ischaemia/reperfusion groups, these were also stained with Evans blue dye, distinguishing areas 'at risk'. Figure 2.3.3.3.1 shows an image of an ischaemia/reperfusion heart section following Evans blue staining. ImageJ (Java) was used to analyse infarct size by computerised planimetry.

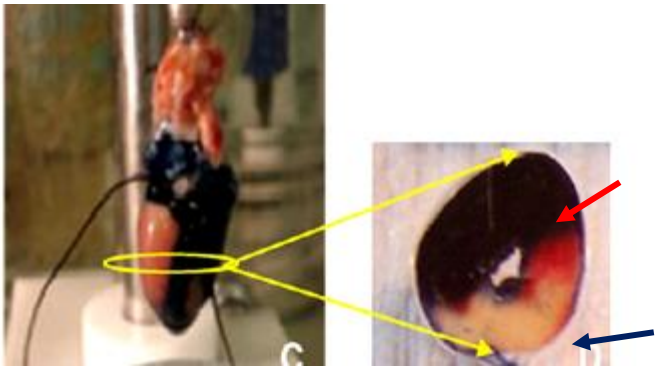

Figure 2.3.3.3.1: Transverse heart slice obtained following Evans blue dye and TTC staining. *The red arrow depicts the area 'at risk' which is dyed with Evans blue, the blue arrow highlights the infarcted area, which is seen as pale (Bell, Mocanu and Yellon 2011).*

2.3.4 Tissue Collection for Western Blot Analysis

Myocardial tissue from the treatment groups as per section 2.3.3.1, were collected. RNaseZap® was used to decontaminate all surfaces before tissue excision, the left ventricle was excised from each heart and divided into two; all tissues were rapidly frozen in liquid nitrogen before storage at -80°C. Approximately 50 mg of the excised left ventricle was homogenised using an IKA Ultra-Turrax® T 25 basic disperser, set to a speed of 21,500 RPM, with lysis buffer (100 mM NaCl, 10 mM Tris base - pH 8.0, 1 mM EDTA - pH 8.0, 2 mM sodium pyrophosphate, 2 mM NaF, 2 mM β-glycerophosphate, Sigma*FAST*™ protease inhibitor cocktail tablets – 1 tablet/100ml and PhosStop™ - 1 tablet/10ml). Homogenised tissue was centrifuged for 10 minutes at 11,000 RPM at 4°C to obtain the supernatant, which was transferred into 1.5ml microcentrifuge tubes. The protein content of each sample was calculated using the Pierce™ BCA assay kit (Thermo Fisher Scientific, UK), as described in section 2.5.1. Samples were diluted with an equal volume of Laemmli buffer (250 mM Tris-HCl – pH 6.8, 10% glycerol, 0.006% bromophenol blue, 4% SDS, β-mercaptoethanol – pH 6.8) and incubated at 100°C for 5 minutes before being stored at -20°C. All samples were defrosted on ice prior to use and diluted further using Laemmli buffer to obtain a protein concentration of 50 µg.

2.3.5 Tissue Collection for RNA Analysis and Isolation

Tissue was obtained as described in section 2.3.4. Tissue was homogenised in TRIsure reagent (Bioline, UK) for RNA extraction, followed by 5 minute incubation at room temperature for phase separation. Chloroform was added to homogenised tissue (0.2ml/ml of TRIsure) and vigorously shaken for 15 seconds, incubated for 3 minutes at room temperature, then centrifuged (12000 x g, 15 minutes) at 4°C to separate into the different phases; organic phase, interphase and aqueous layer containing RNA. The aqueous layer was used to precipitate RNA, by addition of ice cold isopropyl alcohol (0.5ml/ml of TRIsure) and incubated at room temperature for 10 minutes. Samples were centrifuged (12000 x g, 10 minutes) at 4°C. The pellet obtained was washed with 75% ethanol (1ml/ml of TRIsure) mixed, and centrifuged (7500 x g, 5 minutes) at 4°C. Pellets were airdried and dissolved in diethyl pyrocarbonate (DEPC)-treated water (10 µl) before analysis of RNA content. The RNA content of each sample was calculated using a Nanodrop One spectrophotometer. 260/280 ratios of 2.0 were assumed to be pure RNA; samples which had low ratios indicative of impurity were discarded and repeated.

2.4 Adult Rat Ventricular Cardiomyocyte Isolation

2.4.1 Isolation Protocol

Hearts were obtained as described in section 2.3.1, and mounted onto a modified Langendorff set up with a mechanical pump set at a constant flow of 10 ml/min, perfused with a modified calcium free Krebs buffer (KRH - 119.9 mM NaCl, 5.4 mM KCl, 0.49 mM $MgSO_4$, 10 mM Glucose, 19.98 mM Taurine, 5 mM Sodium Pyruvate, 5.06 mM Na_2HPO_4, 11.76 mM KH_2PO_4 oxygenated with 95% O_2 and 5% CO_2, pH 7.4, 37°C). Hearts were perfused with KRH buffer for 3 minutes to ensure cessation of cardiac function, before 5-7 minutes of perfusion with modified digestion buffer (KRH, 0.046% Gibco® Collagenase Type II, 34 µM $CaCl_2$); during digestion, the coronary perfusate was collected and recycled throughout.

Following enzymatic digestion, hearts were removed from the apparatus and the atria were trimmed away. Ventricular tissue was minced and aspirated for a further 5 minutes in digestion buffer at 37°C for complete enzymatic dissociation. The digestion buffer was

filtered through nylon mesh and the filtrate was centrifuged (1200 RPM, 2 minutes) at room temperature, and repeated if necessary. The pellet was resuspended in restoration buffer composed of modified Krebs buffer (KRH, 5 mM Creatine, 50 µM $CaCl_2$, 1% BSA, 1% Pen/Strep) and maintained at 37°C; 3.4 µl of 1 M $CaCl_2$ was added in five stages over a period of 20 minutes to gradually increase the calcium concentration to 1.13 M, reducing the risk of calcium overload. Figure 2.5.1.1 depicts isolated cardiomyocytes in an inverted microscope.

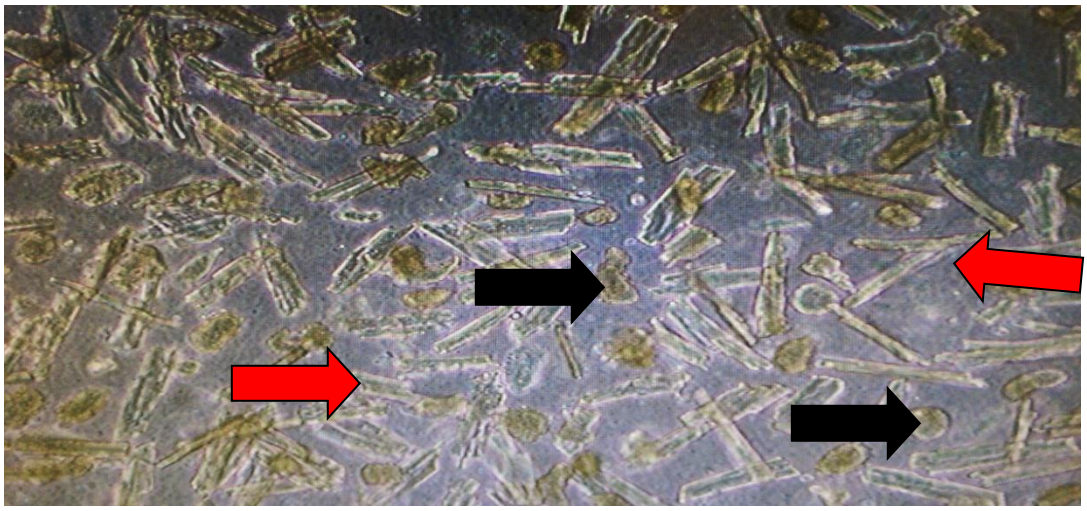

Figure 2.4.1.1: Isolated cardiomyocytes under an inverted microscope following enzymatic digestion. *The red arrows indicate viable cardiomyocytes which appear elongated and striated, and the black arrows show non-viable cardiomyocytes, in hypercontracture.*

2.4.2 Experimental Protocol

Cardiomyocytes were incubated with Tiotropium bromide (10 µM – 0.1 nM) or Tiotropium bromide (1 nM) ± acetylcholine (100 nM), Resveratrol (10 µM), KN-93 (400 nM), Wortmannin (100 nM) and Z-DEVD-FMK (70 nM) diluted in restoration buffer. The normoxic control group was composed of cardiomyocytes and restoration buffer only, at a 1:1 ratio. Approximately 50,000 cells were plated per well with a final volume of 1 ml. Cells

were then incubated for 4 hours in a Nuaire incubator at 37°C with humidified air and 5% CO_2.

2.4.3 Cleaved caspase-3 analysis of cardiomyocytes via flow cytometry

Following section 2.4.2, cells were transferred into microfuge tubes and centrifuged (1200 RPM, 2 minutes) at room temperature. The supernatant was removed, and samples were resuspended and incubated in 1x phosphate buffered saline (PBS – pH 7.4). An equal volume of 6% formaldehyde was added, giving a final concentration of 3%. Cells were incubated at 37°C for 10 minutes, followed by 1 minute on ice. Cells were centrifuged (1200 RPM, 2 minutes) at room temperature and resuspended in 90% ice-cold methanol. Cells were placed in -80°C, or on ice for 30 minutes before immediate use.

Following methanol fixation and permeabilisation of the cell membrane, samples were prepared with cleaved caspase-3 $_{(Asp175)}$ (Alexa Fluor® 488). Cells were washed with incubation buffer (0.5% BSA in 1x PBS) twice and centrifuged (1200 RPM, 2 minutes) at 4°C. Cardiomyocytes were resuspended in incubation buffer and incubated for 10 minutes at room temperature. The final antibody dilution was prepared following the datasheet at 1:100, diluted in incubation buffer. Following the 10-minute incubation, samples were centrifuged (1200 RPM, 2 minutes) at 4°C, the supernatant was removed, and the samples were resuspended in antibody preparations of 100 µl for 1 hour at room temperature, protected from light. Samples were then centrifuged (1200 RPM, 2 minutes) at 4°C, and washed in incubation buffer, before resuspension in 500 µl of 1x PBS for immediate use on the flow cytometer. The FL-1 channel was used for the Alexa Fluor® 488 antibody, set to count 2,000 – 10,000 cells.

2.4.4 Apoptosis and necrosis detection assay – Annexin V FITC

Following drug incubation, samples were transferred to microfuge tubes, and centrifuged (500 RPM, 2 minutes) at room temperature. The supernatant was removed, and samples were resuspended in 1x binding buffer and centrifuged (500 RPM, 2 minutes) at room temperature. The supernatant was removed, and the samples were resuspended in annexin-V and propidium iodide, diluted in 1x binding buffer (1:100), incubated for 5 minutes away from light. Following incubation, samples were immediately analysed on the flow cytometer using the FITC signal detector (FL-1) for annexin-V, and phycoerythrin

emission (FL-2) for propidium iodide, set to count 10,000 cells. Total apoptosis included early and late apoptosis, which equate to cardiomyocytes grouped in the lower right and upper right quadrants of the density plot respectively (cells stained: annexin V positive/propidium iodide negative AV+, PI- and annexin V positive/propidium iodide positive AV+, PI+ respectively).

2.4.5 Reactive oxygen species detection – CellRox® assay

The negative control, N-acetylcysteine (NAC) was prepared with 245 µl PBS to a stock concentration of 250 mM; the positive control, tert-Butyl hydroperoxide (TBHP) was prepared with 496.8 µl to 50 mM. The SYTOX stain was reconstituted in DMSO to give a stock concentration of 5 µM, as per manufacturer's protocol. Cells were incubated with Tiotropium bromide (1 nM), resveratrol (10 µM), Tiotropium bromide + resveratrol, positive, negative and single stain controls for CellROX and SYTOX. The negative control was incubated with NAC (1000 µM) alone for 1 hour, before the addition of TBHP (200 µM) for the rest of the incubation period, the same as the positive control. In the final hour of the 4 hour incubation period, CellROX reagent (500 nM) was added to each well, apart from the SYTOX only well. The CellROX reagent was reconstiuted in DMSO (250 µM). In the last 15 minutes of the incubation period, SYTOX reagent (5 nM) was added to the wells, apart from the CellROX only well. Cells were assessed using the flow cytometer set to 488 nm excitation; the positive control was used to adjust settings, set to count 10,000 cells.

2.4.6 Intracellular Calcium measurement using Fluo 3-AM

Fluo 3-AM (500 µM) stock solution was reconstituted using DMSO. Following incubation with Tiotropium bromide (1 nM), KN-93 (400 nM), Tiotropium bromide + KN-93, the ionophore, ionomycin (1 µM) and the Ca^{2+} channel blocker, amiloride (100 mM). Cells were pipetted into microfuge tubes and washed with restoration buffer before being resuspended in fresh restoration buffer. Fluo 3-AM (5 µM) was added to the cells incubated at room temperature for 25 minutes, protected from light. Following incubation, cells were centrifuged (300 RPM, 2 minutes) at room temperatrure and resuspended in fresh restoration buffer, and subsequently incubated for 10 minutes at room temperature for the de-esterification stage. Cells were immediately analysed on the FL-1 channel of the flow cytometer at 488 nm excitation, set to count 10,000 cells.

2.5 Western Blot Analysis

2.5.1 Protein Quantification using Bicinchoninic acid assay (BCA)

The protein content of homogenised sample lysates was evaluated using a colorimetric BCA Protein assay kit. Concentrated albumin standards were serially diluted using lysis buffer to obtain a concentration range of 0 – 2000 µg/ml. BCA working reagent was prepared following a 50:1 ratio of reagent A and reagent B. In a 96 well plate, all standards and unknown samples were pipetted at a volume of 10 µl in triplicate, following this, 200 µl of working reagent was added to each well and the plate was gently agitated. Plates were covered to protect from light and incubated for 30 minutes at 37°C. The plate was left to cool to room temperature, before measurement using a plate reader set at 562 nm. The absorbance values were then used to calculate protein content in the unknowns.

2.5.2 Gel Electrophoresis

Following sample collection and homogenisation, outlined in 2.3.4; diluted samples at 50 µg were centrifuged (1800 RPM, 2 minutes) at 4°C, before loading. Precast TGX™ (Tris/glycine) gradient gels were used for all experiments; gels were placed inside of a Mini-PROTEAN™ vertical electrophoresis assembly unit. The chamber and outer tank were filled with running buffer (14.42g/L Glycine, 1.0g/L SDS, 3.03g/L Tris base, pH 8.3) before removal of the gel combs, the samples were then loaded into the wells, with at least one well loaded with a molecular protein marker. The gels were run at 130-150V for 60-75 minutes using a Power-Pac 3000 (Bio-Rad, UK).

2.5.3 Protein Transfer

Following separation of the protein samples, gels were removed carefully from their compartments and sandwiched into Trans-Blot® Turbo™ transfer packs, consisting of filter paper, buffer and a polyvinylidene fluoride (PVDF) membrane. The assembled cassettes were then loaded into the Trans-Blot system (Bio-Rad, UK) and run for mixed molecular weight transfer for 7 minutes when using two mini gels, using the 'Turbo' setting.

2.5.4 Antibody Probing

Membrane blots were incubated at room temperature in blocking buffer (5% w/v milk powder in Tris-buffered saline with 1% Tween 20 - TBS/T, pH 7.4) for 60 minutes on an orbital shaker, with the protein side facing downwards. Following the blocking stage,

membranes were washed with TBS/T three times for 5 minutes each, before proceeding with primary antibody incubation (5% w/v bovine serum albumin (BSA) in TBS/T) on a roller shaker at 4°C overnight. Membranes were then washed (3x) with TBS/T to remove any unbound antibody and incubated for 1 hr at room tempartaure with secondary antibody (anti-rabbit HRP IgG – 1 in 10,000) in blocking solution (5% w/v milk powder in TBS/T) on an orbital shaker. The membranes were then washed again using TBS/T (3x) before continuing.

2.5.5 Visualisation, Densitometry and Quantification

Membranes were placed onto acetate sheets with a 1:1 dilution of SuperSignal West Femto, analysed using a ChemiDoc transluminator for imaging (Bio-Rad, UK) with ImageLab®. Membranes were auto-exposed to detect dense bands corresponding to the protein of interest; images were captured and analysed using ImageJ.

2.6 Real Time Polymerase Chain Reaction (qPCR) using PrimePCR Pathway plates

2.6.1 cDNA synthesis from isolated RNA and assessment of optimal cDNA concentration using GAPDH

Following section 2.3.5, cDNA was synthesised using 1 µl of the Oligo dT $_{(20)}$ primer kit, 1 µl of premixed dNTP solution (10 mM), 1000 ng or a maximum volume of 8 µl of RNA, with a volume made to 10 µl using DEPC water, following the protocol from Invitrogen. The reaction mix was prepared in RNase and DNase free tubes and denatured at 65°C for 5 mins in a thermal cycler and immediately placed on ice before brief centrifuging (quick spin setting). Once on ice, mastermix consisiting of SuperScript Reverse transcriptase (1 µl per reaction), 0.1 M DTT (1 µl per reaction) and 5x First strand buffer (4 µl per reaction) were used. Samples were vortexed, centrifuged (quick spin setting), and incubated at 50°C for 50 minutes, followed by 85°C for 5 minutes to terminate the reaction, before placing the samples on ice. The cDNA was quantified using NanoDrop One.

Following cDNA synthesis, a concentration response was determined for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). cDNA at 1, 3, 10, 30, 50, 80 and 100 ng as well as no template controls (NTC) were prepared in DNase- and RNase-free tubes before being plated into a PCR microplate in triplicate. GAPDH primer (2 µl) and SYBR Green (10 µl) were

added to the cDNA and the volume made up to 20 µl using DEPC treated water. Samples were vortexed and centrifuged (quick spin setting) before plating, plates were sealed then briefly centrifuged (quick spin setting), and set up in the PCR machine with: activation at 95 ^{o}C (2 minute cycle), denaturation at 95^{o}C (5 seconds, 40 cycles), annealing/extension at 60^{o}C (30 seconds, 40 cycles), followed by a melt curve at 65-95^{o}C (5 second cycle). C_t values were analysed and a concentration response curve (not shown) determined 80 ng as optimal.

2.6.2 Gene expression associated with Myocardial Infarction and Oxidative Stress

Following section 2.6.1, cDNA was prepared with SYBR green and DEPC treated water and added to wells pre-prepared with primers, with a total of 20 µl per well. PrimePCR™ pathway plates were used for gene expression analysis. For the Myocardial Infarction plate (96 well), Tiotropium bromide (1 nM) and normoxic samples were plated for 27 genes along with 5 control genes. For the Oxidative Stress plate (96 well), only one 'n' number could be plated to assess 91 genes associated with oxidative stress and 5 control genes. Run files for the two plates were obtained from Bio-Rad, which provided the run instructions for the CFX Connect PCR machine (Bio-Rad, UK).

2.7 Data and Statistical Analysis

Data presented in this study is expressed as the Mean ± standard error of the mean (SEM). Microsoft® Excel, GraphPad Prism 6® and SPSS® (IBM) software were used to statistically analyse the data and produce graphs. The statistical test and the post-hoc test used to analyse the haemodynamics and infarct data were one-way ANOVA and Tukey's test, whereas cardiomyocyte and western blot data were analysed using Fisher's Least Significant Difference (LSD) post-hoc test; qPCR data was evaluated using Student's T test. To assess the difference in the data sets, a p-value of $p<0.05$ was considered statistically significant.

Chapter 3: Pharmacological Profiling of Long Acting Muscarinic Receptor Antagonists (LAMAs)

The primary aim of this study was to elucidate the cardiotoxicity of long acting muscarinic receptor antagonists, notably how Tiotropium bromide induces myocardial damage in *ex vivo* and *in vitro* models of normoxic conditions and ischaemia/reperfusion injury.

(a) Determining the cardiotoxicity of other long acting muscarinic receptor antagonists in *ex vivo* models of myocardial ischaemia/reperfusion injury.

(d) To observe the effect of Tiotropium bromide on gene expression profiles associated with myocardial infarction and oxidative stress.

3.1 Profiling of Glycopyrronium Bromide – in an *in vitro* model of Myocardial Ischaemia/Reperfusion injury

3.1.1 Haemodynamic Data Analysis for Glycopyrronium bromide (10 µM – 1 nM)

Using a whole heart Langendorff model, the effect of Glycopyrronium bromide (10 µM – 1 nM) administration was assessed in conditions of myocardial ischaemia/reperfusion injury, with 20 minutes of stabilisation preceding 35 minutes of regional ischaemia followed by 120 minutes of reperfusion; drug administration lasted the duration of reperfusion, as described in section 2.3.3.2.

Haemodynamic data was recorded for each study; at the end of each experiment, hearts were stained with Evans blue and re-stained for infarct size to risk ratio (%) using the TTC method. Coronary flow (fig 3.1.1.1), left ventricular developed pressure (LVDP, fig 3.1.1.2) and heart rate (fig 3.1.1.3) are shown for Glycopyrronium bromide (10 µM – 1 nM) administration in conditions of ischaemia/reperfusion as well as the ischaemia/reperfusion control group. The data collected for all parameters (fig 3.1.1.1 – 3.1.1.3) following Glycopyrronium bromide (10 µM – 1 nM) administration in ischaemia/reperfusion were statistically analysed at 15, 25, 50, 70 and 160 minutes. The Glycopyrronium bromide (10 µM - 1 nM) groups were analysed with respect to the ischaemia/reperfusion control as well as between each concentration.

There was no statistical significance in coronary flow between Glycopyrronium bromide (10 µM – 1 nM) with respect to the ischaemia/reperfusion control nor between each concentration of Glycopyrronium bromide (10 µM – 1 nM) for coronary flow as shown in figure 3.1.1.1 (p>0.05).

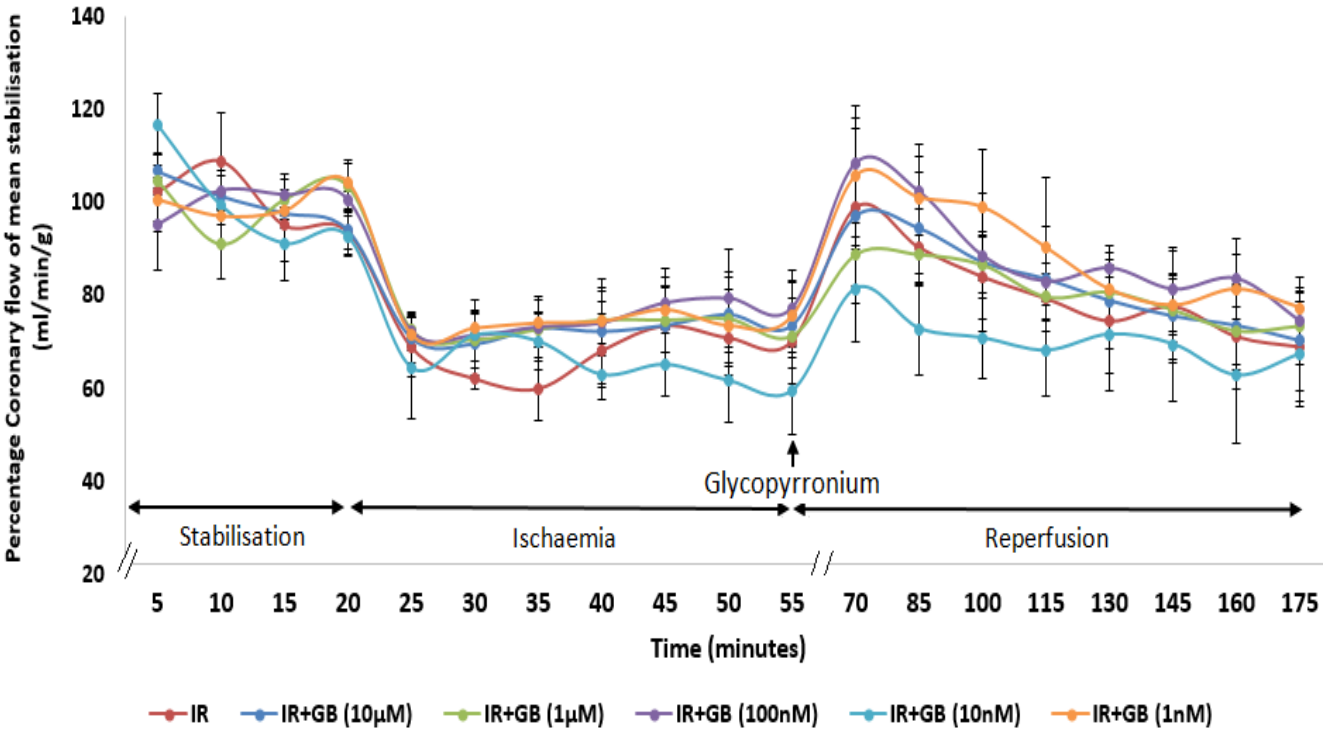

***Figure 3.1.1.1**: **Percentage coronary flow of the mean stabilisation period following Glycopyrronium bromide (10 µM – 1 nM) in ischaemia/reperfusion**. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Glycopyrronium bromide (10 µM – 1 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. The graph represents data collected from a concentration response of Glycopyrronium bromide (GB) (10 µM – 1 nM). Values plotted signify the Mean ± SEM, derived from an n of 4-6 for all groups.*

There was no statistical significance observed for left ventricular developed pressure (LVDP) in figure 3.1.1.2 ($p>0.05$) amongst the Glycopyrronium bromide groups (10 µM – 1 nM) and the ischaemia/reperfusion control.

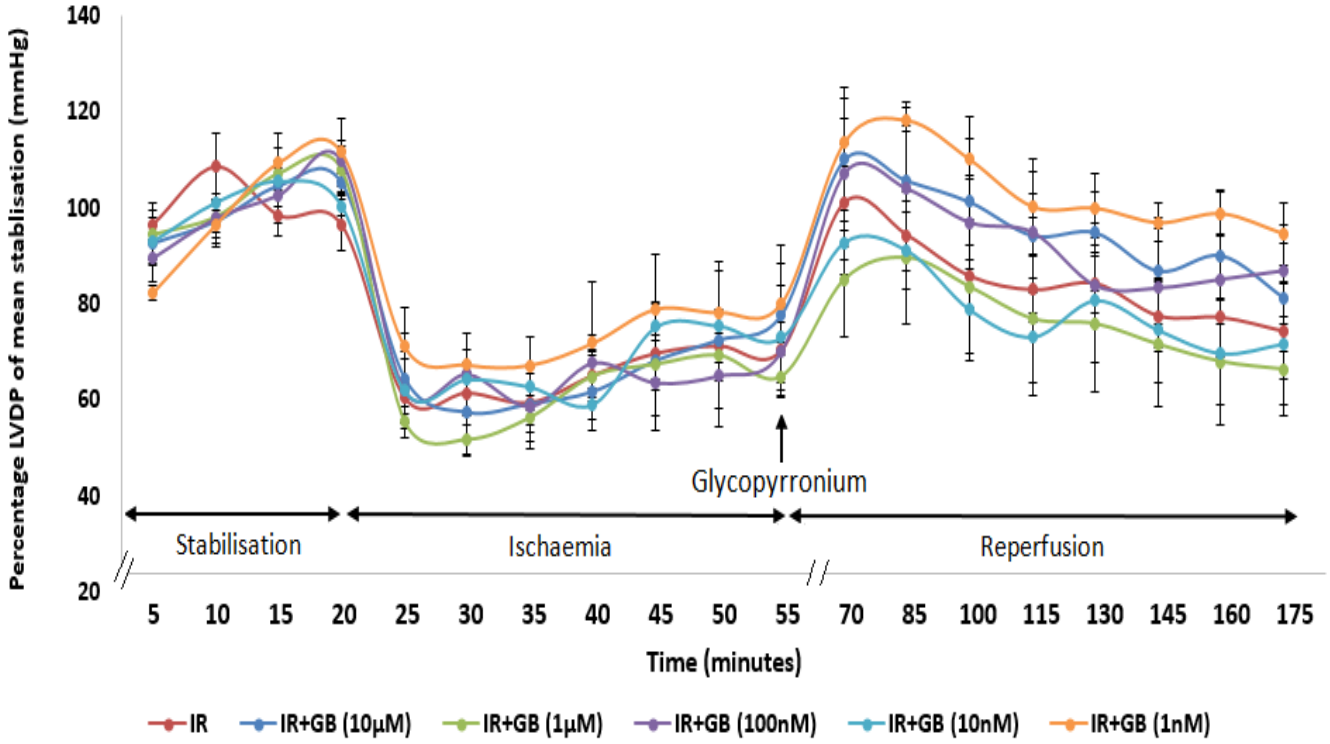

***Figure 3.1.1.2**: **Percentage left ventricular developed pressure (LVDP) of the mean stabilisation period following Glycopyrronium bromide (10 µM – 1 nM) in ischaemia/reperfusion**. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Glycopyrronium bromide (10 µM – 1 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. The graph represents data collected from a concentration response of Glycopyrronium bromide (GB) (10 µM – 1 nM). Values plotted signify the Mean ± SEM, derived from an n of 4-6 for all groups.*

There was no statistical significance observed for heart rate between the Glycopyrronium bromide (10 µM – 1 nM) groups and the ischaemia/reperfusion control at 15, 25, 50, 70 and 160 minutes, in figure 3.1.1.3 ($p>0.05$).

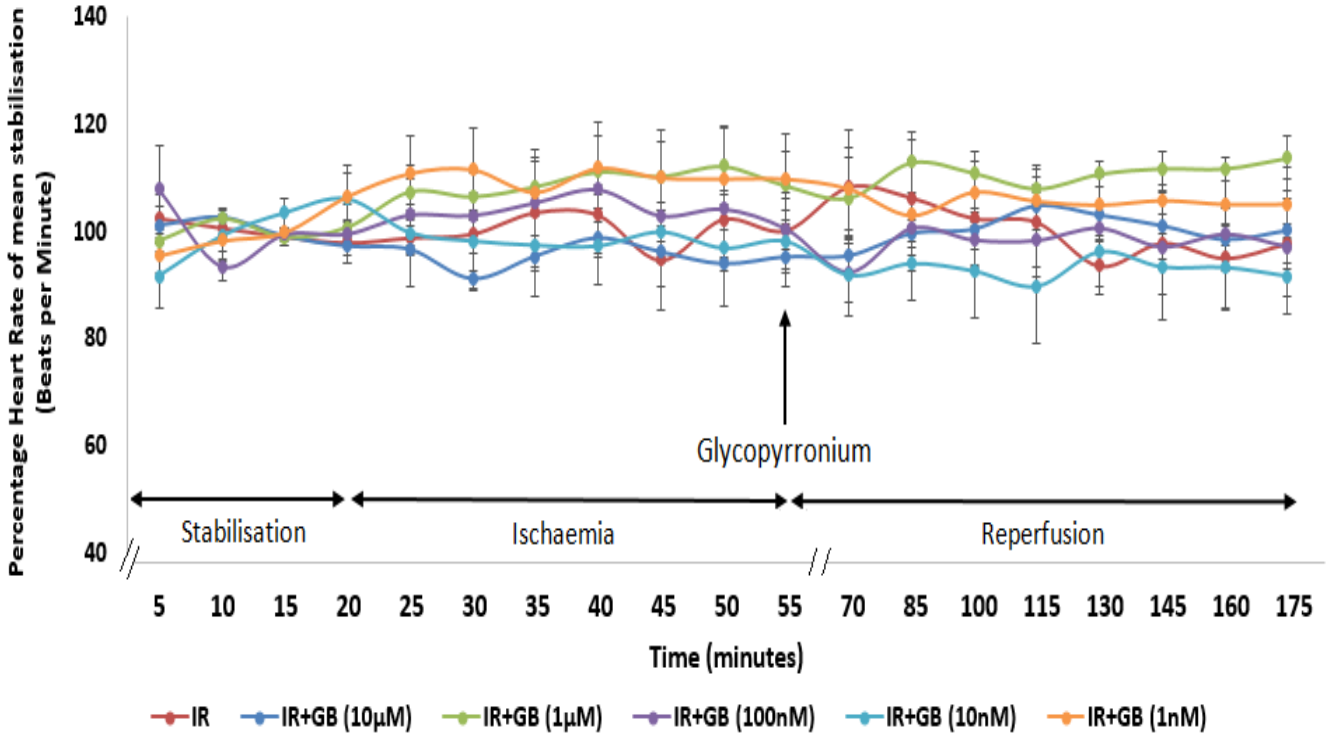

***Figure 3.1.1.3**: **Percentage heart rate of the mean stabilisation period following Glycopyrronium bromide (10 µM – 1 nM) in ischaemia/reperfusion**. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Glycopyrronium bromide (10 µM – 1 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. The graph represents data collected from a concentration response of Glycopyrronium bromide (GB) (10 µM – 1 nM). Values plotted signify the Mean ± SEM, derived from an n of 4-6 for all groups.*

3.1.2 Infarct to Risk Analysis of Glycopyrronium Bromide Administration in an *in vitro* model of Ischaemia/Reperfusion Injury

This study observed the effects of Glycopyrronium bromide (10 µM – 1 nM) administration on infarct to risk ratio (%), in an *in vitro* model of myocardial ischaemia/reperfusion injury. Glycopyrronium bromide (10 µM – 1 nM) was administered at the onset of reperfusion and continued throughout the 120-minute period as described in section 2.3.2.2, followed by Evans blue and TTC staining to delineate ischaemic zones and determine infarct size to risk ratio.

Figure 3.1.2.1 shows the infarct/risk ratios of Glycopyrronium bromide (10 µM – 1 nM) with respect to normoxic and ischaemia/reperfusion (IR) controls. The study shows a significant increase in infarct development between the normoxic control group and the ischaemia/reperfusion control group (10.27 ± 1.94% vs. 50.85 ± 3.93%, $p<0.0001$, fig 3.1.2.1). All concentrations of Glycopyrronium (10 µM – 1 nM) were also significant with respect to the normoxia control group (55.07 ± 2.04% (GB 10 µM), 51.96 ± 2.50% (GB 1 µM), 51.83 ± 5.05% (GB 100 nM), 51.03 ± 3.65% (GB 10 nM) and 50.94 ± 0.38% (GB 1 nM) vs. 10.27 ± 1.94% (Norm), $p<0.001$, fig 3.1.2.1). However, there is no statistically significant difference in infarct development between the concentrations of Glycopyrronium bromide (10 µM – 1 nM) with respect to the ischaemia/reperfusion control, or between each concentration (fig 3.1.2.1, $p>0.05$).

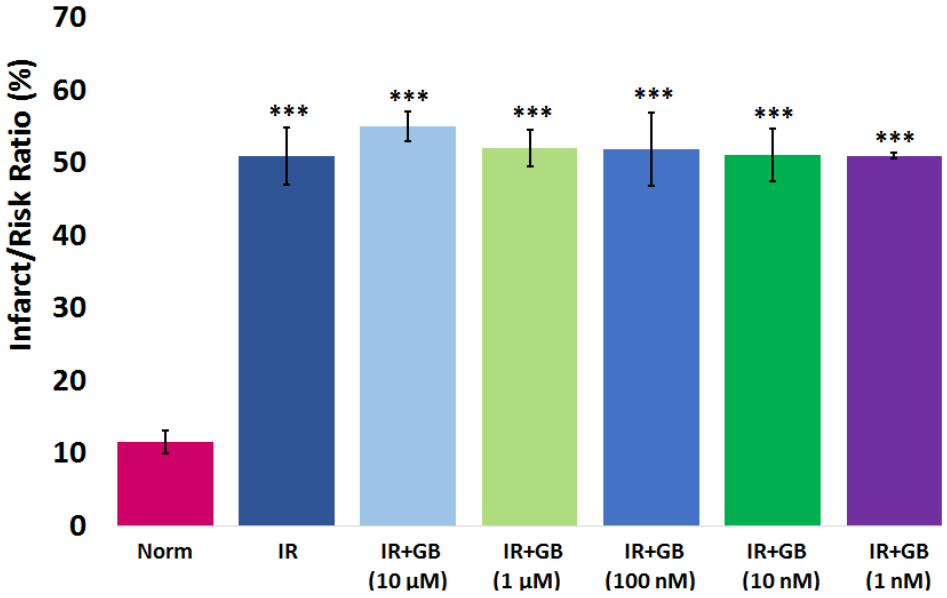

Figure 3.1.2.1: Percentage infarct to risk ratios (%) following Glycopyrronium bromide (10 µM – 1 nM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Glycopyrronium bromide (10 µM – 1 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group and Normoxic (Norm) group. The graph represents data collected from a concentration response of Glycopyrronium bromide (GB) (10 µM – 1 nM). Values plotted signify the Mean ± SEM, derived from an n of 4-6 for all groups. *** vs. Norm, p<0.001.*

3.2 Profiling of Aclidinium Bromide – in an *in vitro* model of Myocardial Ischaemia/Reperfusion injury

3.2.1 Haemodynamic Data Analysis for Aclidinium bromide (10 µM – 1 nM)

Using a whole heart Langendorff model, the effect of Aclidinium bromide (10 µM – 1 nM) administration was assessed in conditions of myocardial ischaemia/reperfusion injury with 20 minutes of stabilisation preceding 35 minutes of regional ischaemia followed by 120 minutes of reperfusion; drug administration lasted the duration of reperfusion, as described in section 2.3.3.2.

Coronary flow (fig 3.2.1.1), left ventricular developed pressure (LVDP, fig 3.2.1.2) and heart rate (fig 3.2.1.3) are shown for Aclidinium bromide (10 µM – 1 nM) administration in ischaemia/reperfusion as well as the ischaemia/reperfusion control group. The data collected for all parameters (fig 3.2.1.1 – 3.2.1.3) following Aclidinium bromide (10 µM – 1 nM) administration in ischaemia/reperfusion were statistically analysed at 15, 25, 50, 70 and 160 minutes. The Aclidinium bromide (10 µM – 1 nM) groups were analysed with respect to the ischaemia/reperfusion control as well as between each concentration.

Figure 3.2.1.1 for coronary flow shows no statistical significance at the onset of reperfusion with Aclidinium bromide (10 µM – 1 nM) ($p>0.05$) between each concentration and also with respect to the ischaemia/reperfusion control.

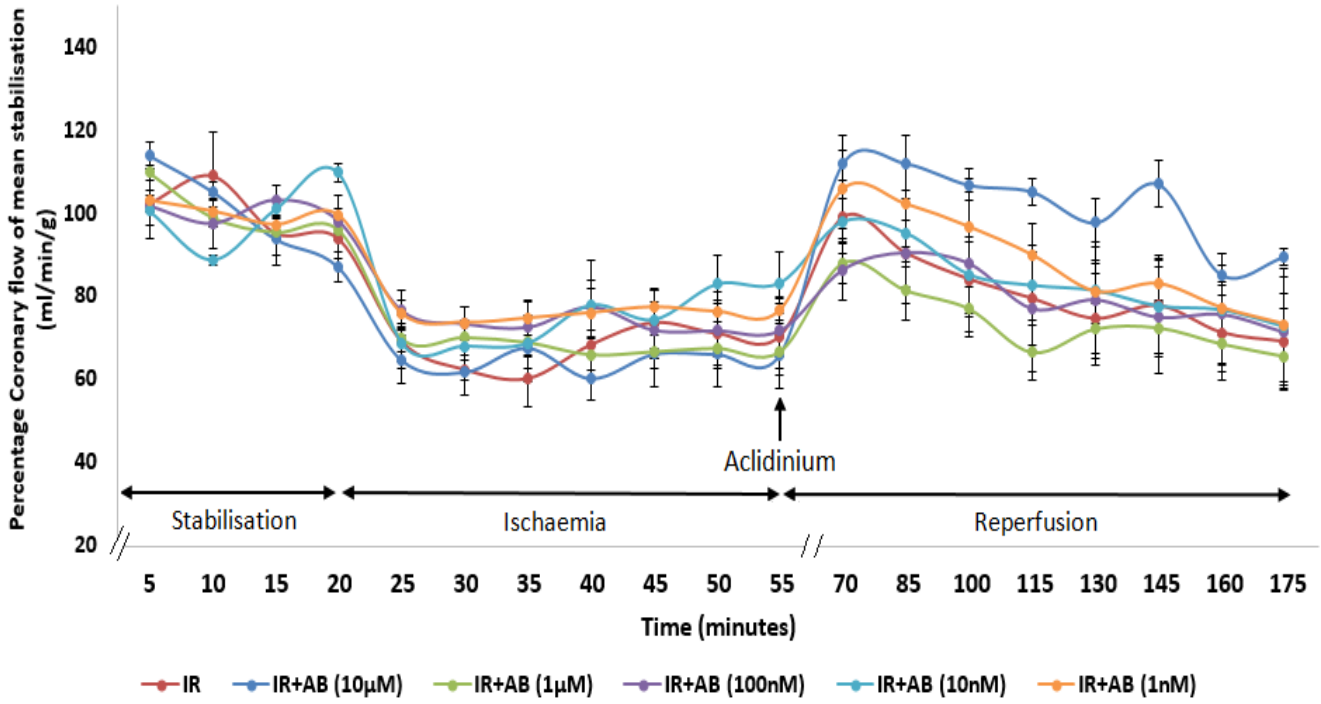

Figure 3.2.1.1: Percentage coronary flow of the mean stabilisation period following Aclidinium bromide (10 µM – 1 nM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Aclidinium bromide (10 µM – 1 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. The graph represents data collected from a concentration response of Aclidinium bromide (GB) (10 µM – 1 nM). Values plotted signify the Mean ± SEM, derived from an n of 4-6 for all groups.*

There was no statistical significance observed for left ventricular developed pressure (LVDP) in figure 3.2.1.2 ($p>0.05$) amongst the Aclidinium bromide groups (10 µM – 1 nM) and the ischaemia/reperfusion control.

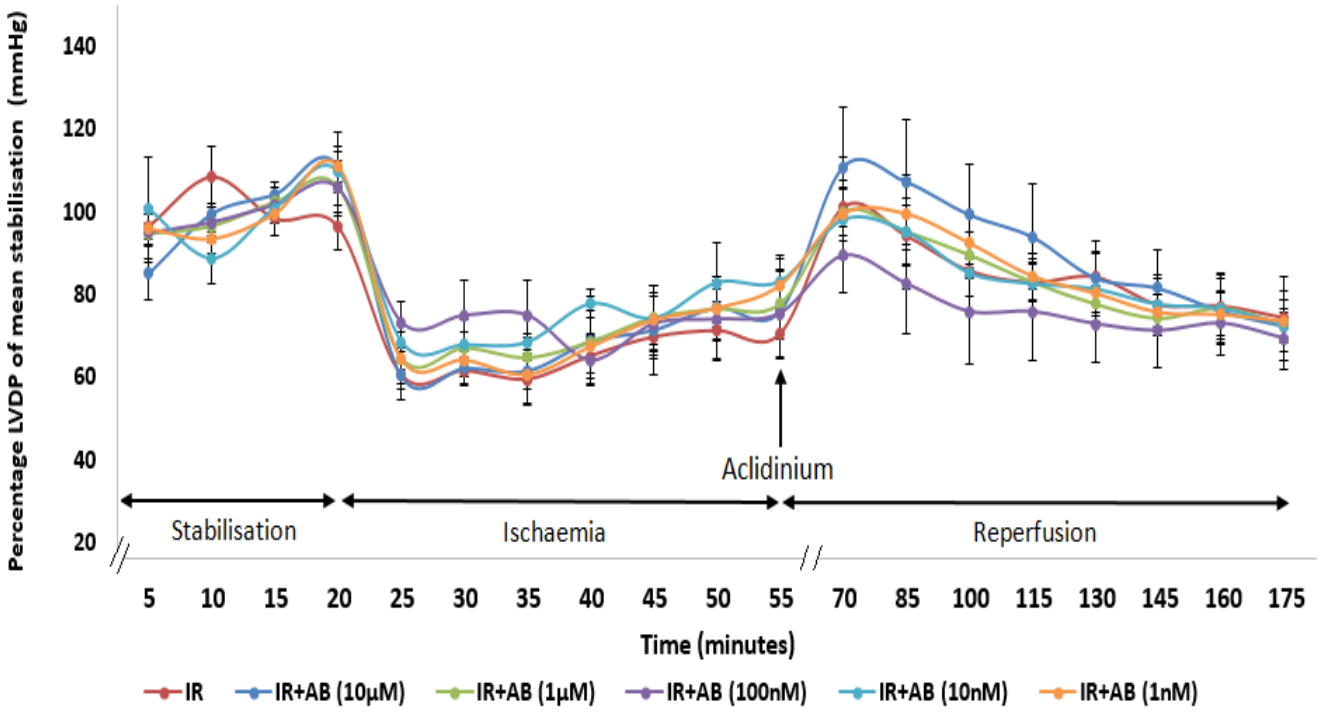

Figure 3.2.1.2: Percentage left ventricular developed pressure (LVDP) of the mean stabilisation period following Aclidinium bromide (10 µM – 1 nM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Aclidinium bromide (10 µM – 1 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. The graph represents data collected from a concentration response of Aclidinium bromide (GB) (10 µM – 1 nM). Values plotted signify the Mean ± SEM, derived from an n of 4-6 for all groups.*

There was no statistical significance observed between the different Aclidinium bromide (10 µM – 1 nM) groups and ischaemia/reperfusion control at 15, 25, 50, 70 and 160 minutes for heart rate, in figure 3.2.1.3 ($p>0.05$).

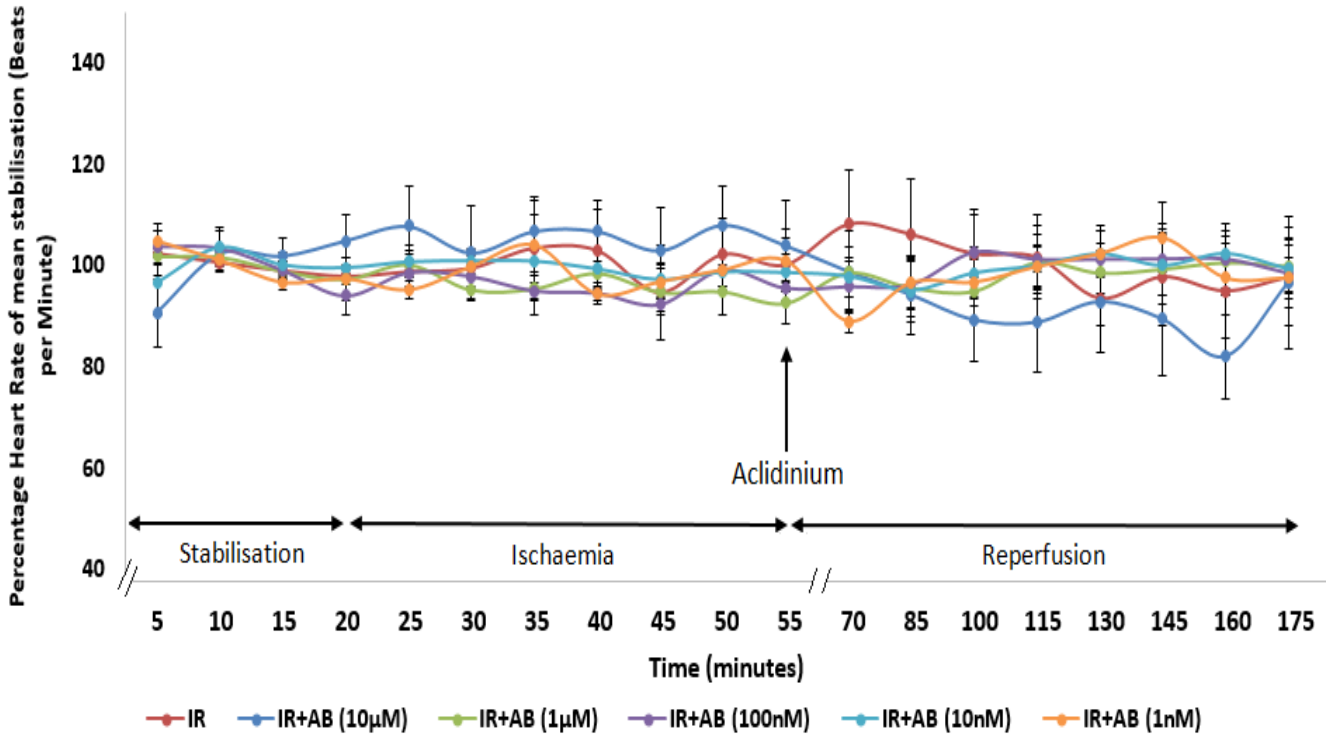

Figure 3.2.1.3: Percentage heart rate of the mean stabilisation period following Aclidinium bromide (10 µM – 1 nM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Aclidinium bromide (10 µM – 1 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. The graph represents data collected from a concentration response of Aclidinium bromide (GB) (10 µM – 1 nM). Values plotted signify the Mean ± SEM, derived from an n of 4-6 for all groups.*

3.2.2 Infarct to Risk Analysis of Aclidinium Bromide Administration in an *in vitro* model of Ischaemia/Reperfusion Injury

This study observed the effects of Aclidinium bromide (10 µM – 1 nM) administration at the onset of reperfusion on infarct to risk ratio (%), in an *in vitro* model of myocardial ischaemia/reperfusion injury.

Figure 3.2.2.1 shows the infarct/risk ratios of Aclidinium bromide (10 µM – 1 nM) with respect to normoxic (Norm) and ischaemia/reperfusion (IR) controls. All concentrations of Aclidinium bromide (10 µM – 1 nM) show an increase in infarct to risk ratio (%) with respect

to the normoxic control ($p<0.001$). Aclidinium bromide at the 10 nM and 1 nM concentrations show a significant increase in infarct size to risk ratio (%) with respect the IR control (67.09 ± 1.78% (AB 10 nM) and 66.15 ± 3.58% (AB 1nM) vs. 50.8 ± 3.93% (IR control), $p<0.01$, fig 3.2.2.1). There are also significances observed between the different Aclidinium bromide (10 µM – 1 nM) concentrations; Aclidinium at 10 nM shows an increase in infarct to risk ratio with respect to Aclidinium at 100 nM concentration (67.09 ± 1.78% (AB 10 nM) vs. 53.07 ± 1.85% (AB 100 nM), $p<0.05$, fig 3.2.2.1).

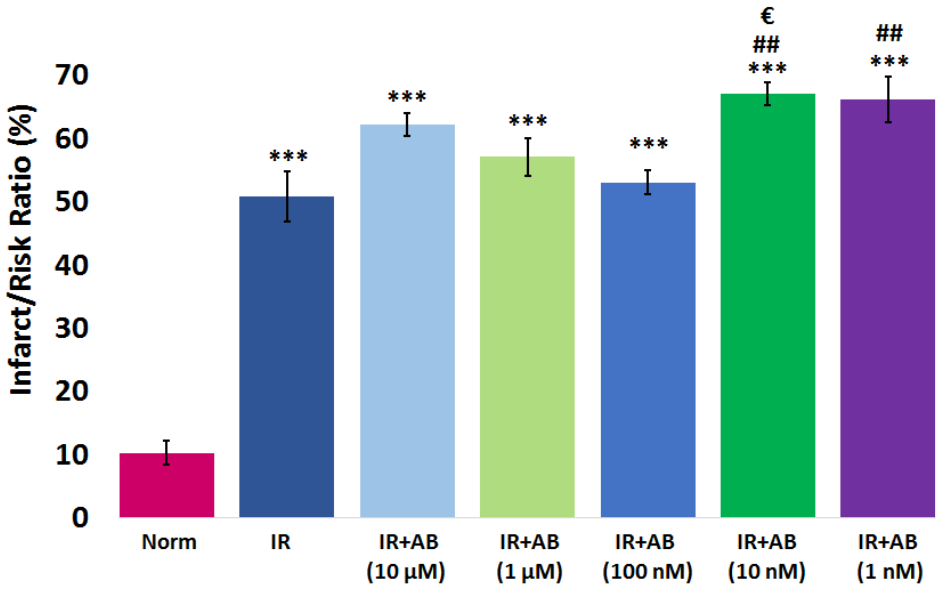

Figure 3.2.2.1: Percentage infarct to risk ratios (%) following Aclidinium bromide (10 µM – 1 nM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Aclidinium bromide (10 µM – 1 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group and Normoxic (Norm) group. The graph represents data collected from a concentration response of Aclidinium bromide (AB) (10 µM – 1 nM). Values plotted signify the Mean ± SEM, derived from an n of 4-6 for all groups. *** vs. Norm, $p<0.001$; ## vs. IR, $p<0.01$; € vs. IR+AB (100 nM), $p<0.05$.*

3.3 Profiling of Umeclidinium Bromide – in an *in vitro* model of Myocardial Ischaemia/Reperfusion injury

3.3.1 Haemodynamic Data Analysis for Umeclidinium bromide (1 µM – 1 nM)

Using a whole heart Langendorff model, the effect of Umeclidinium bromide (1 µM – 1 nM) administration was assessed in conditions of myocardial ischaemia/reperfusion injury with 20 minutes of stabilisation preceding 35 minutes of regional ischaemia followed by 120 minutes of reperfusion; drug administration lasted the duration of reperfusion, as described in section 2.3.3.2.

Coronary flow (fig 3.3.1.1), left ventricular developed pressure (LVDP, fig 3.3.1.2) and heart rate (fig 3.3.1.3) are shown for Umeclidinium bromide (1 µM – 1 nM) administration in conditions of ischaemia/reperfusion as well as the ischaemia/reperfusion control group. The data collected for all parameters (fig 3.3.1.1 – 3.3.1.3) following Umeclidinium bromide (1 µM – 1 nM) administration in ischaemia/reperfusion were statistically analysed at 15, 25, 50, 70 and 160 minutes. The Umeclidinium bromide (1 µM - 1 nM) groups were analysed with respect to the ischaemia/reperfusion control as well as between each concentration.

Figure 3.3.1.1 for coronary flow shows no statistical significance at the onset of reperfusion with Umeclidinium bromide (1 µM – 1 nM) ($p>0.05$) between each concentration and with respect to the ischaemia/reperfusion control.

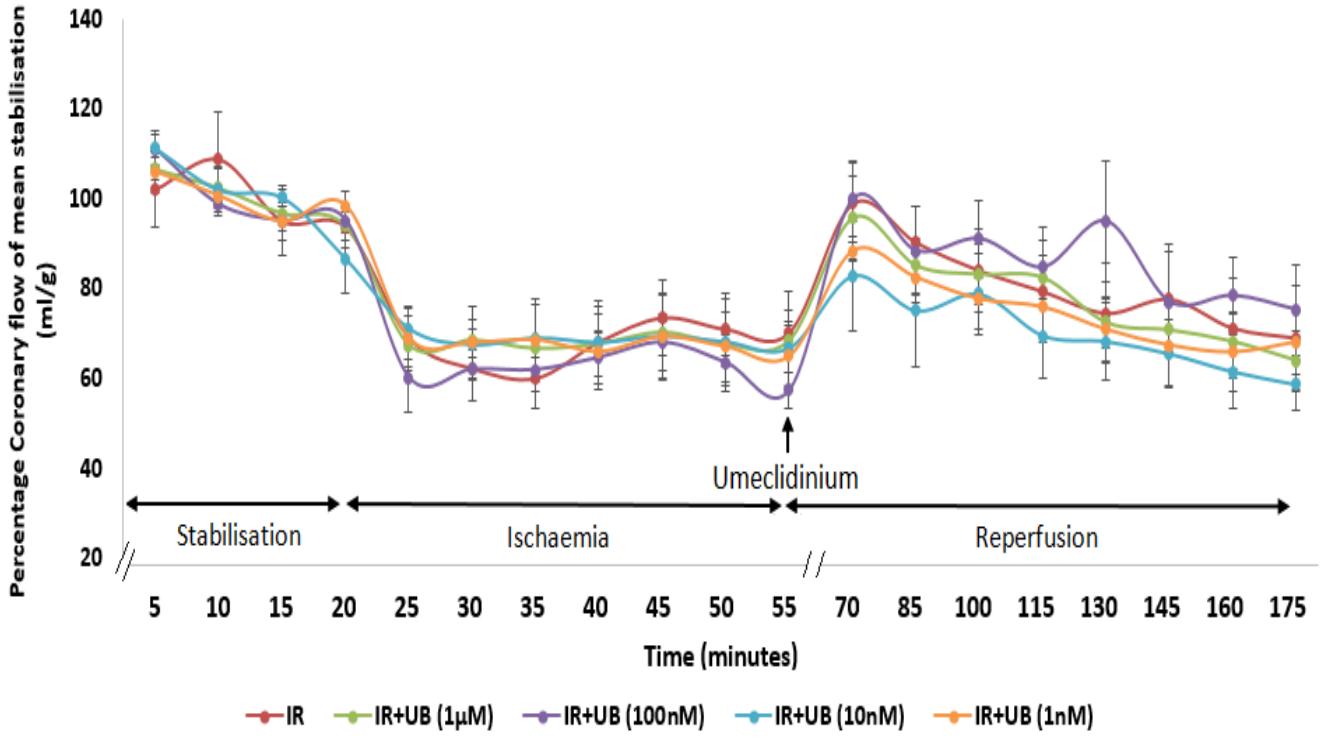

Figure 3.3.1.1: Percentage coronary flow of the mean stabilisation period following Umeclidinium bromide (1 µM – 1 nM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Umeclidinium bromide (1 µM – 1 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. The graph represents data collected from a concentration response of Umeclidinium bromide (UB) (1 µM – 1 nM). Values plotted signify the Mean ± SEM, derived from an n of 4-6 for all groups.*

Figure 3.3.1.2 shows the left ventricular pressure recorded (LVDP) from the Umeclidinium bromide (1 µM – 1 nM) treatment groups. The data does not show any statistical significances amongst the Umeclidinium bromide (1 µM – 1 nM) groups at the time points assessed ($p>0.05$, fig 3.3.1.2) between the different concentrations or with respect to the ischaemia/reperfusion group.

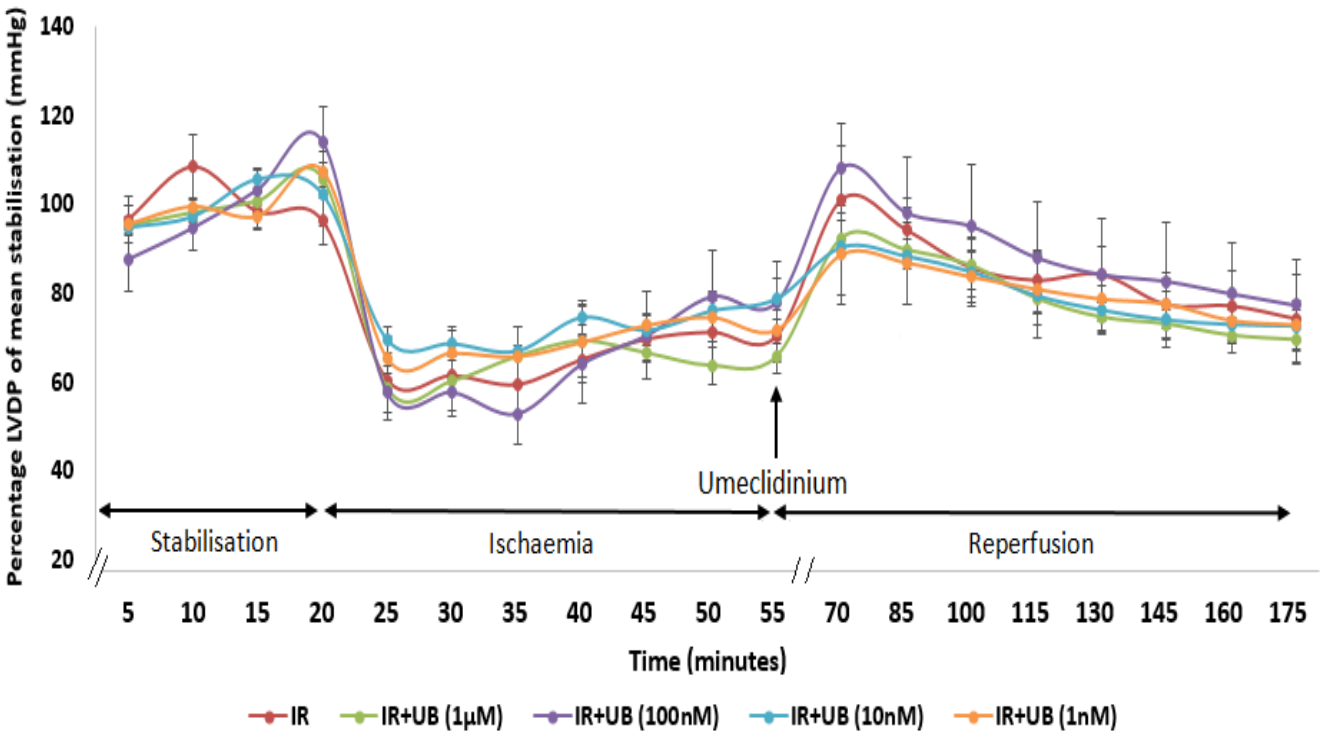

Figure 3.3.1.2: Percentage left ventricular developed pressure (LVDP) of the mean stabilisation period following Umeclidinium bromide (1 µM – 1 nM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Umeclidinium bromide (1 µM – 1 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. The graph represents data collected from a concentration response of Umeclidinium bromide (UB) (1 µM – 1 nM). Values plotted signify the Mean ± SEM, derived from an n of 4-6 for all groups.*

Figure 3.3.1.3 shows heart rate following administration of Umeclidinium bromide (1 µM – 1 nM) following ischaemia/reperfusion. The data shows no statistical significance in heart rate observed with Umeclidinium bromide administration (1 µM – 1 nM) and the ischaemia/reperfusion control group ($p>0.05$, fig 3.3.1.3).

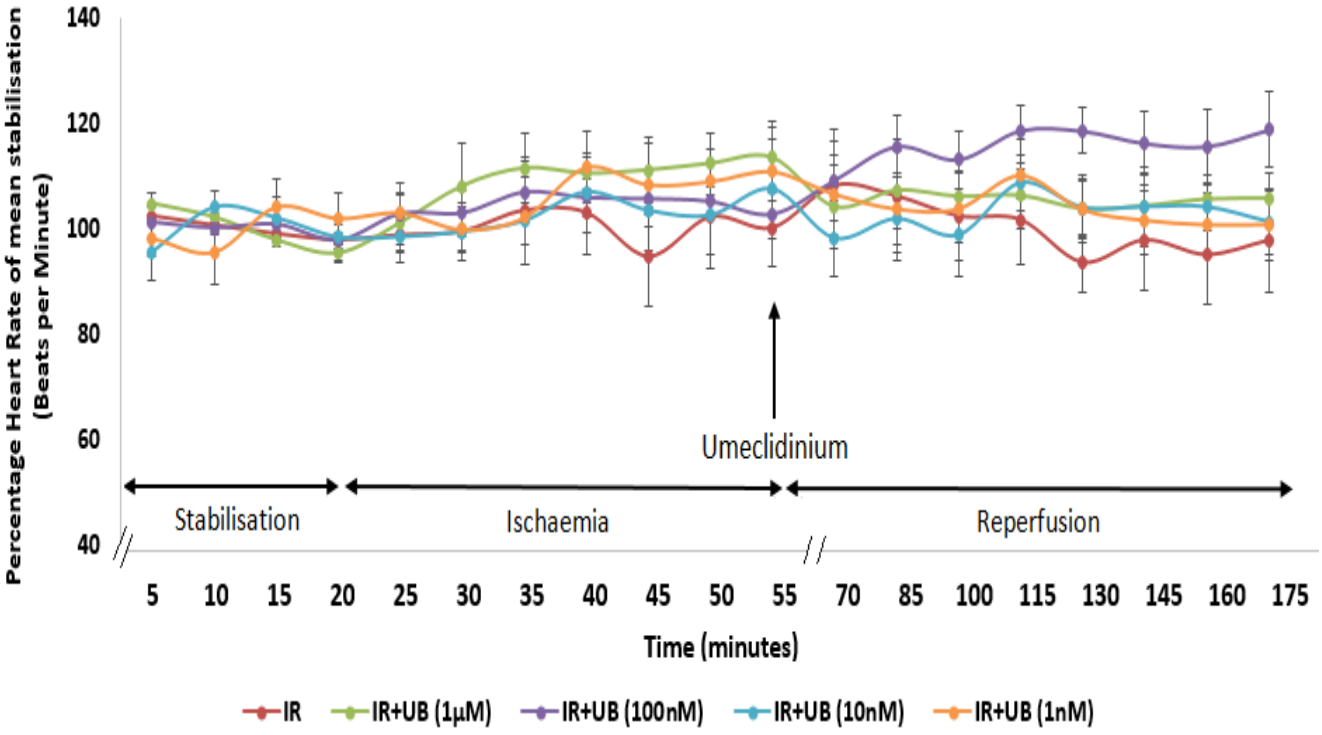

Figure 3.3.1.3: Percentage heart rate of the mean stabilisation period following Umeclidinium bromide (1 µM – 1 nM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Umeclidinium bromide (1 µM – 1 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. The graph represents data collected from a concentration response of Umeclidinium bromide (UB) (1 µM – 1 nM). Values plotted signify the Mean ± SEM, derived from an n of 4-6 for all groups.*

3.3.2. Infarct to Risk Analysis of Umeclidinium Bromide Administration in an *in vitro* model of Ischaemia/Reperfusion Injury

This study observed the effects of Umeclidinium bromide (1 µM – 1 nM) administration at the onset of reperfusion and throughout, on infarct to risk ratio (%), in an *in vitro* model of myocardial ischaemia/reperfusion injury.

Figure 3.3.2.1 shows the infarct/risk ratios of Umeclidinium bromide (1 µM – 1 nM) with respect to normoxic (Norm) and ischaemia/reperfusion (IR) controls. All concentrations of Umeclidinium bromide (1 µM – 1 nM) show an increase in infarct to risk ratio (%) with

respect to the normoxic control (p<0.001). Umeclidinium bromide at 100 nM, 10 nM and 1 nM show a significant increase in infarct size to risk ratio (%) with respect the IR control (60.07 ± 0.70% (UB 100 nM, p<0.05), 69.56 ± 1.03% and 69.86 ± 1.54% (UB 10nM and UB 1nM respectively, p<0.001) vs. 50.8 ± 3.93% (IR control), fig 3.3.2.1). Umeclidinium bromide at 10 nM and 1 nM show a significant increase with respect to Umeclidinium at 1µM (69.56 ± 1.03% (UB 10 nM) and 69.86 ± 1.54% (UB 1 nM) vs. 55.23 ± 0.91% (UB 1µM), p<0.001, fig 3.3.2.1) and Umeclidinium at 100nM (69.56 ± 1.03% (UB 10 nM) and 69.86 ± 1.54% (UB 1 nM) vs. 60.07 ± 0.70% (UB 100 nM), p<0.05, fig 3.3.2.1).

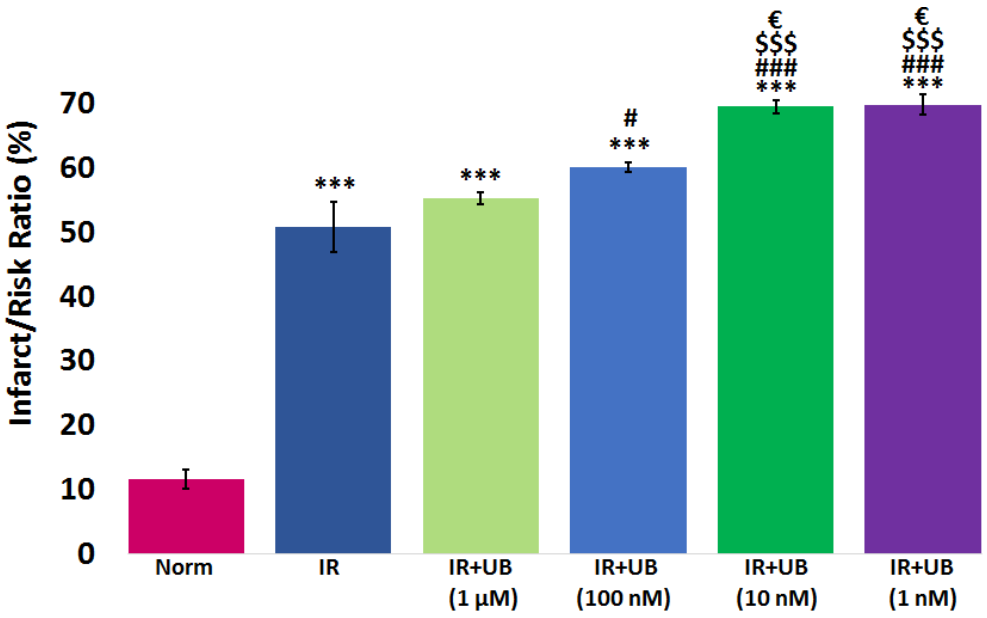

Figure 3.3.2.1: Percentage infarct to risk ratios (%) following Umeclidinium bromide (1 µM – 1 nM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Umeclidinium bromide (1 µM – 1 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group and Normoxic (Norm) group. The graph represents data collected from a concentration response of Umeclidinium bromide (UB) (1 µM – 1 nM). Values plotted signify the Mean ± SEM, derived from an n of 4-6 for all groups. *** vs. Norm, p<0.001; # vs. IR, p<0.05; ### vs. IR, p<0.001; $$$ vs. IR+UB (1 µM), p<0.001; € vs IR+UB (100nM), p<0.05.*

3.4 Profiling of Tiotropium Bromide – in an *in vitro* model of Myocardial Ischaemia/Reperfusion injury

3.4.1 Haemodynamic Data Analysis for Tiotropium bromide (10 µM – 0.1 nM)

Using a whole heart Langendorff model, the effect of Tiotropium bromide (10 µM – 0.1 nM) administration was assessed in conditions of myocardial ischaemia/reperfusion injury with 20 minutes of stabilisation preceding 35 minutes of regional ischaemia followed by 120 minutes of reperfusion; drug administration lasted the duration of reperfusion, as described in section 2.3.3.2.

Coronary flow (fig 3.4.1.1), left ventricular developed pressure (LVDP, fig 3.4.1.2) and heart rate (fig 3.4.1.3) are shown for Tiotropium bromide (10 µM – 0.1 nM) administration in conditions of ischaemia/reperfusion as well as the ischaemia/reperfusion control group. The data collected for all parameters (fig 3.4.1.1 – 3.4.1.3) following Tiotropium bromide (10 µM – 0.1 nM) administration in ischaemia/reperfusion were statistically analysed at 15, 25, 50, 70 and 160 minutes. The Tiotropium bromide (10 µM – 0.1 nM) groups were analysed with respect to the ischaemia/reperfusion control as well as between each concentration.

Figure 3.4.1.1 for coronary flow shows no statistical significance at the onset of reperfusion with Tiotropium bromide (10 µM – 0.1 nM) ($p>0.05$) between each concentration and also with respect to the ischaemia/reperfusion control.

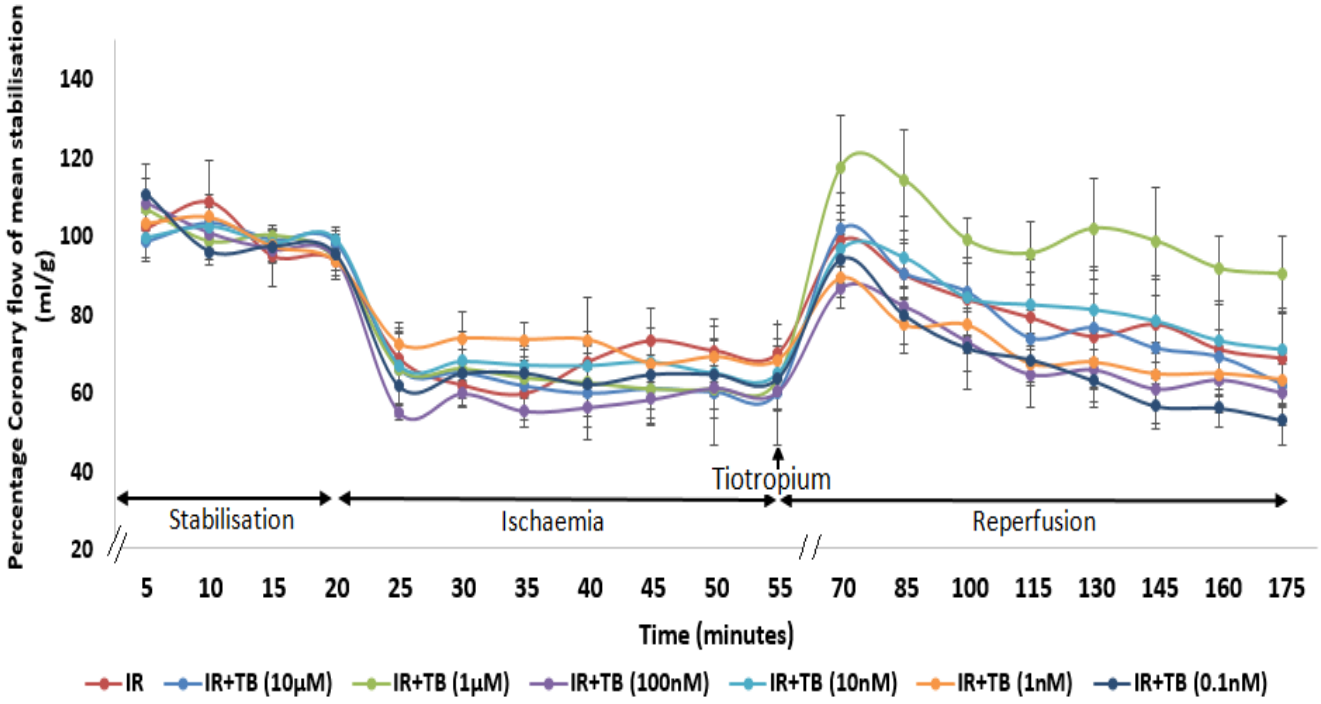

Figure 3.4.1.1: Percentage coronary flow of the mean stabilisation period following Tiotropium bromide (10 µM – 0.1 nM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Tiotropium bromide (10 µM – 0.1 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. The graph represents data collected from a concentration response of Tiotropium bromide (TB) (10 µM – 0.1 nM). Values plotted signify the Mean ± SEM, derived from an n of 4-6 for all groups.*

Figure 3.4.1.2 shows the left ventricular pressure recorded (LVDP) from the Tiotropium bromide (10 µM – 0.1 nM) treatment groups. The data does not show any statistical significances amongst the Tiotropium bromide (10 µM – 0.1 nM) groups at the time points assessed ($p>0.05$, fig 3.4.1.2) between the different concentrations or with respect to the ischaemia/reperfusion group.

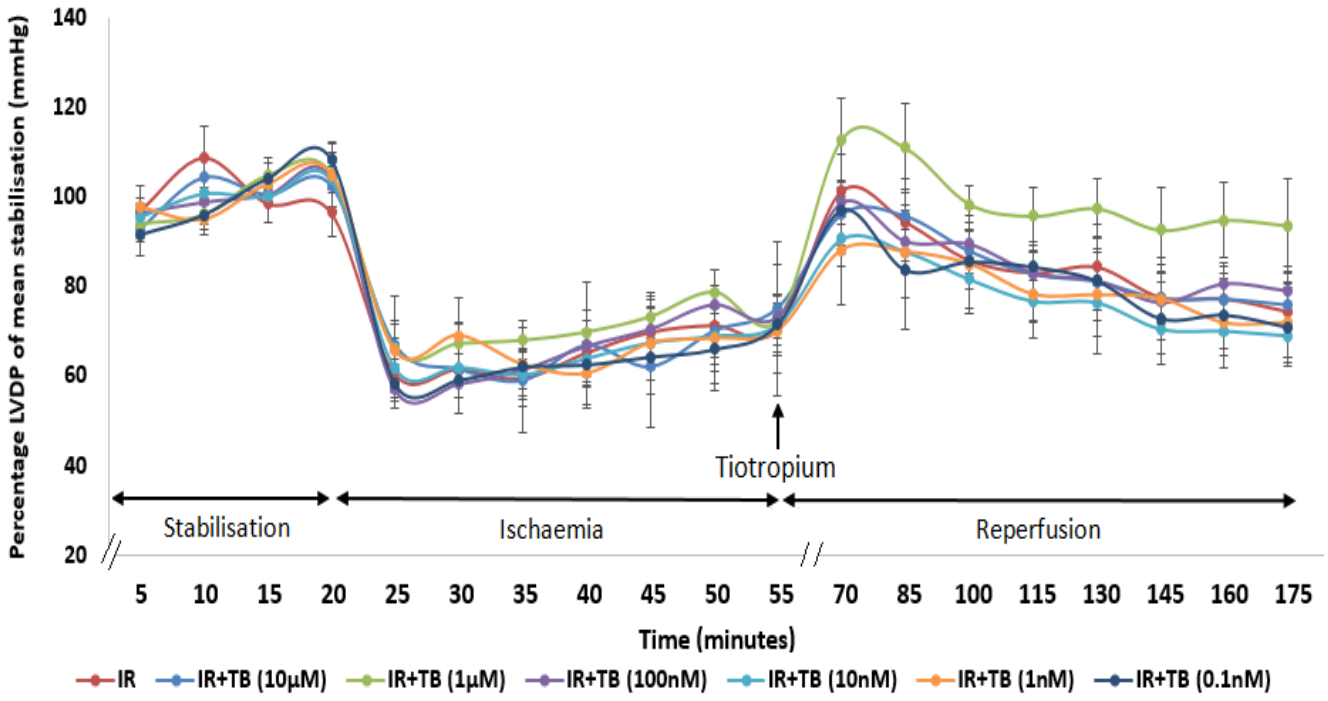

Figure 3.4.1.2: Percentage left ventricular developed pressure (LVDP) of the mean stabilisation period following Tiotropium bromide (10 µM – 0.1 nM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Tiotropium bromide (10 µM – 0.1 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. The graph represents data collected from a concentration response of Tiotropium bromide (TB) (10 µM – 0.1 nM). Values plotted signify the Mean ± SEM, derived from an n of 4-6 for all groups.*

Figure 3.4.1.3 shows heart rate following administration of Tiotropium bromide (10 µM – 0.1 nM) following ischaemia/reperfusion. The data shows no statistical significance in heart rate observed with Tiotropium bromide administration (10 µM – 0.1 nM) and the ischaemia/reperfusion control group ($p>0.05$, fig 3.4.1.3).

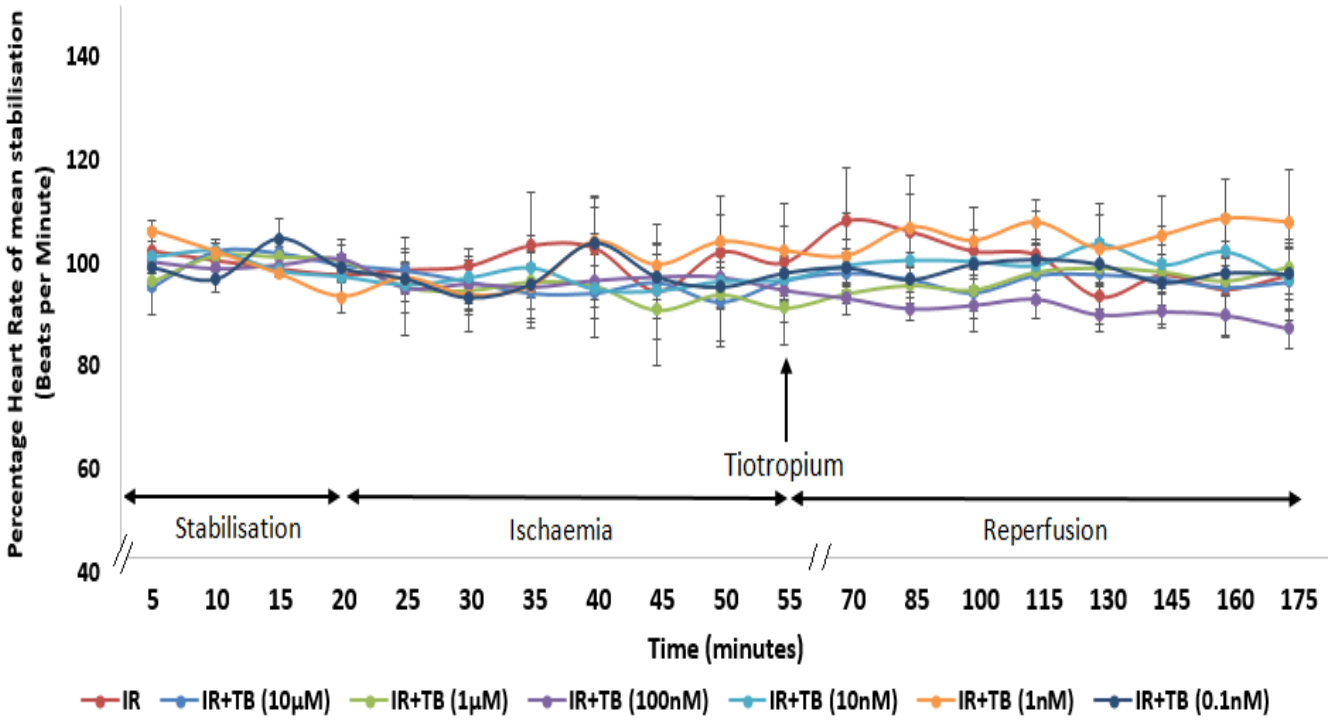

Figure 3.4.1.3: Percentage heart rate of the mean stabilisation period following Tiotropium bromide (10 µM – 0.1 nM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Tiotropium bromide (10 µM – 0.1 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. The graph represents data collected from a concentration response of Tiotropium bromide (TB) (10 µM – 0.1 nM). Values plotted signify the Mean ± SEM, derived from an n of 4-6 for all groups.*

3.4.2 Infarct to Risk Analysis of Tiotropium Bromide Administration in an *in vitro* model of Ischaemia/Reperfusion Injury

This study observed the effects of Tiotropium bromide (10 µM – 0.1 nM) administration at the onset of reperfusion and throughout, on infarct to risk ratio (%), in an *in vitro* model of myocardial ischaemia/reperfusion injury.

Figure 3.4.2.1 shows the infarct/risk ratios of Tiotropium bromide (10 µM – 0.1 nM) with respect to normoxic (Norm) and ischaemia/reperfusion (IR) controls. All concentrations of Tiotropium bromide (10 µM – 0.1 nM) show an increase in infarct to risk ratio (%) with

respect to the normoxic control ($p<0.001$, fig 3.4.2.1). Tiotropium bromide (100 nM) shows a significant increase in infarct size to risk ratio (%) with respect to the IR control (64.03 ± 1.06% vs. 50.8 ± 3.93% (IR control), $p<0.05$, fig 3.4.2.1). Tiotropium (10 nM) (67.36 ± 3.01% vs. 50.8 ± 3.93% (IR control), $p<0.001$, fig 3.4.2.1) and (1 nM) (65.42 ± 3.00% vs. 50.8 ± 3.93% (IR control), $p<0.01$, fig 3.4.2.1) also show a significant increase in infarct to risk ratio with respect to the IR control.

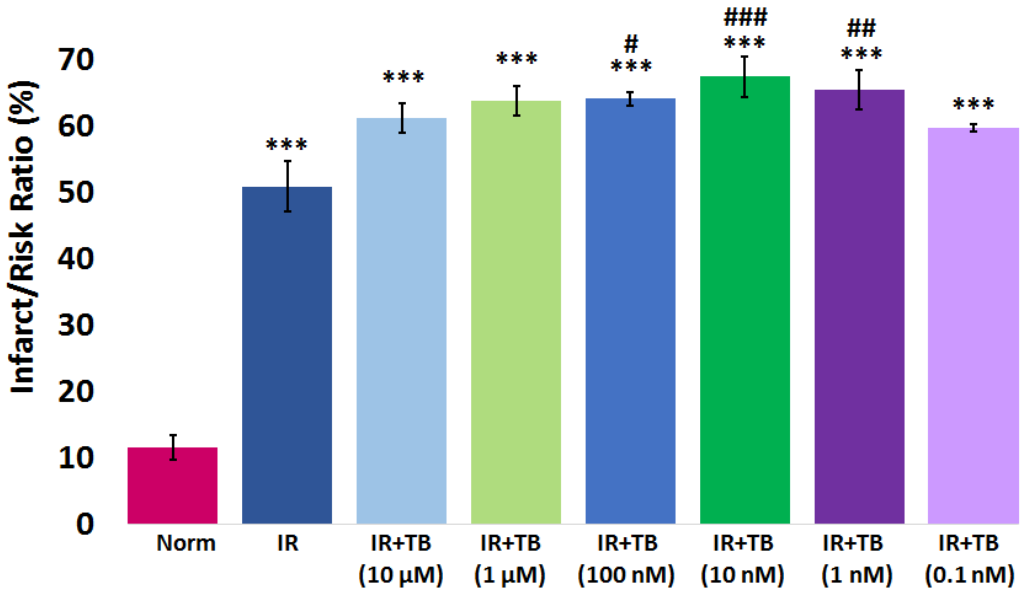

Figure 3.4.2.1: Percentage infarct to risk ratios (%) following Tiotropium bromide (10 µM – 0.1 nM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Tiotropium bromide (10 µM – 0.1 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group and Normoxic (Norm) group. The graph represents data collected from a concentration response of Tiotropium bromide (TB) (10 µM – 0.1 nM). Values plotted signify the Mean ± SEM, derived from an n of 4-6 for all groups. *** vs. Norm, $p<0.001$; # vs. IR, $p<0.05$; ## vs. IR, $p<0.01$; ### vs. IR, $p<0.001$.*

3.5 Tiotropium bromide in Normoxic Conditions in a Whole heart model

3.5.1 Haemodynamic Data Analysis for Tiotropium bromide (10 nM – 0.1 nM)

Tiotropium bromide (10 nM – 0.1 nM) was administered following a 20-minute stabilisation period and perfused throughout the rest of the experimental protocol for 155 minutes, as described in section 2.3.3.1. The data for all parameters (fig 3.5.1.1 – 3.5.1.3) following Tiotropium bromide (10 nM – 0.1 nM), were statistically analysed at 15, 25, 50, 70 and 160 minutes. Tiotropium bromide (10 nM – 0.1 nM) was compared to the normoxic control and each concentration.

The coronary flow (fig 3.5.1.1) data shows significance between Tiotropium bromide (0.1 nM and 10 nM) at 70 minutes. Tiotropium bromide (0.1 nM) shows an increase in coronary flow with respect to Tiotropium bromide (10 nM) (70 minutes: 109.34 ± 18.18% (TB 0.1 nM) vs. 73.57 ± 6.61% (TB 10nM), $p<0.05$, fig 3.5.1.1).

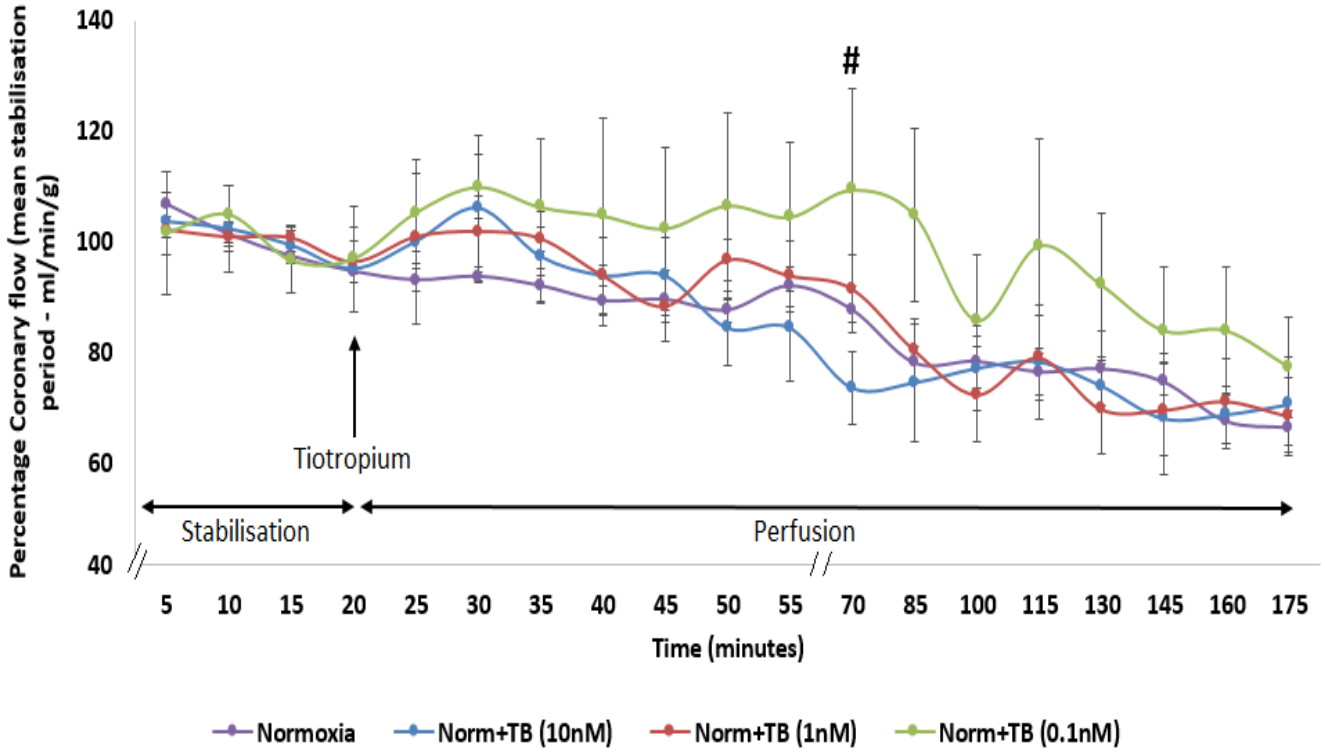

Figure 3.5.1.1: Percentage coronary flow of the mean stabilisation period following administration with Tiotropium bromide (10 nM – 0.1 nM). *Langendorff hearts were subjected to a 20-minute period of stabilisation perfused with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (10 nM – 0.1 nM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 3-5 for all groups. # Norm+TB (0.1nM) vs. Norm+TB (10nM), p<0.05.*

Figure 3.5.1.2 shows left ventricular developed pressure (LVDP) following Tiotropium bromide (10 nM – 0.1 nM) administration in normoxic conditions. The analysed data shows statistical significance observed at the 160 minute time point; Tiotropium at 10 nM shows an increase in LVDP with respect to the normoxic control (160 minutes: 100.11 ± 5.43% vs. 83.19 ± 2.89% (normoxia), $p<0.05$, fig 3.5.1.2). Tiotropium at 0.1 nM also shows a significant increase in LVDP with respect to the normoxic control (160 minutes: 104.47 ± 1.19% vs. 83.19 ± 2.89% (normoxia), $p<0.05$, fig 3.5.1.2). Significance is also observed between Tiotropium at the 10 nM and 0.1 nM concentrations with respect to Tiotropium at 1 nM, where Tiotropium at 10 nM and 0.1 nM show a significant increase in LVDP (160

minutes: 100.11 ± 5.43% (TB 10 nM) and 104.47 ± 1.19% (TB 0.1 nM) vs. 76.54 ± 6.11% (TB 1nM), p<0.01, fig 3.5.1.2).

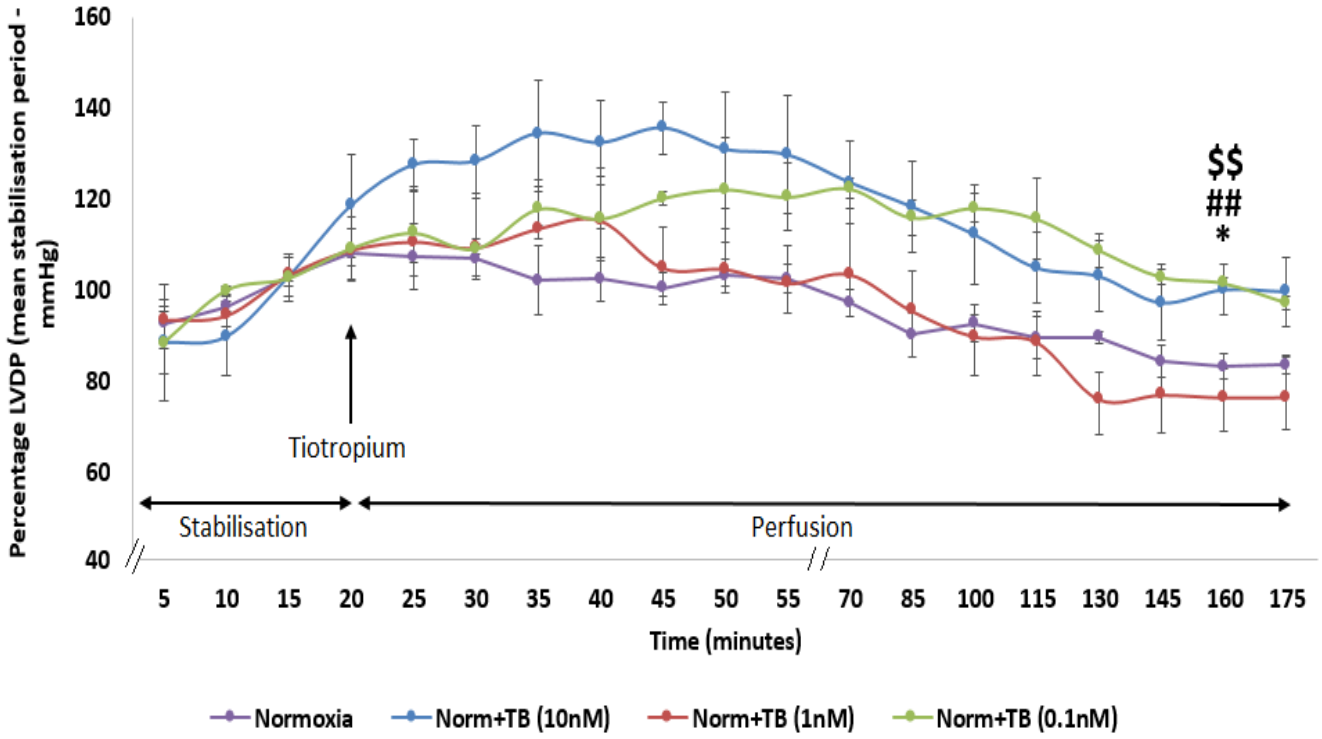

Figure 3.5.1.2: Percentage left ventricular developed pressure (LVDP) of the mean stabilisation period following administration with Tiotropium bromide (10 nM – 0.1 nM). *Langendorff hearts were subjected to a 20-minute period of stabilisation perfused with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (10 nM – 0.1 nM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 3-5 for all groups. * Norm+TB (10nM) and Norm+TB (1nM) vs. Normoxia, p<0.05; ## Norm+TB (1nM) vs. Norm+TB (10nM), p<0.01; $$ Norm+TB (0.1nM) vs. Norm+TB (1nM), p<0.01.*

Figure 3.5.1.3 shows heart rate following administration of Tiotropium bromide (10 nM – 0.1 nM) in normoxic conditions. The data shows no statistical significance in heart rate observed with Tiotropium bromide administration (10 nM – 0.1 nM) and the normoxic control group (p>0.05, fig 3.5.1.3).

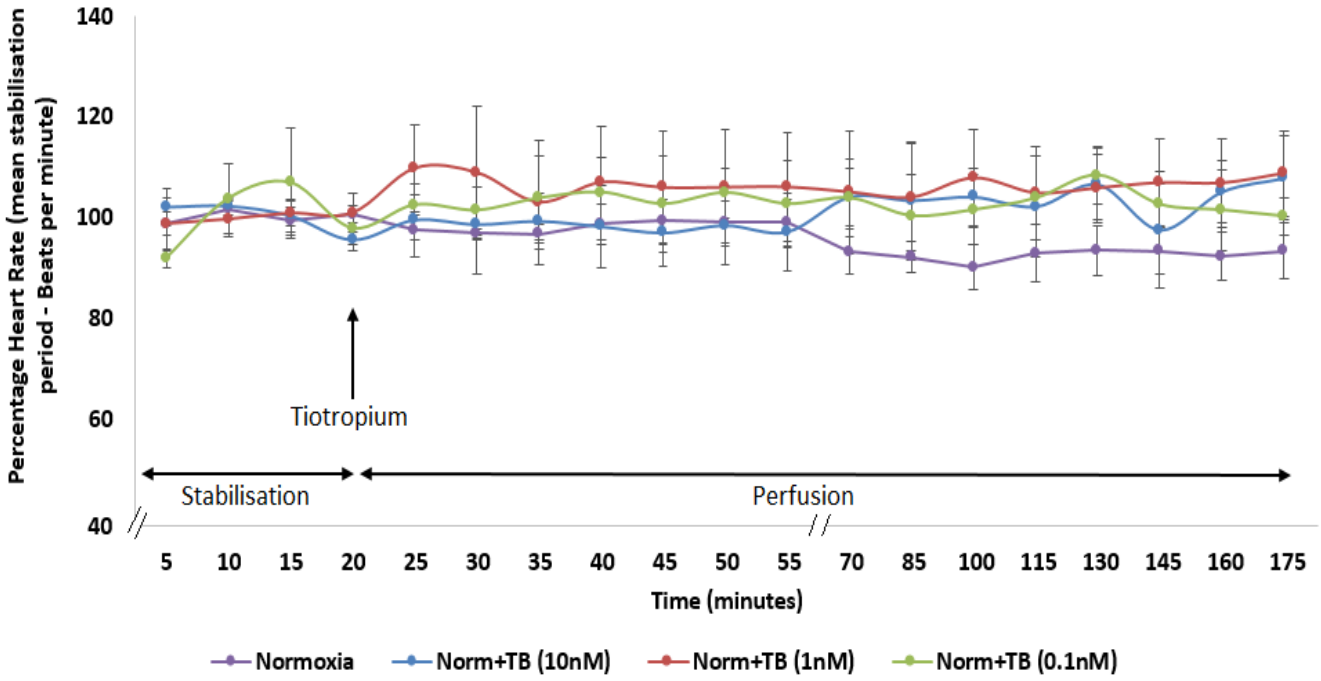

Figure 3.5.1.3: Percentage heart rate of the mean stabilisation period following administration with Tiotropium bromide (10 nM – 0.1 nM). *Langendorff hearts were subjected to a 20-minute period of stabilisation perfused with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (10 nM – 0.1 nM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 3-5 for all groups.*

3.5.2. Infarct to Risk analysis of Tiotropium bromide administration following stabilisation in Normoxic conditions

This study observed the effects of Tiotropium bromide (10 nM – 0.1 nM) administration on infarct size (%), in normoxic conditions. Tiotropium bromide (10 nM – 0.1 nM) was administered following 20 minutes of stabilisation and continued for 155 minutes; hearts were subjected to TTC staining to determine infarct size.

Figure 3.5.2.1 shows the infarct size of Tiotropium bromide (10 nM – 0.1 nM) with respect to normoxic (Norm) controls. The study shows that there is a significant increase in infarct size following Tiotropium bromide (10 nM – 0.1 nM) administration with respect to the normoxic control. Tiotropium at 10 nM and 0.1 nM show a similar increase in infarct size

compared to the normoxic control (17.55 ± 0.98% (TB 10 nM) and 17.19 ± 0.37% (TB 0.1 nM, $p<0.01$) vs. 10.27 ± 1.94% (Norm), $p<0.001$, fig 3.5.2.1). Tiotropium (1 nM) shows a slightly greater increase in infarct size compared to the normoxic control (18.69 ± 1.79% vs. 10.28 ± 1.74%, $p<0.001$, fig 3.5.2.1).

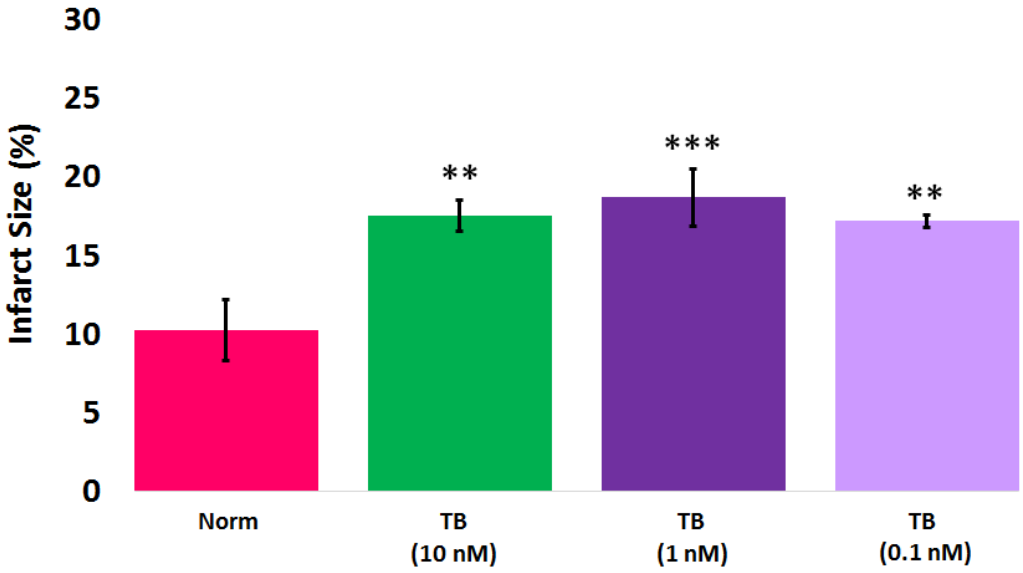

Figure 3.5.2.1: Percentage infarct size (%) following Tiotropium bromide (10 nM – 1 nM) in normoxic conditions*. Langendorff hearts were subjected to a 20-minute period of stabilisation with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (10 nM – 0.1 nM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 3-5 for all groups. ** TB (10nM) and TB (0.1nM) vs. Norm, p<0.01, *** TB (1nM) vs. Norm, p<0.001*

3.6 Tiotropium bromide mediated signalling in Adult rat ventricular cardiomyocytes

3.6.1 Effect of Tiotropium bromide on the expression profile of genes involved in myocardial ischaemia

Figure 3.6.1.1 shows Ppargc1α, Bax, Stat-3, Casp-3 and VEGF-A gene expression typically involved in myocardial infarction, following Tiotropium bromide (1 nM) administration in normoxic conditions. All genes were normalised using the ΔΔ Ct method to the normoxic control samples which represent a value of 1. Statistically significant decreases were observed with the expression of the Ppargc1a (0.19 ± 0.26 vs. 1.0 ± 0.0, $p<0.05$, fig 3.6.1.1) and Bax genes (0.34 ± 0.02 vs. 1.0 ± 0.0, $p<0.001$, fig 3.6.1.1) with respect to normoxic controls following GAPDH normalisation. The Stat-3 and VEGF-A genes show a non-significant decrease in gene expression following Tiotropium (1 nM) administration with respect to normoxic controls (0.25 ± 0.36 (Stat-3) and 0.21 ± 0.27 (VEGF-A) vs. 1.0 ± 0.0, $p>0.05$, fig 3.6.1.1); caspase 3 shows a non-significant increase in gene expression following Tiotropium (1 nM) administration with respect to normoxic controls (1.78 ± 0.94 (Casp-3) vs. 1.0 ± 0.0, $p>0.05$, fig 3.6.1.1).

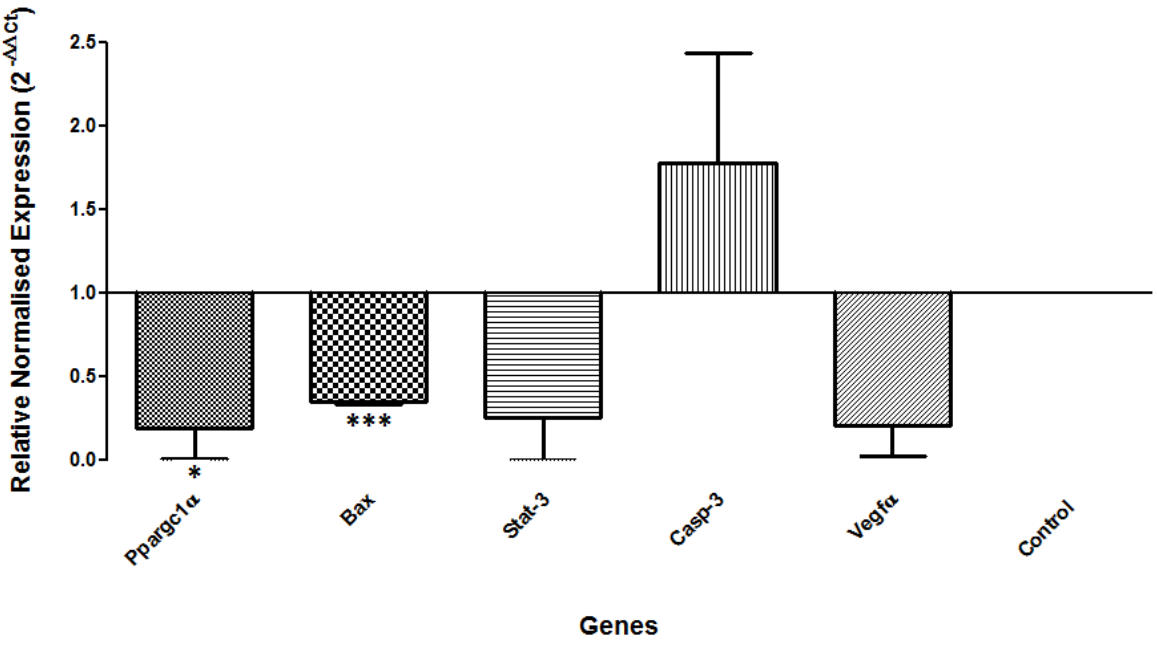

Figure 3.6.1.1: Relative normalised gene expression levels of Ppargc1 α, Bax, Stat-3, Casp-3 and Vegfα following Tiotropium bromide (1 nM) in a whole heart Langendorff model of normoxic conditions*. Whole heart tissue was obtained following 120 minutes of Tiotropium (1 nM) administration and assessed for gene expression using the Myocardial Infarction Prime PCR pathway plate. GAPDH was used to normalise the gene expression levels using the ΔΔ Ct method and compared against the normoxic control samples. Data is presented as the Mean ± SEM, n of 2. * vs. Control, p<0.05 and *** vs. Control, p<0.001.*

3.6.2 Role of Tiotropium bromide in affecting cell death via Annexin-V/Propidium iodide staining

Cardiomyocytes were incubated with Tiotropium bromide (10 µM – 0.1 nM) or control for a period of 4 hours before staining with Annexin-V and Propidium iodide (PI), and analysed on the flow cytometer using the FL-1 and FL-2 channels. Propidium iodide was used as a counter stain to differentiate between necrotic and dead apoptotic cells.

Figure 3.6.2.1 shows the percentage of total apoptosis in cardiomyocyte treated with Tiotropium bromide (10 µM – 0.1 nM) in normoxic conditions. Figure 3.6.2.1 shows a concentration dependent relationship with total apoptosis following Tiotropium bromide (10 µM – 0.1 nM), where the highest concentration (10 µM) shows a significant percentage

increase in total apoptosis with respect to the normoxic control (149.85 ± 25.1% vs. 100 ± 0.0%, $p<0.05$, fig 3.6.2.1). Tiotropium bromide at 1 µM – 0.1 nM shows a decreasing but non-significant trend in percentage total apoptosis with respect to normoxic controls (135.25 ± 18.33% (TB 1 µM), 129.69 ± 21.40% (TB 100 nM), 114.62 ± 15.89% (TB 10 nM), 117.2 ± 8.85% (TB 1 nM), 115.71 ± 18.59% (TB 0.1 nM) vs. 100 ± 0.0% (normoxia), $p>0.05$, fig 3.6.2.1). Analysis of necrosis data showed no significant difference between Tiotropium bromide (10 µM – 0.1 nM) and normoxic conditions (data not shown, $p>0.05$).

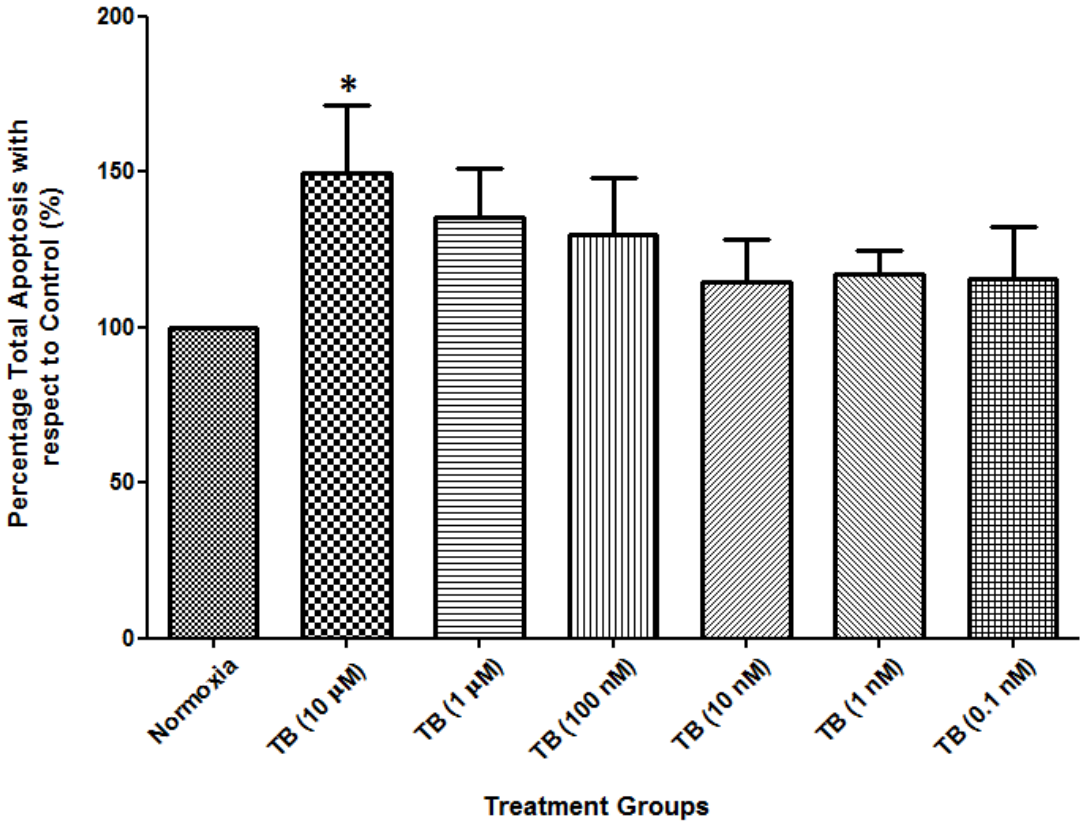

Figure 3.6.2.1: Percentage of total apoptotic cardiomyocytes (%) following treatment with Tiotropium bromide (TB 10 µM – 0.1 nM) in normoxic conditions, expressed as a percentage of the normoxia control*. Cardiomyocytes were treated for 4 hours in normoxic conditions with Tiotropium bromide (10 µM – 0.1 nM), apart from the normoxia control group, before staining with Annexin-V/Propidium iodide and analysed using the FL-1 and FL-2 channels of the flow cytometer. Data is presented as the Mean ± SEM, n of 4-5. * vs. Normoxia, $p<0.05$.*

Chapter 4: Role of Adjunctive Therapies in Reversing Tiotropium Mediated Cardiotoxicity

The primary aim of this study was to elucidate the cardiotoxicity of long acting muscarinic receptor antagonists, notably how Tiotropium bromide induces myocardial damage in *ex vivo* and *in vitro* models of normoxic conditions and ischaemia/reperfusion injury.

(c) Elucidating the function of muscarinic, PI3K-Akt, caspase-3 and the mPTP in Tiotropium bromide mediated myocardial damage.

4.1 Effect of the muscarinic receptor agonist, Acetylcholine in the presence or absence of Tiotropium bromide in Normoxia and Ischaemia/Reperfusion whole heart models

4.1.1 Haemodynamic Data Analysis

4.1.1.1 Haemodynamic in Normoxic conditions for Tiotropium ± Acetylcholine

Using a whole heart Langendorff model, the haemodynamic data for Tiotropium bromide (1 nM) administration in the presence or absence of acetylcholine (100 nM) following 20 minutes of stabilisation in normoxic conditions are depicted in figures 4.1.1.1.1 – 4.1.1.1.3. Tiotropium bromide (1nM) ± acetylcholine (100nM) was administered following a 20-minute stabilisation period and perfused throughout the rest of the experimental protocol for a period of 155 minutes, as described in section 2.3.3.1. The data collected for all parameters (fig 4.1.1.1.1 – 4.1.1.1.3) following Tiotropium bromide (1nM) ± acetylcholine (100nM) administration were statistically analysed at 15, 25, 50, 70 and 160 minutes. The Tiotropium bromide (1nM) ± acetylcholine (100nM) groups were analysed with respect to the normoxic control as well as between each treatment group.

Tiotropium bromide (1nM) alone and acetylcholine (100nM) alone show a significant increase in coronary flow with respect to the co-administered Tiotropium bromide (1nM) + acetylcholine (100nM) group, at 25 minutes (25 minutes: 100.86 ± 4.72% (TB 1nM) and 98.47 ± 3.58% (ACh 100nM) vs. 83.72 ± 3.43% (TB 1nM + ACh 100nM), $p<0.05$, fig 4.1.1.1.1).

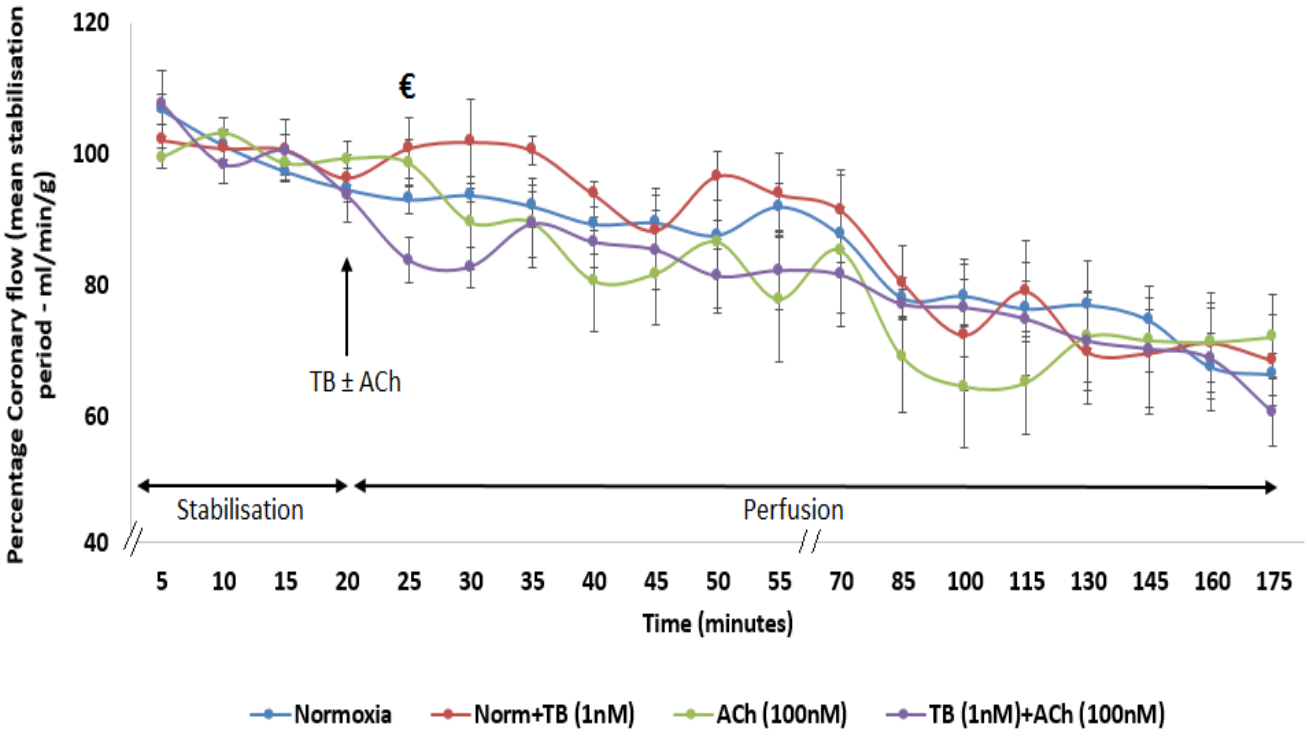

Figure 4.1.1.1.1: Percentage coronary flow of the mean stabilisation period following administration with Tiotropium bromide (1nM) ± acetylcholine (100nM)*. Langendorff hearts were subjected to a 20-minute period of stabilisation perfused with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (1nM) ± acetylcholine (100nM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups. € Norm+TB (1nM) and ACh (100nM) vs. TB (1nM) + ACh (100nM), p<0.05.*

Figure 4.1.1.1.2 shows left ventricular developed pressure (LVDP) following Tiotropium bromide (1nM) ± acetylcholine (100nM) administration in normoxic conditions. The data does not show any statistical significances amongst the Tiotropium bromide (1nM) ± acetylcholine (100nM) groups at the time points assessed ($p>0.05$, fig 4.1.1.1.2) between the groups or with respect to the normoxic control.

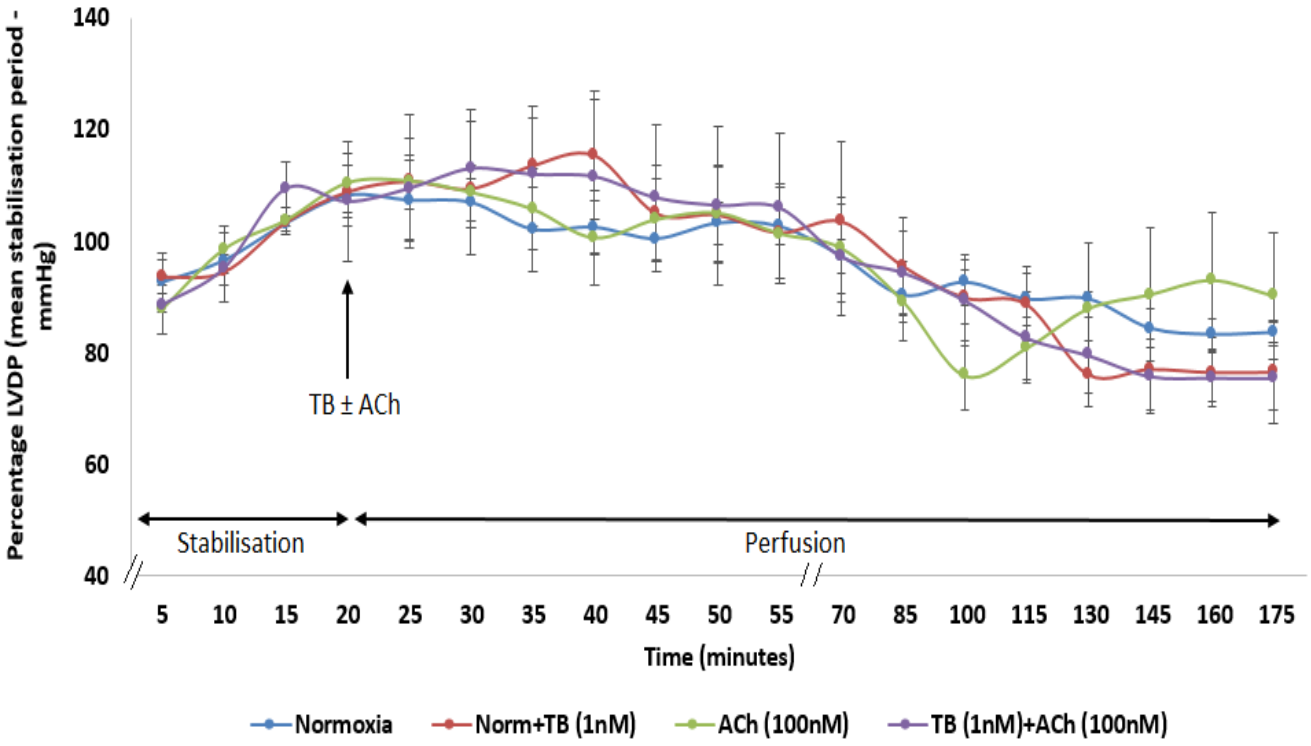

Figure 4.1.1.1.2: Percentage left ventricular developed pressure (LVDP) of the mean stabilisation period following administration with Tiotropium bromide (1nM) ± acetylcholine (100nM)*. Langendorff hearts were subjected to a 20-minute period of stabilisation perfused with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (1nM) ± acetylcholine (100nM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups.*

Figure 4.1.1.1.3 shows heart rate following administration of Tiotropium bromide (1nM) ± acetylcholine (100nM) in normoxic conditions. The data shows no statistical significance in heart rate observed with Tiotropium bromide (1nM) ± acetylcholine (100nM) and the normoxic control group ($p>0.05$, fig 4.1.1.1.3).

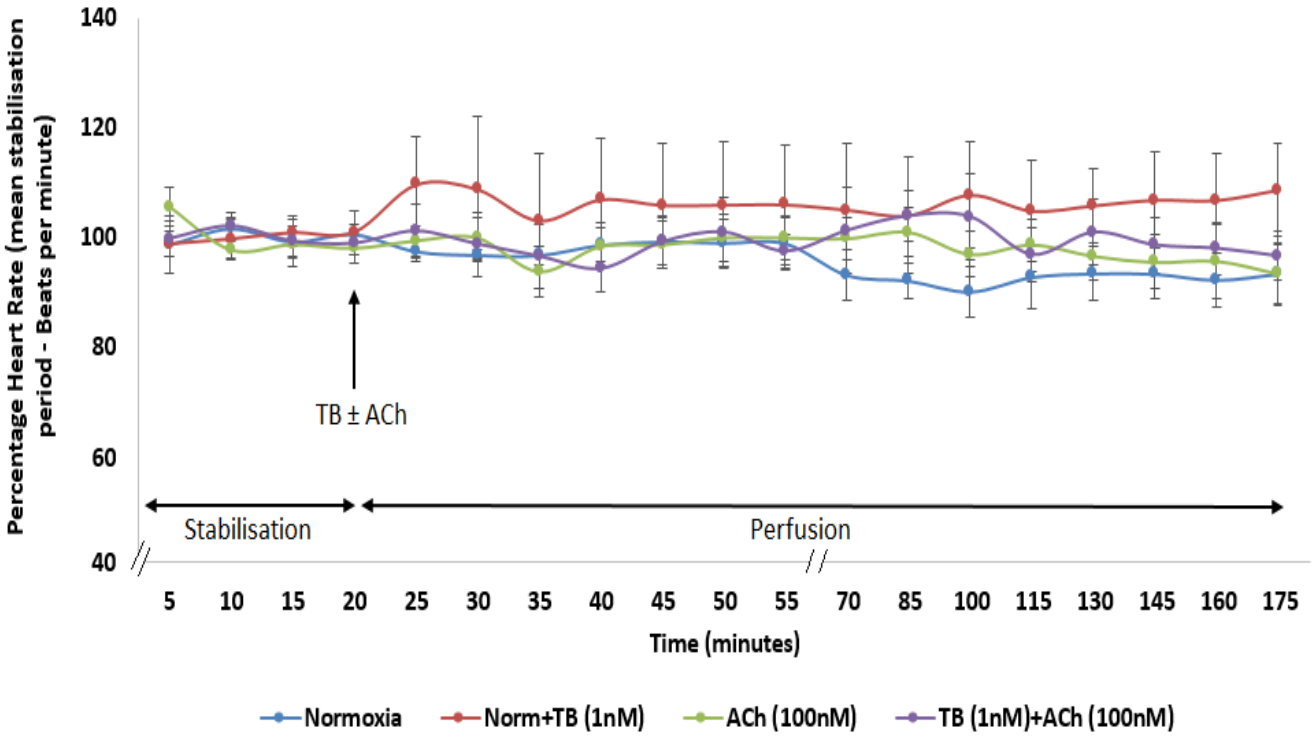

Figure 4.1.1.1.3: Percentage heart rate of the mean stabilisation period following administration with Tiotropium bromide (1nM) ± acetylcholine (100nM)*. Langendorff hearts were subjected to a 20-minute period of stabilisation perfused with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (1nM) ± acetylcholine (100nM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups.*

4.1.1.2 Haemodynamics in Ischaemia/Reperfusion for Tiotropium ± Acetylcholine

The effect of Tiotropium bromide (1 nM) administration in the presence or absence of acetylcholine (100 nM), was assessed in conditions of myocardial ischaemia/reperfusion injury with 20 minutes of stabilisation preceding 35 minutes of regional ischaemia followed by 120 minutes of reperfusion; drug administration lasted the duration of reperfusion, as described in section 2.3.3.2.

Haemodynamic data was recorded for each study; at the end of each experiment, hearts were stained with Evans blue and retained for infarct analysis using the TTC method. Coronary flow (fig 4.1.1.2.1), left ventricular developed pressure (LVDP, fig 4.1.1.2.2) and heart rate (fig 4.1.1.2.2.3) are shown for Tiotropium bromide (1 nM) ± acetylcholine (100

nM) administration in conditions of ischaemia/reperfusion as well as the ischaemia/reperfusion control group. The data collected for all parameters (fig 4.1.1.2.1 – 4.1.1.2.3) following Tiotropium bromide (1 nM) ± acetylcholine (100 nM) administration in ischaemia/reperfusion were statistically analysed at 15, 25, 50, 70 and 160 minutes.

Figure 4.1.1.2.1 for coronary flow shows no statistical significance at the onset of reperfusion with Tiotropium bromide (1 nM) ± acetylcholine (100 nM) ($p>0.05$) between each group and also with respect to the ischaemia/reperfusion control.

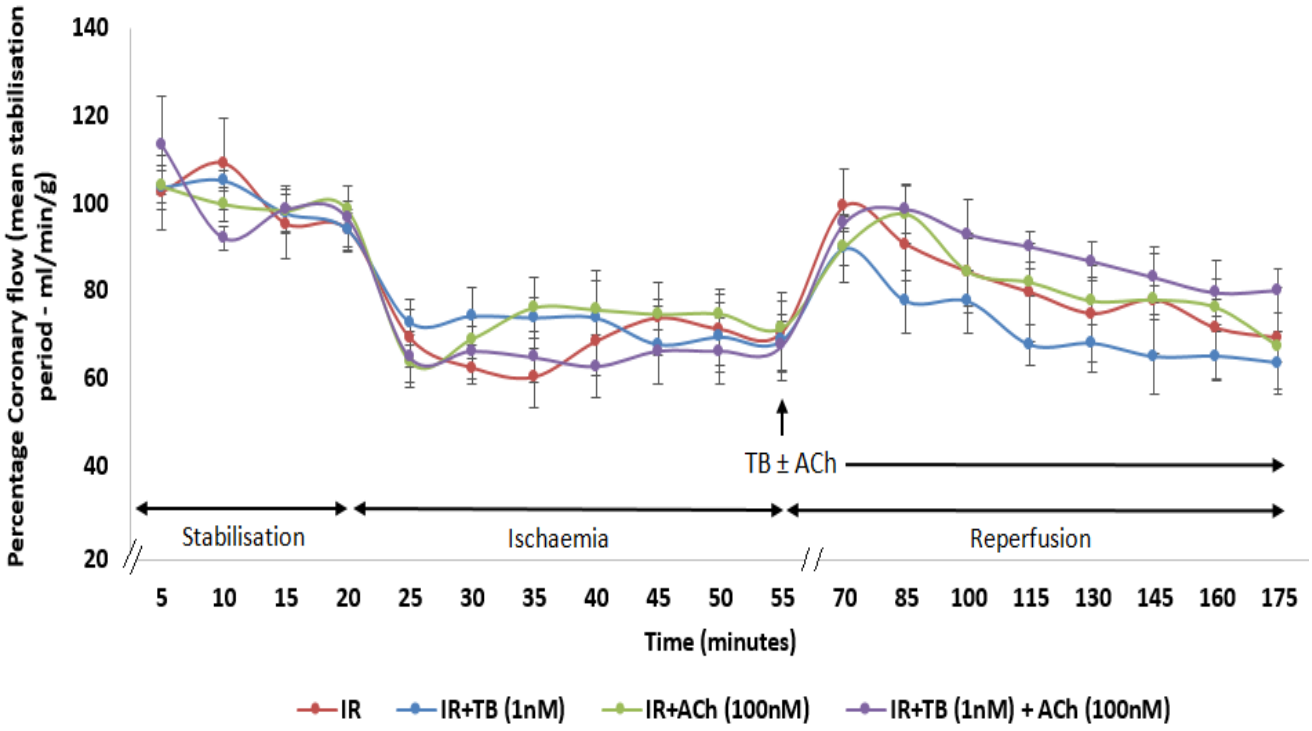

Figure 4.1.1.2.1: Percentage coronary flow of the mean stabilisation period following Tiotropium bromide (1 nM) ± acetylcholine (100 nM) in ischaemia/reperfusion. *Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Tiotropium bromide (1 nM) ± acetylcholine (100 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups.*

Figure 4.1.1.2.2 shows the left ventricular pressure recorded (LVDP) from the Tiotropium bromide (1 nM) ± acetylcholine (100 nM) treatment groups. The data does not show any

statistical significances amongst the Tiotropium bromide (1 nM) ± acetylcholine (100 nM) groups at the time points assessed (p>0.05, fig 4.1.1.2.2) between the different groups or with respect to the ischaemia/reperfusion group.

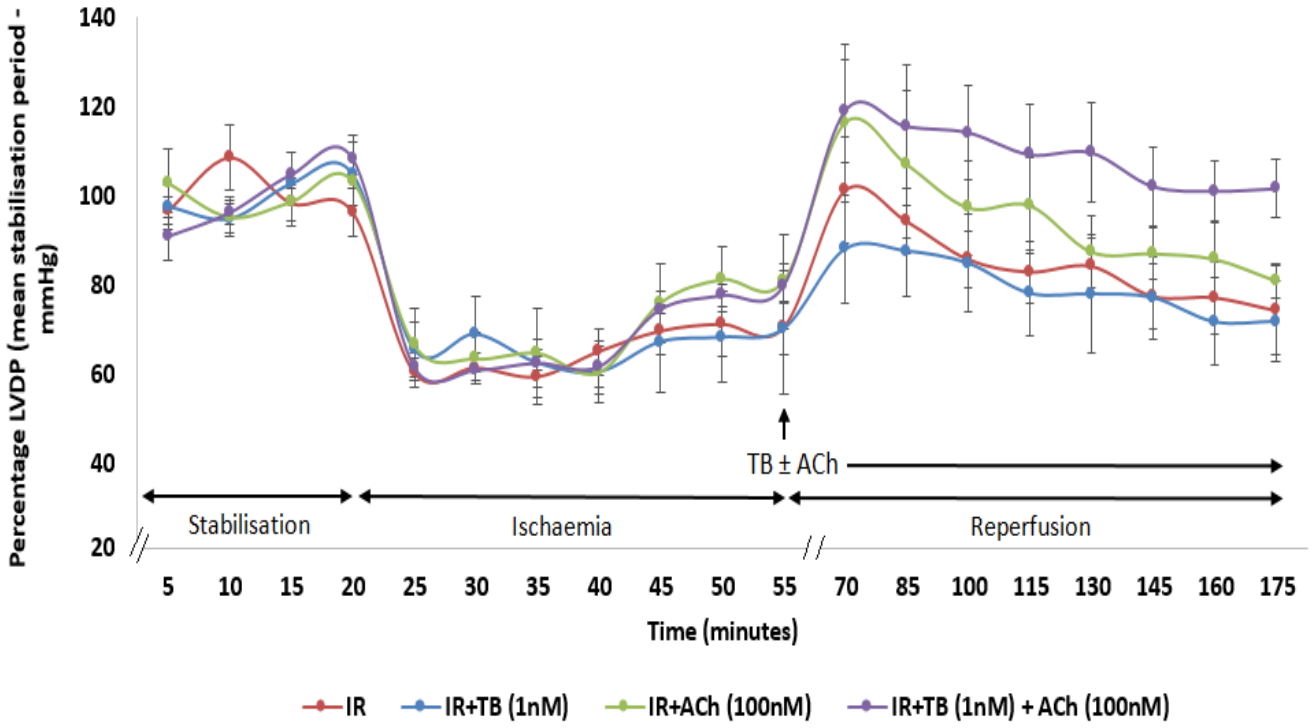

Figure 4.1.1.2.2: Percentage left ventricular developed pressure (LVDP) of the mean stabilisation period following Tiotropium bromide (1 nM) ± acetylcholine (100 nM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Tiotropium bromide (1 nM) ± acetylcholine (100 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups.*

Figure 4.1.1.2.3 shows heart rate following administration of Tiotropium bromide (1 nM) ± acetylcholine (100 nM) following ischaemia/reperfusion. The data shows no statistical significance in heart rate observed with Tiotropium bromide (1 nM) ± acetylcholine (100 nM) and the ischaemia/reperfusion control group (p>0.05, fig 4.1.1.2.3).

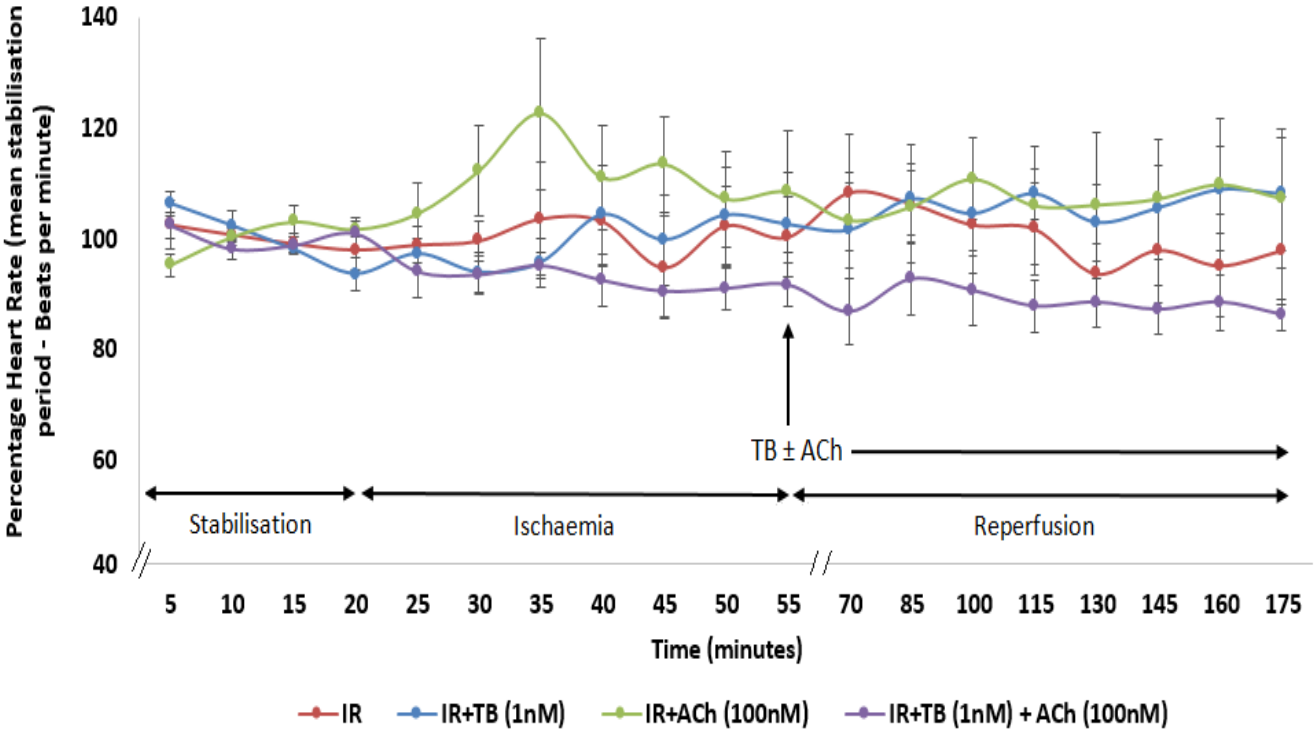

Figure 4.1.1.2.3: Percentage heart rate of the mean stabilisation period following Tiotropium bromide (1 nM) ± acetylcholine (100 nM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Tiotropium bromide (1 nM) ± acetylcholine (100 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups.*

4.1.2 Infarct size analysis of Tiotropium ± Acetylcholine administration

4.1.2.1 Infarct Analysis in Normoxic conditions for Tiotropium ± Acetylcholine

This study observed the effects of Tiotropium bromide (1 nM) ± acetylcholine (100 nM) administration on infarct size (%), in normoxic conditions. Tiotropium bromide (1 nM) ±

acetylcholine (100 nM) was administered following 20 minutes of stabilisation and continued for 155 minute; hearts were subjected to TTC staining to determine infarct.

Figure 4.1.2.1.1 shows the infarct size of Tiotropium bromide (TB 1 nM) ± acetylcholine (ACh 100 nM) with respect to normoxic (Norm) controls. The study shows that there is a significant difference in infarct size following TB (1 nM) ± ACh (100 nM) administration with respect to the normoxic control. Tiotropium (1 nM) shows an increase in infarct size compared to the normoxic control (18.69 ± 1.79% vs. 10.28 ± 1.74%, $p<0.001$, fig 4.1.2.1.1). The administration of acetylcholine (100 nM) shows no significance with respect to the normoxic control ($p>0.05$, fig 4.1.2.1.1), however a significant decrease in infarct size is observed with respect to Tiotropium (1 nM) (10.74 ± 1.02% (ACh 100nM) vs. 18.69 ± 1.79% (TB 1nM), $p<0.01$, fig 4.1.2.1.1). The decrease in infarct size following acetylcholine (100 nM) administration is also observed upon co-administration with Tiotropium (1 nM) with respect to the Tiotropium (1 nM) alone group (13.31 ± 0.96% (TB 1nM + ACh 100nM) vs. 18.69 ± 1.79% (TB 1nM), $p<0.05$, fig 4.1.2.1.1).

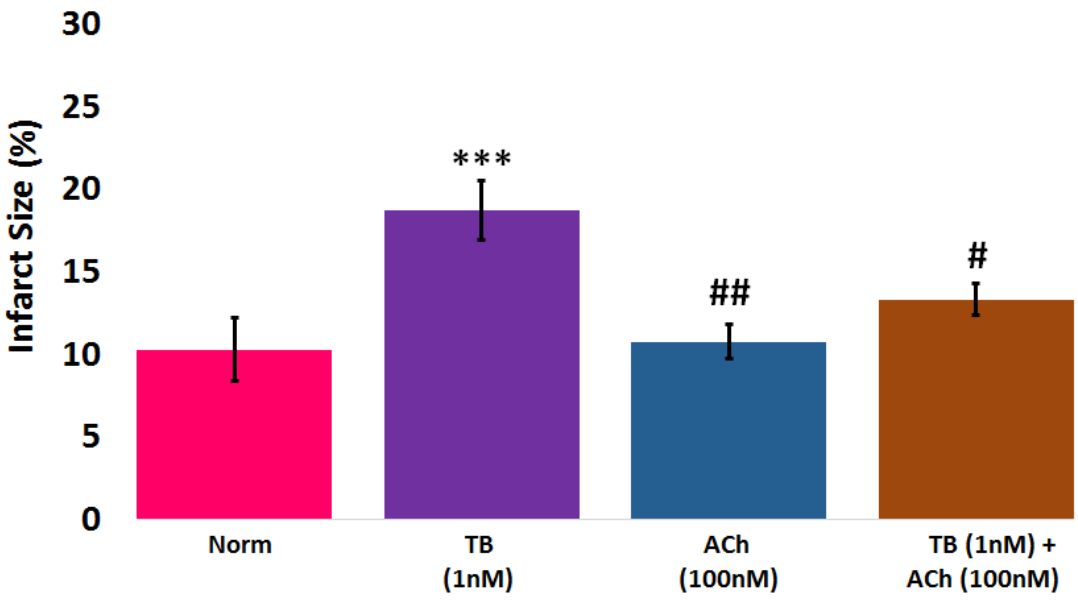

Figure 4.1.2.1.1: Percentage infarct size (%) following Tiotropium bromide (1 nM) ± acetylcholine (100 nM) in normoxic conditions. *Langendorff hearts were subjected to a 20-minute period of stabilisation with Krebs-Henseleit buffer, thereafter switched to*

*Tiotropium bromide (1 nM) ± acetylcholine (100 nM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, n = 4-5. *** TB (1nM) vs. Norm, p<0.001; # TB (1nM) + ACh (100nM) vs. TB (1nM), p<0.05 and ## ACh (100nM) vs. TB (1nM), p<0.01.*

4.1.2.2 Infarct Analysis in Ischaemia/Reperfusion for Tiotropium ± Acetylcholine

This study observed the effects of Tiotropium bromide (1 nM) ± acetylcholine (100 nM) administration on infarct to risk ratio (%), in an *in vitro* model of myocardial ischaemia/reperfusion. Tiotropium bromide (1 nM) ± acetylcholine (100 nM) was administered at the onset of reperfusion and continued throughout the 120-minute period following 20 minutes of stabilisation and 35 minutes of regional ischaemia as described in section 2.3.2.2, followed by Evans blue dye staining to delineate ischaemic zones, and subjected to TTC staining to determine infarct size to risk ratio (%).

Figure 4.1.2.2.1 shows the infarct/risk ratios of Tiotropium bromide (1 nM) ± acetylcholine (100 nM) with respect to the ischaemia/reperfusion (IR) control. The study showed that Tiotropium bromide (1 nM) administration results in a significant increase in infarct to risk ratio (%) with respect to the ischaemia/reperfusion control (65.42 ± 3.00% (TB 1nM) vs. 50.85 ± 3.93 (IR), p<0.01, fig 4.1.2.2.1). There is a significant decrease in infarct to risk ratio with acetylcholine (100 nM) alone with respect to Tiotropium (1 nM) alone (39.97 ± 0.94% (ACh 100nM) vs. 65.42 ± 3.00% (TB 1nM), p<0.001, fig 4.1.2.2.1). Tiotropium (1 nM) + acetylcholine (100 nM) administration also shows a significant decrease in infarct to risk ratio with respect to Tiotropium (1 nM) (46.05 ± 0.30% (TB 1nM + ACh 100nM) vs. 65.42 ± 3.00% (TB 1nM), p<0.01, fig 4.1.2.2.1).

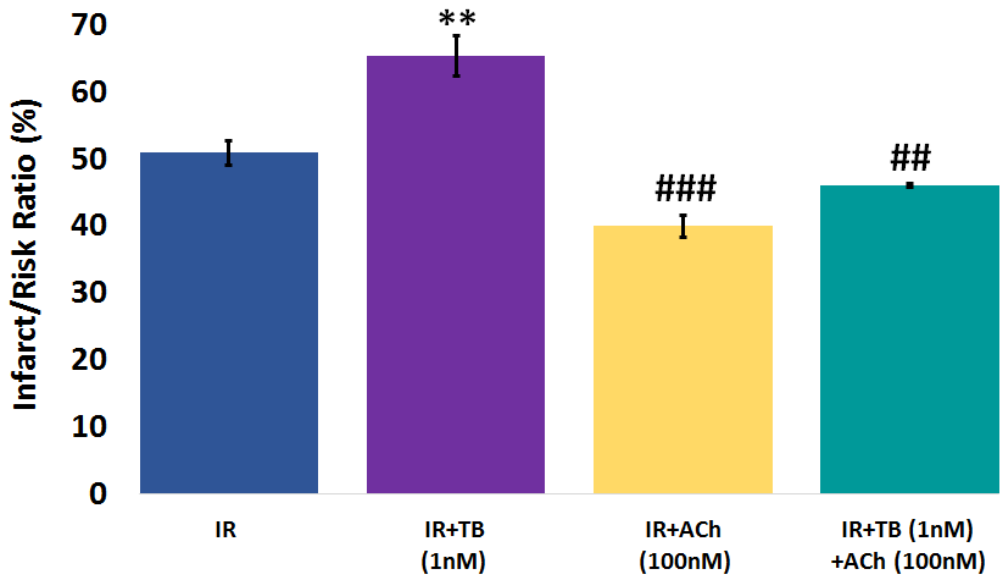

Figure 4.1.2.2.1: Percentage infarct to risk ratios (%) following Tiotropium bromide (1 nM) ± acetylcholine (100 nM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Tiotropium bromide (1 nM) ± acetylcholine (100 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups. ** vs. IR, p<0.05; ## vs. IR+TB (1nM), p<0.01 and ### vs. IR+TB (1nM), p<0.001.*

4.1.3 Effect of Tiotropium bromide administration on Phospho-Akt (Ser473) expression in the presence or absence of Acetylcholine

P-Akt (Ser473) activation was assessed using western blotting for phosphorylated Akt (Ser473) in cardiac tissue to establish the role of Akt in Tiotropium bromide (1 nM) ± acetylcholine (100 nM) mediated cardiotoxicity. Figure 4.1.3.1 shows the expression pattern observed following normoxia and Tiotropium bromide (1 nM) ± acetylcholine (100 nM). All drugs were administered following stabilisation in a modified Langendorff model, for a period of 155 minutes; hearts were immediately removed following the end of the protocol period and the left ventricle was excised and snap-frozen as described in section 2.3.4.

The data shows a significant increase in phosphorylated Akt (Ser473) following Tiotropium (1 nM) administration with respect to the normoxic control (88.03 ± 5.62% (TB 1nM) vs. 36.17 ± 5.13% (Norm), p<0.0001, fig 4.1.3.1). Acetylcholine (100 nM) shows a significant decrease in p-Akt (Ser473) with respect to the Tiotropium (1 nM) mediated increase (47.71 ± 4.47% (ACh 100nM) vs. 88.03 ± 5.62% (TB 1nM), p<0.0001, fig 4.1.3.1). This increase in p-Akt (Ser473) is attenuated upon co-administration of acetylcholine (100 nM) with Tiotropium (1 nM) with respect to Tiotropium (1 nM) (47.30 ± 8.08% (TB 1nM + ACh 100nM) vs. 88.03 ± 5.62% (TB 1nM), p<0.0001, fig 4.1.3.1).

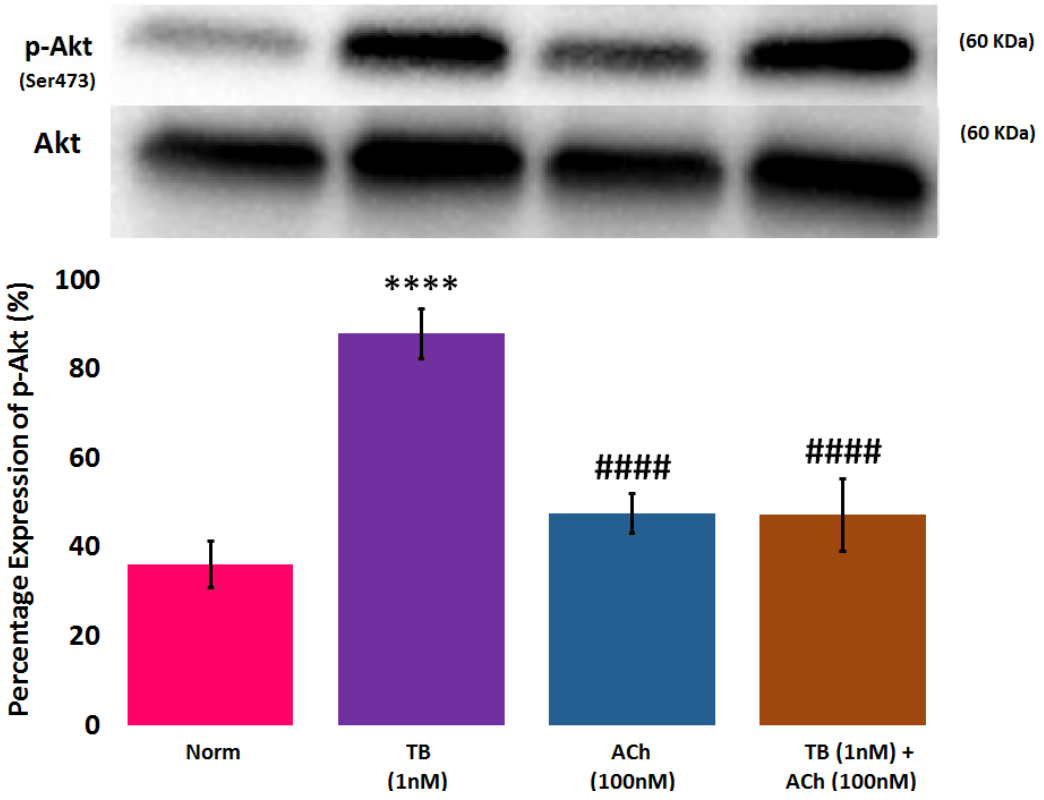

Figure 4.1.3.1: Percentage expression (%) of phosphorylated Akt (Ser473) as a percentage of total Akt following Tiotropium bromide (1 nM) ± acetylcholine (100 nM) in normoxic conditions*. Langendorff hearts were perfused with either Krebs-Henseleit buffer (Norm) or Tiotropium bromide (1 nM) ± acetylcholine (100 nM). Values plotted signify the Mean ± SEM, derived from an n of 3 for all groups. **** vs. Norm, p<0.0001 and #### vs. TB (1nM), p<0.0001.*

4.1.4 Role of Acetylcholine in Tiotropium bromide mediated apoptotic cell death

Cardiomyocytes were incubated with Tiotropium bromide (1 nM) ± acetylcholine (100 nM) and the normoxic controls respectively for a period of 4 hours before staining with Annexin-V and Propidium iodide (PI), and analysed on the flow cytometer using the FL-1 and FL-2 channels. Propidium iodide was used as a counter stain to differentiate between necrotic and dead apoptotic cells.

Figure 4.1.4.1 shows the percentage of total apoptosis in cardiomyocyte treated with Tiotropium bromide (1 nM) ± acetylcholine (100 nM) in normoxic conditions. Tiotropium bromide (1 nM) shows an observable but non-significant increase in total apoptosis with respect to the normoxic control (117.2 ± 8.85% (TB 1 nM) vs. 100 ± 0.0% (normoxia), $p>0.05$, fig 4.1.4.1). However, acetylcholine (100 nM) administration shows a decrease in total apoptosis with respect to Tiotropium (1 nM) (83.92 ± 9.9% (ACh 100nM) vs. 117.2 ± 8.85% (TB 1 nM), $p<0.05$, fig 4.1.4.1). The effect of acetylcholine (100 nM) on total apoptosis is masked upon co-administration with Tiotropium (1 nM), which shows an increase in total apoptosis with respect to acetylcholine (100 nM) alone (119.96 ± 9.95% (TB 1nM + ACh 100nM) vs. 83.92 ± 9.9% (ACh 100nM), $p<0.05$, fig 4.1.4.1). Data for necrosis did not show any significant difference between any of the groups ($p>0.05$, data not shown).

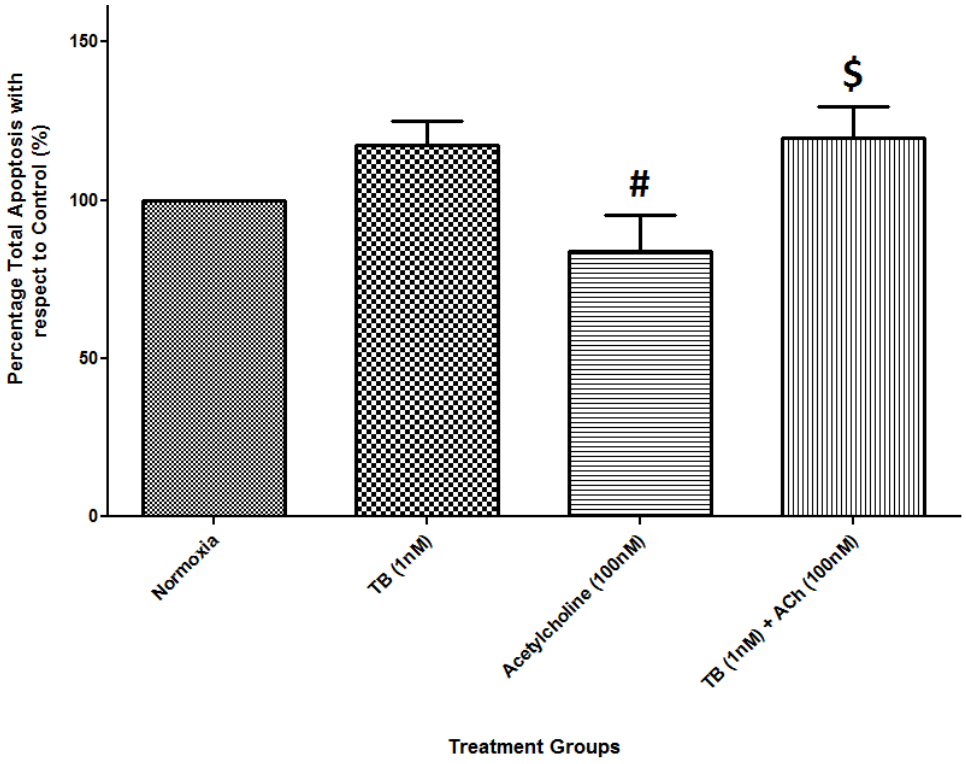

Figure 4.1.4.1: Percentage of total apoptotic cardiomyocytes (%) following treatment with Tiotropium bromide (1 nM) ± acetylcholine (100 nM) in normoxic conditions, expressed as a percentage of the normoxia control*. Cardiomyocytes were treated for 4 hours in normoxic conditions with Tiotropium bromide (1 nM) ± acetylcholine (100 nM), apart from the normoxia control group, before staining with Annexin-V/Propidium iodide and analysed using the FL-1 and FL-2 channels of the flow cytometer. Data is presented as the Mean ± SEM, n of 3-4. # vs. TB (1nM), p<0.05 and $ vs. Acetylcholine (100nM), p<0.05.*

4.2 Effect of the PI3K inhibitor, Wortmannin in the presence or absence of Tiotropium bromide in Normoxia and Ischaemia/Reperfusion whole heart models

4.2.1 Haemodynamic Data Analysis

4.2.1.1 Haemodynamic in Normoxic conditions for Tiotropium ± Wortmannin

Tiotropium bromide (1nM) ± wortmannin (100nM) was administered following a 20-minute stabilisation period and perfused throughout the rest of the experimental protocol for a period of 155 minutes, as described in section 2.3.3.1. The data for all parameters (fig 4.2.1.1.1 – 4.2.1.1.3) following Tiotropium bromide (1nM) ± wortmannin (100nM) were analysed at 15, 25, 50, 70 and 160 minutes. Tiotropium bromide (1nM) ± wortmannin (100nM) was compared to the normoxic control and each concentration.

The coronary flow (fig 4.2.1.1.1) shows that Tiotropium bromide (1nM) ± wortmannin (100nM) alone show no significance with respect to the normoxic control or each other.

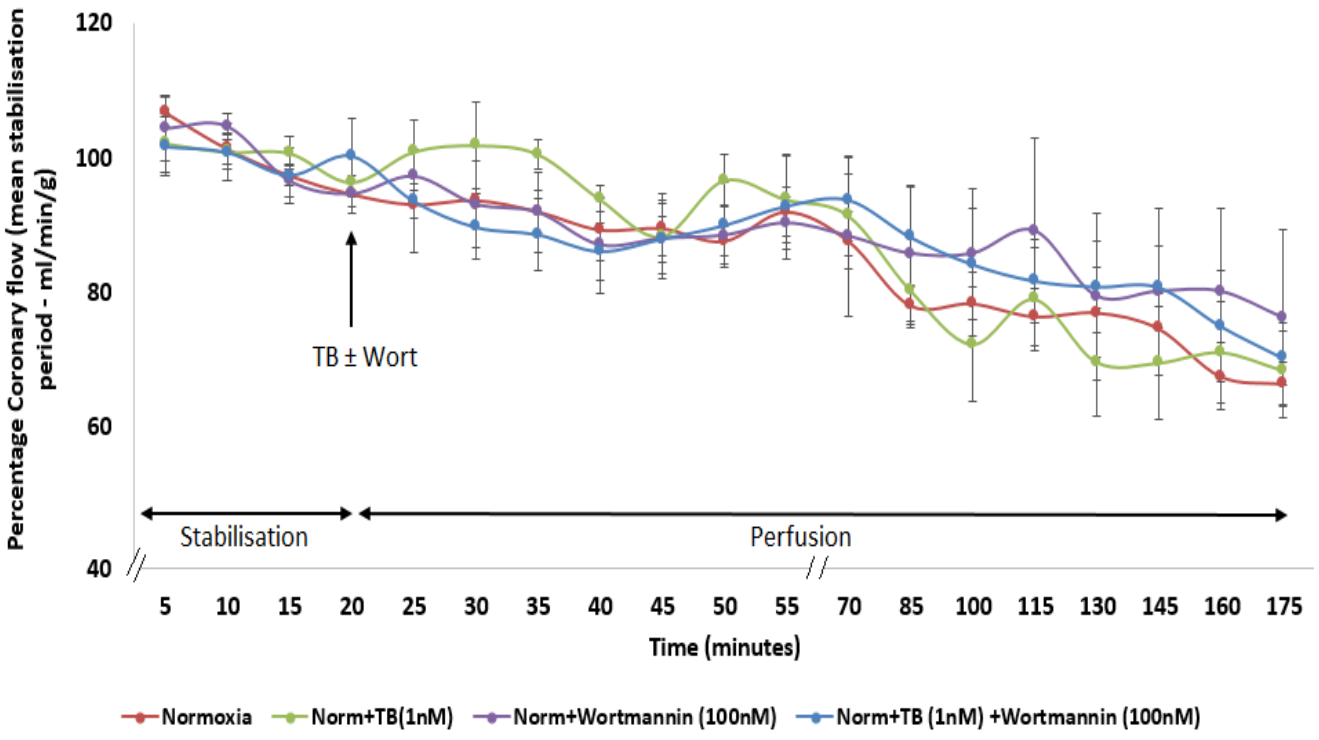

Figure 4.2.1.1.1: Percentage coronary flow of the mean stabilisation period following administration with Tiotropium bromide (1nM) ± wortmannin (100nM)*. Langendorff hearts were subjected to a 20-minute period of stabilisation perfused with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (1nM) ± wortmannin (100nM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups.*

Figure 4.2.1.1.2 shows left ventricular developed pressure (LVDP) following Tiotropium bromide (1nM) ± wortmannin (100nM) administration in normoxic conditions. The data does not show any statistical significances amongst the Tiotropium bromide (1nM) ±

wortmannin (100nM) groups at the time points assessed (p>0.05, fig 4.2.1.1.2) between the groups or with respect to the normoxic control.

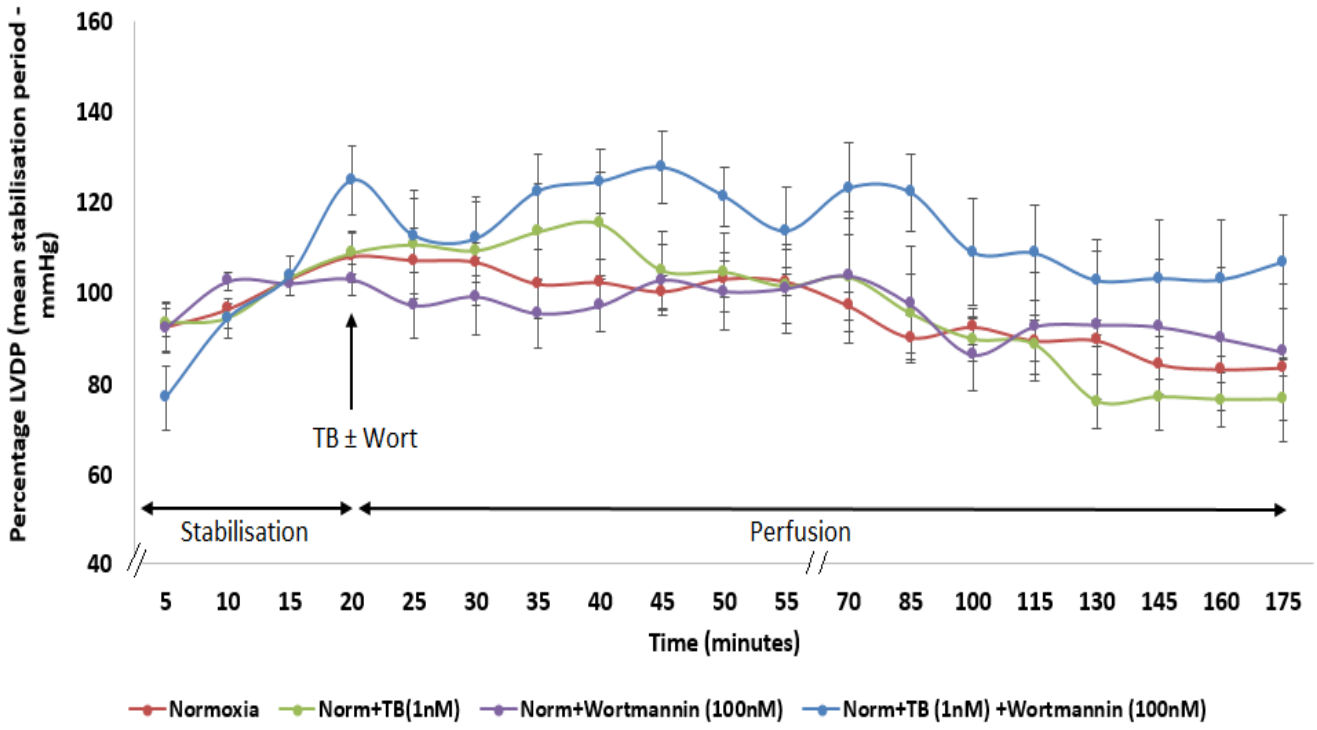

Figure 4.2.1.1.2: Percentage left ventricular developed pressure (LVDP) of the mean stabilisation period following administration with Tiotropium bromide (1nM) ± wortmannin (100nM)*. Langendorff hearts were subjected to a 20-minute period of stabilisation perfused with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (1nM) ± wortmannin (100nM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups.*

Figure 4.2.1.1.3 shows heart rate following administration of Tiotropium bromide (1nM) ± wortmannin (100nM) in normoxic conditions. The data shows no statistical significance in heart rate observed with Tiotropium bromide (1nM) ± wortmannin (100nM) and the normoxic control group (p>0.05, fig 4.2.1.1.3).

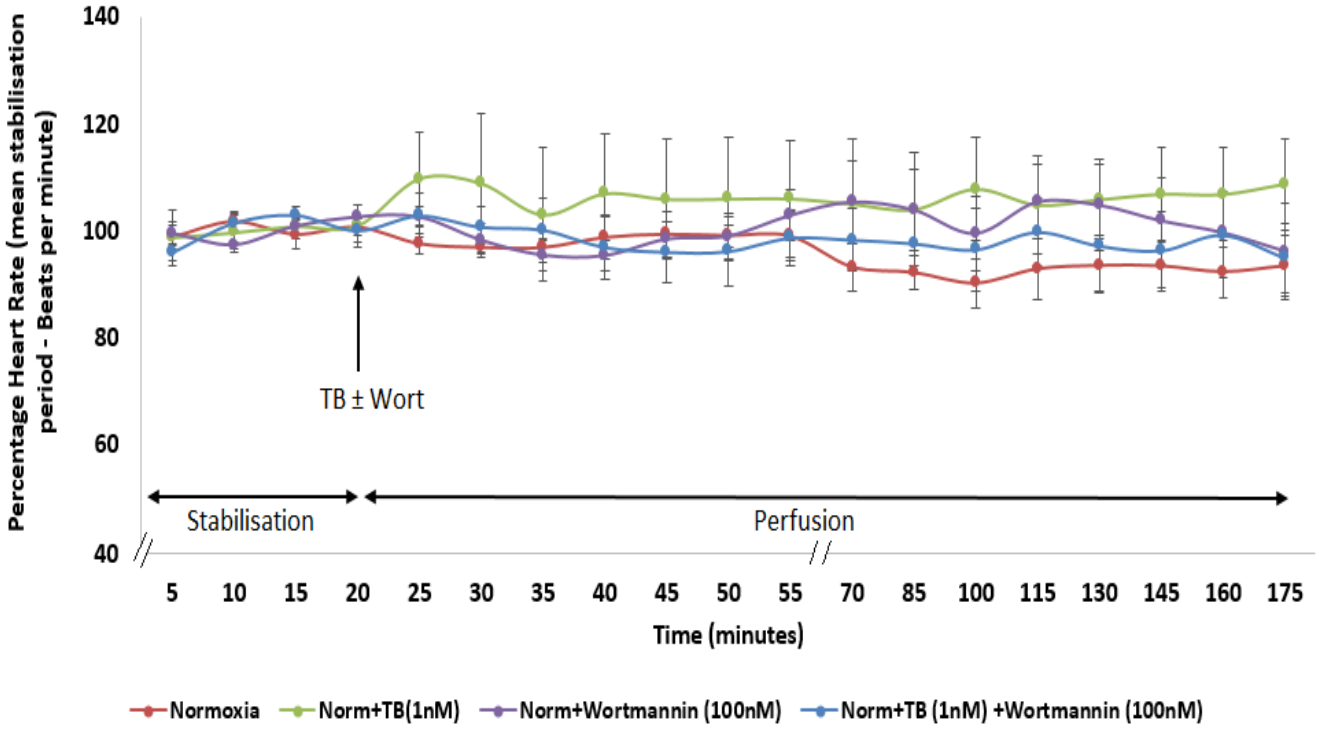

Figure 4.2.1.1.3: Percentage heart rate of the mean stabilisation period following administration with Tiotropium bromide (1nM) ± wortmannin (100nM). *Langendorff hearts were subjected to a 20-minute period of stabilisation perfused with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (1nM) ± wortmannin (100nM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups.*

4.2.1.2 Haemodynamic in Ischaemia/Reperfusion for Tiotropium ± Wortmannin

The effect of Tiotropium bromide (1 nM) administration in the presence or absence of wortmannin (100 nM), was assessed in conditions of myocardial ischaemia/reperfusion injury with 20 minutes of stabilisation preceding 35 minutes of regional ischaemia followed by 120 minutes of reperfusion; drug administration lasted the duration of reperfusion, as described in section 2.3.3.2.

Coronary flow (fig 4.2.1.2.1), left ventricular developed pressure (LVDP, fig 4.2.1.2.2) and heart rate (fig 4.2.1.2.2.3) are shown for Tiotropium bromide (1 nM) ± wortmannin (100 nM) administration in conditions of ischaemia/reperfusion as well as the ischaemia/reperfusion control group. The data collected for all parameters (fig 4.2.1.2.1 –

4.2.1.2.3) following Tiotropium bromide (1 nM) ± wortmannin (100 nM) administration in ischaemia/reperfusion were statistically analysed at 15, 25, 50, 70 and 160 minutes.

Figure 4.2.1.2.1 for coronary flow shows no statistical significance at the onset of reperfusion with Tiotropium bromide (1 nM) ± wortmannin (100 nM) ($p>0.05$) between each group and also with respect to the ischaemia/reperfusion control.

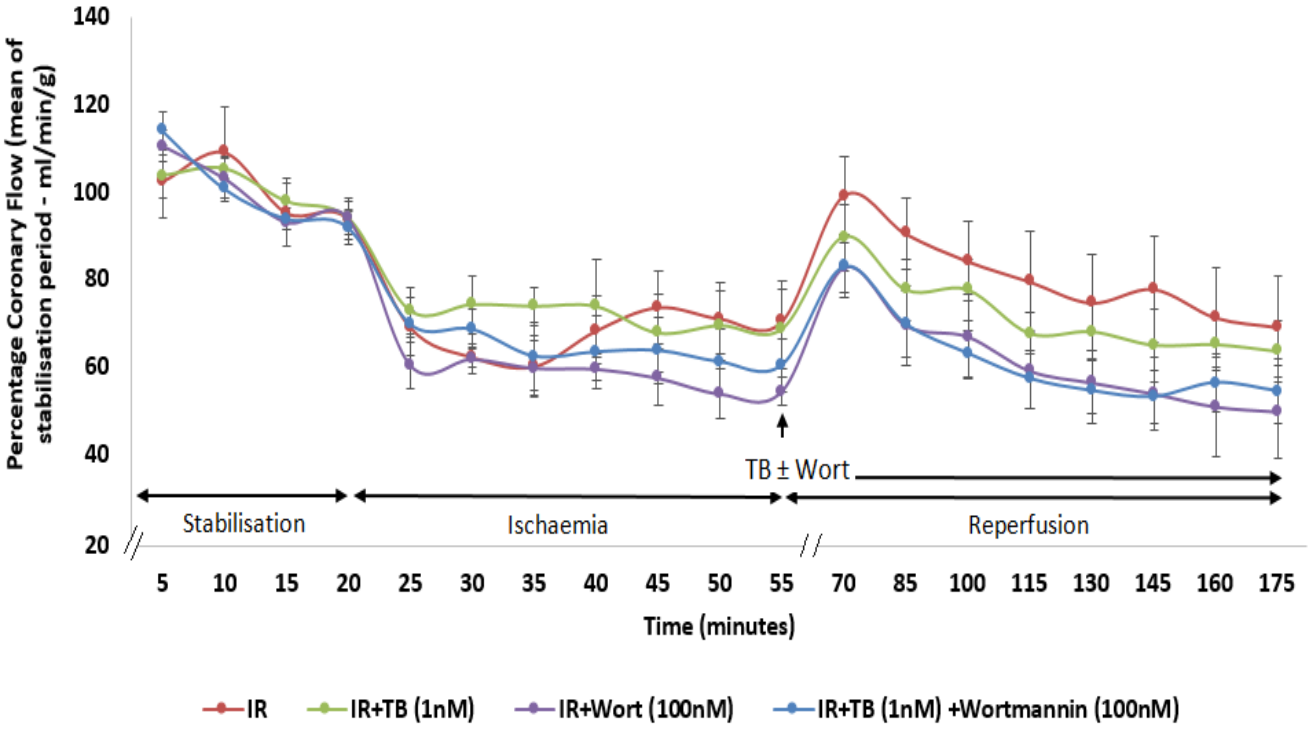

Figure 4.2.1.2.1: Percentage coronary flow of the mean stabilisation period following Tiotropium bromide (TB 1 nM) ± wortmannin (100 nM) in ischaemia/reperfusion. *Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Tiotropium bromide (1 nM) ± wortmannin (100 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups.*

Figure 4.2.1.2.2 shows the left ventricular pressure recorded (LVDP) from the Tiotropium bromide (1 nM) ± wortmannin (100 nM) treatment groups. The data does not show any statistical significances amongst the Tiotropium bromide (1 nM) ± wortmannin (100 nM)

groups at the time points assessed (p>0.05, fig 4.2.1.2.2) between the different groups or with respect to the ischaemia/reperfusion group.

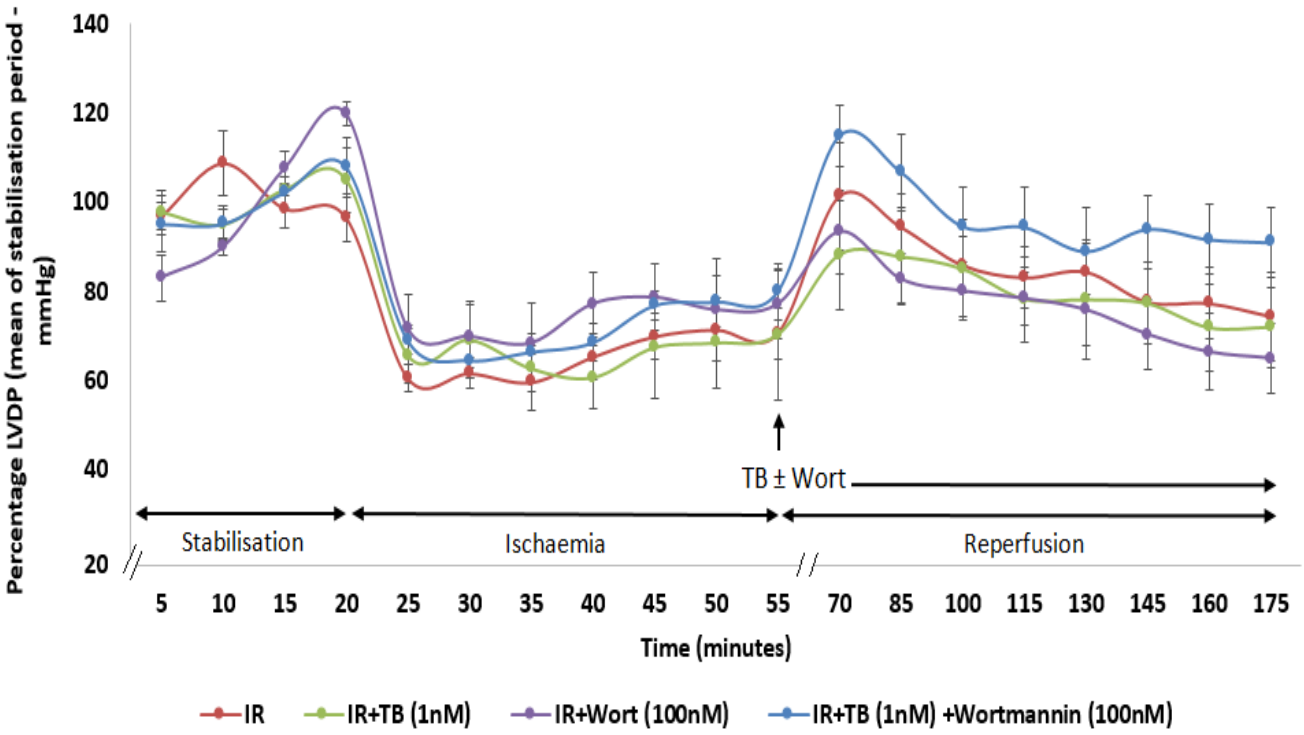

Figure 4.2.1.2.2: Percentage left ventricular developed pressure (LVDP) of the mean stabilisation period following Tiotropium bromide (TB 1 nM) ± wortmannin (100 nM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Tiotropium bromide (1 nM) ± wortmannin (100 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups.*

Figure 4.2.1.2.3 shows heart rate following administration of Tiotropium bromide (1 nM) ± wortmannin (100 nM) following ischaemia/reperfusion. The data shows no statistical significance in heart rate observed with Tiotropium bromide (1 nM) ± wortmannin (100 nM) and the ischaemia/reperfusion control group (p>0.05, fig 4.2.1.2.3).

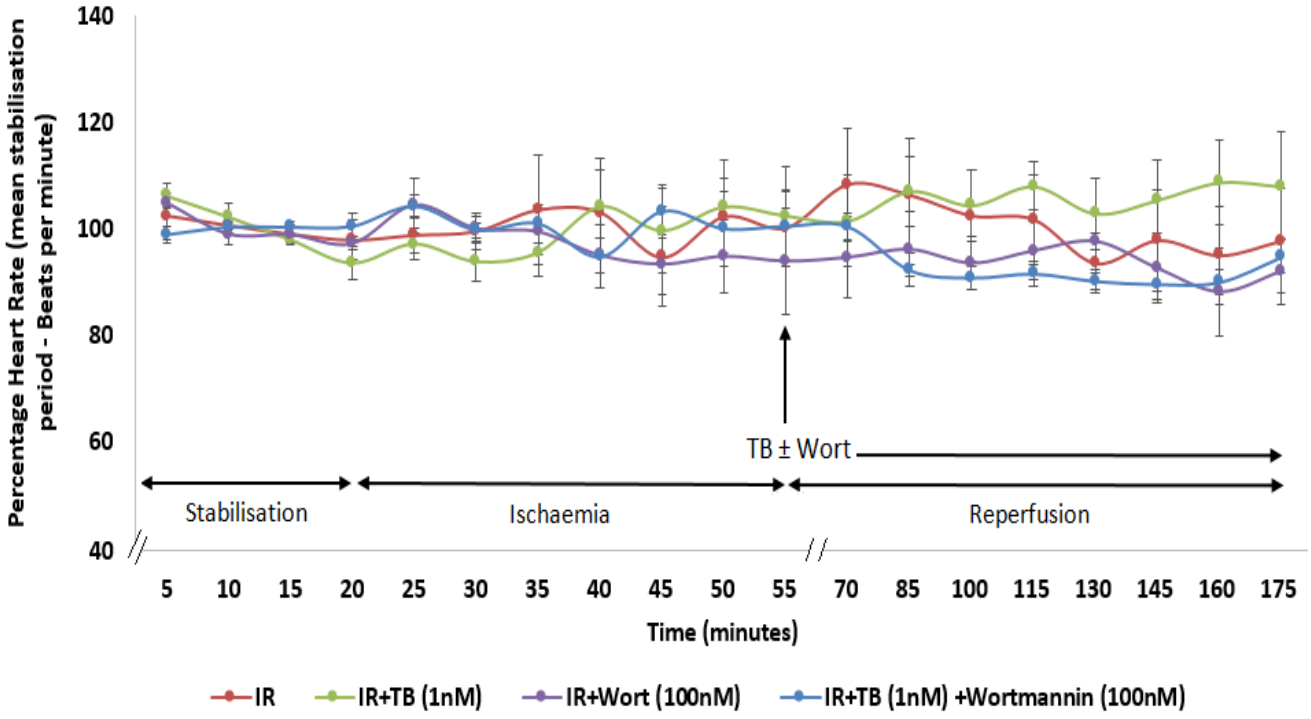

Figure 4.2.1.2.3: Percentage heart rate of the mean stabilisation period following Tiotropium bromide (TB 1 nM) ± wortmannin (100 nM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Tiotropium bromide (1 nM) ± wortmannin (100 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups.*

4.2.2 Infarct size analysis of Tiotropium ± Wortmannin administration

4.2.2.1 Infarct Analysis in Normoxic conditions for Tiotropium ± Wortmannin

This study observed the effects of Tiotropium bromide (1 nM) ± wortmannin (100 nM) administration on infarct size (%), in normoxic conditions. Figure 4.2.2.1.1 shows the infarct size of Tiotropium bromide (1 nM) ± wortmannin (100 nM) with respect to normoxic (Norm) controls. There is a significant difference in infarct size (%) following Tiotropium bromide (1 nM) ± wortmannin (100 nM) administration with respect to the normoxic control. The administration of wortmannin (100 nM) shows no significance in infarct size (%) with respect to the normoxic control ($p>0.05$), however a significant decrease is

observed with respect to the Tiotropium (1 nM) group (9.83 ± 0.75% (Wort 100nM) vs. 18.69 ± 1.79% (TB 1nM), p<0.001, fig 4.2.2.1.1). Wortmannin (100 nM) co-administration with Tiotropium (1 nM) shows no significant difference with respect to the other groups (p>0.05, fig 4.2.2.1.1).

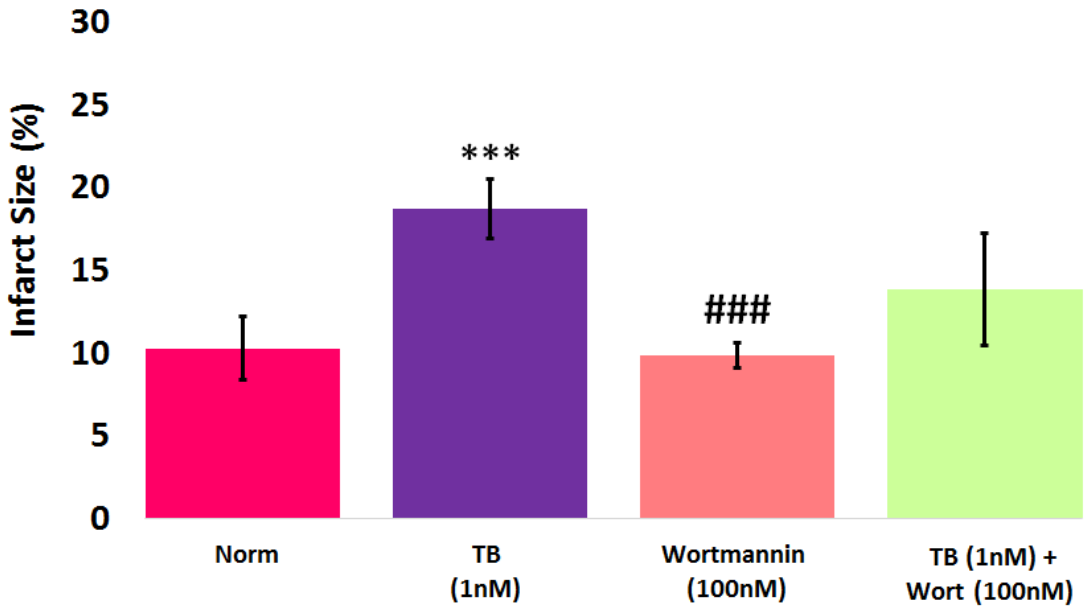

Figure 4.2.2.1.1: Percentage infarct to risk ratios (%) following Tiotropium bromide (1 nM) ± wortmannin (100 nM) in normoxic conditions*. Langendorff hearts were subjected to a 20-minute period of stabilisation with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (1 nM) ± wortmannin (100 nM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups. *** TB (1nM) vs. Norm, p<0.001; ### Wortmannin (100nM) vs. TB (1nM), p<0.001.*

4.2.2.2 Infarct Analysis in Ischaemia/Reperfusion for Tiotropium ± Wortmannin

This study observed the effects of Tiotropium bromide (TB 1 nM) ± wortmannin (Wort 100 nM) administration on infarct to risk ratio (%), in an *in vitro* model of myocardial ischaemia/reperfusion. Figure 4.2.2.2.1 shows the infarct/risk ratios of TB (1 nM) ± Wort (100 nM) with respect to the ischaemia/reperfusion (IR) control. The study showed that there is no significance in infarct size to risk ratio (%) observed upon administration of

wortmannin (100 nM) with or without Tiotropium bromide (1 nM) with respect to the ischaemia/reperfusion control or Tiotropium (1 nM) (50.6 ± 3.83% (Wort 100nM) and 55.94 ± 8.14% (TB 1nM + Wort 100nM) vs. 50.85 ± 3.93 (IR) and 65.42 ± 3.00% (TB 1nM), p>0.05, fig 4.2.2.2.1).

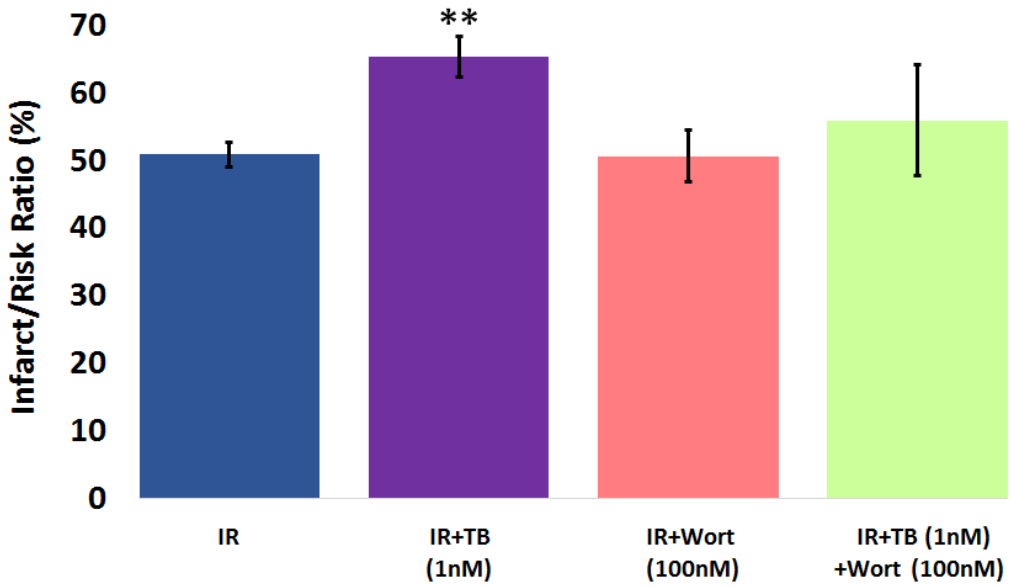

Figure 4.2.2.2.1: Percentage infarct to risk ratios (%) following Tiotropium bromide (1 nM) ± wortmannin (100 nM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Tiotropium bromide (1 nM) ± wortmannin (100 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups. ** vs. IR, p<0.01.*

4.2.3 Effect of Tiotropium bromide administration on Phospho-Akt (Ser473) expression in the presence or absence of Wortmannin

P-Akt (Ser473) activation was assessed using western blotting for phosphorylated Akt (Ser473) in cardiac tissue to establish the role of Akt in Tiotropium bromide (1 nM) ± wortmannin (100 nM) mediated cardiotoxicity. Figure 4.2.3.1 shows the expression pattern observed following normoxia and Tiotropium bromide (1 nM) ± wortmannin (100 nM).

Wortmannin (100 nM) administration blocks p-Akt $_{(Ser473)}$ phosphorylation with respect to the normoxic control and Tiotropium (1 nM) mediated increase (4.26 ± 0.78% (Wort 100nM) vs. 36.17 ± 5.13% (Norm) and 88.03 ± 5.62% (TB 1nM), $p<0.05$ and $p<0.0001$ respectively, fig 4.2.3.1). Tiotropium (1 nM) mediated p-Akt $_{(Ser473)}$ upregulation is attenuated upon co-administration of wortmannin (100 nM) with respect to Tiotropium (1 nM) alone (15.09 ± 3.06% (TB 1nM + Wort 100nM) vs. 88.03 ± 5.62% (TB 1nM), $p<0.0001$, fig 4.2.3.1).

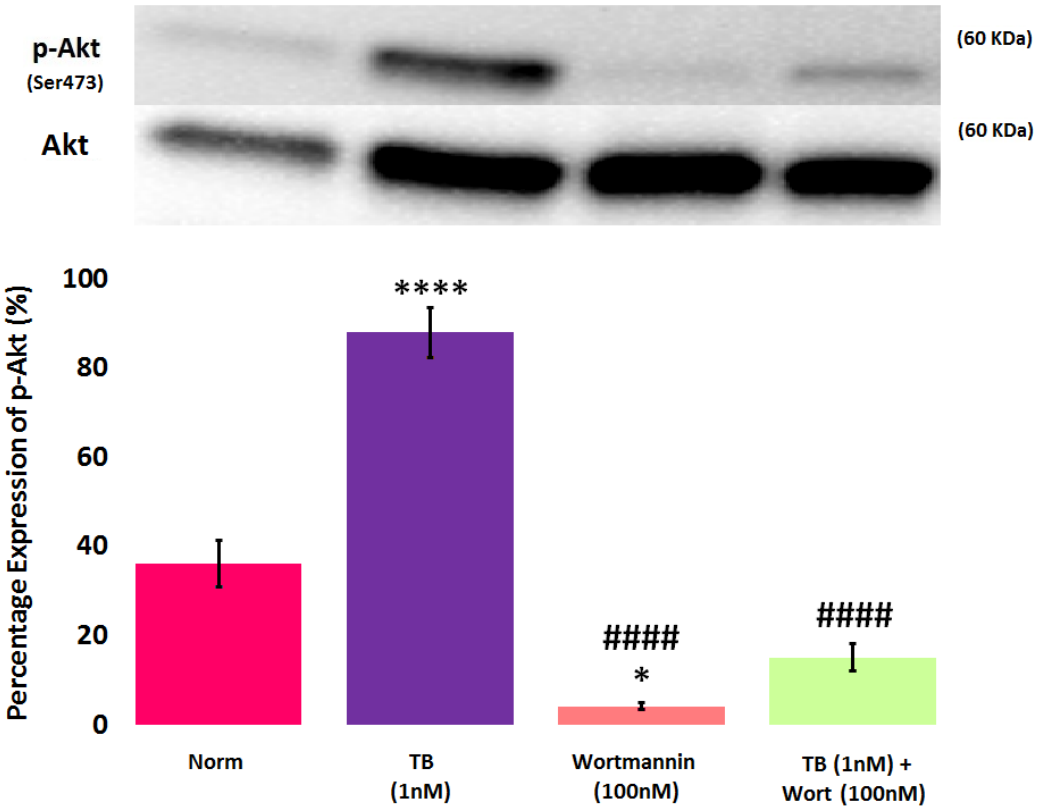

Figure 4.2.3.1: Percentage expression (%) of phosphorylated Akt $_{(Ser473)}$ as a percentage of total Akt following Tiotropium bromide (1 nM) ± wortmannin (100 nM) in normoxic conditions*. Langendorff hearts were perfused with either Krebs-Henseleit buffer (Norm) or Tiotropium bromide (1 nM) ± wortmannin (100 nM). Values plotted signify the Mean ± SEM, derived from an n of 3 for all groups. * vs. Norm, $p<0.05$; **** vs. Norm, $p<0.0001$ and #### vs. TB (1nM), $p<0.0001$.*

4.2.4 Role of Wortmannin in Tiotropium bromide mediated apoptotic cell death

Cardiomyocytes were incubated with Tiotropium bromide (1 nM) ± wortmannin (100 nM) and the normoxic controls respectively for a period of 4 hours before staining with Annexin-V and Propidium iodide (PI), and analysed on the flow cytometer using the FL-1 and FL-2 channels. Propidium iodide was used as a counter stain to differentiate between necrotic and dead apoptotic cells.

Figure 4.2.4.1 shows the percentage of total apoptosis in cardiomyocyte treated with Tiotropium bromide (1 nM) ± wortmannin (100 nM) in normoxic conditions. Tiotropium bromide (1 nM) shows an observable but non-significant increase in total apoptosis with respect to the normoxic control (117.2 ± 8.85% (TB 1 nM) vs. 100 ± 0.0% (normoxia), $p>0.05$, fig 4.2.4.1). However, wortmannin (100 nM) administration shows a decrease in total apoptosis with respect to Tiotropium (1 nM) (88.21 ± 12.64% (Wort 100nM) vs. 117.2 ± 8.85% (TB 1 nM), $p<0.05$, fig 4.2.4.1). The effect of wortmannin (100 nM) when co-administered with Tiotropium (1 nM) reverses the apoptotic effect of Tiotropium (1 nM) alone (85.93 ± 11.37% (TB 1nM + Wort 100nM) vs. 117.2 ± 8.85% (TB 1 nM), $p<0.05$, fig 4.2.4.1), but shows no significant difference to the other groups ($p>0.05$, fig 4.2.4.1). Data for necrosis did not show any significant difference between any of the groups ($p>0.05$, data not shown).

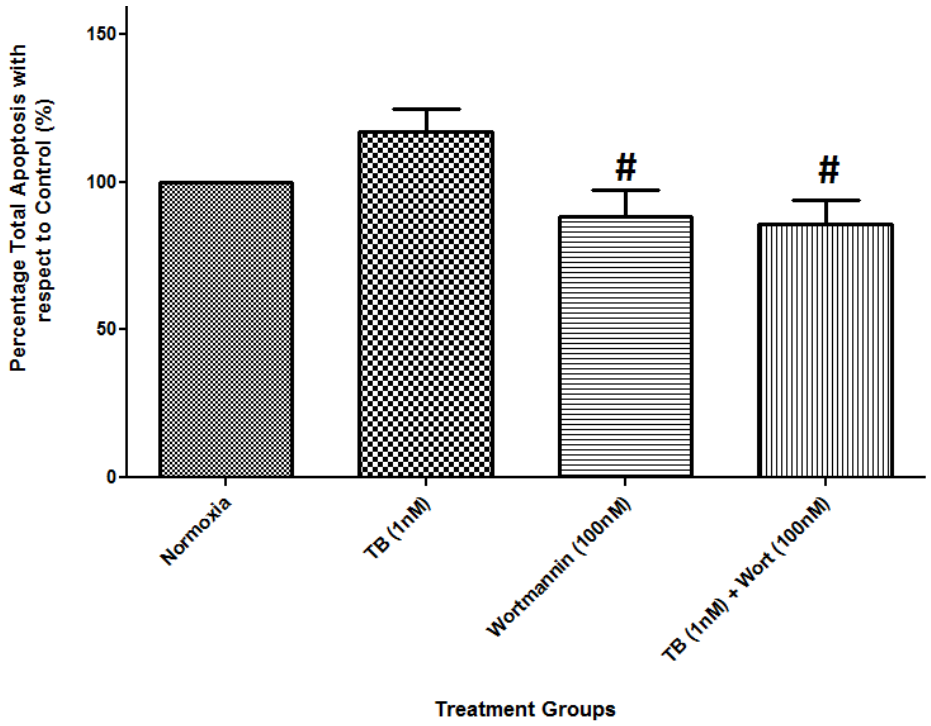

Figure 4.2.4.1: Percentage of total apoptotic cardiomyocytes (%) following treatment with Tiotropium bromide (1 nM) ± wortmannin (100 nM) in normoxic conditions, expressed as a percentage of the normoxia control*. Cardiomyocytes were treated for 4 hours in normoxic conditions with Tiotropium bromide (1 nM) ± wortmannin (100 nM), apart from the normoxia control group, before staining with Annexin-V/Propidium iodide and analysed using the FL-1 and FL-2 channels of the flow cytometer. Data is presented as the Mean ± SEM, n of 4. # vs. TB (1nM), p<0.05.*

4.3 Effect of the caspase-3 inhibitor, Z-DEVD-FMK in the presence or absence of Tiotropium bromide in Normoxia and Ischaemia/Reperfusion whole heart models

4.3.1 Haemodynamic Data Analysis

4.3.1.1 Haemodynamic in Normoxic conditions for Tiotropium ± Z-DEVD-FMK

Tiotropium bromide (1nM) ± Z-DEVD-FMK (140nM) was administered following a 20-minute stabilisation period and perfused throughout the rest of the experimental protocol

for a period of 155 minutes, as described in section 2.3.3.1. The data for all parameters (fig 4.3.1.1.1 – 4.2.1.1.3) following Tiotropium bromide (1nM) ± Z-DEVD-FMK (140nM) were analysed at 15, 25, 50, 70 and 160 minutes. Tiotropium bromide (1nM) ± Z-DEVD-FMK (140nM) was compared to the normoxic control and each concentration.

The coronary flow (fig 4.3.1.1.1) data shows that the administration of Z-DEVD-FMK (140 nM) show a significant increase in coronary flow with respect to normoxic control, at 25, 50 and 70 minutes (25 minutes: 109.64 ± 6.69% (Z-DEVD 140nM) vs. 93.03 ± 2.16% (Norm); 50 minutes: 108.31 ± 8.17% (Z-DEVD 140nM) vs. 87.57 ± 2.24% (Norm); 70 minutes: 109.31 ± 5.90% (Z-DEVD 140nM) vs. 87.68 ± 4.23% (Norm), $p<0.05$ respectively, fig 4.3.1.1.1).

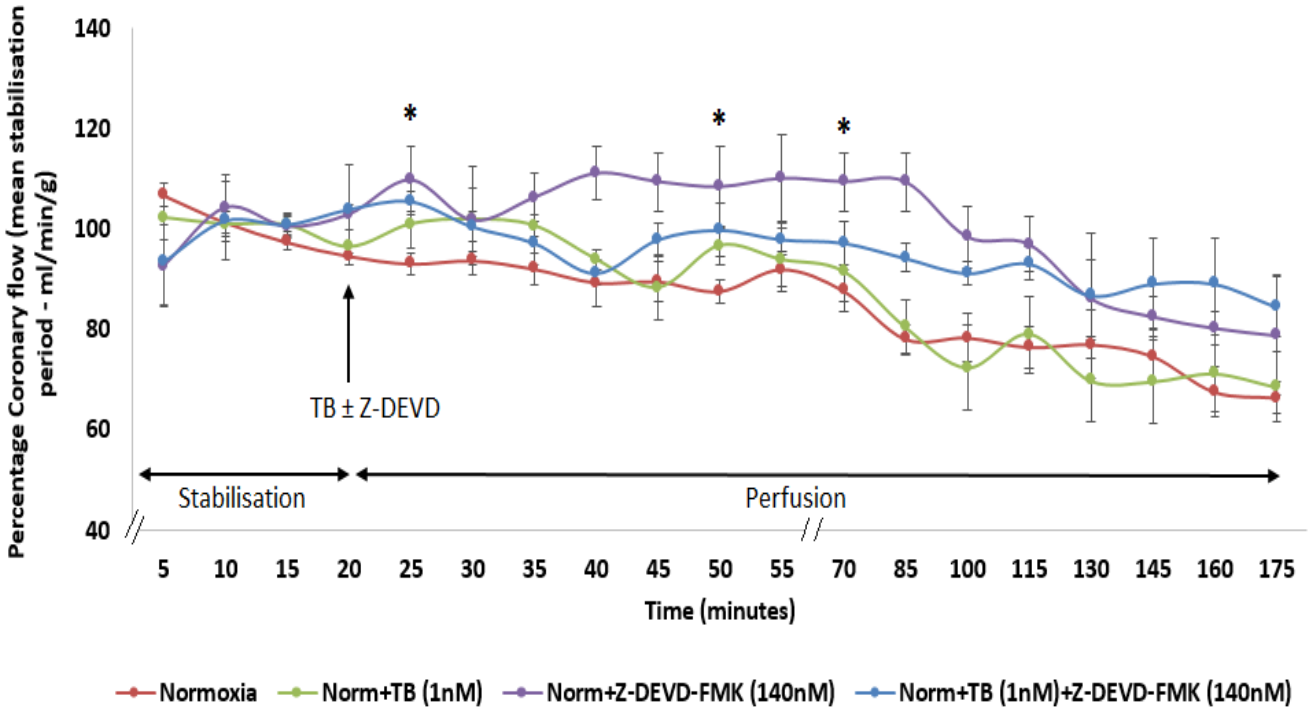

Figure 4.3.1.1.1: Percentage coronary flow of the mean stabilisation period following administration with Tiotropium bromide (1nM) ± Z-DEVD-FMK (140nM). *Langendorff*

*hearts were subjected to a 20-minute period of stabilisation perfused with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (1nM) ± Z-DEVD-FMK (140nM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 3-5 for all groups. * Z-DEVD-FMK (140nM) vs. Norm, p<0.05.*

Figure 4.3.1.1.2 shows left ventricular developed pressure (LVDP) following Tiotropium bromide (1nM) ± Z-DEVD-FMK (140nM) administration in normoxic conditions. The data does not show any statistical significances amongst the Tiotropium bromide (1nM) ± Z-DEVD-FMK (140nM) groups at the time points assessed (p>0.05, fig 4.3.1.1.2) between the groups or with respect to the normoxic control.

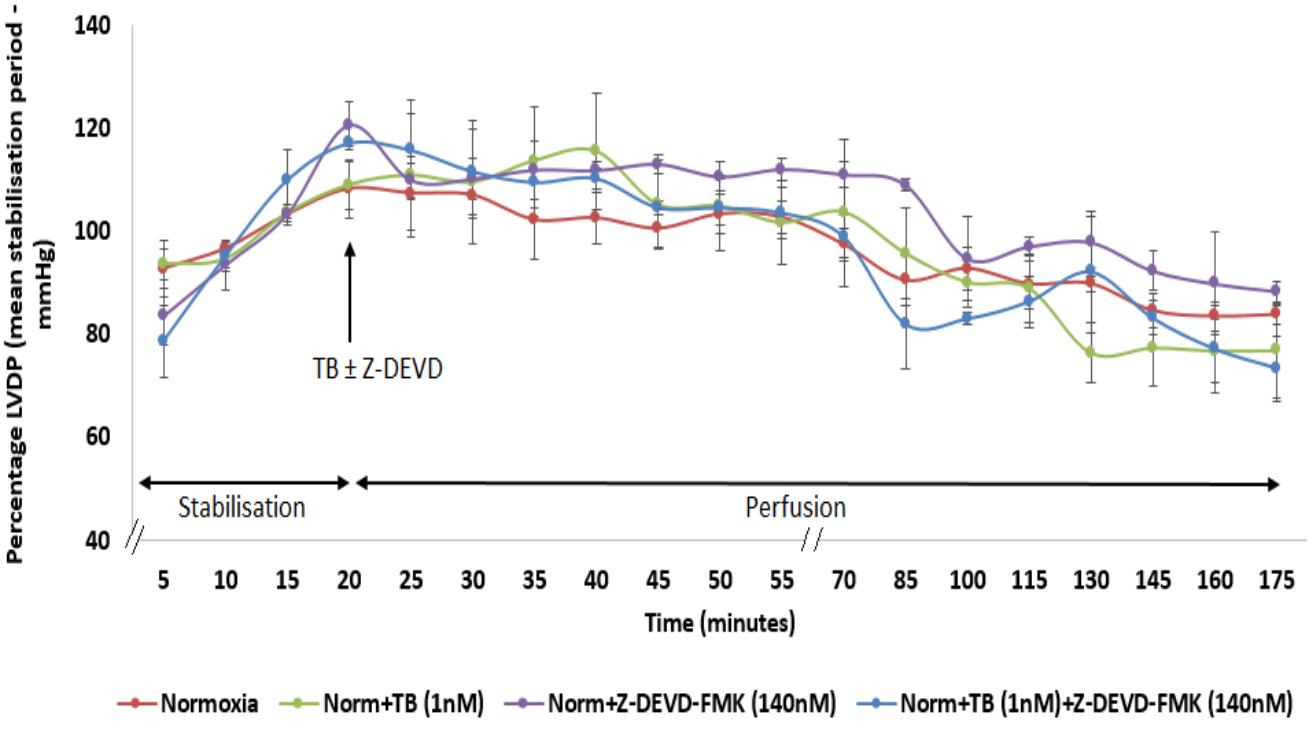

Figure 4.3.1.1.2: Percentage left ventricular developed pressure (LVDP) of the mean stabilisation period following administration with Tiotropium bromide (1nM) ± Z-DEVD-FMK (140nM)*. Langendorff hearts were subjected to a 20-minute period of stabilisation perfused with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (1nM) ± Z-DEVD-FMK (140nM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 3-5 for all groups.*

Figure 4.3.1.1.3 shows heart rate following administration of Tiotropium bromide (1nM) ± Z-DEVD-FMK (140nM) in normoxic conditions. The data shows no statistical significance in heart rate observed with Tiotropium bromide (1nM) ± Z-DEVD-FMK (140nM) and the normoxic control group ($p>0.05$, fig 4.3.1.1.3).

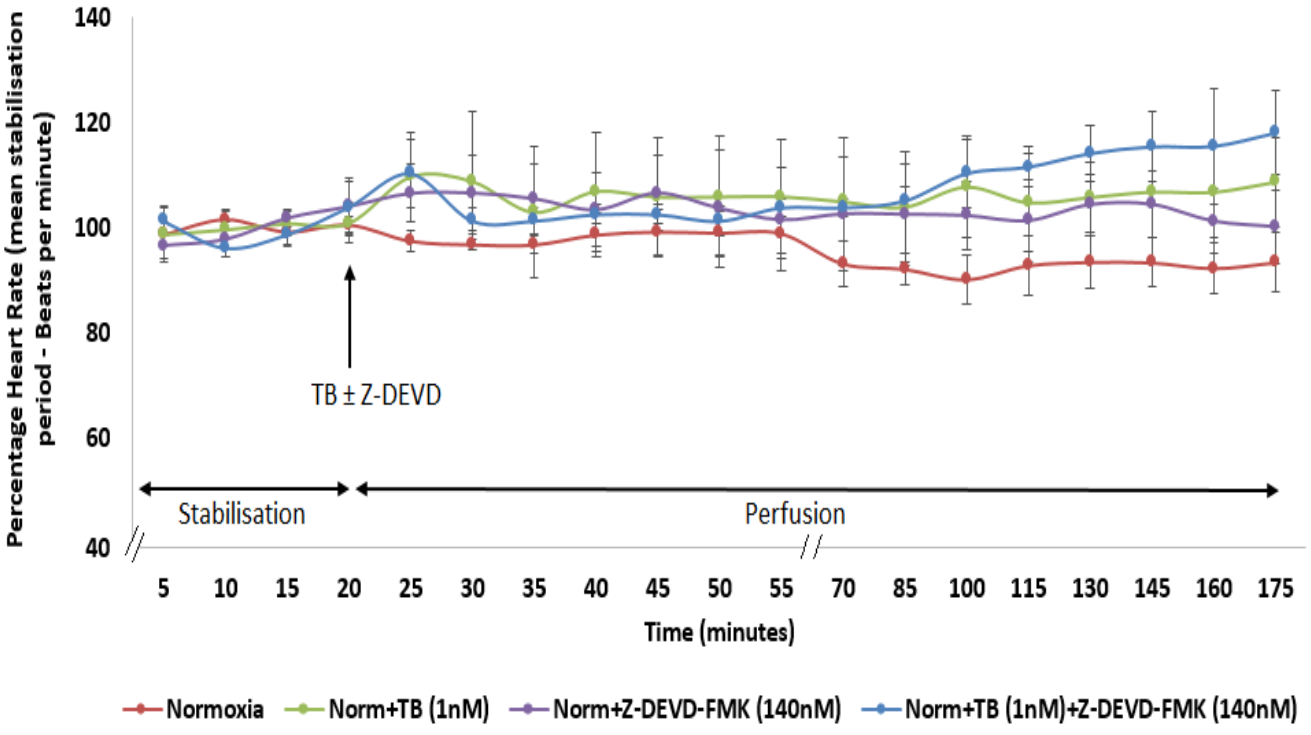

Figure 4.3.1.1.3: Percentage heart rate of the mean stabilisation period following administration with Tiotropium bromide (1nM) ± Z-DEVD-FMK (140nM). *Langendorff hearts were subjected to a 20-minute period of stabilisation perfused with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (1nM) ± Z-DEVD-FMK (140nM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 3-5 for all groups.*

4.3.1.2 Haemodynamic in Ischaemia/Reperfusion for Tiotropium ± Z-DEVD-FMK

The effect of Tiotropium bromide (1 nM) administration in the presence or absence of Z-DEVD-FMK (70 nM), was assessed in conditions of myocardial ischaemia/reperfusion injury with 20 minutes of stabilisation preceding 35 minutes of regional ischaemia followed by 120 minutes of reperfusion; drug administration lasted the duration of reperfusion, as described in section 2.3.3.2.

Coronary flow (fig 4.3.1.2.1), left ventricular developed pressure (LVDP, fig 4.3.1.2.2) and heart rate (fig 4.3.1.2.2.3) are shown for Tiotropium bromide (1 nM) ± Z-DEVD-FMK (70 nM) administration in conditions of ischaemia/reperfusion as well as the ischaemia/reperfusion control group. The data collected for all parameters (fig 4.3.1.2.1 – 4.3.1.2.3) following Tiotropium bromide (1 nM) ± Z-DEVD-FMK (70 nM) administration in ischaemia/reperfusion were statistically analysed at 15, 25, 50, 70 and 160 minutes.

Figure 4.3.1.2.1 for coronary flow shows no statistical significance at the onset of reperfusion with Tiotropium bromide (1 nM) ± Z-DEVD-FMK (70 nM) ($p>0.05$) between each group and also with respect to the ischaemia/reperfusion control.

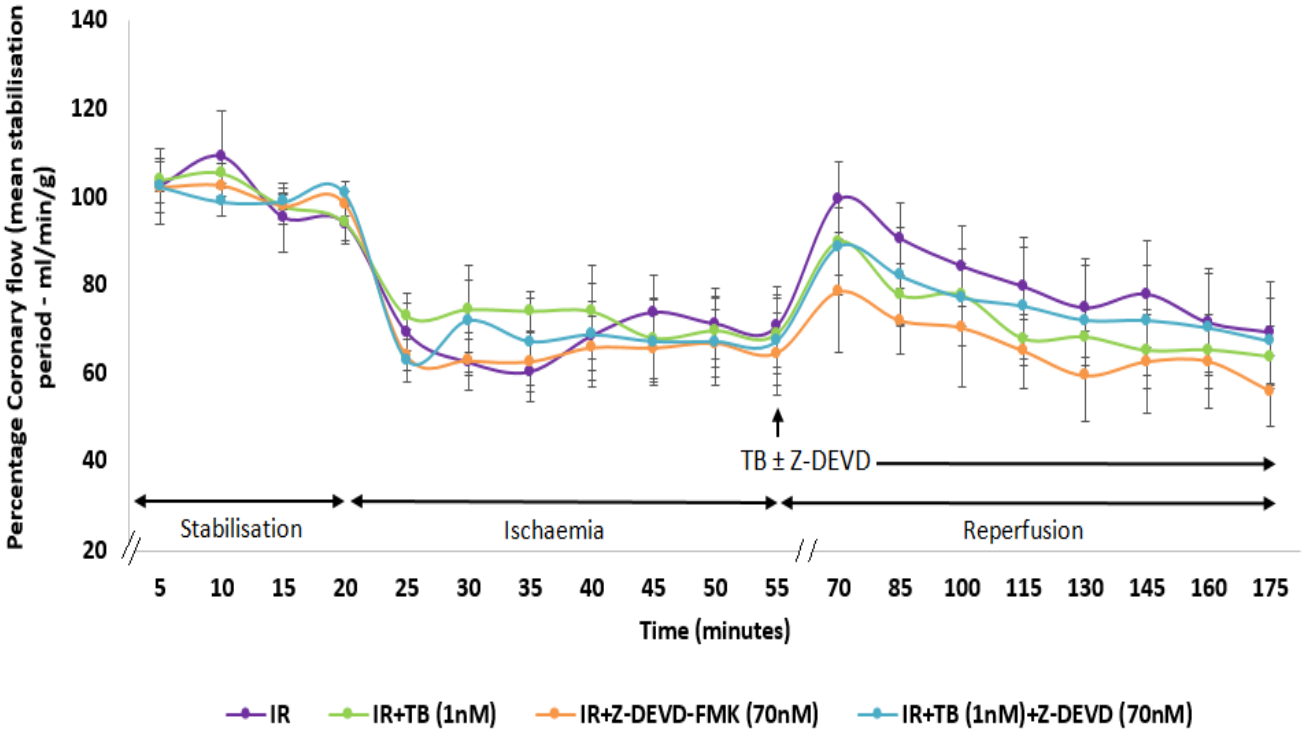

Figure 4.3.1.2.1: Percentage coronary flow of the mean stabilisation period following Tiotropium bromide (TB 1 nM) ± Z-DEVD-FMK (70 nM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Tiotropium bromide (TB 1 nM) ± Z-DEVD-FMK (70 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups.*

Figure 4.3.1.2.2 shows the left ventricular pressure recorded (LVDP) from the Tiotropium bromide (1 nM) ± Z-DEVD-FMK (70 nM) treatment groups. The data does not show any statistical significances amongst the Tiotropium bromide (1 nM) ± Z-DEVD-FMK (70 nM) groups at the time points assessed ($p>0.05$, fig 4.3.1.2.2) between the different groups or with respect to the ischaemia/reperfusion group.

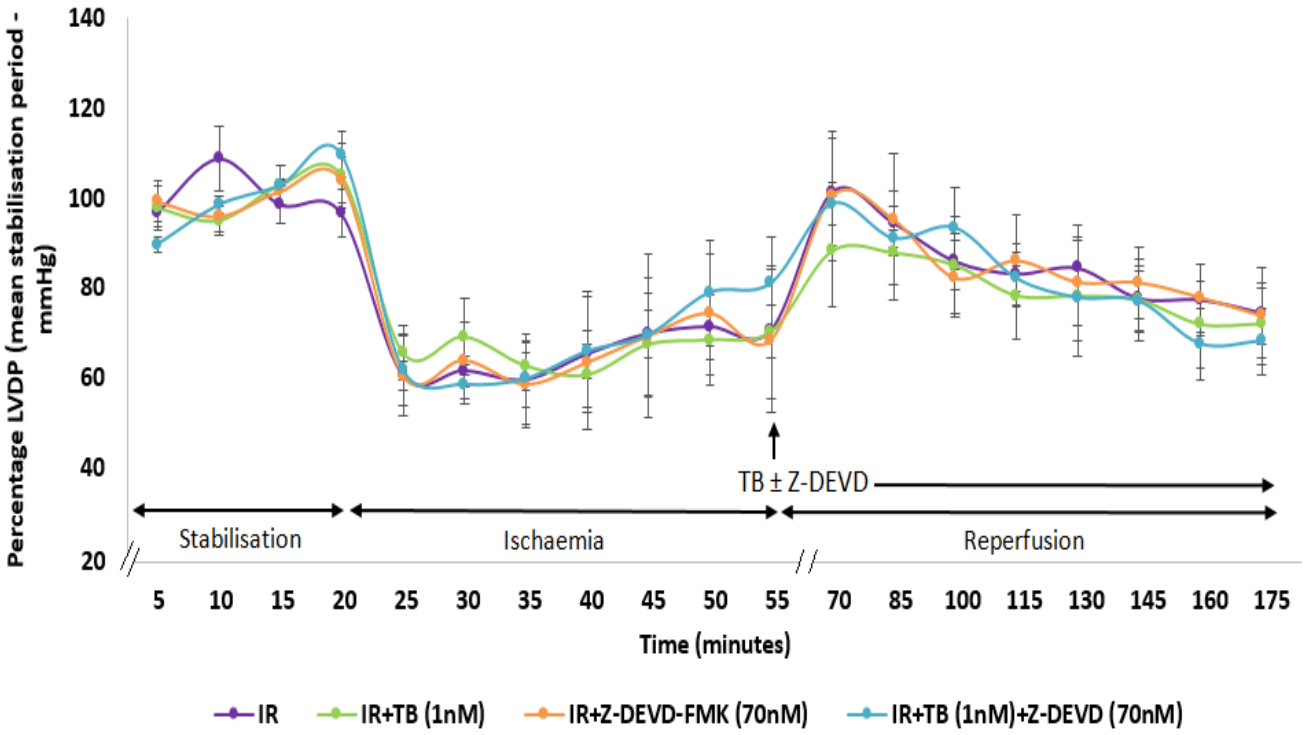

Figure 4.3.1.2.2: Percentage left ventricular developed pressure (LVDP) of the mean stabilisation period following Tiotropium bromide (TB 1 nM) ± Z-DEVD-FMK (70 nM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Tiotropium bromide (TB 1 nM) ± Z-DEVD-FMK (70 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups.*

Figure 4.3.1.2.3 shows heart rate following administration of Tiotropium bromide (1 nM) ± Z-DEVD-FMK (70 nM) following ischaemia/reperfusion. The data shows no statistical

significance in heart rate observed with Tiotropium bromide (1 nM) ± Z-DEVD-FMK (70 nM) and the ischaemia/reperfusion control group (p>0.05, fig 4.3.1.2.3).

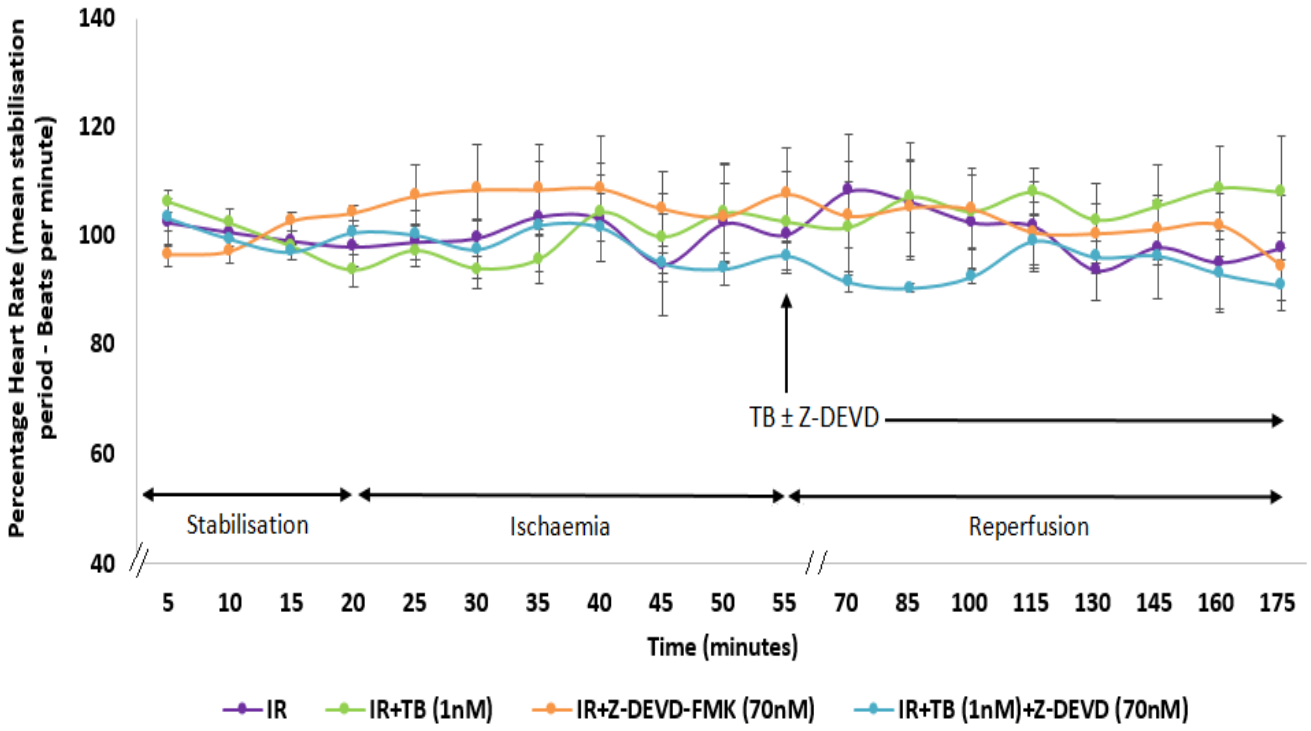

Figure 4.3.1.2.3: Percentage heart rate of the mean stabilisation period following Tiotropium bromide (TB 1 nM) ± Z-DEVD-FMK (70 nM) in ischaemia/reperfusion. *Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Tiotropium bromide (TB 1 nM) ± Z-DEVD-FMK (70 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups.*

4.3.2 Infarct size analysis of Tiotropium ± Z-DEVD-FMK administration

4.3.2.1 Infarct Analysis in Normoxic conditions for Tiotropium ± Z-DEVD-FMK

This study observed the effects of Tiotropium bromide (1 nM) ± Z-DEVD-FMK (140 nM) administration on infarct size (%), in normoxic conditions. Tiotropium bromide (1 nM) ± Z-DEVD-FMK (140 nM) was administered following 20 minutes of stabilisation and continued for 155 minute; hearts were subjected to TTC staining to determine infarct size.

Figure 4.3.2.1.1 shows the infarct size of Tiotropium bromide (1 nM) ± wortmannin (100 nM) with respect to normoxic (Norm) controls. The study shows that there is a significant difference in infarct size following Tiotropium bromide (1 nM) ± Z-DEVD-FMK (140 nM) administration with respect to the normoxic control. The administration of Z-DEVD-FMK (140 nM) shows a significant decrease in infarct size (%) with respect to the Tiotropium (1 nM) group (8.66 ± 2.84% (Z-DEVD-FMK 140nM) vs. 18.69 ± 1.79% (TB 1nM), p<0.001, fig 4.3.2.1.1). Z-DEVD-FMK (140 nM) co-administration with Tiotropium (1 nM) sustains the significant decrease in infarct size with respect to Tiotropium (1 nM) (8.74 ± 2.41% (TB 1nM + Z-DEVD 140nM) vs. 18.69 ± 1.79% (TB 1nM), p<0.001, fig 4.3.2.1.1).

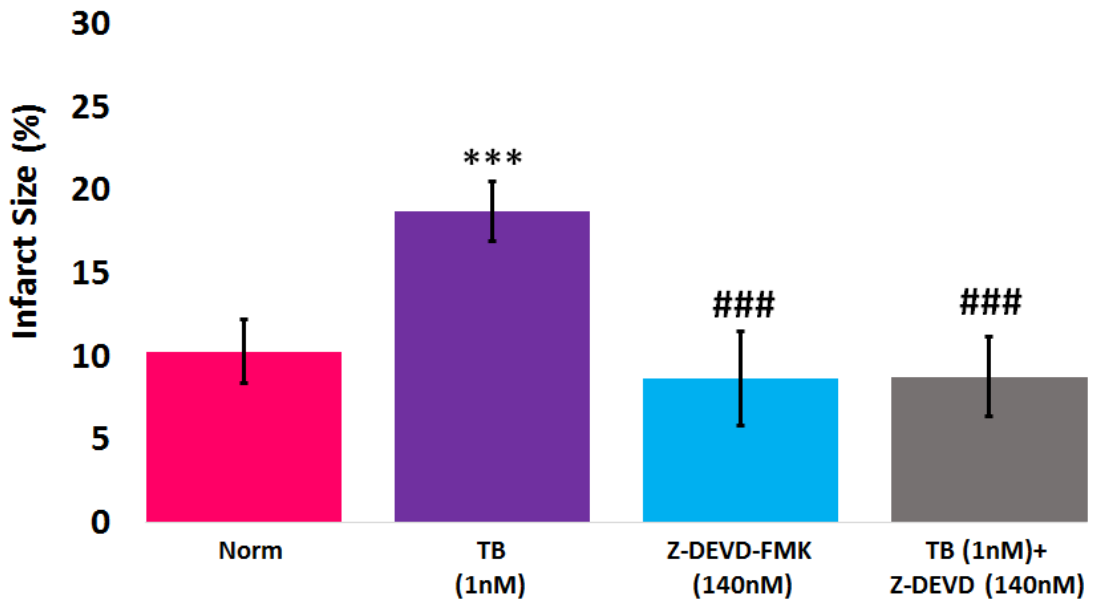

Figure 4.3.2.1.1: Percentage infarct to risk ratios (%) following Tiotropium bromide (1 nM) ± Z-DEVD-FMK (140 nM) in normoxic conditions*. Langendorff hearts were subjected to a 20-minute period of stabilisation with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (1 nM) ± Z-DEVD-FMK (140 nM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups. *** TB (1nM) vs. Norm, p<0.001; ### Z-DEVD-FMK (140nM) and TB (1nM) + Z-DEVD (140nM) vs. TB (1nM), p<0.001.*

4.3.2.2 Infarct Analysis in Ischaemia/Reperfusion for Tiotropium ± Z-DEVD-FMK

This study observed the effects of Tiotropium bromide (1 nM) ± Z-DEVD-FMK (70 nM) administration on infarct to risk ratio (%), in an *in vitro* model of myocardial ischaemia/reperfusion. Figure 4.3.2.2.1 shows the infarct/risk ratios of Tiotropium bromide (1 nM) ± Z-DEVD-FMK (70 nM) with respect to the ischaemia/reperfusion (IR) control. Administration of Z-DEVD-FMK (70 nM) shows a reversal in the infarct to risk ratio observed with ischaemia/reperfusion and Tiotropium (1 nM) alone (19.87 ± 3.63% (Z-DEVD-FMK 70nM) vs. 50.85 ± 3.93% (IR) and 65.42 ± 3.00% (TB 1nM), $p<0.001$ respectively, fig 4.3.2.2.1). Tiotropium bromide (1 nM) co-administered with Z-DEVD-FMK (70 nM) shows an abrogated infarct to risk ratio with respect to Tiotropium (1 nM) alone (43.70 ± 1.79% (TB 1nM + Z-DEVD 70nM) vs. 65.42 ± 3.00% (TB 1nM), $p<0.01$, fig 4.3.2.2.1); however the Tiotropium (1 nM) + Z-DEVD-FMK (70 nM) group shows an increase in infarct to risk ratio with respect to Z-DEVD-FMK (70 nM) alone (43.70 ± 1.79% (TB 1nM + Z-DEVD 70nM) vs. 19.87 ± 3.63% (Z-DEVD-FMK 70nM), $p<0.001$, fig 4.3.2.2.1).

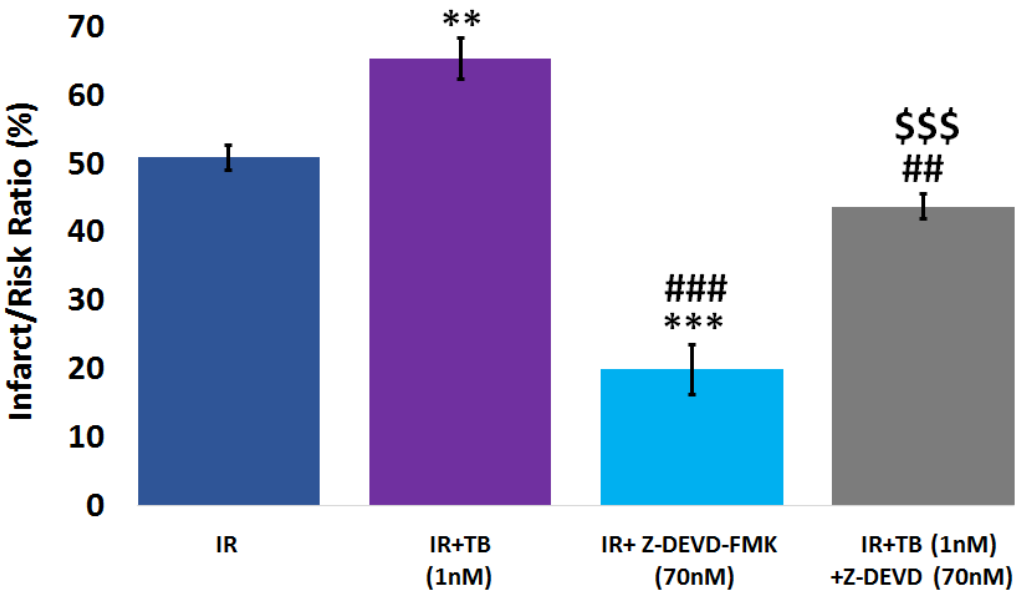

Figure 4.3.2.2.1: Percentage infarct to risk ratios (%) following Tiotropium bromide (1 nM) ± Z-DEVD-FMK (70 nM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Tiotropium bromide (1 nM) ± Z-DEVD-FMK (70 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups. ** vs. IR, p<0.01; *** vs. IR, p<0.001, ## vs. IR+TB (1nM), p<0.01; ### vs. IR+TB (1nM), p<0.001 and $$ vs. IR+Z-DEVD-FMK (70nM), p<0.01.*

4.3.3 Role of Z-DEVD-FMK in Tiotropium bromide mediated apoptotic cell death

Cardiomyocytes were incubated with Tiotropium bromide (1 nM) ± Z-DEVD-FMK (70 nM) and the normoxic controls respectively for a period of 4 hours before staining with Annexin-V and Propidium iodide (PI), and analysed on the flow cytometer using the FL-1 and FL-2 channels. Propidium iodide was used as a counter stain to differentiate between necrotic and dead apoptotic cells.

Figure 4.3.3.1 shows the percentage of total apoptosis in cardiomyocyte treated with Tiotropium bromide (1 nM) ± Z-DEVD-FMK (70 nM) in normoxic conditions. Tiotropium bromide (1 nM) shows a non-significant increase in total apoptosis with respect to the normoxic control (117.2 ± 8.85% (TB 1 nM) vs. 100 ± 0.0% (normoxia), p>0.05, fig 4.3.3.1).

Z-DEVD-FMK (70 nM) administration shows a decrease in total apoptosis with respect to Tiotropium bromide (1 nM) and the normoxic control (75.7 ± 6.72% (Z-DEVD-FMK 70nM) vs. 117.2 ± 8.85% (TB 1nM) and 100 ± 0.0% (Norm), $p<0.001$ and $p<0.01$ respectively, fig 4.3.3.1). Co-administration of Z-DEVD-FMK (70 nM) with Tiotropium bromide (1 nM) suppresses the effect of Tiotropium bromide (1 nM) alone (79.53 ± 4.92% (TB 1nM + Z-DEVD 70nM) vs. 117.2 ± 8.85% (TB 1 nM), $p<0.001$, fig 4.3.3.1), and shows a significant decrease with respect to the normoxic control (79.53 ± 4.92% (TB 1nM + Z-DEVD 70nM) vs. 100 ± 0.0% (Norm), $p<0.05$, fig 4.3.3.1). Data for necrosis did not show any significant difference between any of the groups ($p>0.05$, data not shown).

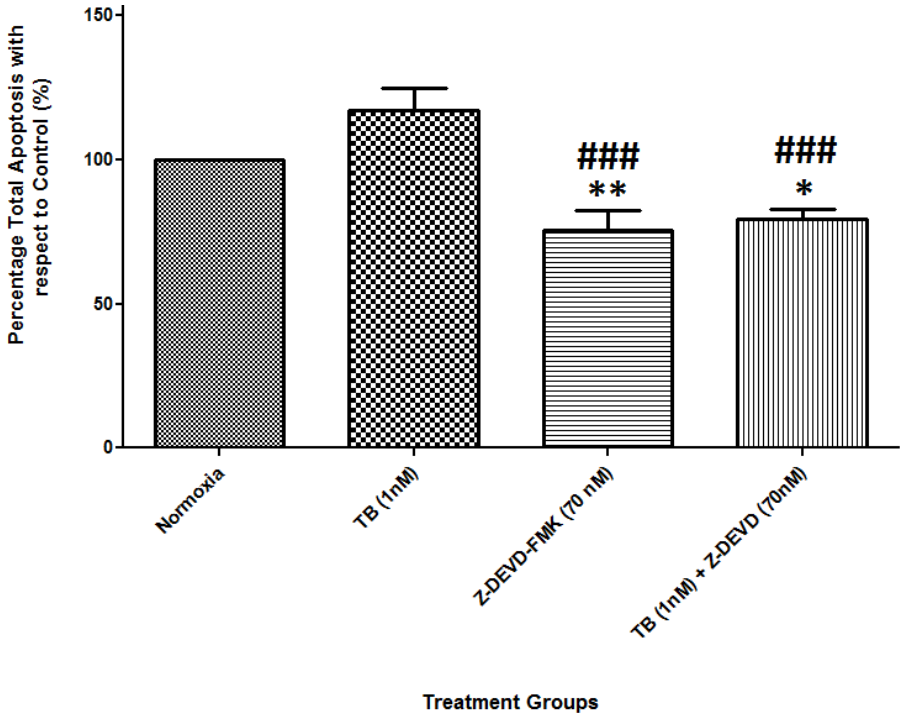

Figure 4.3.3.1: Percentage of total apoptotic cardiomyocytes (%) following treatment with Tiotropium bromide (1 nM) ± Z-DEVD-FMK (70 nM) in normoxic conditions, expressed as a percentage of the normoxia control*. Cardiomyocytes were treated for 4 hours in normoxic conditions with Tiotropium bromide (1 nM) ± Z-DEVD-FMK (70 nM), apart from the normoxia control group, before staining with Annexin-V/Propidium iodide and analysed using the FL-1 and FL-2 channels of the flow cytometer. Data is presented as the Mean ± SEM, n of 3-4. * vs. Normoxia, p<0.05; ** vs. Normoxia, p<0.01; ### vs. TB (1 nM), p<0.001.*

4.3.4 The effect of Tiotropium bromide on Caspase-3 activity

Cardiomyocytes were incubated with acetylcholine (100 nM), Tiotropium (1 nM) ± Z-DEVD (70 nM) or control for 4 hours before methanol fixation prior to staining with anti-cleaved caspase-3 $_{(Asp175)}$ and analysed on the flow cytometer using the FL-1 channel.

Figure 4.3.4.1 shows the percentage of cleaved caspase-3 $_{(Asp175)}$ in cardiomyocyte treated with Tiotropium bromide (1 nM) ± Z-DEVD-FMK (70 nM) or acetylcholine (100 nM) in normoxic conditions. Tiotropium bromide (1 nM) significantly increases cleaved caspase-3 $_{(Asp175)}$ compared to control (195.0 ± 30.38% (TB 1nM) vs. 100 ± 0.0% (Control), $p<0.01$, fig 4.3.4.1). Acetylcholine (100 nM) shows no significance with respect to the control ($p>0.05$, fig 4.3.4.1), but shows a significant decrease in cleaved caspase-3 with respect to Tiotropium bromide (1 nM) (83.14 ± 17.38% (ACh 100nM) vs. 195.0 ± 30.38% (TB 1nM), $p<0.01$, fig 4.3.3.1). Z-DEVD-FMK (70 nM) significantly inhibits cleaved caspase-3 $_{(Asp175)}$ compared to the control and Tiotropium bromide (1 nM) (11.65 ± 6.08% (Z-DEVD-FMK 70nM) vs. 100 ± 0.0% (Control) and 195.0 ± 30.38% (TB 1nM), $p<0.05$ and $p<0.001$ respectively, fig 4.3.4.1). Co-administration of Tiotropium bromide (1 nM) + Z-DEVD-FMK (70 nM) sustains inhibition compared to Tiotropium bromide (1 nM) (46.43 ± 4.38% (TB 1nM + Z-DEVD 70nM) vs. 195.0 ± 30.38% (TB 1nM), $p<0.001$, fig 4.3.4.1).

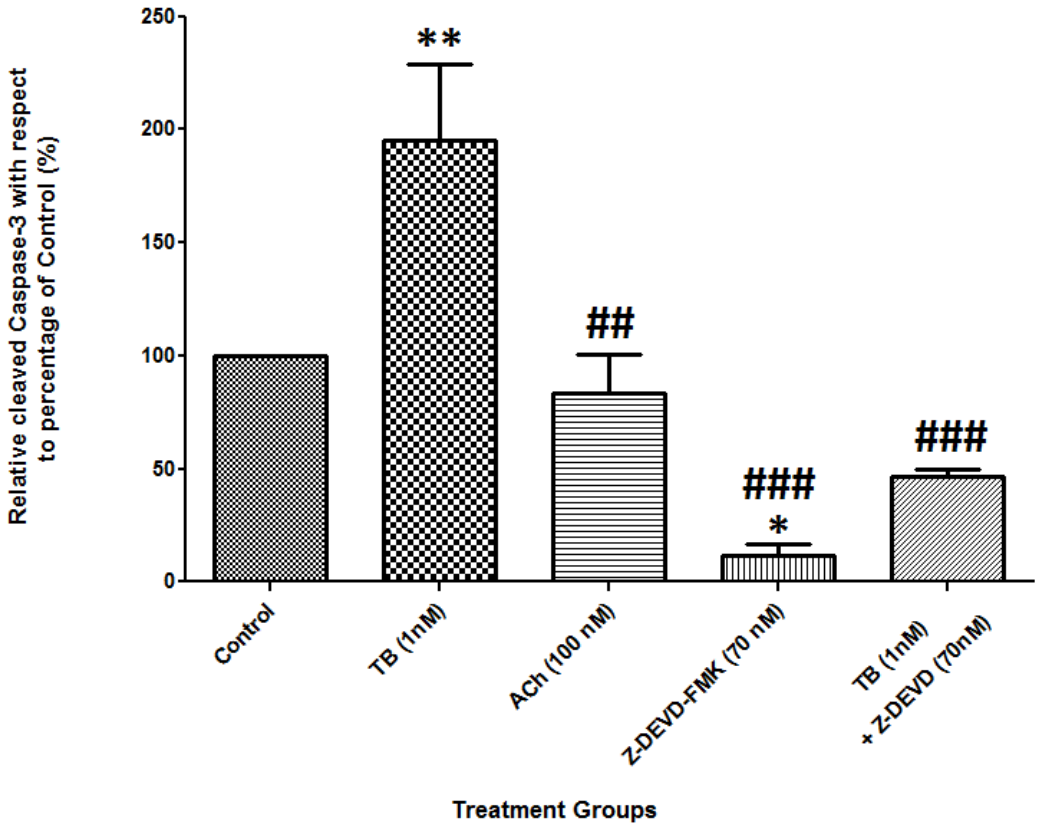

Figure 4.3.4.1: Percentage of cleaved caspase-3 $_{(Asp175)}$ in cardiomyocytes (%) following treatment with Tiotropium bromide (1 nM) ± Z-DEVD-FMK (70 nM) or acetylcholine (ACh 100 nM) expressed as a percentage of the normoxia control*. Cardiomyocytes were treated for 4 hours in normoxic conditions with Tiotropium bromide (1 nM) ± Z-DEVD-FMK (70 nM) or acetylcholine (ACh 100 nM), apart from the normoxia control group. Data is presented as the Mean ± SEM, n of 3-6. * vs. Normoxia, p<0.05; ** vs. Normoxia, p<0.01; ## vs. TB (1 nM), p<0.01; ### vs. TB (1 nM), p<0.001.*

4.4 Effect of mPTP inhibitor, Cyclosporin A in the presence or absence of Tiotropium bromide in Normoxia and Ischaemia/Reperfusion whole heart models

4.4.1 Haemodynamic Data Analysis

4.4.1.1 Haemodynamic in Normoxic conditions for Tiotropium ± Cyclosporin A (CsA)

Tiotropium bromide (1nM) ± CsA (200nM) was administered following a 20-minute stabilisation period and perfused throughout the rest of the experimental protocol for a

period of 155 minutes, as described in section 2.3.3.1. The data for all parameters (fig 4.4.1.1.1 – 4.4.1.1.3) following Tiotropium bromide (1nM) ± CsA (200nM) were analysed at 15, 25, 50, 70 and 160 minutes. Tiotropium bromide (1nM) ± CsA (200nM) was compared to the normoxic control and each concentration.

The coronary flow (fig 4.4.1.1.1) shows that Tiotropium bromide (1nM) ± cyclosporin A (200nM) alone shows no significance with respect to the normoxic control or each other.

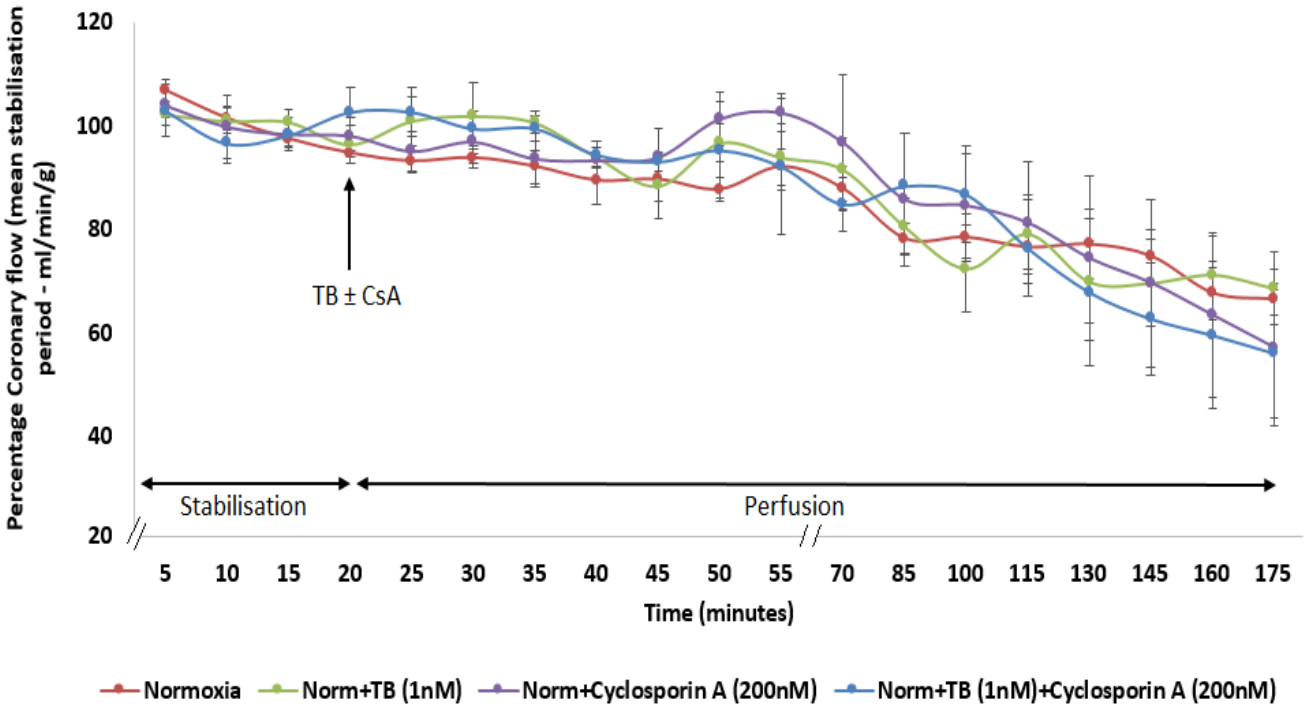

Figure 4.4.1.1.1: Percentage coronary flow of the mean stabilisation period following administration with Tiotropium bromide (1nM) ± cyclosporin A (200nM). *Langendorff hearts were subjected to a 20-minute period of stabilisation perfused with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (1nM) ± cyclosporin A (200nM) perfusion*

for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 3-5 for all groups.

Figure 4.4.1.1.2 shows left ventricular developed pressure (LVDP) following Tiotropium bromide (1nM) ± cyclosporin A (200nM) administration in normoxic conditions. The data does not show any statistical significances amongst the Tiotropium bromide (1nM) ± cyclosporin A (200nM) groups at the time points assessed ($p>0.05$, fig 4.4.1.1.2) between the groups or with respect to the normoxic control.

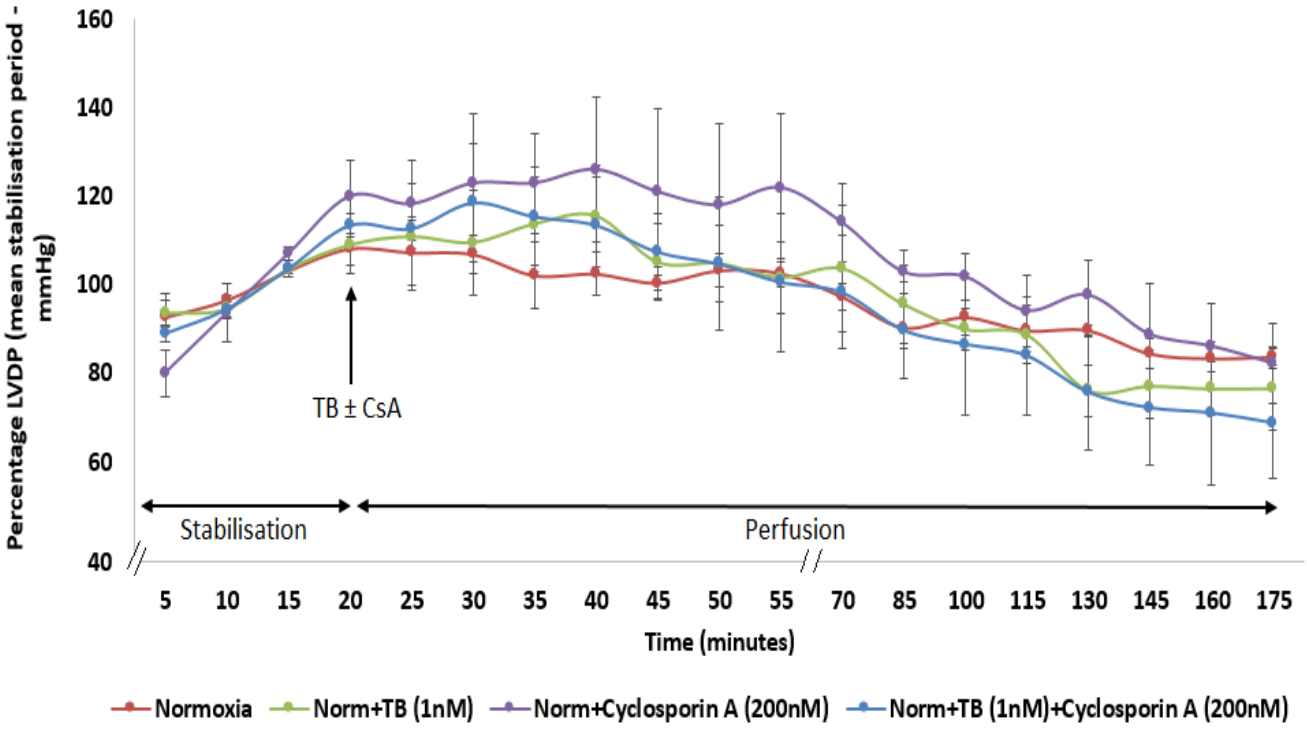

Figure 4.4.1.1.2: Percentage left ventricular developed pressure (LVDP) of the mean stabilisation period following administration with Tiotropium bromide (1nM) ± cyclosporin A (200nM)*. Langendorff hearts were subjected to a 20-minute period of stabilisation perfused with Krebs-Henseleit buffer, thereafter switched to Tiotropium*

bromide (1nM) ± cyclosporin A (200nM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 3-5 for all groups.

Figure 4.4.1.1.3 shows heart rate following administration of Tiotropium bromide (1nM) ± cyclosporin A (200nM) in normoxic conditions. The data shows no statistical significance in heart rate observed with Tiotropium bromide (1nM) ± cyclosporin A (200nM) and the normoxic control group ($p>0.05$, fig 4.4.1.1.3).

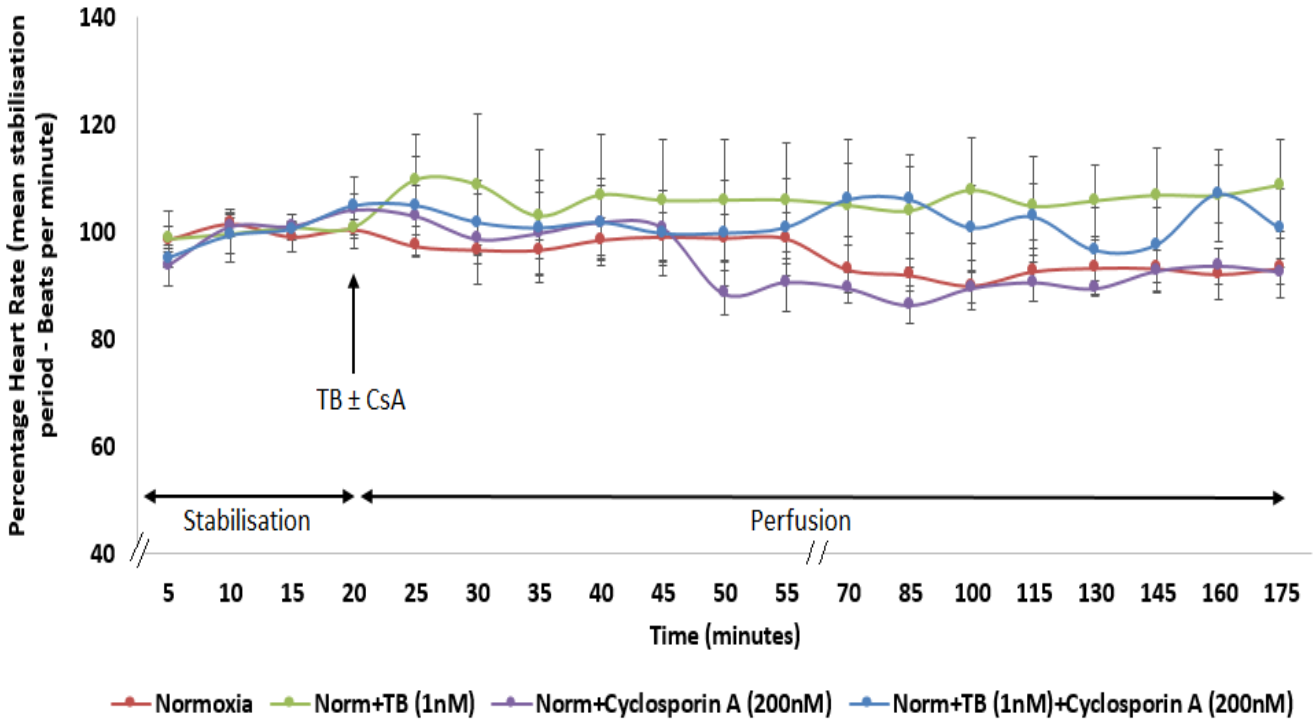

Figure 4.4.1.1.3: Percentage heart rate of the mean stabilisation period following administration with Tiotropium bromide (1nM) ± cyclosporin A (200nM). *Langendorff hearts were subjected to a 20-minute period of stabilisation perfused with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (1nM) ± cyclosporin A (200nM) perfusion*

for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 3-5 for all groups.

4.4.1.2 Haemodynamic in Ischaemia/Reperfusion for Tiotropium ± Cyclosporin A

The effect of Tiotropium bromide (1 nM) administration in the presence or absence of cyclosporin A (200 nM), was assessed in conditions of myocardial ischaemia/reperfusion injury with 20 minutes of stabilisation preceding 35 minutes of regional ischaemia followed by 120 minutes of reperfusion; drug administration lasted the duration of reperfusion, as described in section 2.3.3.2.

Coronary flow (fig 4.4.1.2.1), left ventricular developed pressure (LVDP, fig 4.4.1.2.2) and heart rate (fig 4.4.1.2.2.3) are shown for Tiotropium bromide (1 nM) ± cyclosporin A (200 nM) administration in conditions of ischaemia/reperfusion as well as the ischaemia/reperfusion control group. The data collected for all parameters (fig 4.4.1.2.1 – 4.4.1.2.3) following Tiotropium bromide (1 nM) ± cyclosporin A (200 nM) administration in ischaemia/reperfusion were statistically analysed at 15, 25, 50, 70 and 160 minutes.

Figure 4.4.1.2.1 for coronary flow shows no statistical significance at the onset of reperfusion with Tiotropium bromide (1 nM) ± cyclosporin A (200 nM) ($p>0.05$) between each group and also with respect to the ischaemia/reperfusion control.

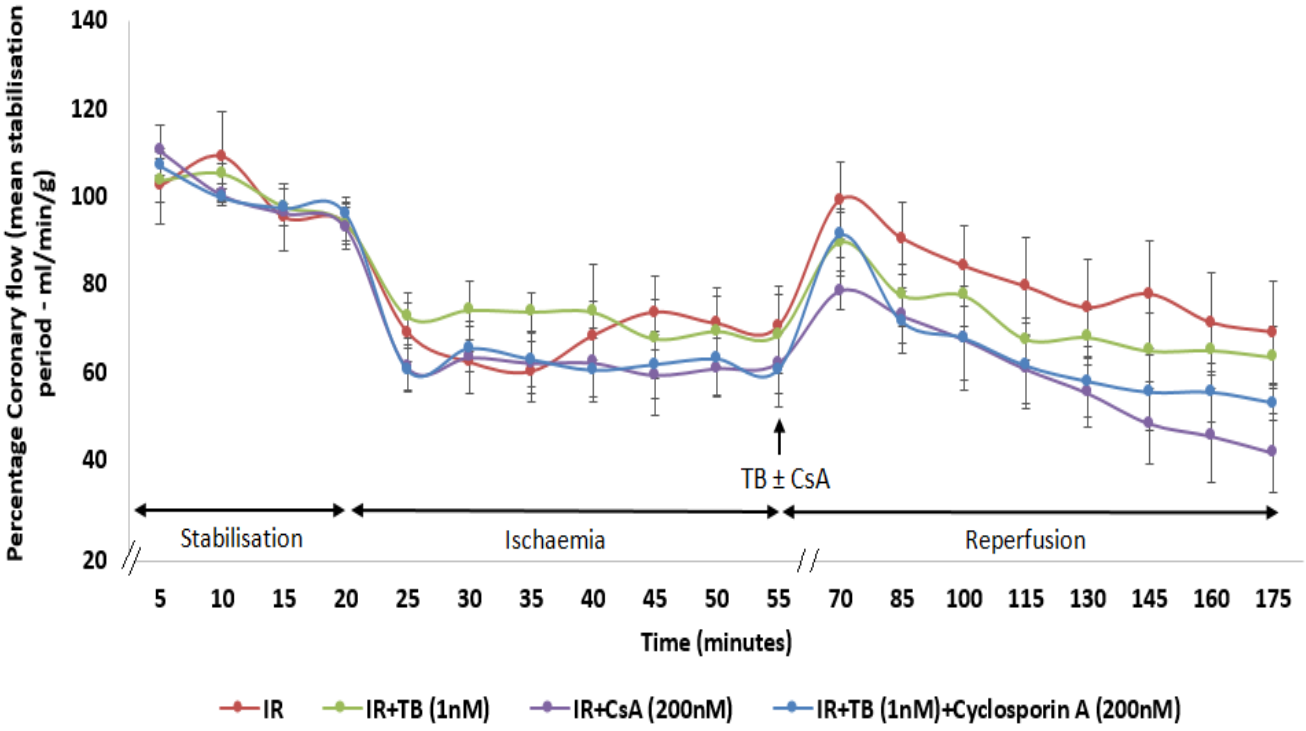

Figure 4.4.1.2.1: Percentage coronary flow of the mean stabilisation period following Tiotropium bromide (TB 1 nM) ± cyclosporin A (CsA 200 nM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Tiotropium bromide (TB 1 nM) ± cyclosporin A (200 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups.*

Figure 4.4.1.2.2 shows the left ventricular pressure recorded (LVDP) from the Tiotropium bromide (1 nM) ± cyclosporin A (200 nM) treatment groups. The data shows a statistically significant increase in LVDP with cyclosporin A (200 nM) compared to Tiotropium bromide

(1 nM) at 70 minutes (70 minutes: 132.71 ± 10.45% (CsA 200nM) vs. 88.06 ± 12.20% (TB 1nM), p<0.05, fig 4.4.1.2.2).

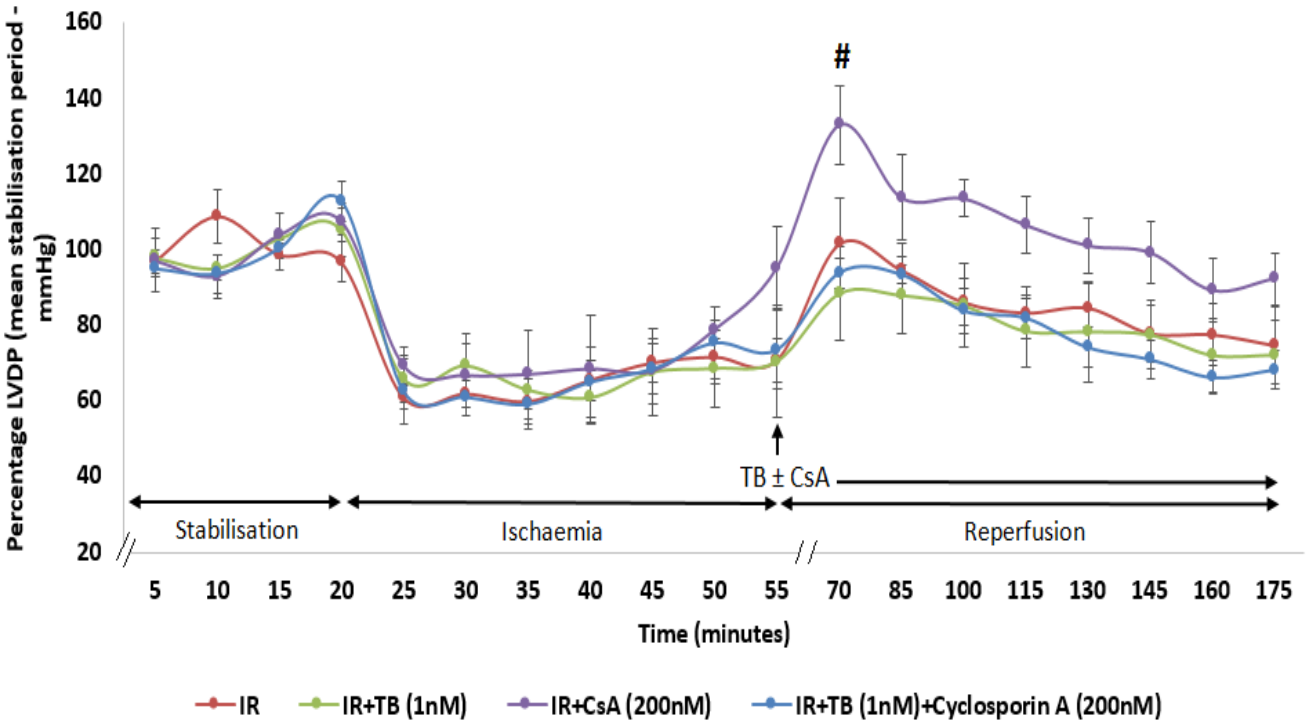

Figure 4.4.1.2.2: Percentage left ventricular developed pressure (LVDP) of the mean stabilisation period following Tiotropium bromide (TB 1 nM) ± cyclosporin A (CsA 200 nM) in ischaemia/reperfusion*. Langendorff hearts were subjected to 20-minute of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Tiotropium bromide (TB 1 nM) ± cyclosporin A (200 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups. # vs. IR+TB (1nM), p<0.05.*

Figure 4.4.1.2.3 shows heart rate following administration of Tiotropium bromide (1 nM) ± cyclosporin A (200 nM) following ischaemia/reperfusion. The data shows no statistical significance in heart rate observed with Tiotropium bromide (1 nM) ± cyclosporin A (200 nM) and the ischaemia/reperfusion control group ($p>0.05$, fig 4.4.1.2.3).

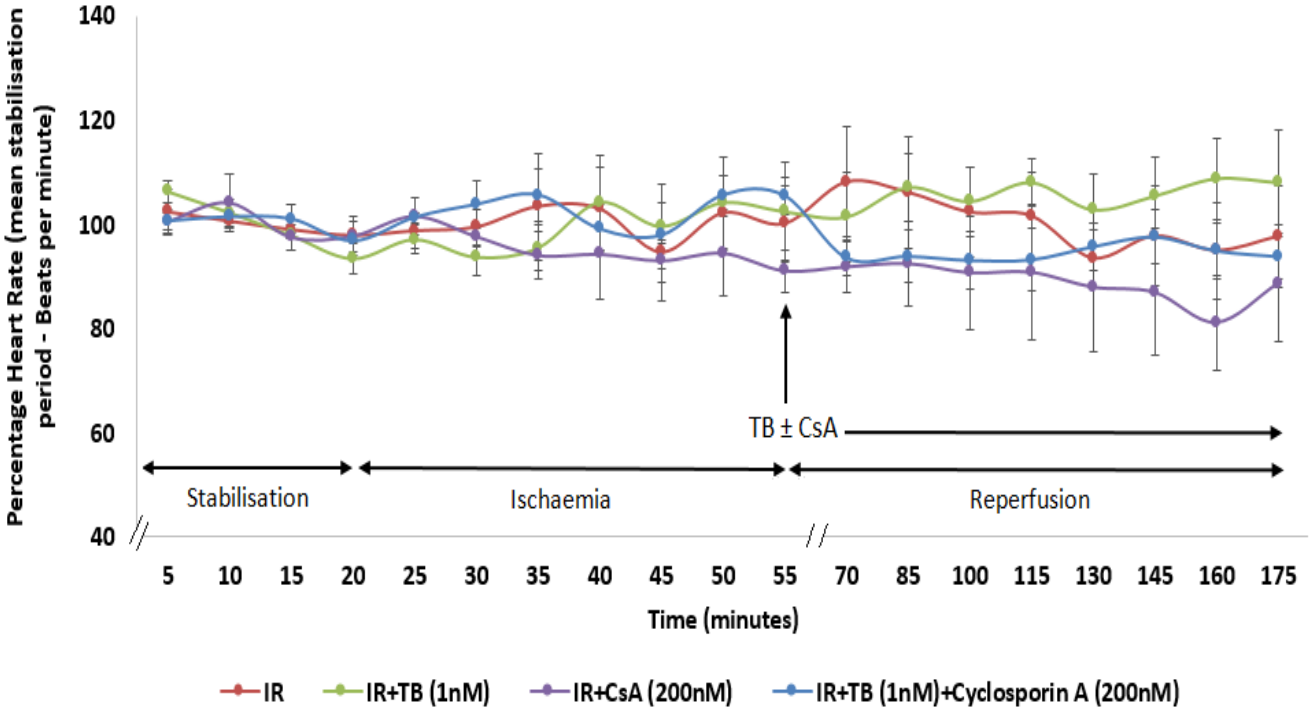

Figure 4.4.1.2.3: Percentage heart rate of the mean stabilisation period following Tiotropium bromide (TB 1 nM) ± cyclosporin A (CsA 200 nM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Tiotropium bromide (TB 1 nM) ± cyclosporin A (200 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups.*

4.4.2 Infarct size analysis of Tiotropium ± Cyclosporin A administration

4.4.2.1 Infarct Analysis in Normoxic conditions for Tiotropium ± Cyclosporin A

This study observed the effects of Tiotropium bromide (1 nM) ± cyclosporin A (200 nM) administration on infarct size (%) in normoxic conditions. Figure 4.4.2.1.1 shows infarct size

following Tiotropium bromide (1 nM) ± cyclosporin A (200 nM) with respect to normoxic (Norm) controls. A significant difference in infarct size following Tiotropium bromide (1 nM) ± cyclosporin A (200 nM) administration is shown with respect to the normoxic control. The administration of cyclosporin A (200 nM) shows a significant decrease in infarct size (%) with respect to Tiotropium (1 nM) (5.14 ± 0.95% (cyclosporin A 200nM) vs. 18.69 ± 1.79% (TB 1nM), $p<0.001$, fig 4.4.2.1.1). Cyclosporin A (200 nM) co-administration with Tiotropium (1 nM) sustains the significant decrease in infarct size with respect to Tiotropium (1 nM) (8.41 ± 2.13% (TB 1nM + CsA 200nM) vs. 18.69 ± 1.79% (TB 1nM), $p<0.001$, fig 4.4.2.1.1).

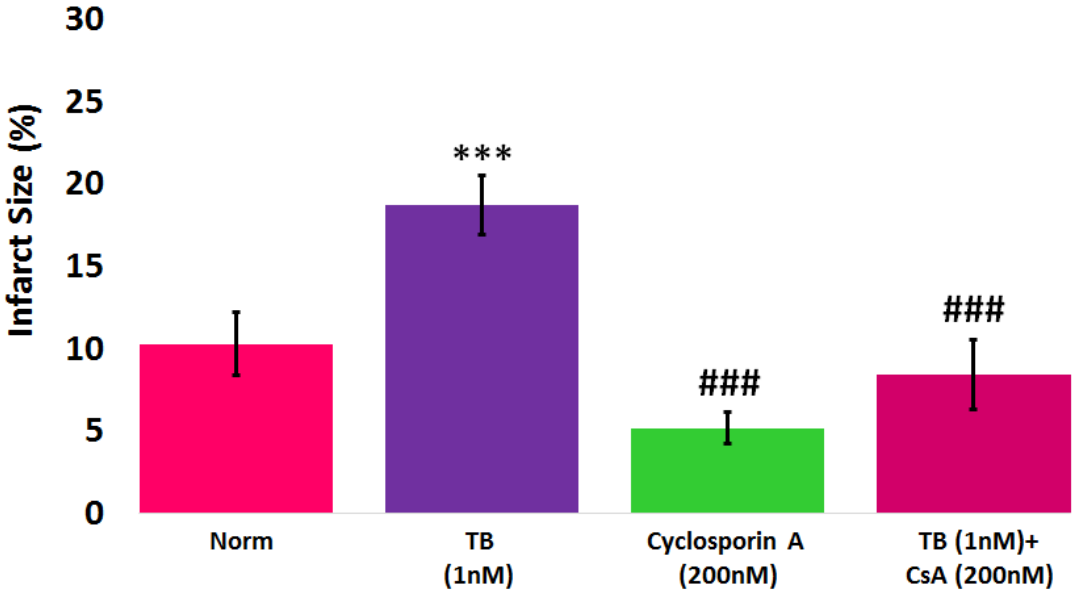

Figure 4.2.2.1.1: Percentage infarct to risk ratios (%) following Tiotropium bromide (1 nM) ± cyclosporin A (200 nM) in normoxic conditions*. Langendorff hearts were subjected to a 20-minute period of stabilisation with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (1 nM) ± cyclosporin A (200 nM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups. *** TB (1nM) vs. Norm, $p<0.001$; ### Cyclosporin A (200nM) and TB (1nM) + CsA (200nM) vs. TB (1nM), $p<0.001$.*

4.4.2.2 Infarct Analysis in Ischaemia/Reperfusion for Tiotropium ± Cyclosporin A

This study observed the effects of Tiotropium bromide (1 nM) ± cyclosporin A (200 nM) administration on infarct to risk ratio (%), in an *in vitro* model of myocardial ischaemia/reperfusion.

Figure 4.4.2.2.1 shows the infarct/risk ratios of Tiotropium bromide (1 nM) ± cyclosporin A (200 nM) with respect to the ischaemia/reperfusion (IR) control. Administration of cyclosporin A (200 nM) shows a reversal in the infarct to risk ratio observed with ischaemia/reperfusion and Tiotropium (1 nM) alone (7.16 ± 1.23% (cyclosporin A 200nM) vs. 50.85 ± 3.93% (IR) and 65.42 ± 3.00% (TB 1nM), $p<0.001$ respectively, fig 4.4.2.2.1). Tiotropium bromide (1 nM) co-administered with cyclosporin A (200 nM) also shows an abrogated infarct to risk ratio with respect to the ischaemia/reperfusion control and Tiotropium (1 nM) alone (16.34 ± 0.78% (TB 1nM + CsA 200nM) vs. 50.85 ± 3.93% (IR) and 65.42 ± 3.00% (TB 1nM), $p<0.001$, fig 4.4.2.2.1).

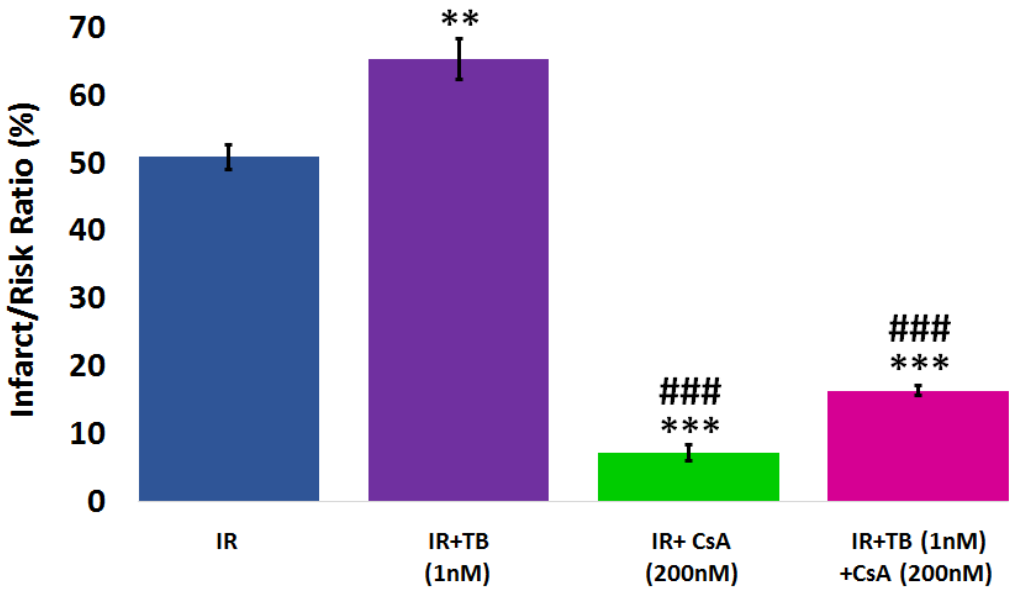

Figure 4.4.2.2.1: Percentage infarct to risk ratios (%) following Tiotropium bromide (1 nM) ± cyclosporin A (200 nM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Tiotropium bromide (1 nM) ± cyclosporin A (CsA 200 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups. ** vs. IR p<0.01; *** vs. IR p<0.001; ### vs. IR+TB (1nM), p<0.001.*

Chapter 5: Role of reactive oxygen species in Tiotropium mediated signalling in *ex vivo* and *in vitro* Cardiac models

The primary aim of this study was to elucidate the cardiotoxicity of long acting muscarinic receptor antagonists, notably how Tiotropium bromide induces myocardial damage in *ex vivo* and *in vitro* models of normoxic conditions and ischaemia/reperfusion injury.

(b) Understanding the effect of Tiotropium bromide on … oxidative stress in *ex vivo* and *in vitro* normoxic conditions via … the antioxidant, resveratrol.

(d) To observe the effect of Tiotropium bromide on gene expression profiles associated with myocardial infarction and oxidative stress.

5.1 Effect of the antioxidant, Resveratrol on Tiotropium bromide mediated cardiotoxicity in Normoxic conditions and Ischaemia/Reperfusion whole heart models

5.1.1 Haemodynamic Data Analysis

5.1.1.1 Haemodynamic in Normoxic conditions for Tiotropium ± Resveratrol

Using a whole heart Langendorff model, the haemodynamic data for Tiotropium bromide (1 nM) administration in the presence or absence of resveratrol (10 µM) following 20 minutes of stabilisation in normoxic conditions are depicted in figures 5.1.1.1.1 – 5.1.1.1.3. Tiotropium bromide (1nM) ± resveratrol (10 µM) was administered following a 20-minute stabilisation period and perfused throughout the rest of the experimental protocol for a period of 155 minutes, as described in section 2.3.3.1. The data collected for all parameters (fig 5.1.1.1.1 – 5.1.1.1.3) following Tiotropium bromide (1nM) ± resveratrol (10 µM) administration were statistically analysed at 15, 25, 50, 70 and 160 minutes. The Tiotropium bromide (1nM) ± resveratrol (10 µM) groups were analysed with respect to the normoxic control as well as between each treatment group.

The coronary flow (fig 5.1.1.1.1) data shows that the Tiotropium bromide (1nM) + resveratrol (10 µM) group shows a significant increase in coronary flow with respect to the normoxic control, at 50 and 160 minutes (50 minutes: 113.33 ± 5.93% (TB 1nM + Resv 10µM) vs. 87.57 ± 2.24% (Norm), $p<0.05$; 160 minutes: 93.63 ± 7.78% (TB 1nM + Resv 10µM) vs. 67.48 ± 4.99% (Norm), $p<0.05$, fig 5.1.1.1.1).

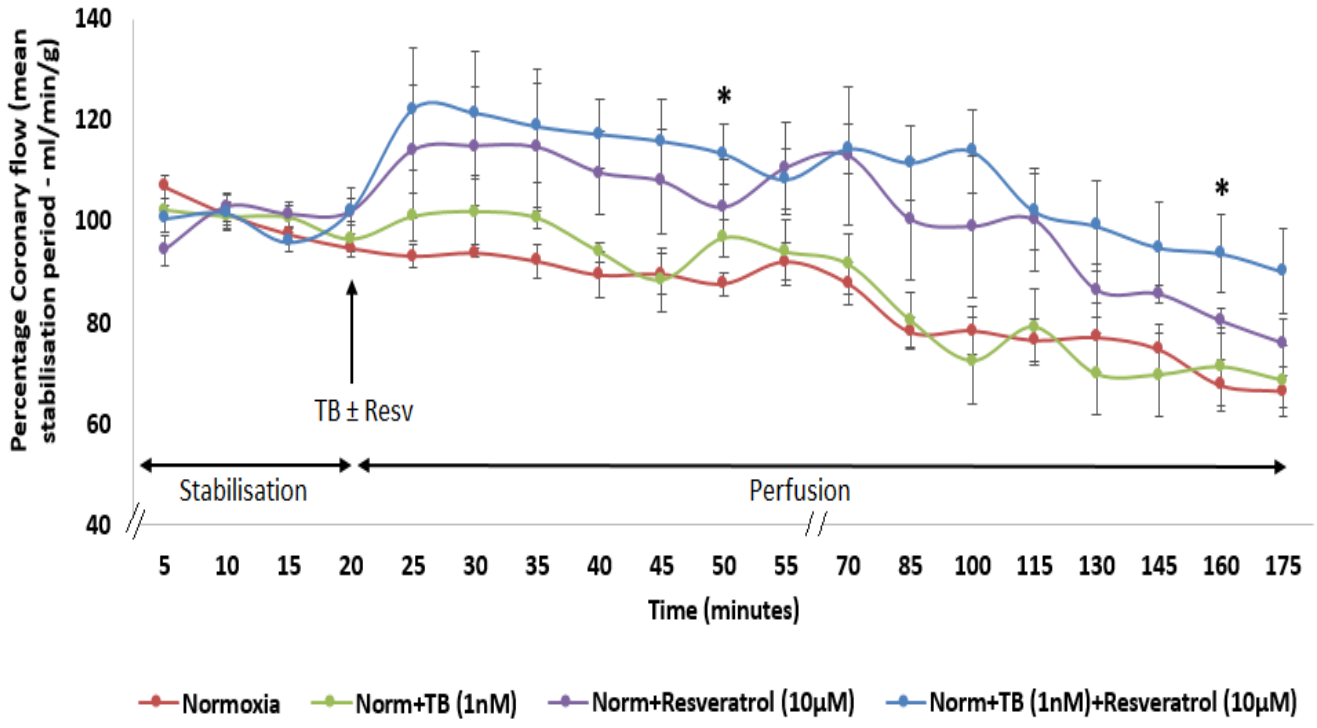

Figure 5.1.1.1.1: Percentage coronary flow of the mean stabilisation period following administration with Tiotropium bromide (1nM) ± resveratrol (10 µM)*. Langendorff hearts were subjected to a 20-minute period of stabilisation perfused with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (1nM) ± resveratrol (10 µM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 4-6 for all groups.* TB (1nM) + Resv (10µM) vs. Normoxia, p<0.05.*

Figure 5.1.1.1.2 shows left ventricular developed pressure (LVDP) following Tiotropium bromide (1nM) ± resveratrol (10 µM) administration in normoxic conditions. The data does not show any statistical significances amongst the Tiotropium bromide (1nM) ± resveratrol (10 µM) groups at the time points assessed (p>0.05, fig 5.1.1.1.2) between the groups or with respect to the normoxic control.

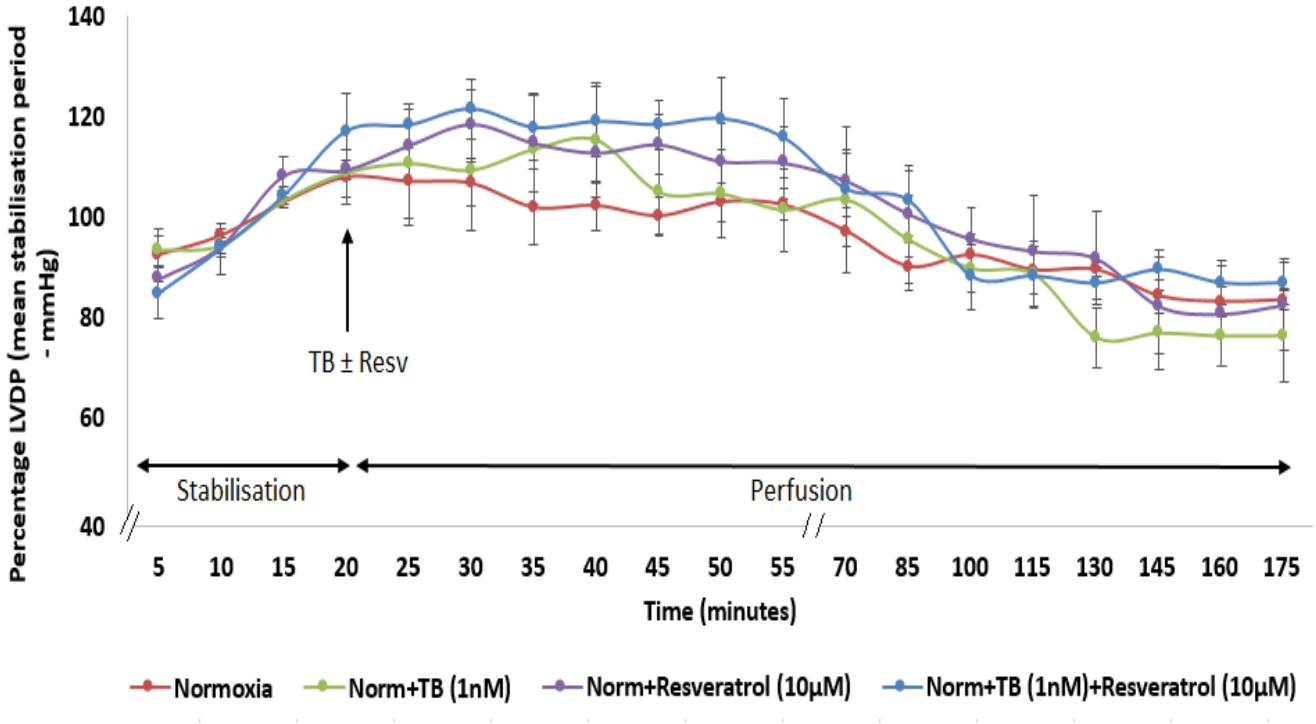

Figure 5.1.1.1.2: Percentage left ventricular developed pressure (LVDP) of the mean stabilisation period following administration with Tiotropium bromide (1nM) ± resveratrol (10 µM)*. Langendorff hearts were subjected to a 20-minute period of stabilisation perfused with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (1nM) ± resveratrol (10 µM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 4-6 for all groups.*

Figure 5.1.1.1.3 shows heart rate following administration of Tiotropium bromide (1nM) ± resveratrol (10 µM) in normoxic conditions. The data shows no statistical significance in heart rate observed with Tiotropium bromide (1nM) ± resveratrol (10 µM) and the normoxic control group ($p>0.05$, fig 5.1.1.1.3).

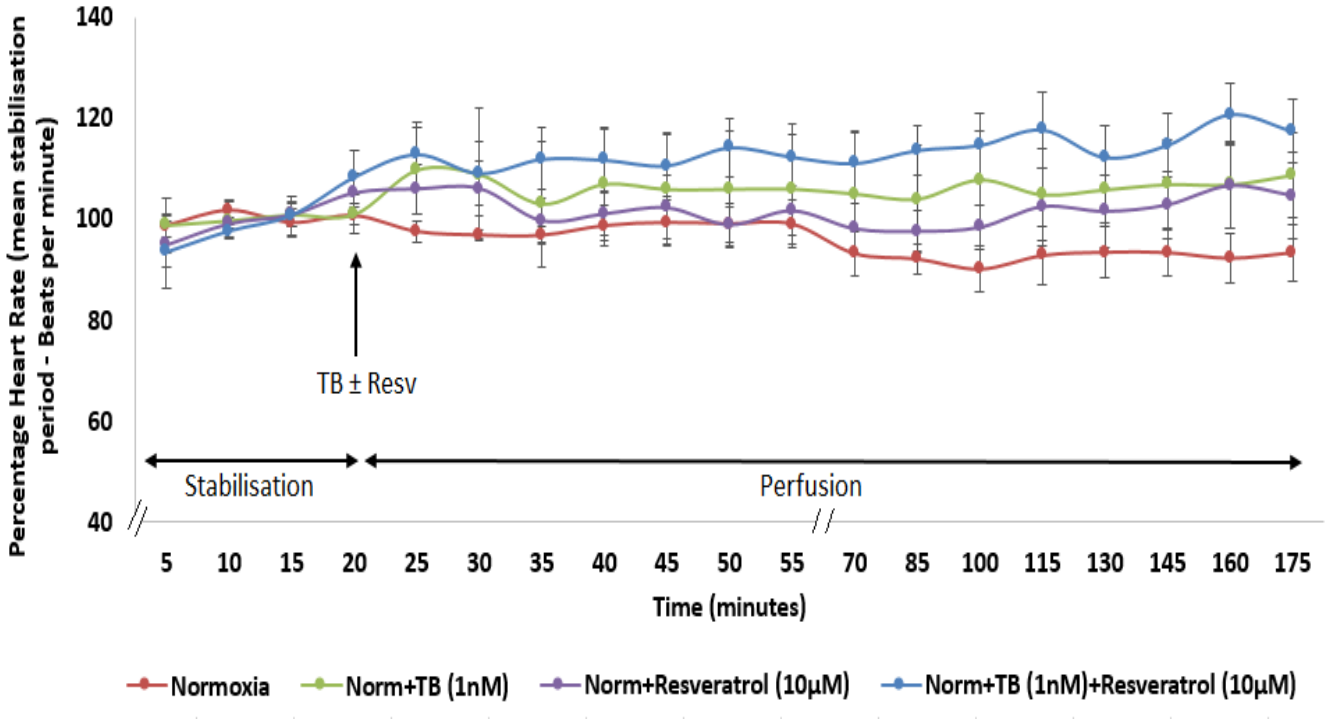

Figure 5.1.1.1.3: Percentage heart rate of the mean stabilisation period following administration with Tiotropium bromide (1nM) ± resveratrol (10 µM)*. Langendorff hearts were subjected to a 20-minute period of stabilisation perfused with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (1nM) ± resveratrol (10 µM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 4-6 for all groups.*

5.1.1.2 Haemodynamic in Ischaemia/Reperfusion for Tiotropium ± Resveratrol

The effect of Tiotropium bromide (1 nM) administration in the presence or absence of resveratrol (10 µM), was assessed in conditions of myocardial ischaemia/reperfusion injury with 20 minutes of stabilisation preceding 35 minutes of regional ischaemia followed by 120 minutes of reperfusion; drug administration lasted the duration of reperfusion.

Haemodynamic data was recorded for each study; at the end of each experiment, hearts were stained with Evans blue and retained for infarct analysis using the TTC method. Coronary flow (fig 5.1.1.2.1), left ventricular developed pressure (LVDP, fig 5.1.1.2.2) and heart rate (fig 5.1.1.2.3) are shown for Tiotropium bromide (1 nM) ± resveratrol (10 µM)

administration in conditions of ischaemia/reperfusion as well as the ischaemia/reperfusion control group. The data collected for all parameters (fig 5.1.1.2.1 – 5.1.1.2.3) following Tiotropium bromide (1 nM) ± resveratrol (10 µM) administration in ischaemia/reperfusion were statistically analysed at 15, 25, 50, 70 and 160 minutes.

Figure 5.1.1.2.1 for coronary flow shows no statistical significance at the onset of reperfusion with Tiotropium bromide (1 nM) ± resveratrol (10 µM) ($p>0.05$) between each group and also with respect to the ischaemia/reperfusion control.

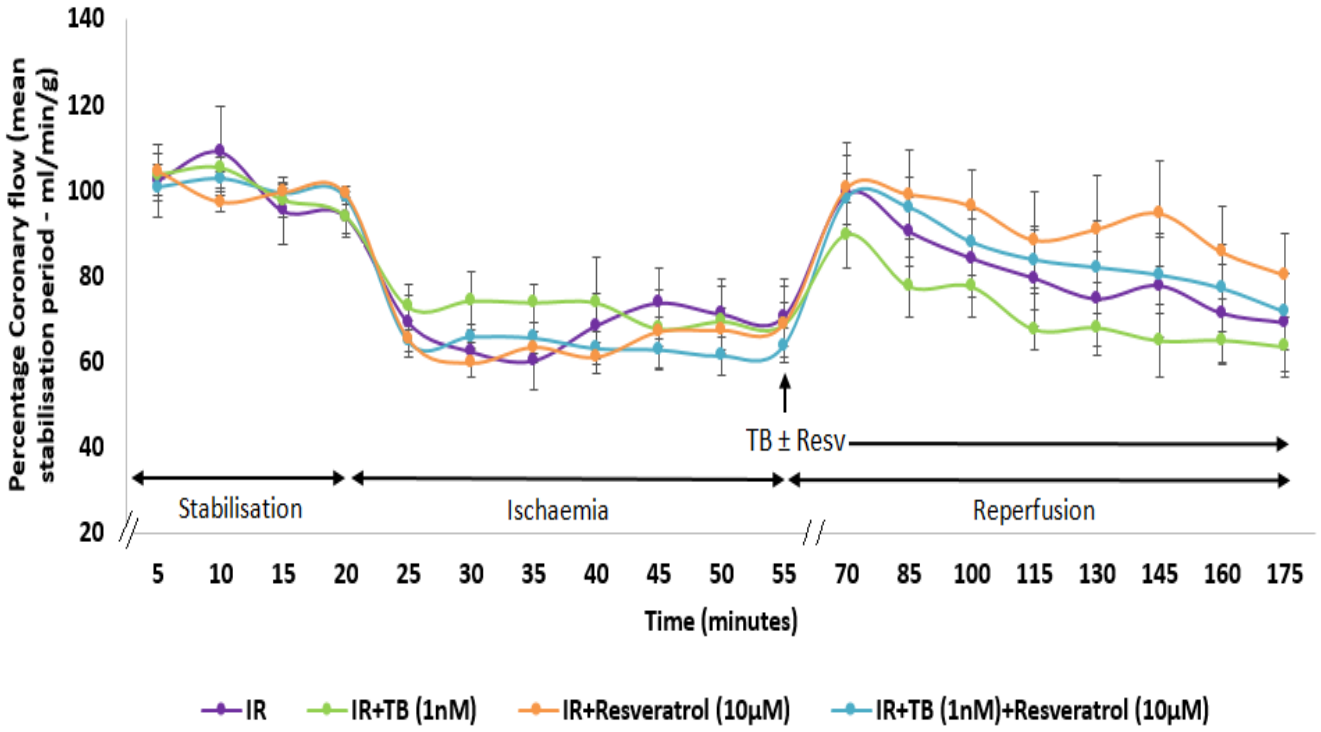

Figure 5.1.1.2.1: Percentage coronary flow of the mean stabilisation period following Tiotropium bromide (1 nM) ± resveratrol (10 µM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Tiotropium bromide (1 nM) ± resveratrol (10 µM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. Values plotted signify the Mean ± SEM, derived from an n of 4-6 for all groups.*

Figure 5.1.1.2.2 shows the left ventricular pressure recorded (LVDP) from the Tiotropium bromide (1 nM) ± resveratrol (10 µM) treatment groups. The data does not show any statistical significances amongst the Tiotropium bromide (1 nM) ± resveratrol (10 µM)

groups at the time points assessed (p>0.05, fig 5.1.1.2.2) between the different groups or with respect to the ischaemia/reperfusion group.

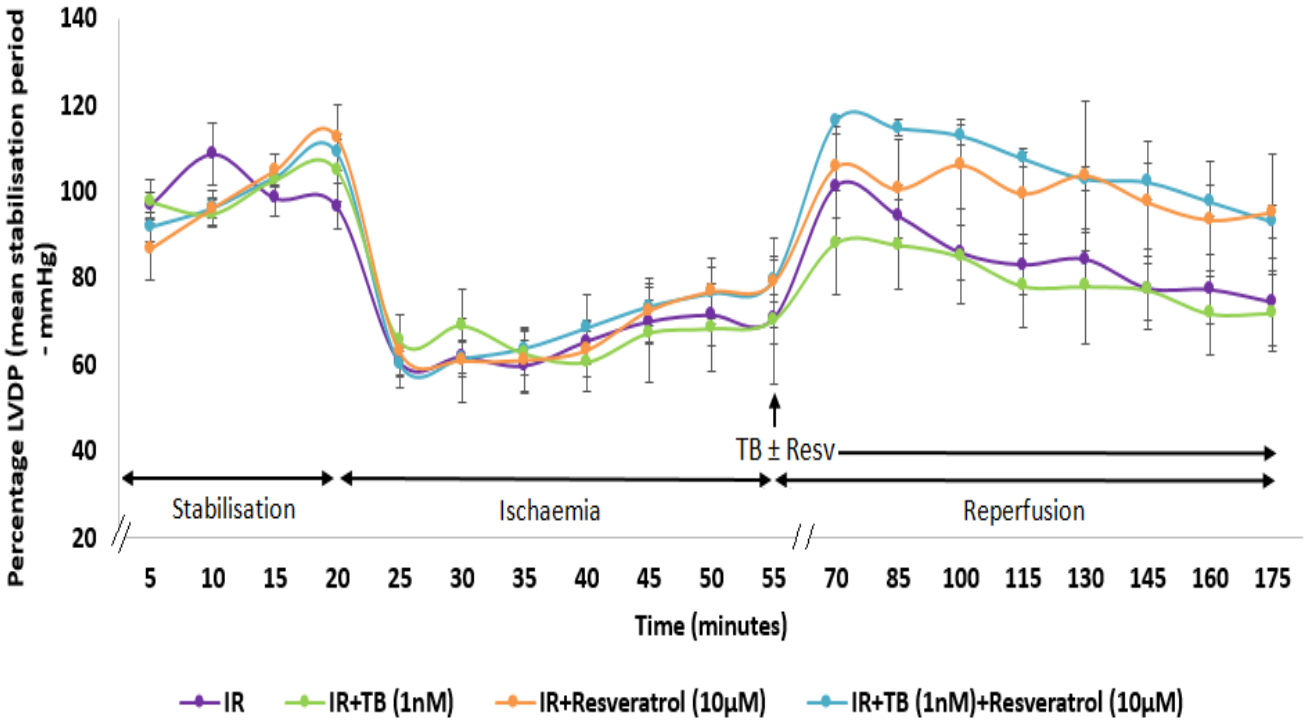

Figure 5.1.1.2.2: Percentage left ventricular developed pressure (LVDP) of the mean stabilisation period following Tiotropium bromide (1 nM) ± resveratrol (10 µM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Tiotropium bromide (1 nM) ± resveratrol (10 µM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. Values plotted signify the Mean ± SEM, derived from an n of 4-6 for all groups.*

Figure 5.1.1.2.3 shows heart rate following administration of Tiotropium bromide (1 nM) ± resveratrol (10 µM) following ischaemia/reperfusion. The data shows no statistical significance in heart rate observed with Tiotropium bromide (1 nM) ± resveratrol (10 µM) and the ischaemia/reperfusion control group (p>0.05, fig 5.1.1.2.3).

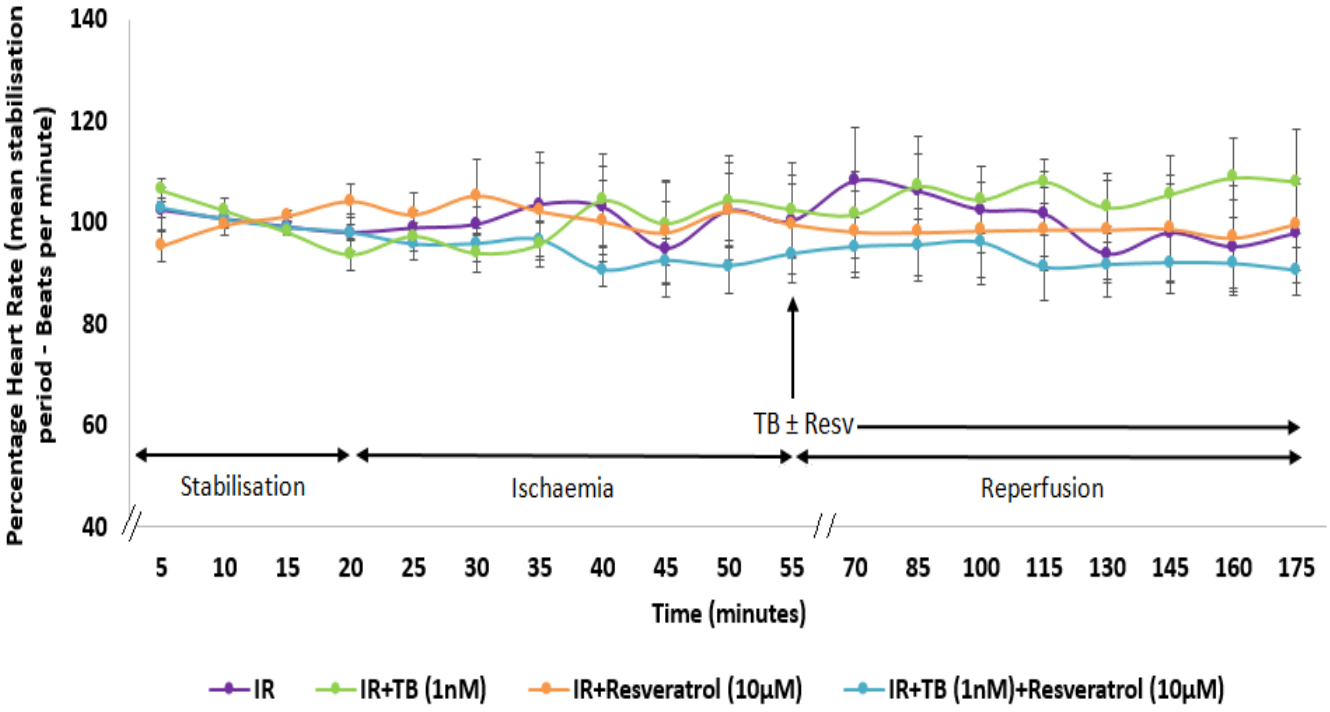

Figure 5.1.1.2.3: Percentage heart rate of the mean stabilisation period following Tiotropium bromide (1 nM) ± resveratrol (10 µM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Tiotropium bromide (1 nM) ± resveratrol (10 µM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. Values plotted signify the Mean ± SEM, derived from an n of 4-6 for all groups.*

5.1.2 Infarct to Risk analysis of Tiotropium Bromide following Resveratrol administration in normoxia and ischaemia/reperfusion

5.1.2.1 Infarct to Risk analysis in Normoxic conditions for Tiotropium ± Resveratrol

This study observed the effects of Tiotropium bromide (1 nM) ± resveratrol (10 µM) administration on infarct size (%), in normoxic conditions. Tiotropium bromide (1 nM) ± resveratrol (10 µM) was administered following 20 minutes of stabilisation and continued

for 155 minutes, as described in section 2.3.3.2; hearts were subjected to TTC staining to determine infarct size.

Figure 5.1.2.1.1 shows the infarct size of Tiotropium bromide (1 nM) ± resveratrol (10 µM) with respect to normoxic (Norm) controls. The study shows that there is a significant difference in infarct size following Tiotropium bromide (1 nM) ± resveratrol (10 µM) administration with respect to the normoxic control. Tiotropium (1 nM) shows an increase in infarct size compared to the normoxic control (18.69 ± 1.79% vs. 10.28 ± 1.74%, $p<0.001$, fig 5.1.2.1.1). The administration of resveratrol (10 µM) shows no significance with respect to the normoxic control ($p>0.05$), however a significant decrease is observed with respect to the Tiotropium (1 nM) group (12.28 ± 1.50% (Resv 10µM) vs. 18.69 ± 1.79% (TB 1nM), $p<0.01$, fig 5.1.2.1.1). The decrease in infarct size following resveratrol (10 µM) administration is sustained upon co-administration with Tiotropium (1 nM) with respect to the Tiotropium (1 nM) alone group (11.99 ± 1.71% (TB 1nM + Resv 10µM) vs. 18.69 ± 1.79% (TB 1nM), $p<0.01$, fig 5.1.2.1.1).

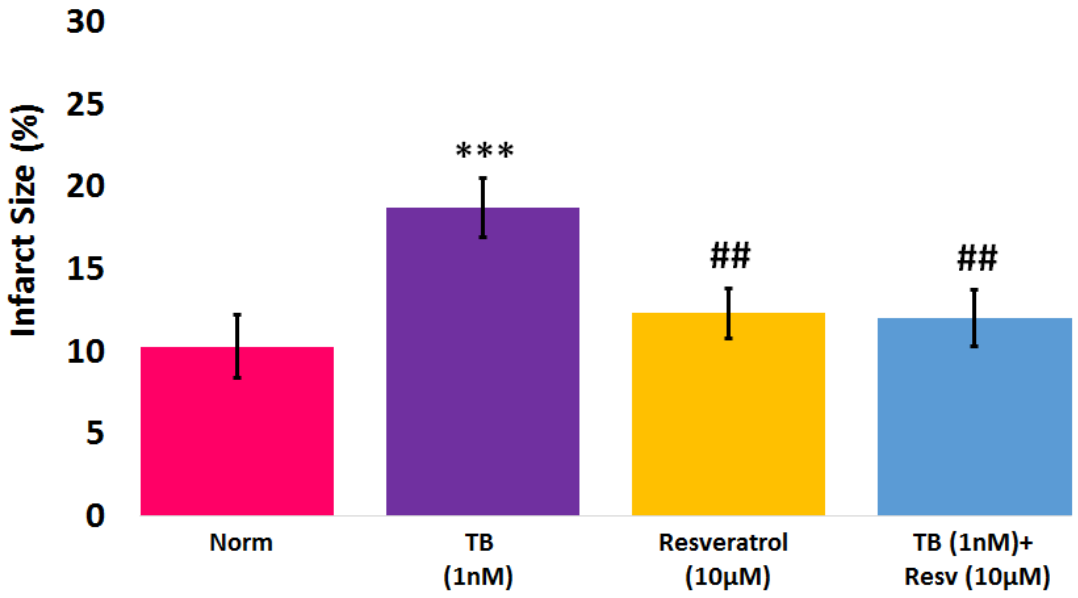

Figure 5.1.2.1.1: Percentage infarct to risk ratios (%) following Tiotropium bromide (1 nM) ± resveratrol (10 µM) in normoxic conditions*. Langendorff hearts were subjected to a 20-minute period of stabilisation with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (1 nM) ± resveratrol (10 µM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 4-6 for all groups. *** TB (1nM) vs. Norm, p<0.001; ## Resveratrol (10µM) and TB (1nM) + Resv (10µM) vs. TB (1nM), p<0.01.*

5.1.2.2 Infarct to Risk analysis of Ischaemia/Reperfusion for Tiotropium ± Resveratrol

This study observed the effects of Tiotropium bromide (1 nM) ± resveratrol (10 µM) administration on infarct to risk ratio (%), in an *in vitro* model of myocardial ischaemia/reperfusion. Tiotropium bromide (1 nM) ± resveratrol (10 µM) was administered at the onset of reperfusion and continued throughout the 120-minute period following 20 minutes of stabilisation and 35 minutes of regional ischaemia as described in section 2.3.2.2, followed by Evans blue dye staining to delineate ischaemic zones, and subjected to TTC staining to determine infarct size to risk ratio.

Figure 5.1.2.2.1 shows the infarct/risk ratios of Tiotropium bromide (1 nM) ± resveratrol (10 µM) with respect to the ischaemia/reperfusion (IR) control. The study showed that Tiotropium bromide (1 nM) administration results in a significant increase in infarct to risk ratio with respect to the ischaemia/reperfusion control (65.42 ± 3.00% (TB 1nM) vs. 50.85 ± 3.93 (IR), p<0.01, fig 5.1.2.2.1). There is a significant decrease in infarct to risk ratio with resveratrol (10 µM) administration with respect to Tiotropium (1 nM) (35.24 ± 0.40% (Resv 10µM) vs. 65.42 ± 3.00% (TB 1nM), p<0.001, fig 5.1.2.2.1). Tiotropium (1 nM) + resveratrol (10 µM) administration also shows a significant decrease in infarct to risk ratio with respect to Tiotropium (1 nM) (39.10 ± 0.89% (TB 1nM + Resv 10µM) vs. 65.42 ± 3.00% (TB 1nM), p<0.01, fig 5.1.2.2.1).

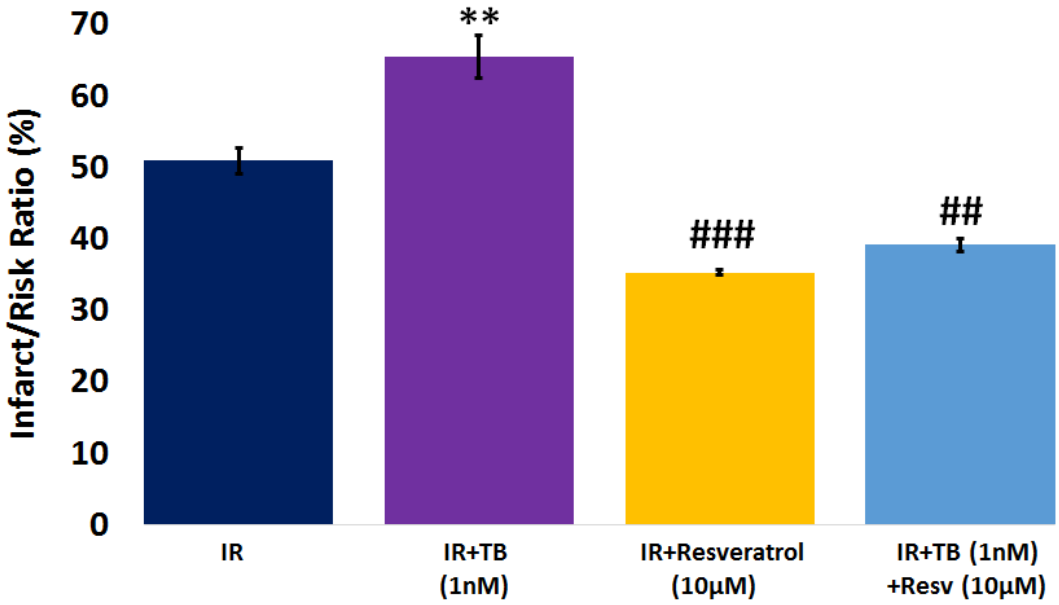

Figure 5.1.2.2.1: Percentage infarct to risk ratios (%) following Tiotropium bromide (1 nM) ± resveratrol (10 µM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Tiotropium bromide (1 nM) ± resveratrol (10 µM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. Values plotted signify the Mean ± SEM, derived from an n of 4-6 for all groups. ** vs. IR, p<0.05; ## vs. IR+TB (1nM), p<0.01 and ### vs. IR+TB (1nM), p<0.001.*

5.2 Effect of Tiotropium Bromide on Akt expression in the presence or absence of Resveratrol in Normoxic conditions

5.2.1 Effect of Tiotropium bromide administration on Phospho-Akt (Ser473) expression in the presence or absence of Resveratrol

P-Akt (Ser473) activation was assessed using western blotting for phosphorylated Akt (Ser473) in cardiac tissue to establish the role of Akt in Tiotropium bromide (1 nM) mediated cardiotoxicity ± resveratrol (10 µM). Figure 5.2.1.1 shows the expression pattern observed following normoxia and Tiotropium bromide (1 nM) ± resveratrol (10 µM). All drugs were administered following stabilisation in a modified Langendorff model, for a period of 155

minutes; hearts were immediately removed following the end of the protocol period and the left ventricle was excised and snap-frozen as described.

The data shows a significant increase in phosphorylated Akt $_{(Ser473)}$ following Tiotropium (1 nM) administration with respect to the normoxic control (88.03 ± 5.62% (TB 1nM) vs. 36.17 ± 5.13% (Norm), $p<0.0001$, fig 5.2.1.1). Resveratrol (10 µM) shows a significant decrease in p-Akt $_{(Ser473)}$ with respect to the Tiotropium (1 nM) mediated increase (65.73 ± 8.50% (Resv 10µM) vs. 88.03 ± 5.62% (TB 1nM), $p<0.05$, fig 5.2.1.1); however, resveratrol (10 µM) administration shows a significant increase in p-Akt $_{(Ser473)}$ with respect to the normoxic control (65.73 ± 8.50% (Resv 10µM) vs. 36.17 ± 5.13% (Norm), $p<0.01$, fig 5.2.1.1). Tiotropium (1 nM) mediated increase in p-Akt $_{(Ser473)}$ is attenuated upon co-administration of resveratrol (10 µM) with respect to Tiotropium (1 nM) alone (61.05 ± 5.34% (TB 1nM + Resv 10µM) vs. 88.03 ± 5.62% (TB 1nM), $p<0.01$, fig 5.2.1.1); however, it shows significantly greater p-Akt $_{(Ser473)}$ than the normoxic control (61.05 ± 5.34% (TB 1nM + Resv 10µM) vs. 36.17 ± 5.13% (Norm), $p<0.01$, fig 5.2.1.1).

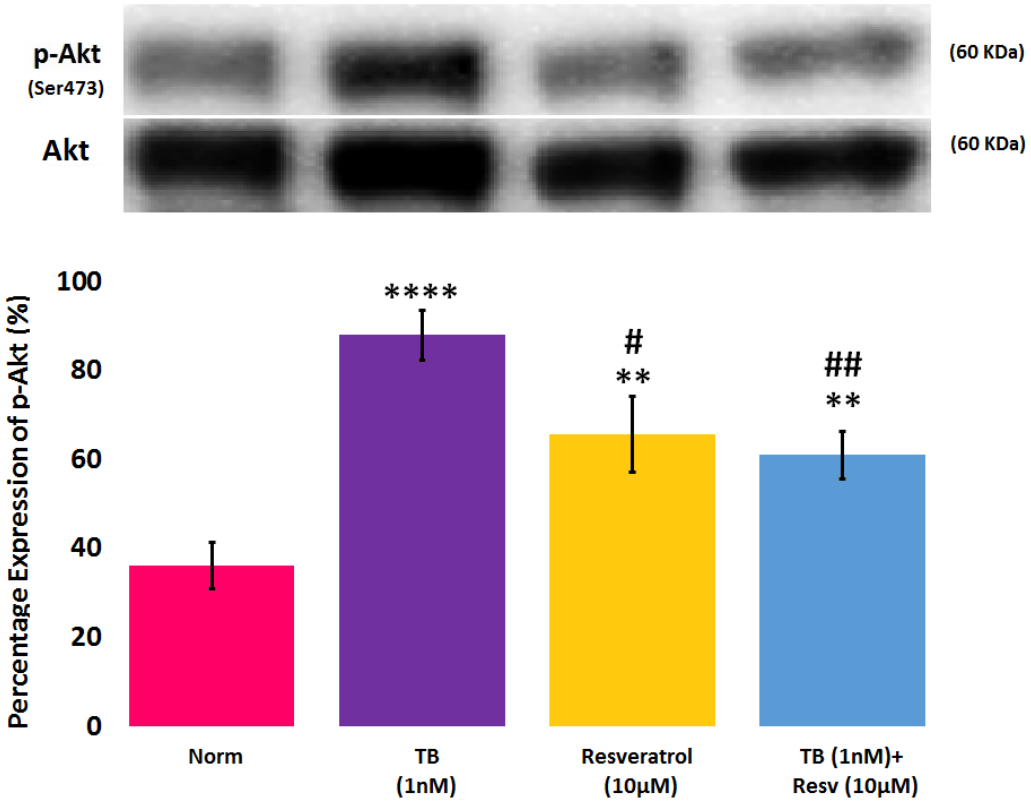

Figure 5.2.1.1: Percentage expression (%) of phosphorylated Akt (Ser473) as a percentage of total Akt following Tiotropium bromide (1 nM) ± resveratrol (10 µM) in normoxic conditions*. Langendorff hearts were perfused with either Krebs-Henseleit buffer (Norm) or Tiotropium bromide (1 nM) ± resveratrol (10 µM). Values plotted signify the Mean ± SEM, derived from an n of 3 for all groups. ** vs. Norm, p<0.01; **** vs. Norm, p<0.0001; ## vs. TB (1nM), p<0.01 and # vs. TB (1nM), p<0.05.*

5.3 Effect of Tiotropium bromide in the presence or absence of Resveratrol on Reactive Oxygen Species as a marker of oxidative stress

Cardiomyocytes were incubated with Tiotropium bromide (1 nM) ± resveratrol (10 µM) and the normoxic controls respectively for a period of 4 hours before staining with CellROX green for 1 hour and analysed on the flow cytometer using the FL-1 channel. SYTOX red was used as a counter stain to differentiate dead cells.

Figure 5.3.1 shows the percentage of CellROX in cardiomyocyte treated with Tiotropium bromide (1 nM) ± resveratrol (10 µM) in normoxic conditions. Tiotropium bromide (1 nM)

shows no observable difference in ROS production with respect to the normoxic control (99.52 ± 4.67% (TB 1nM) vs. 100 ± 0.0% (Norm), p>0.05, fig 5.4.1). However, resveratrol (10 µM) administration shows a significant decrease in ROS production with respect to the normoxic control and Tiotropium (1 nM) (84.27 ± 4.68% (Resv 10µM) vs. 100 ± 0.0% (Norm) and 99.52 ± 4.67% (TB 1nM), p<0.05, fig 5.3.1). Tiotropium (1 nM) ± resveratrol (10 µM) shows a significant increase in ROS production with respect to resveratrol (10 µM) alone (98.15 ± 10.21% (TB 1nM + Resv 10µM) vs. 84.27 ± 4.68% (Resv 10µM), p>0.01, fig 5.3.1).

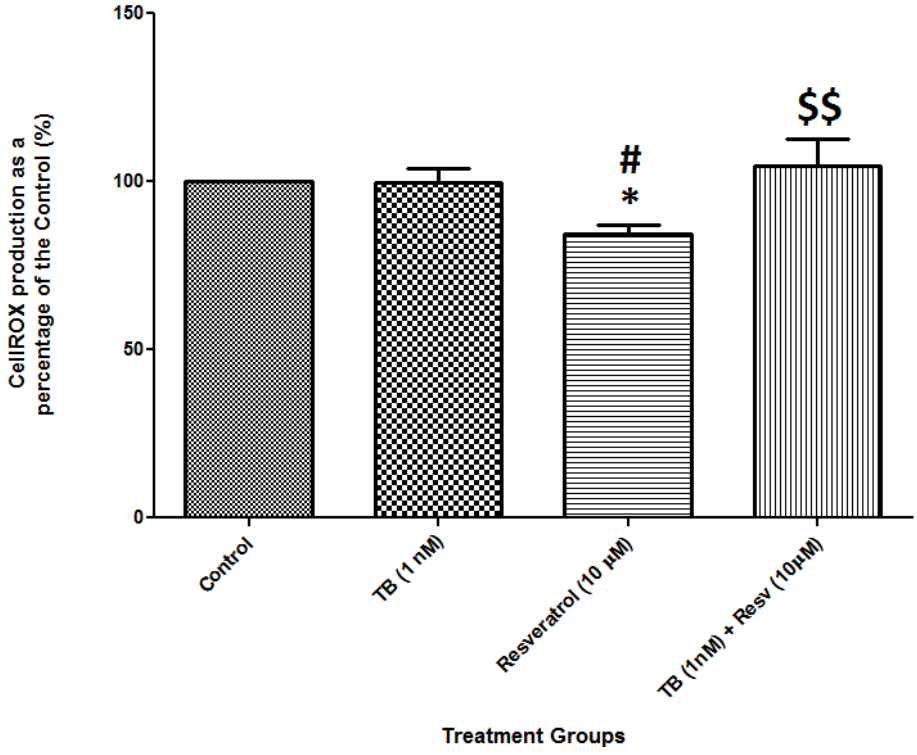

Figure 5.3.1: Percentage of CellROX production in cardiomyocytes (%) following treatment with Tiotropium bromide (1 nM) ± resveratrol (10 µM) in normoxic conditions, expressed as a percentage of the normoxia control*. Cardiomyocytes were treated for 4 hours in normoxic conditions with Tiotropium bromide (1 nM) ± resveratrol (10 µM), apart from the normoxia control group, before staining with CellROX and analysed using the FL-1 channel of the flow cytometer. Data is presented as the Mean ± SEM, n of 3-7. * vs. Control, p<0.05; # vs. TB (1nM), p<0.05 and $$ vs. Resveratrol (10µM), p<0.01.*

5.4 Role of Tiotropium bromide in presence or absence of Resveratrol on cardiomyocyte death

Cardiomyocytes were incubated with Tiotropium bromide (1 nM) ± resveratrol (10 µM) and the normoxic controls respectively for a period of 4 hours before staining with Annexin-V and Propidium iodide (PI) and analysed on the flow cytometer using the FL-1 and FL-2 channels. Propidium iodide was used as a counter stain to differentiate between necrotic and dead apoptotic cells.

Figure 5.4.1 shows the percentage of total apoptosis in cardiomyocytes treated with Tiotropium bromide (1 nM) ± resveratrol (10 µM) in normoxic conditions. Tiotropium bromide (1 nM) shows an observable but non-significant increase in total apoptosis with respect to the normoxic control (117.2 ± 8.85% (TB 1 nM) vs. 100 ± 0.0% (normoxia), $p>0.05$, fig 5.4.1). However, resveratrol (10 µM) administration shows a decrease in total apoptosis with respect to Tiotropium (1 nM) (79.97 ± 7.79% (Resv 10µM) vs. 117.2 ± 8.85% (TB 1 nM), $p<0.05$, fig 5.4.1). Tiotropium (1 nM) + resveratrol (10 µM) shows an observable but non-significant decrease in total apoptosis with respect to Tiotropium (1 nM) alone (97.48 ± 28.71% (TB 1nM + Resv 10µM) vs. 117.2 ± 8.85% (TB 1 nM), $p>0.05$, fig 5.4.1). Data for necrosis did not show any significant difference between any of the groups ($p>0.05$, data not shown).

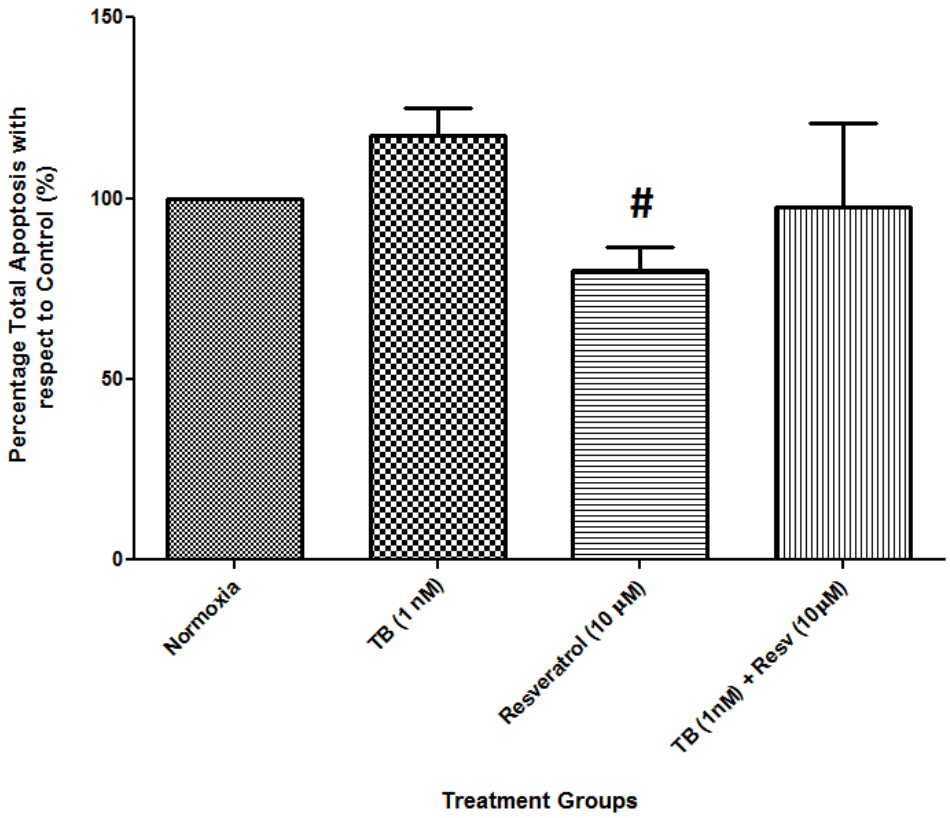

Figure 5.4.1: Percentage of total apoptotic cardiomyocytes (%) following treatment with Tiotropium bromide (1 nM) ± resveratrol (10 µM) in normoxic conditions, expressed as a percentage of the normoxia control. *Cardiomyocytes were treated for 4 hours in normoxic conditions with Tiotropium bromide (1 nM) ± resveratrol (10 µM), apart from the normoxia control group, before staining with Annexin-V/Propidium iodide and analysed using the FL-1 and FL-2 channels of the flow cytometer. Data is presented as the Mean ± SEM, n of 3-4. # vs. TB (1nM), p<0.05.*

5.5 Expression profile of genes involved in Oxidative Stress following Tiotropium Bromide administration

Figure 5.5.1 shows the gene expression profile of Nfe2l2, Casp-3, Akt1, Gpx4, Hrt1, Prkcδ, Gstπ1, Prdx4, Prkcζ, Tp53, Cyba, Atf2, Lcn2, Ldha, Prkaα1, Sod2 and Ucp2 typically involved in oxidative stress, following Tiotropium bromide (1 nM) administration in normoxic conditions, all genes were normalised using the ΔΔ Ct method to normoxic control samples which represent a value of 1.

Several of the genes show statistically significant decreases with respect to the control samples. The Nfe2l2, Akt1, Tp53 and Prkaα1 show a similar significant decrease in gene expression following Tiotropium (1 nM) administration with respect to the normoxic control following GAPDH normalisation (0.18 ± 0.25 (Nfe2l2), 0.16 ± 0.20 (Akt1), 0.37 ± 0.13 (Tp53) and 0.15 ± 0.21 (Prkaα1) vs. 1.0 ± 0.0, $p<0.05$ respectively, fig 5.5.1). The Gstπ1, Cyba, Atf2 and Lcn2 also show a significant decrease in gene expression following Tiotropium (1 nM) (0.18 ± 0.06 (Gstπ1), 0.22 ± 0.04 (Cyba), 0.08 ± 0.11 (Atf2) and 0.66 ± 0.02 (Lcn2) vs. 1.0 ± 0.0, $p<0.01$, fig 5.5.1). The greatest significant decrease in gene expression is observed with the Prkcζ gene following Tiotropium (1 nM) administration with respect to normoxic controls (0.33 ± 0.00 (Prkcζ) vs. 1.0 ± 0.0, $p<0.001$, fig 5.5.1). Although the Gpx4, Hprt1 and Ldha genes shows an observable increase in expression, these are non-significant with respect to normoxic controls (2.08 ± 1.29 (Gpx4), 2.11 ± 0.76 (Hprt1) and 3.24 ± 1.98 (Ldha) vs. 1.0 ± 0.0, $p>0.05$, fig 5.5.1). Similarly, although the Casp-3, Prkcδ, Prdx4, Sod2 and Ucp2 genes show an observable decrease in expression, these are non-significant with respect to normoxic controls (0.86 ± 0.59 (Casp-3), 0.54 ± 0.35 (Prkcδ), 0.66 ± 0.12 (Prdx4), 0.23 ± 0.33 (Sod2) and 0.92 ± 0.61 (Ucp2) vs. 1.0 ± 0.0, $p>0.05$, fig 5.5.1).

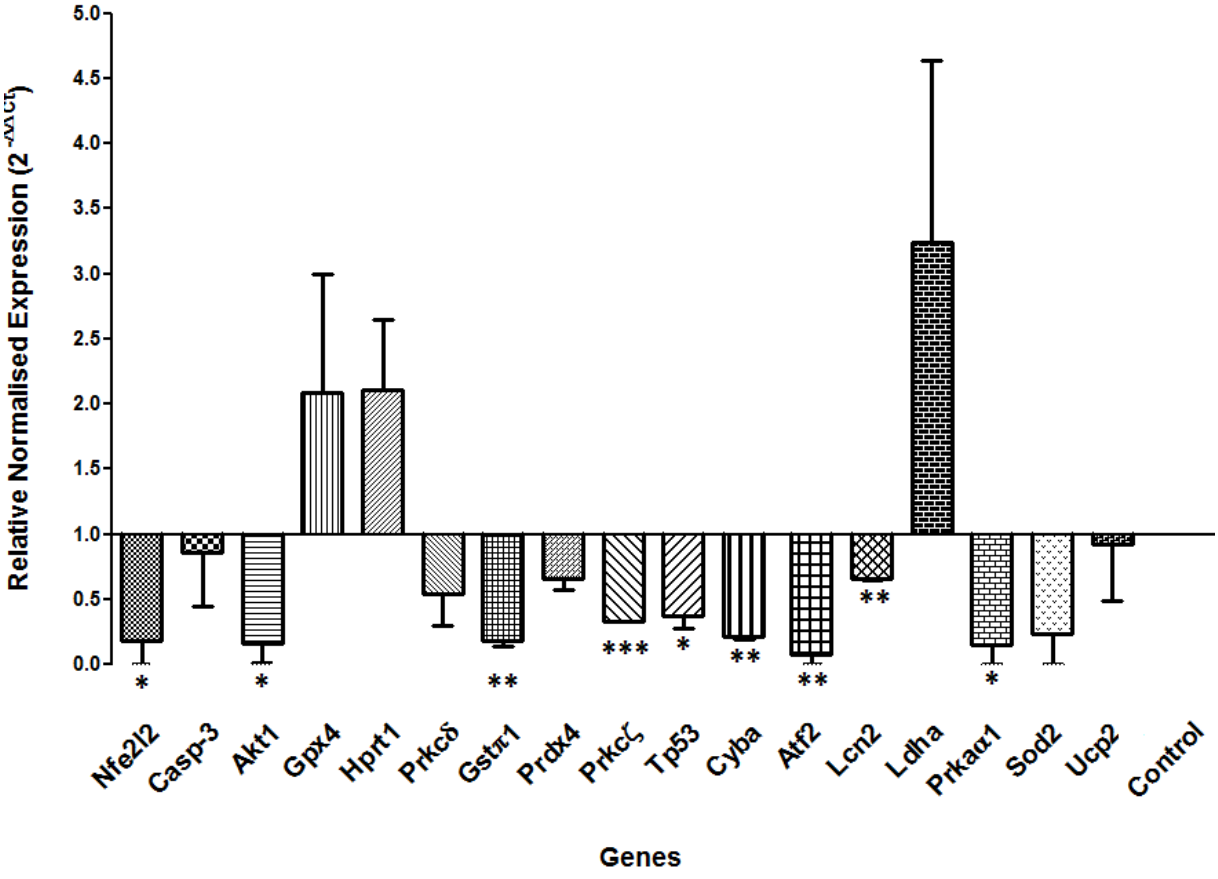

Figure 5.5.1: Relative normalised gene expression levels of various genes involved in oxidative stress following Tiotropium bromide (1 nM) in a whole heart Langendorff model of normoxic conditions*. Whole heart tissue was obtained following 120 minutes of Tiotropium (1 nM) administration and assessed for gene expression using the Myocardial Infarction Prime PCR pathway plate. GAPDH was used as a house keeping gene to normalise the gene expression levels using the ΔΔ Ct method and compared against the normoxic control samples. Data is presented as the Mean ± SEM, n of 2. * vs. Control, p<0.05; ** vs. Control, p<0.01 and *** vs. Control, p<0.001.*

Chapter 6: The role of Calcium signalling in Tiotropium mediated cardiotoxicity in *ex vivo* and *in vitro* Cardiac models

The primary aim of this study was to elucidate the cardiotoxicity of long acting muscarinic receptor antagonists, notably how Tiotropium bromide induces myocardial damage in *ex vivo* and *in vitro* models of normoxic conditions and ischaemia/reperfusion injury.

(b) Understanding the effect of Tiotropium bromide on calcium signalling … in *ex vivo* and *in vitro* normoxic conditions via Ca^{2+}/calmodulin kinase II…

6.1 Effect of the Ca^{2+} channel blocker, Nifedipine on Tiotropium mediated cardiotoxicity in Normoxic cardiac conditions

6.1.1 Haemodynamic Data Analysis in Normoxic conditions

Using a whole heart Langendorff model, the haemodynamic data for Tiotropium bromide (1 nM) administration in the presence or absence of nifedipine (1 nM) following 20 minutes of stabilisation in normoxic conditions are depicted in figures 6.1.1.1 – 6.1.1.3. Tiotropium bromide (1nM) ± nifedipine (1 nM) was administered following a 20-minute stabilisation period and perfused throughout the rest of the experimental protocol for a period of 155 minutes, as described in section 2.3.3.1. The data collected for all parameters (fig 6.1.1.1 – 6.1.1.3) following Tiotropium bromide (1nM) ± nifedipine (1 nM) administration were statistically analysed at 15, 25, 50, 70 and 160 minutes. The Tiotropium bromide (1nM) ± nifedipine (1 nM) groups were analysed with respect to the normoxic control as well as between each treatment group.

The coronary flow (fig 6.1.1.1) data shows that the Tiotropium bromide (1nM) + nifedipine (1 nM) group shows a significant decrease in coronary flow with respect to nifedipine (1 nM) at 50 and 70 minutes (50 minutes: 80.44 ± 6.02% (TB 1nM + Nif 1nM) vs. 99.98 ± 7.30% (Nif 1nM), $p<0.05$; 70 minutes: 71.31 ± 8.83% (TB 1nM + Nif 1nM) vs. 98.98 ± 3.00% (Nif 1nM), $p<0.01$, fig 6.1.1.1).

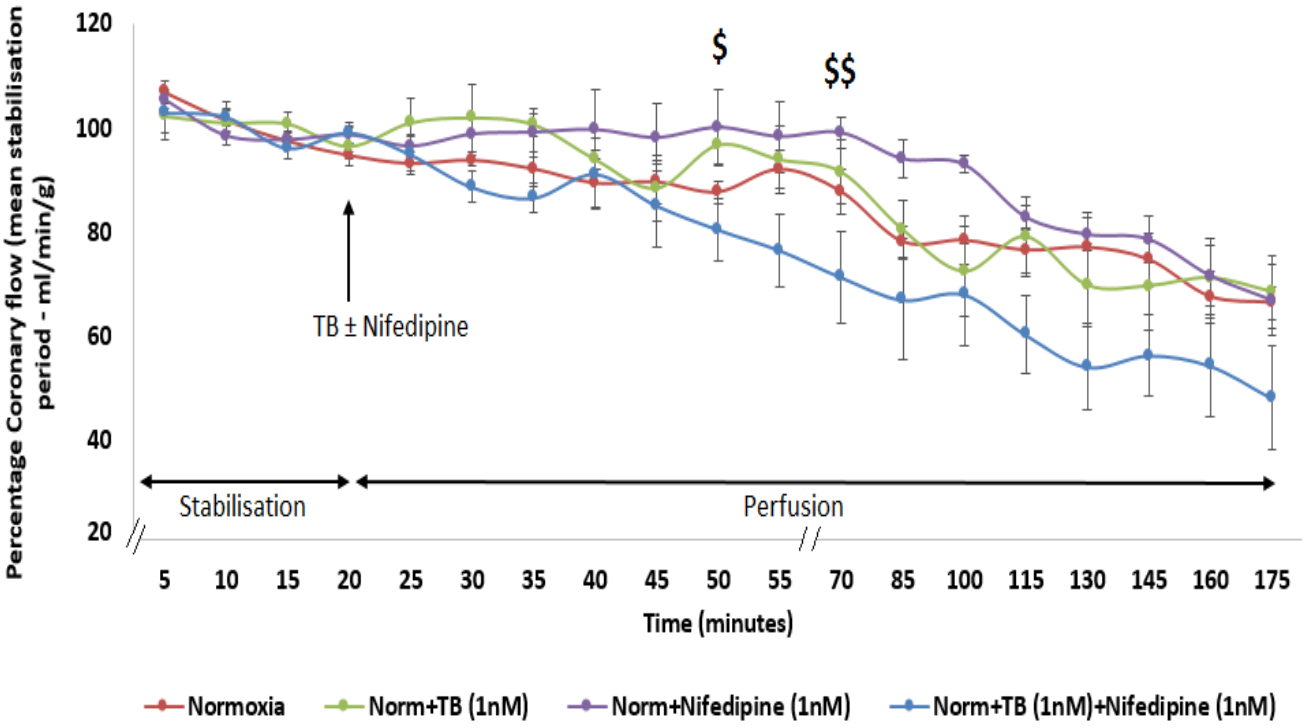

Figure 6.1.1.1: Percentage coronary flow of the mean stabilisation period following administration with Tiotropium bromide (1nM) ± nifedipine (1 nM)*. Langendorff hearts were subjected to a 20-minute period of stabilisation perfused with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (1nM) ± nifedipine (1 nM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups. $ TB (1nM) + Nifedipine (1nM) vs. Nifedipine (1nM), p<0.05; $$ TB (1nM) + Nifedipine (1nM) vs. Nifedipine (1nM), p<0.01.*

Figure 6.1.1.2 shows left ventricular developed pressure (LVDP) following Tiotropium bromide (1nM) ± nifedipine (1 nM) administration in normoxic conditions. The data does not show any statistical significances amongst the Tiotropium bromide (1nM) ± nifedipine (1 nM) groups at the time points assessed (p>0.05, fig 6.1.1.2) between the groups or with respect to the normoxic control.

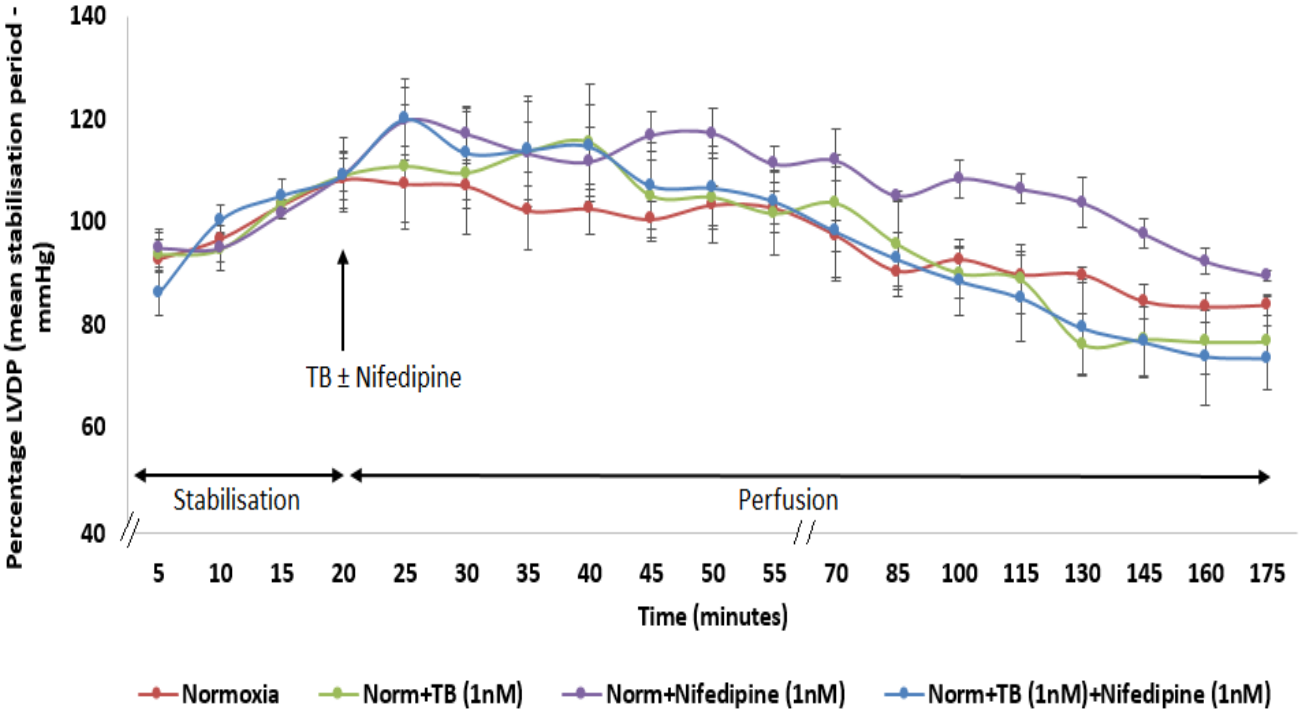

Figure 6.1.1.2: Percentage left ventricular developed pressure (LVDP) of the mean stabilisation period following administration with Tiotropium bromide (1nM) ± nifedipine (1 nM)*. Langendorff hearts were subjected to a 20-minute period of stabilisation perfused with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (1nM) ± nifedipine (1 nM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups.*

Figure 6.1.1.3 shows heart rate following administration of Tiotropium bromide (1nM) ± nifedipine (1 nM) in normoxic conditions. The data shows no statistical significance in heart rate observed with Tiotropium bromide (1nM) ± nifedipine (1 nM) and the normoxic control group ($p>0.05$, fig 6.1.1.3).

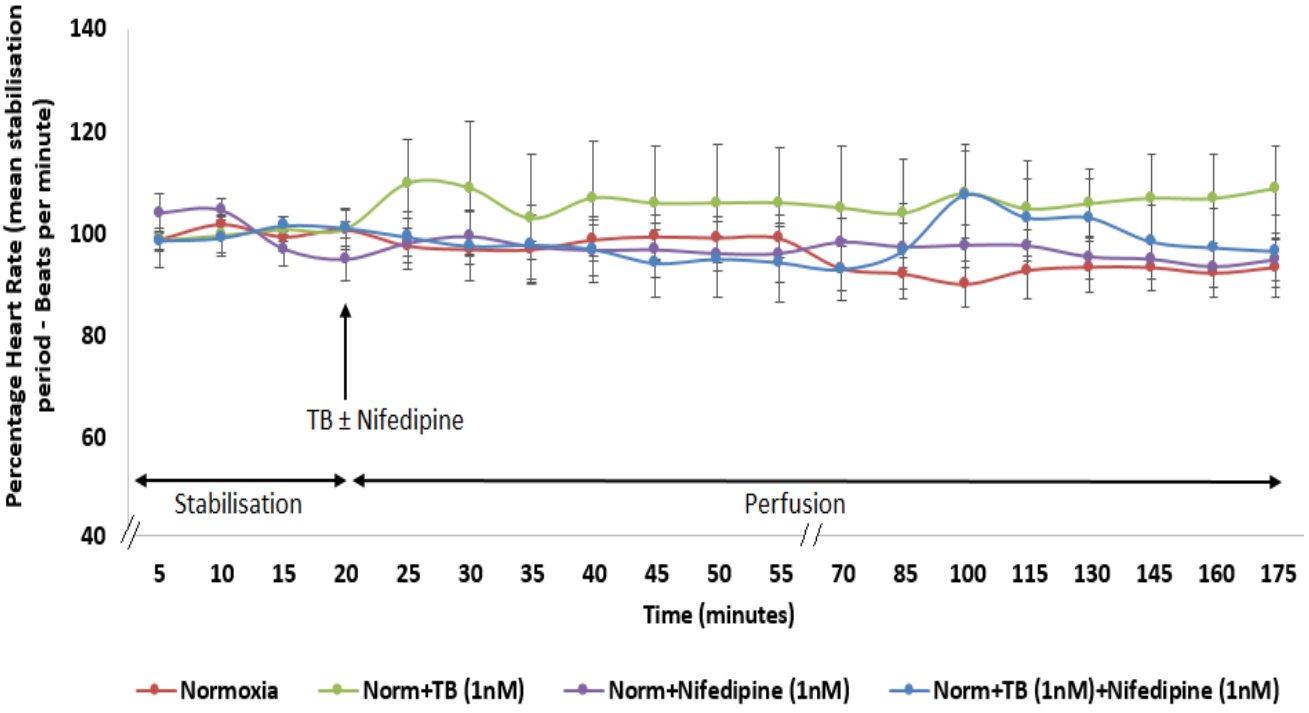

Figure 6.1.1.3: Percentage heart rate of the mean stabilisation period following administration with Tiotropium bromide (1nM) ± nifedipine (1 nM)*. Langendorff hearts were subjected to a 20-minute period of stabilisation perfused with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (1nM) ± nifedipine (1 nM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups.*

6.1.2 Infarct size analysis following Nifedipine administration in the presence or absence of Tiotropium bromide

This study observed the effects of Tiotropium bromide (1 nM) ± nifedipine (1 nM) administration on infarct size (%), in normoxic conditions. Tiotropium bromide (1 nM) ± nifedipine (1 nM) was administered following 20 minutes of stabilisation and continued for 155 minutes; hearts were subjected to TTC staining to determine infarct size.

Figure 6.1.2.1 shows the infarct/risk ratios of Tiotropium bromide (1 nM) ± nifedipine (1 nM) with respect to normoxic (Norm) controls. The study shows that there is a significant difference in infarct size following Tiotropium bromide (1 nM) ± nifedipine (1 nM) administration with respect to the normoxic control. Tiotropium (1 nM) shows an increase

in infarct size compared to the normoxic control (18.69 ± 1.79% vs. 10.28 ± 1.74%, p<0.001, fig 6.1.2.1). The administration of nifedipine (1 nM) shows no significance with respect to the normoxic control (p>0.05), however a significant decrease is observed with respect to the Tiotropium (1 nM) group (6.32 ± 0.77% (Nif 1nM) vs. 18.69 ± 1.79% (TB 1nM), p<0.001, fig 6.1.2.1). The decrease in infarct size following nifedipine (1 nM) administration is sustained upon co-administration with Tiotropium (1 nM) with respect to Tiotropium (1 nM) alone (11.32 ± 1.66% (TB 1nM + Nif 1nM) vs. 18.69 ± 1.79% (TB 1nM), p<0.01, fig 6.1.2.1).

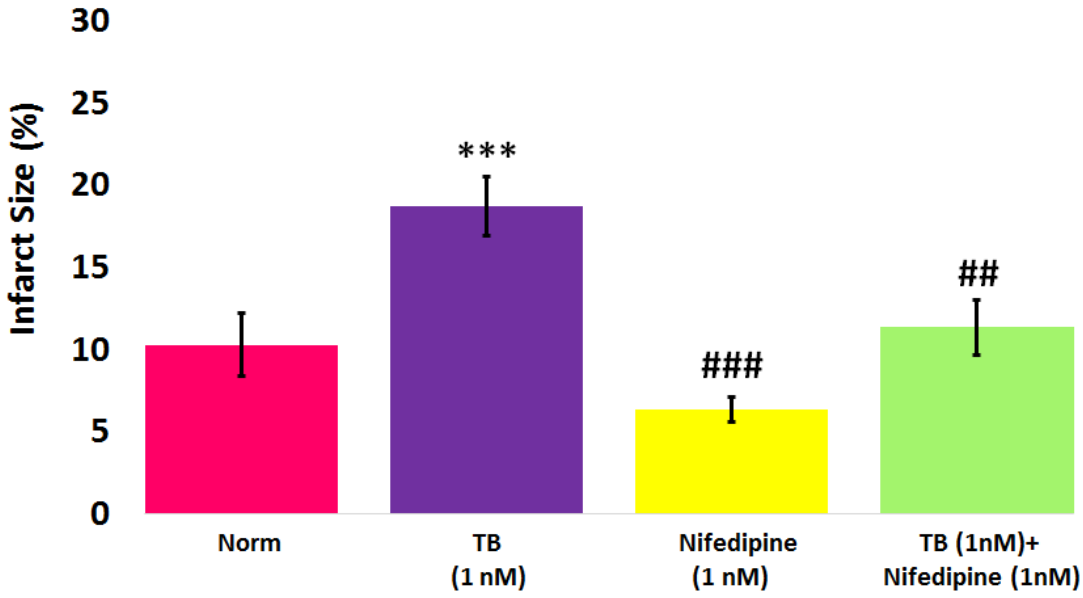

Figure 6.1.2.1: Percentage infarct size (%) following Tiotropium bromide (1 nM) ± nifedipine (1 nM) in normoxic conditions*. Langendorff hearts were subjected to a 20-minute period of stabilisation with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (1 nM) ± nifedipine (1 nM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups. *** TB (1nM) vs. Norm, p<0.001; ## Nifedipine (1 nM) vs. TB (1nM), p<0.01 and ### Nifedipine (1nM) vs. TB (1nM), p<0.001.*

6.2 Effect of the specific Ca^{2+}/Calmodulin Kinase II inhibitor, KN-93 on Tiotropium bromide induced cardiotoxicity in Normoxia and Ischaemia/Reperfusion whole heart models

6.2.1 Haemodynamic Data Analysis

6.2.1.1 Haemodynamic in Normoxic conditions for Tiotropium ± KN-93

Tiotropium bromide (1nM) ± KN-93 (400nM) was administered following a 20-minute stabilisation period and perfused throughout the rest of the experimental protocol for a period of 155 minutes, as described in section 2.3.3.1. The data for all parameters (fig 6.2.1.1.1 – 6.2.1.1.3) following Tiotropium bromide (1nM) ± KN-93 (400nM) were analysed at 15, 25, 50, 70 and 160 minutes. Tiotropium bromide (1nM) ± KN-93 (400nM) was compared to the normoxic control and each group.

The coronary flow (fig 6.2.1.1.1) shows that Tiotropium bromide (1nM) ± KN-93 (400 nM) show no significant difference in coronary flow with respect to other groups ($p>0.05$, fig 6.2.1.1.1).

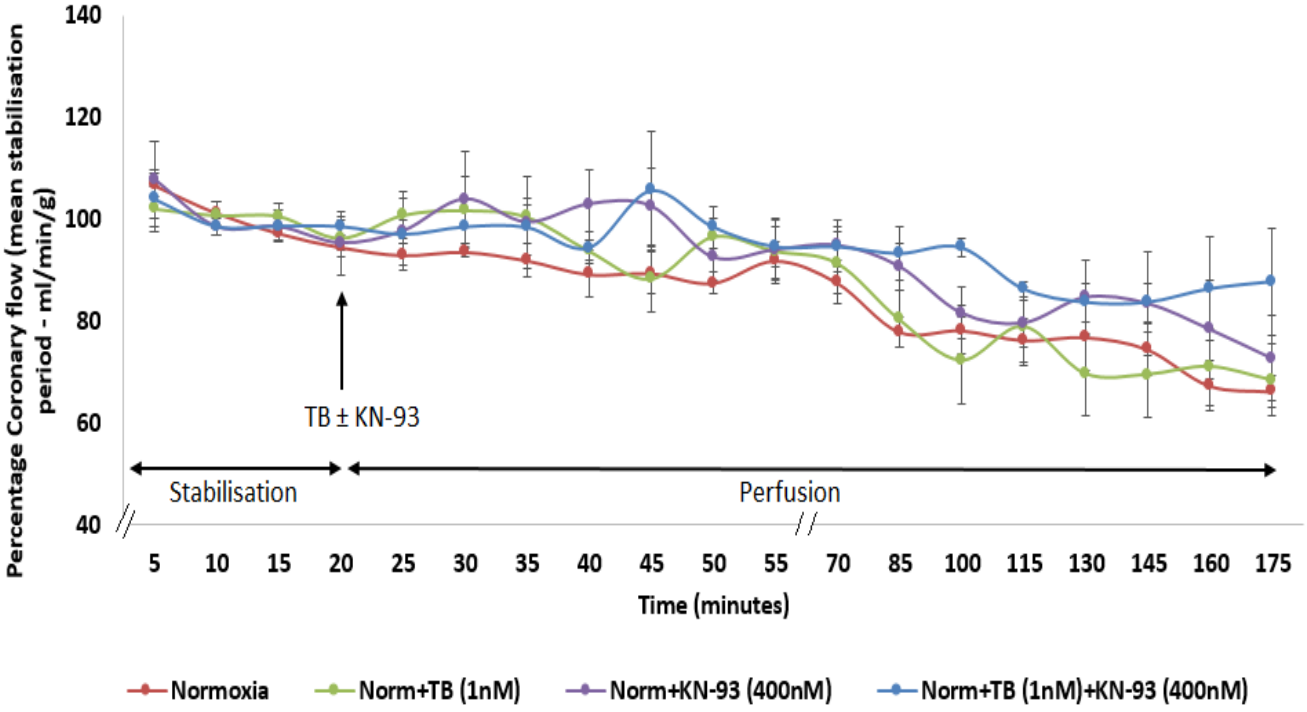

Figure 6.2.1.1.1: Percentage coronary flow of the mean stabilisation period following administration with Tiotropium bromide (1nM) ± KN-93 (400 nM)*. Langendorff hearts were subjected to a 20-minute period of stabilisation perfused with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (1nM) ± KN-93 (400 nM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups.*

Figure 6.2.1.1.2 shows left ventricular developed pressure (LVDP) following Tiotropium bromide (1nM) ± KN-93 (400 nM) administration in normoxic conditions. The data shows statistical significances amongst the Tiotropium bromide (1nM) ± KN-93 (400 nM) groups at 50 and 160 minutes. At 50 minutes, there is a significant increase in coronary flow following Tiotropium (1 nM) + KN-93 (400 nM) with respect to the KN-93 (400 nM) alone group (131.35 ± 12.62% (TB 1nM + KN-93 400nM) vs. 91.75 ± 6.36% (KN-93 400nM), $p<0.05$, fig 6.2.1.1.2). At 160 minutes, the Tiotropium (1 nM) + KN-93 (400 nM) group shows a significant increase in coronary flow with respect to all other groups (103.47 ± 7.28% (TB 1nM + KN-93 400nM) vs. 83.19 ± 2.89% (Norm, $p<0.05$), 76.45 ± 6.11% (TB 1nM) and 77.14 ± 3.01% (KN-93 400nM), $p<0.01$ respectively, fig 6.2.1.1.2).

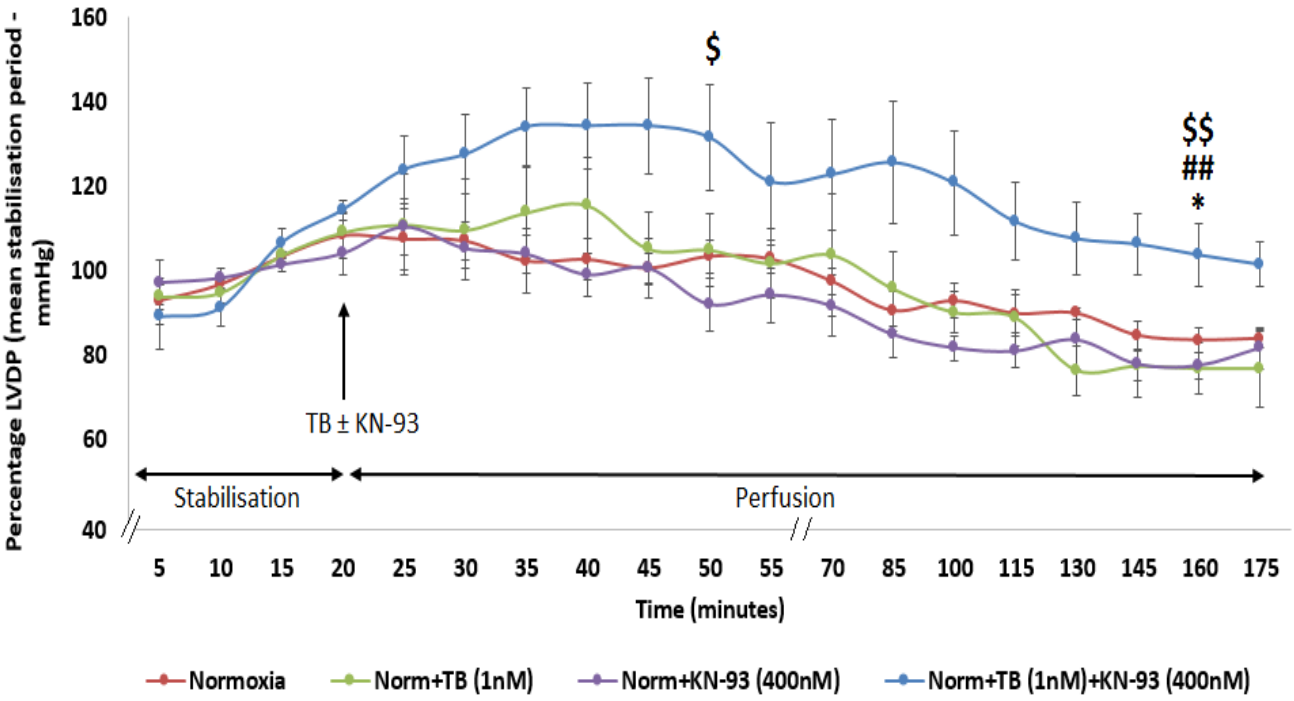

Figure 6.2.1.1.2: Percentage left ventricular developed pressure (LVDP) of the mean stabilisation period following administration with Tiotropium bromide (1nM) ± KN-93 (400 nM). *Langendorff hearts were subjected to a 20-minute period of stabilisation perfused with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (1nM) ± KN-93 (400 nM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups. * vs. Normoxia, p<0.05; ## vs. Norm+TB (1nM), p<0.01; $ vs. Norm+KN-93 (400nM), p<0.05 and $$ vs. Norm+KN-93 (400nM), p<0.01.*

Figure 6.2.1.1.3 shows heart rate following administration of Tiotropium bromide (1nM) ± KN-93 (400 nM) in normoxic conditions. The data shows no statistical significance in heart rate observed with Tiotropium bromide (1nM) ± KN-93 (400 nM) and the normoxic control group (p>0.05, fig 6.2.1.1.3).

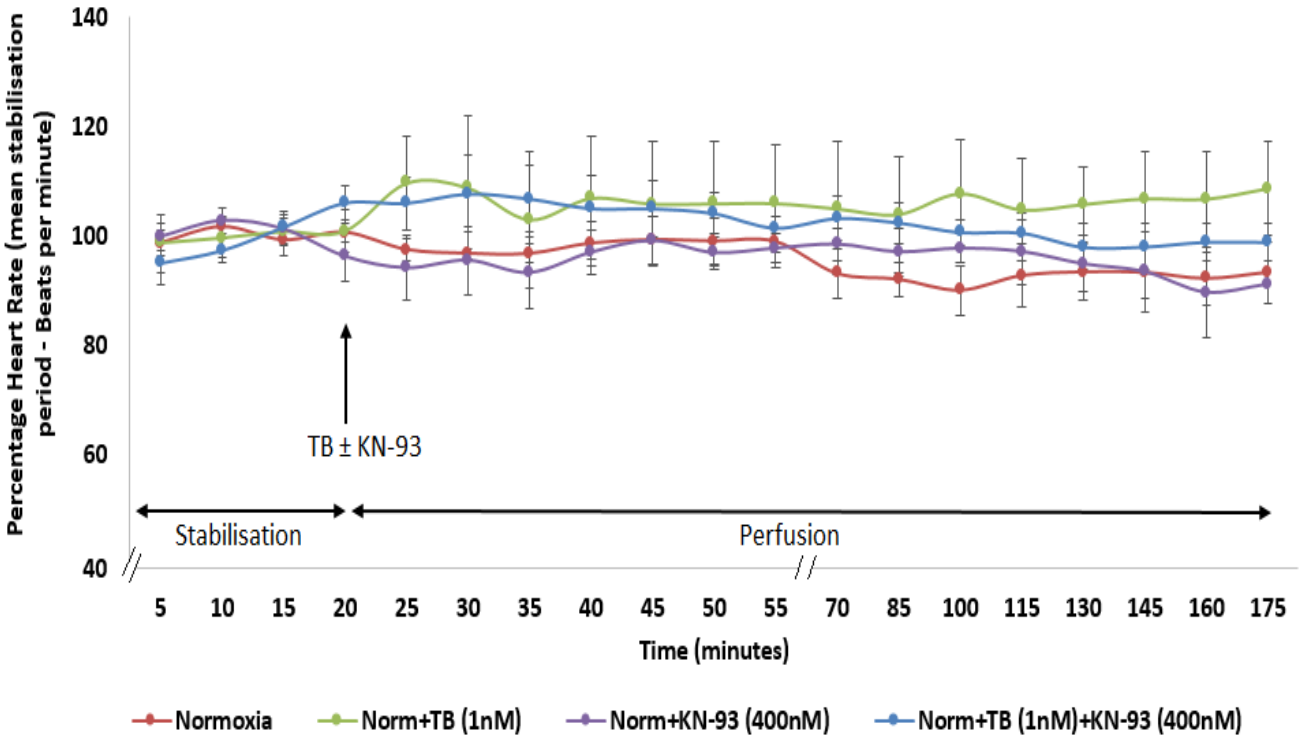

Figure 6.2.1.1.3: Percentage heart rate of the mean stabilisation period following administration with Tiotropium bromide (1nM) ± KN-93 (400 nM)*. Langendorff hearts were subjected to a 20-minute period of stabilisation perfused with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (1nM) ± KN-93 (400 nM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups.*

6.2.1.2 Haemodynamics in Ischaemia/Reperfusion for Tiotropium ± KN-93

The effect of Tiotropium bromide (1 nM) administration in the presence or absence of KN-93 (400 nM), was assessed in conditions of myocardial ischaemia/reperfusion injury with 20 minutes of stabilisation preceding 35 minutes of regional ischaemia followed by 120 minutes of reperfusion; drug administration lasted the duration of reperfusion, as described in section 2.3.3.2.

Haemodynamic data was recorded for each study; at the end of each experiment, hearts were stained with Evans blue and retained for infarct analysis using the TTC method. Coronary flow (fig 6.2.1.2.1), left ventricular developed pressure (LVDP, fig 6.2.1.2.2) and

heart rate (fig 6.2.1.2.3) are shown for Tiotropium bromide (1 nM) ± KN-93 (400 nM) administration in conditions of ischaemia/reperfusion as well as the ischaemia/reperfusion control group. The data collected for all parameters (fig 6.2.1.2.1 – 6.2.1.2.3) following Tiotropium bromide (1 nM) ± KN-93 (400 nM) administration in ischaemia/reperfusion were statistically analysed at 15, 25, 50, 70 and 160 minutes.

Figure 6.2.1.2.1 for coronary flow shows no statistical significance at the onset of reperfusion with Tiotropium bromide (1 nM) ± KN-93 (400 nM) ($p>0.05$) between each group and also with respect to the ischaemia/reperfusion control.

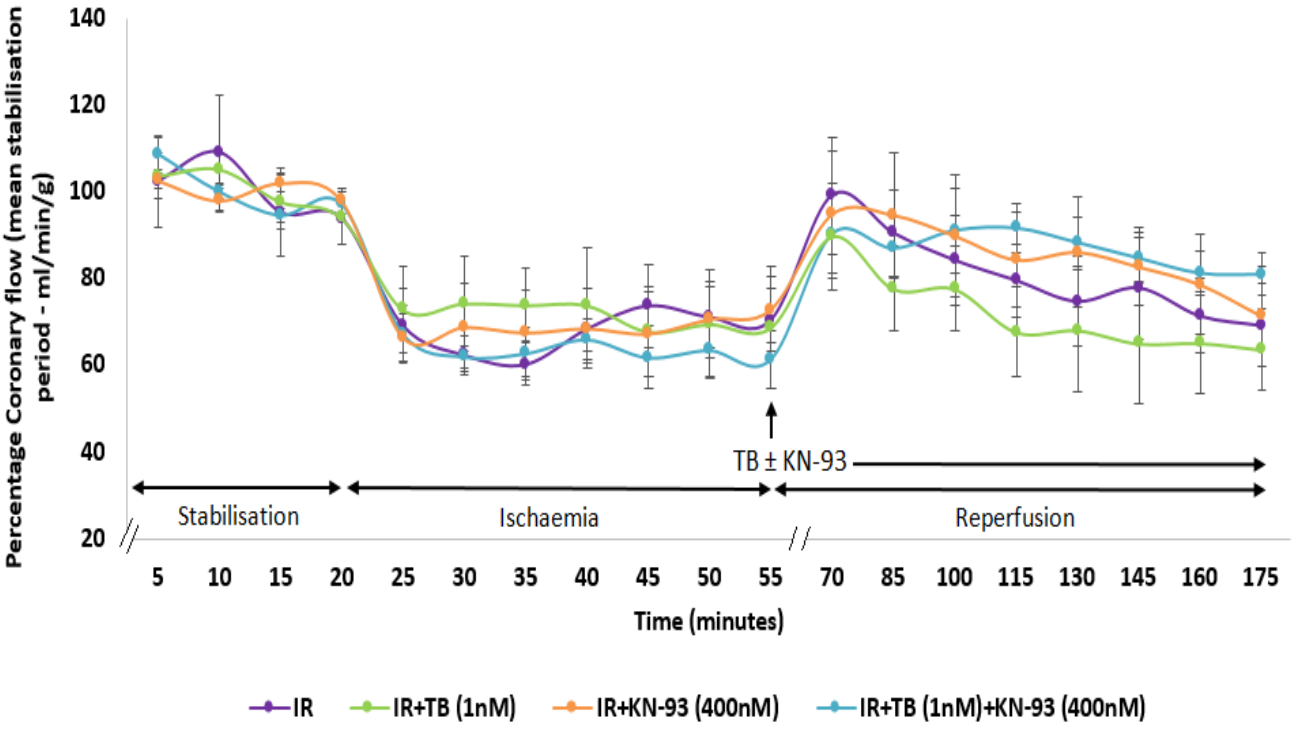

Figure 6.2.1.2.1: Percentage coronary flow of the mean stabilisation period following Tiotropium bromide (1 nM) ± KN-93 (400 nM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Tiotropium bromide (1 nM) ± KN-93 (400 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups.*

Figure 6.2.1.2.2 shows the left ventricular pressure recorded (LVDP) from the Tiotropium bromide (1 nM) ± KN-93 (400 nM) treatment groups. The data does not show any statistical significances amongst the Tiotropium bromide (1 nM) ± KN-93 (400 nM) groups at the time points assessed ($p>0.05$, fig 6.2.1.2.2) between the different groups or with respect to the ischaemia/reperfusion group.

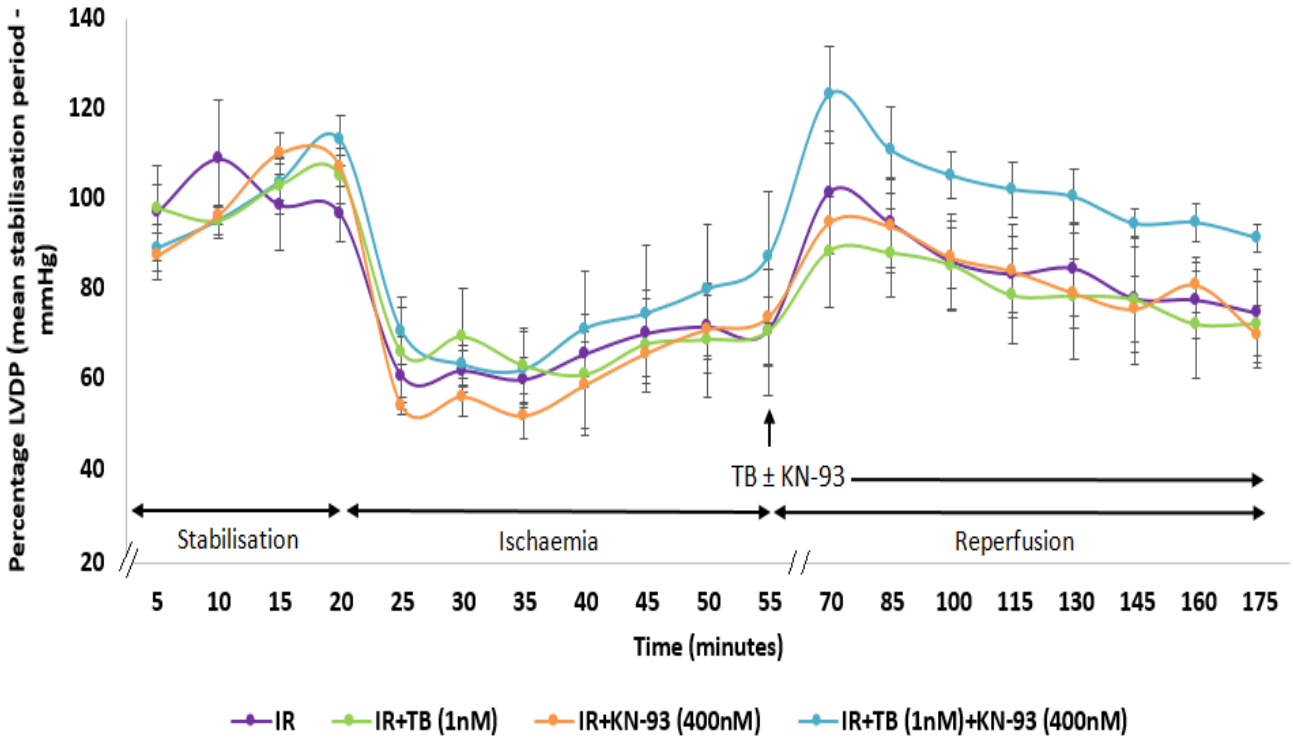

Figure 6.2.1.2.2: Percentage left ventricular developed pressure (LVDP) of the mean stabilisation period following Tiotropium bromide (1 nM) ± KN-93 (400 nM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Tiotropium bromide (1 nM) ± KN-93 (400 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups.*

Figure 6.2.1.2.3 shows heart rate following administration of Tiotropium bromide (1 nM) ± KN-93 (400 nM) following ischaemia/reperfusion. The data shows no statistical significance

in heart rate observed with Tiotropium bromide (1 nM) ± KN-93 (400 nM) and the ischaemia/reperfusion control group ($p>0.05$, fig 6.2.1.2.3).

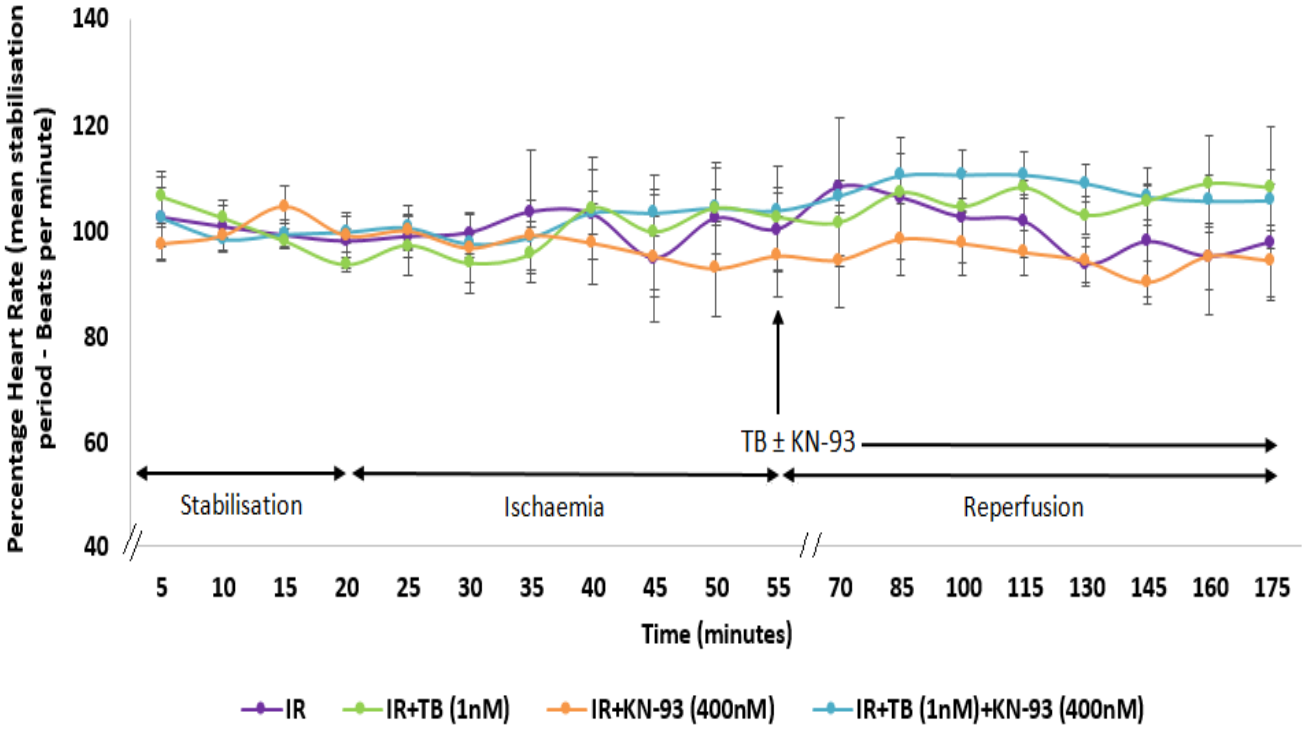

Figure 6.2.1.2.3: Percentage heart rate of the mean stabilisation period following Tiotropium bromide (1 nM) ± KN-93 (400 nM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Tiotropium bromide (1 nM) ± KN-93 (400 nM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups.*

6.2.2 Infarct to Risk analysis of Tiotropium bromide in the presence or absence of KN-93

6.2.2.1 Infarct to Risk analysis in Normoxic conditions for Tiotropium ± KN-93

This study observed the effects of Tiotropium bromide (1 nM) ± KN-93 (400 nM) administration on infarct size (%), in normoxic conditions. Tiotropium bromide (1 nM) ±

KN-93 (400 nM) was administered following 20 minutes of stabilisation and continued for 155 minutes; hearts were subjected to TTC staining to determine infarct size.

Figure 6.2.2.1.1 shows the infarct size of Tiotropium bromide (1 nM) ± KN-93 (400 nM) with respect to normoxic (Norm) controls. The study shows that there is a significant difference in infarct size following Tiotropium bromide (1 nM) ± KN-93 (400 nM) administration with respect to the normoxic control. Tiotropium (1 nM) shows an increase in infarct size compared to the normoxic control (18.69 ± 1.79% vs. 10.28 ± 1.74%, $p<0.001$, fig 6.2.2.1.1). The administration of KN-93 (400 nM) shows no significance with respect to the normoxic control ($p>0.05$), however a significant decrease is observed with respect to the Tiotropium (1 nM) group (9.50 ± 0.90% (KN-93 400nM) vs. 18.69 ± 1.79% (TB 1nM), $p<0.01$, fig 6.2.2.1.1). Tiotropium (1 nM) + KN-93 (400 nM) administration also shows a significant decrease in infarct size with respect to Tiotropium (1 nM) (12.81 ± 2.81% (TB 1nM + KN-93 400nM) vs. 18.69 ± 1.79% (TB 1nM), $p<0.05$, fig 6.2.2.1.1).

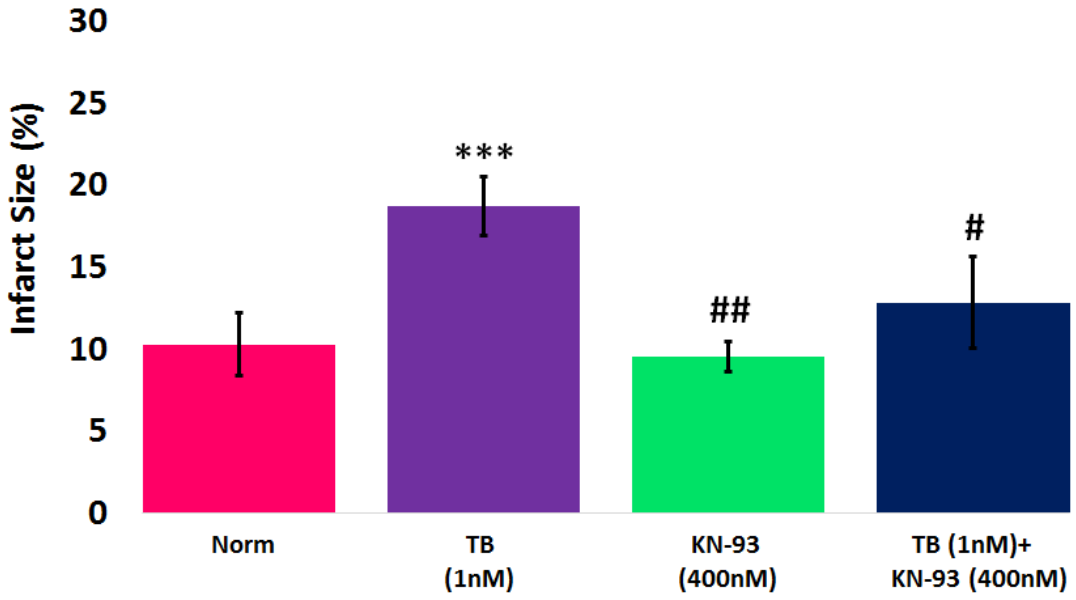

Figure 6.2.2.1.1: Percentage infarct to risk ratios (%) following Tiotropium bromide (1 nM) ± KN-93 (400 nM) in normoxic conditions*. Langendorff hearts were subjected to a 20-minute period of stabilisation with Krebs-Henseleit buffer, thereafter switched to Tiotropium bromide (1 nM) ± KN-93 (400 nM) perfusion for 155-minutes. All groups were subjected to drug treatment apart from the normoxia group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups. *** TB (1nM) vs. Norm p<0.001; # TB (1nM) + KN-93 (400nM) vs. TB (1nM), p<0.05 and ## KN-93 (400nM) vs. TB (1nM), p<0.01*

6.2.2.2 Infarct to Risk analysis in Ischaemia/Reperfusion for Tiotropium ± KN-93

This study observed the effects of Tiotropium bromide (1 nM) ± KN-93 (400 nM) administration on infarct to risk ratio (%), in an *in vitro* model of myocardial ischaemia/reperfusion injury. Tiotropium bromide (1 nM) ± KN-93 (400 nM) was administered at the onset of reperfusion and continued throughout the 120-minute period following 20 minutes of stabilisation and 35 minutes of regional ischaemia as described in section 2.3.2.2, followed by Evans blue dye staining to delineate ischaemic zones, and subjected to TTC staining to determine infarct size to risk ratio.

Figure 6.2.2.2.1 shows the infarct/risk ratios of Tiotropium bromide (1 nM) ± KN-93 (400 nM) with respect to the ischaemia/reperfusion (IR) control. The study showed that Tiotropium bromide (1 nM) administration results in a significant increase in infarct to risk

ratio with respect to the ischaemia/reperfusion control (65.42 ± 3.00% (TB 1nM) vs. 50.85 ± 3.93 (IR), p<0.01, fig 6.2.2.2.1). There is a significant decrease in infarct to risk ratio with KN-93 (400 nM) administration with respect to Tiotropium (1 nM) (48.20 ± 4.62% (KN-93 400nM) vs. 65.42 ± 3.00% (TB 1nM), p<0.05, fig 6.2.2.2.1). Tiotropium (1 nM) + KN-93 (400 nM) administration also shows a significance decrease in infarct to risk ratio with respect to Tiotropium (1 nM) alone (47.36 ± 3.88% (TB 1nM + KN-93 400nM) vs. 65.42 ± 3.00% (TB 1nM), p<0.05, fig 6.2.2.2.1).

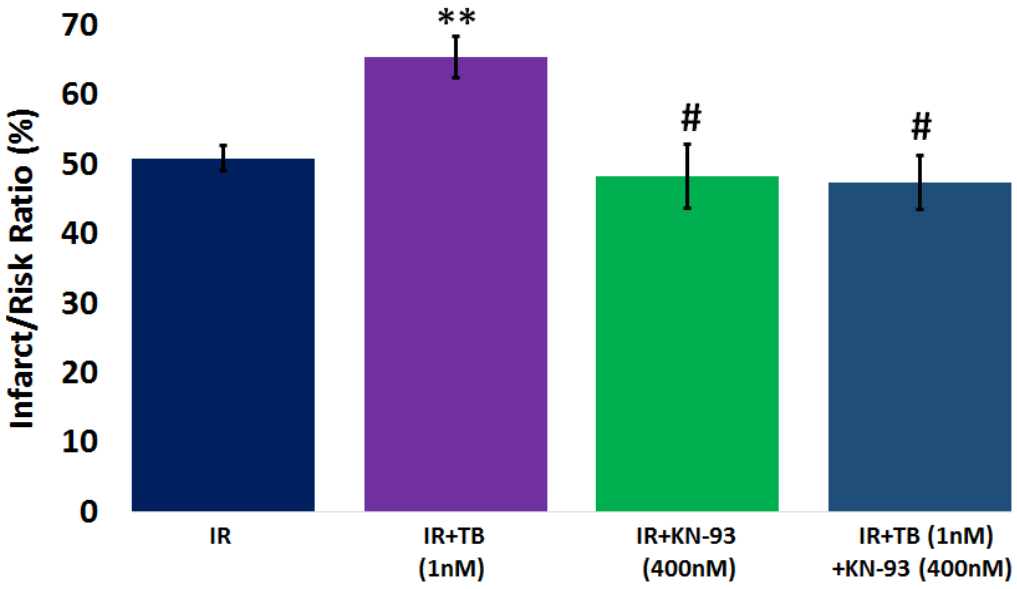

Figure 6.2.2.2.1: Percentage infarct to risk ratios (%) following Tiotropium bromide (1 nM) ± resveratrol (10 µM) in ischaemia/reperfusion*. Langendorff hearts were subjected to a 20-minute period of stabilisation, thereafter subjected to 35 minutes of ischaemia before 120 minutes of Tiotropium bromide (1 nM) ± resveratrol (10 µM) throughout reperfusion. All groups were subjected to drug treatment apart from the 'IR' (ischaemia/reperfusion) group. Values plotted signify the Mean ± SEM, derived from an n of 4-5 for all groups. ** vs. IR p<0.01 and # vs. IR+TB (1nM), p<0.05.*

6.3 Effect of Tiotropium Bromide on p-Akt $_{(Ser473)}$ expression in the presence or absence of KN-93 in Normoxic conditions

6.3.1 Effect of Tiotropium bromide administration on Phospho-Akt $_{(Ser473)}$ expression in the presence or absence of KN-93

P-Akt $_{(Ser473)}$ activation was assessed using western blotting for phosphorylated Akt $_{(Ser473)}$ in cardiac tissue to establish the role of Akt in Tiotropium bromide (1 nM) ± KN-93 (400 nM) mediated cardiotoxicity. Figure 6.3.1.1 shows the expression pattern observed following normoxia and Tiotropium bromide (1 nM) ± KN-93 (400 nM). All drugs were administered following stabilisation in a modified Langendorff model, for a period of 155 minutes; hearts were immediately removed following the end of the protocol period and the left ventricle was excised and snap-frozen as described.

The data shows a significant increase in phosphorylated Akt $_{(Ser473)}$ following Tiotropium (1 nM) administration with respect to the normoxic control (88.03 ± 5.62% (TB 1nM) vs. 36.17 ± 5.13% (Norm), $p<0.0001$, fig 6.3.1.1). KN-93 (400 nM) shows a significant decrease in p-Akt $_{(Ser473)}$ with respect to the Tiotropium (1 nM) mediated increase (57.21 ± 4.69% (KN-93 400nM) vs. 88.03 ± 5.62% (TB 1nM), $p<0.01$, fig 6.3.1.1); however, KN-93 (400 nM) administration shows a significant increase in p-Akt $_{(Ser473)}$ with respect to the normoxic control (57.21 ± 4.69% (KN-93 400nM) vs. 36.17 ± 5.13% (Norm), $p<0.05$, fig 6.3.1.1). Tiotropium (1 nM) mediated increase in p-Akt $_{(Ser473)}$ is attenuated upon co-administration of KN-93 (400 nM) with respect to Tiotropium (1 nM) alone (60.51 ± 4.92% (TB 1nM + KN-93 400nM) vs. 88.03 ± 5.62% (TB 1nM), $p<0.01$, fig 6.3.1.1); however, it shows significantly greater p-Akt $_{(Ser473)}$ than the normoxic control (60.51 ± 4.92% (TB 1nM + KN-93 400nM) vs. 36.17 ± 5.13% (Norm), $p<0.01$, fig 6.3.1.1).

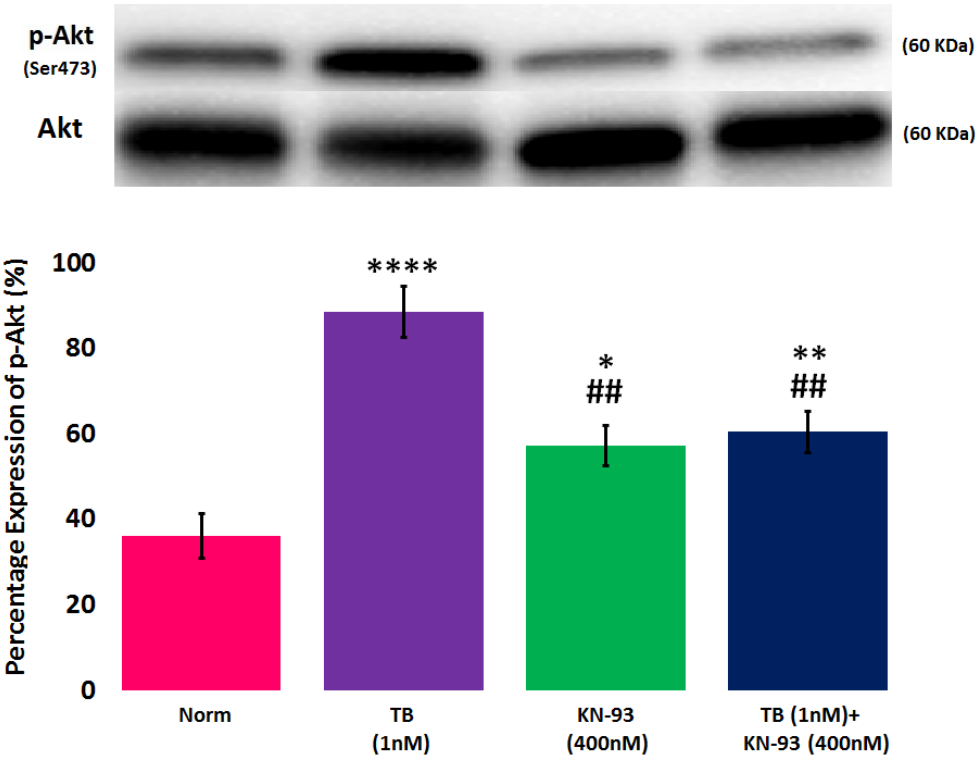

Figure 6.3.1.1: Percentage expression (%) of phosphorylated Akt *(Ser473)* ***as a percentage of total Akt following Tiotropium bromide (1 nM) ± KN-93 (400 nM) in normoxic conditions****. Langendorff hearts were perfused with either Krebs-Henseleit buffer (Norm) or Tiotropium bromide (1 nM) ± KN-93 (400 nM). Values plotted signify the Mean ± SEM, derived from an n of 3 for all groups. * vs. Norm, p<0.05; ** vs. Norm, p<0.01; **** vs. Norm, p<0.0001; ## vs. TB (1nM), p<0.01.*

6.4 Effect of Tiotropium Bromide on the expression of phosphorylated Ca^{2+}/Calmodulin Kinase II (CaMKII) $_{(Thr286)}$ in the presence or absence of KN-93

CaMKII activation was assessed using western blotting for phosphorylated CaMKII $_{(Thr286)}$ in cardiac tissue to establish the role of CaMKII in Tiotropium bromide (1 nM) mediated cardiotoxicity ± KN-93 (400 nM). Figure 6.4.1 shows the expression pattern of p-CaMKII $_{(Thr286)}$ observed following normoxia and Tiotropium bromide (1 nM) ± KN-93 (400 nM). All drugs were administered following stabilisation in a modified Langendorff setup, for a period of 155 minutes; hearts were immediately removed following the end of the protocol period and the left ventricle was excised and snap-frozen as described.

The data shows a significant increase in phosphorylated CaMKII $_{(Thr286)}$ following Tiotropium (1 nM) administration with respect to the normoxic control (86.46 ± 8.43% (TB 1nM) vs. 43.73 ± 5.71% (Norm), $p<0.0001$, fig 6.4.1). KN-93 (400 nM) shows a significant decrease in p-CaMKII $_{(Thr286)}$ with respect to Tiotropium (1 nM) (51.84 ± 8.11% (KN-93 400nM) vs. 86.46 ± 8.43% (TB 1nM), $p<0.01$, fig 6.3.2.1). The increase in p-CaMKII $_{(Thr286)}$ observed with Tiotropium (1 nM) is attenuated upon co-administration with KN-93 (400 nM) with respect to Tiotropium (1 nM) alone (59.58 ± 8.94% (TB 1nM + KN-93 400nM) vs. 86.46 ± 8.43% (TB 1nM), $p<0.05$, fig 6.3.2.1).

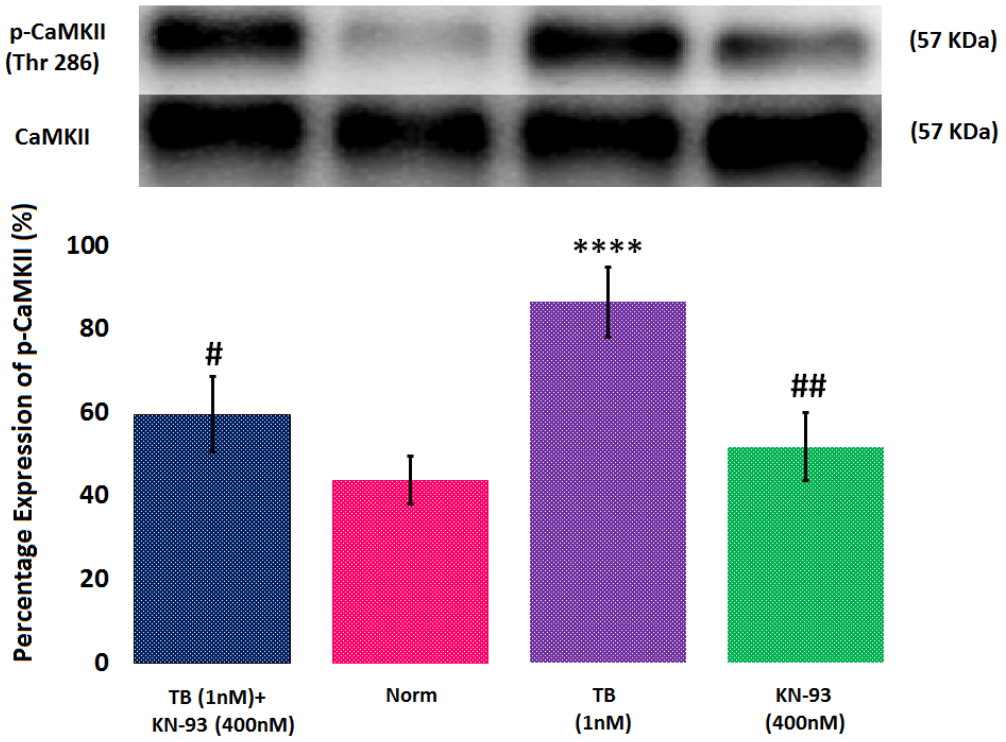

Figure 6.4.1: Percentage expression (%) of phosphorylated CaMKII $_{(Thr286)}$ as a percentage of total CaMKII following Tiotropium bromide (1 nM) ± KN-93 (400 nM) in normoxic conditions*. Langendorff hearts were perfused with either Krebs-Henseleit buffer (Norm) or Tiotropium bromide (1 nM) ± KN-93 (400 nM). Values plotted signify the Mean ± SEM, derived from an n of 3 for all groups. **** vs. Norm, $p<0.0001$; # vs. TB (1nM), $p<0.05$ and ## vs. TB (1nM), $p<0.01$.*

6.5 Release of intracellular Ca^{2+} in cardiomyocytes following Tiotropium bromide administration in the presence or absence of KN-93

Cardiomyocytes were incubated with Tiotropium bromide (1 nM) ± KN-93 (400 nM), amiloride (100 mM) as a negative control, ionomycin (1 µM) as a positive control and the normoxic controls respectively for a period of 4 hours before staining with the Ca^{2+} indicator, Fluo-3AM and analysed on the flow cytometer using the FL-1 channel.

Figure 6.5.1 shows the percentage of Fluo-3AM as an indicator of intracellular Ca^{2+} in cardiomyocytes treated with Tiotropium bromide (1 nM) ± KN-93 (400 nM) in normoxic conditions. Tiotropium bromide (1 nM) shows a significant increase in Fluo-3AM with respect to the normoxic control (130.63 ± 1.65% (TB 1nM) vs. 100 ± 0.0% (Norm), $p<0.05$, fig 6.5.1). KN-93 (400 nM) administration shows an observable but non-significant decrease in Fluo-3AM with respect to Tiotropium (1 nM) alone, Tiotropium (1 nM) + KN-93 (400 nM) and ionomycin (1 µM) (108.01 ± 8.59% (KN-93 400nM) vs. 130.63 ± 1.65% (TB 1nM), 124.34 ± 22.30% (TB 1nM + KN-93 400nM) and 135.69 ± 9.17% (Iono 1µM), $p>0.05$, fig 6.5.1). Amiloride (100 mM) shows a significant decrease in Fluo-3AM with respect to Tiotropium (1 nM) alone and TB (1 nM) + KN-93 (400 nM) (93.92 ± 10.01% (Amil 100mM) vs. 130.63 ± 1.65% (TB 1nM) and 124.34 ± 22.30% (TB 1nM + KN-93 400nM), $p<0.05$, fig 6.5.1). Ionomycin (1 µM) shows a significant increase in Fluo-3AM with respect to the normoxic control and amiloride (100 mM) groups (135.69 ± 9.17% (Iono 1µM) vs. 100 ± 0.0% (Norm) and 93.92 ± 10.01% (Amil 100mM), $p<0.01$, fig 6.5.1).

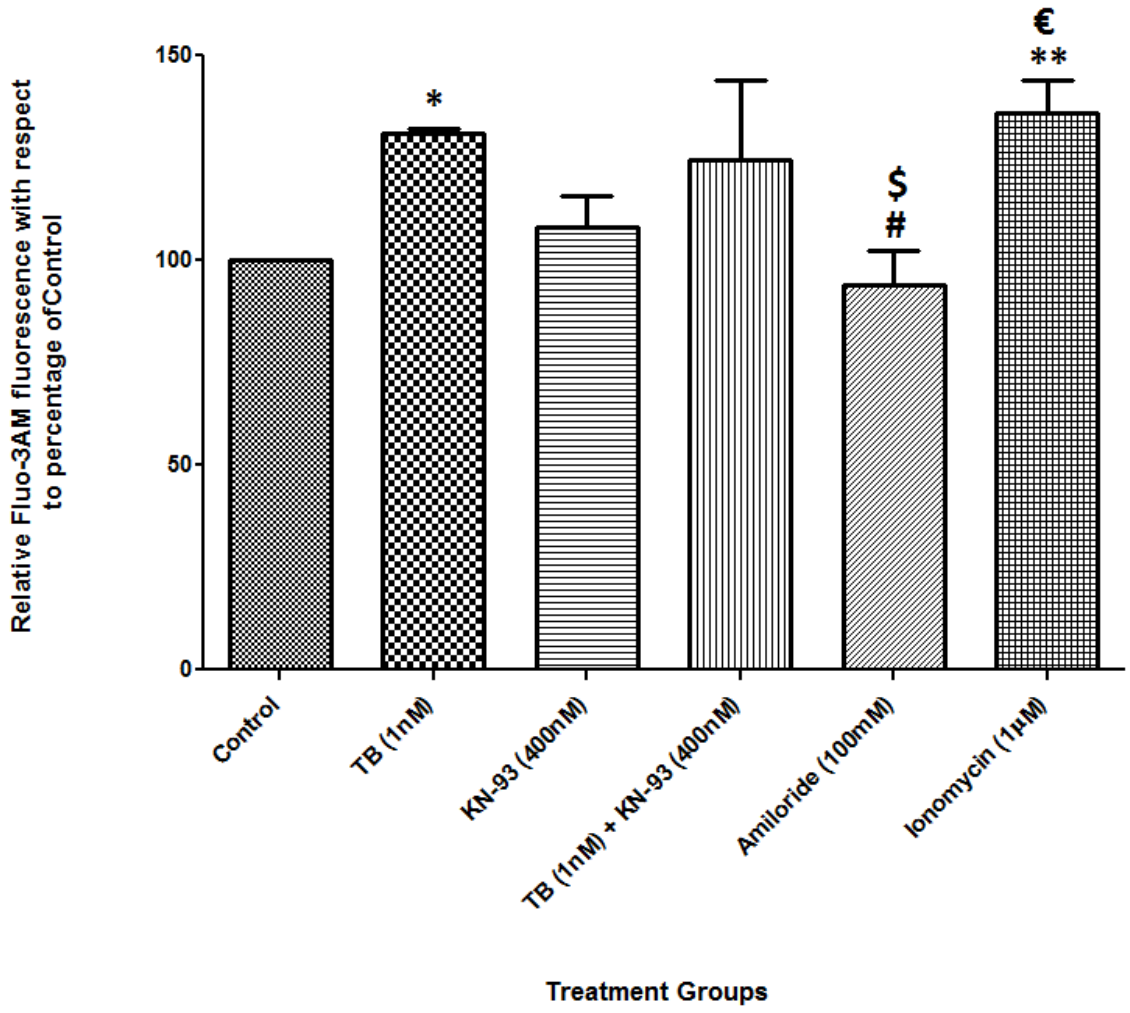

Figure 6.5.1: Percentage fluorescence of Fluo-3AM in cardiomyocytes (%) following treatment with Tiotropium bromide (1 nM) ± KN-93 (400 nM) in normoxic conditions, expressed as a percentage of the normoxia control. *Cardiomyocytes were treated for 4 hours in normoxic conditions with Tiotropium bromide (1 nM) ± KN-93 (400 nM), apart from the normoxia control group, before staining with Fluo-3AM and analysed using the FL-1 channel of the flow cytometer. Data is presented as the Mean ± SEM, n of 3-6. * vs. Control, p<0.05; ** vs. Control, p<0.01; # vs. TB (1nM), p<0.05; $ vs. TB (1nM) + KN-93 (400nM), p<0.05 and € vs. Amiloride (100mM), p<0.05.*

6.6 Role of Tiotropium bromide in presence or absence of KN-93 on cardiomyocyte death

Cardiomyocytes were incubated with Tiotropium bromide (1 nM) ± KN-93 (400 nM) and the normoxic controls respectively for a period of 4 hours before staining with Annexin-V and Propidium iodide (PI) and analysed on the flow cytometer using the FL-1 and FL-2 channels. Propidium iodide was used as a counter stain to differentiate between necrotic and dead apoptotic cells.

Figure 6.6.1 shows the percentage of total apoptosis in cardiomyocyte treated with Tiotropium bromide (1 nM) ± KN-93 (400 nM) in normoxic conditions. Tiotropium bromide (1 nM) shows an observable but non-significant increase in total apoptosis with respect to the normoxic control (117.2 ± 8.85% (TB 1 nM) vs. 100 ± 0.0% (normoxia), $p>0.05$, fig 6.6.1). KN-93 (400 nM) administration does not show any significant change in total apoptosis with respect to the other groups (106.83 ± 14.94% (KN-93 400nM) vs. 100 ± 0.0% (Norm), 117.2 ± 8.85% (TB 1 nM) and 107.16 ± 14.02% (TB 1nM + KN-93 400nM), $p>0.05$, fig 6.6.1). Although the KN-93 (400 nM) and Tiotropium (1 nM) + KN-93 (400 nM) groups show an observable decrease with respect to Tiotropium (1 nM), this is not significant ($p>0.05$, fig 6.6.1). Data for necrosis did not show any significant difference between any of the groups ($p>0.05$, data not shown).

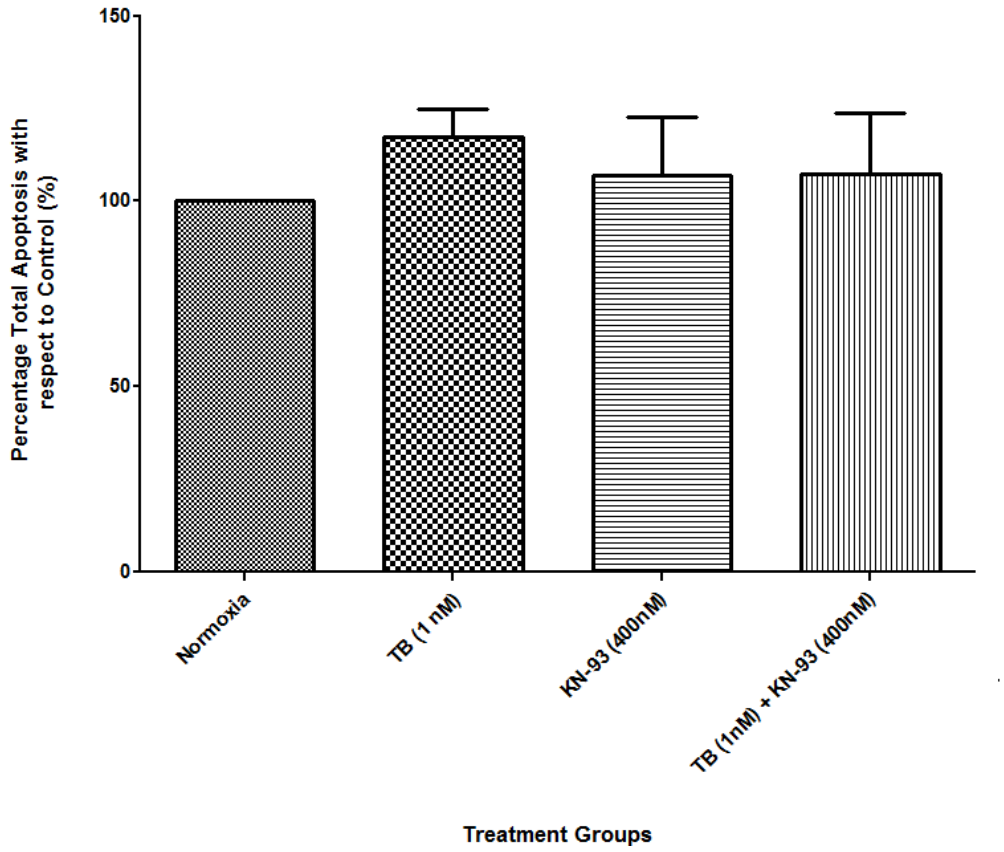

Figure 6.6.1: Percentage of total apoptotic cardiomyocytes (%) following treatment with Tiotropium bromide (1 nM) ± KN-93 (400 nM) in normoxic conditions, expressed as a percentage of the normoxia control*. Cardiomyocytes were treated for 4 hours in normoxic conditions with Tiotropium bromide (1 nM) ± KN-93 (400 nM), apart from the normoxia control group, before staining with Annexin-V/Propidium iodide and analysed using the FL-1 and FL-2 channels of the flow cytometer. Data is presented as the Mean ± SEM, n of 3-4.*

Chapter 7: General Discussion

Chapter 3 Summary - Pharmacological Profiling of Long Acting Muscarinic Receptor Antagonists (LAMAs)

This chapter observed the effect of Long acting muscarinic receptor antagonists (LAMAs) in ischaemia/reperfusion injury. LAMAs show little effect on haemodynamic function in *ex vivo* models of myocardial ischaemia/reperfusion injury; all four LAMAs show no statistical significance with respect to the ischaemia/reperfusion control nor amongst concentrations. However, analysis of infarct size to risk ratio showed a significant increase in infarct size with administration of Aclidinium (10 – 1 nM), Tiotropium (10 µM – 0.1 nM) and Umeclidinium bromide (100 – 1 nM) at reperfusion amongst various concentrations. Glycopyrronium bromide (10 µM – 1 nM) did not show any statistical significance with infarct size or haemodynamic parameters. In normoxic conditions, Tiotropium bromide (10 – 0.1 nM) significantly increases infarct size with respect to the normoxic control; haemodynamics (coronary flow and left ventricular developed pressure) also show a significant change with respect to normoxic controls and between concentrations. Analysis of genes associated with myocardial infarction show a decrease in pro-apoptotic genes (Bax and caspase-3) and a decrease in genes associated with mitochondrial biosynthesis with Tiotropium bromide at 1 nM. Analysis of apoptotic cell death show significance only with Tiotropium at 10 µM, with a concentration dependent trend in cell death.

Chapter 4 Summary - Role of Adjunctive Therapies in Reversing Tiotropium Mediated Cardiotoxicity

This chapter investigated the effect of several cardioprotective therapies on abrogating Tiotropium bromide mediated infarct size and the effect on various signalling pathways. The muscarinic agonist, acetylcholine (100 nM) shows little change in haemodynamic function when administered alone in normoxic conditions, however lower the coronary flow when combined with Tiotropium. Acetylcholine significantly reduces infarct size when co-administered with Tiotropium bromide, this is also observed with phosphorylated Akt where Tiotropium shows a significant increase in Akt phosphorylation however this is abrogated upon acetylcholine co-administration. Although acetylcholine dampens infarct size in ischaemia/reperfusion and normoxic conditions and reduces Akt phosphorylation,

there is no protective effect observed upon apoptosis when co-administered with Tiotropium. The PI3K inhibitor was also used to determine the role of the PI3K-Akt signalling pathway in Tiotropium bromide mediated cardiotoxicity. Wortmannin (100 nM) showed no significant effect on haemodynamic parameters, interestingly wortmannin did not affect Tiotropium bromide mediated infarct size but significantly attenuated Akt phosphorylation, alone and co-administered with Tiotropium. Wortmannin also significantly abrogated apoptotic cell death, alone and combined with Tiotropium. The caspase-3 inhibitor, Z-DEVD-FMK (70/140 nM) was also investigated to understand the role of caspase-3 activation in Tiotropium bromide mediated cardiotoxicity. When administered alone, Z-DEVD-FMK showed an increase in coronary flow in normoxic conditions; additionally, Z-DEVD-FMK abrogated infarct size under ischaemia/reperfusion and normoxic conditions comparably, attenuated apoptotic cell death and showed a significant reversal in Tiotropium bromide mediated cleaved caspase-3. Lastly, the mitochondrial permeability transition pore (mPTP) inhibitor cyclosporin A (200 nM) was found to increase coronary flow in ischaemia/reperfusion when administered alone and significantly attenuated infarct size in ischaemia/reperfusion and normoxic conditions, alone and combined with Tiotropium.

Chapter 5 Summary – Role of reactive oxygen species in Tiotropium mediated signalling in *ex vivo* and *in vitro* Cardiac models

The effect of the dietary antioxidant, resveratrol (10 µM) was also investigated on Tiotropium bromide mediated cardiotoxicity. Resveratrol administration showed an increase in coronary flow in normoxic conditions and showed a significant decrease in infarct size when co-administered with Tiotropium bromide in ischaemia/reperfusion and normoxic conditions. Resveratrol also showed a decrease in Akt phosphorylation compared to Tiotropium alone, however when combined a greater decrease in Akt was observed. Interestingly, analysis of reactive oxygen species (ROS) production in cardiomyocytes showed no effect on ROS following Tiotropium bromide. Although resveratrol showed a significant decrease in ROS, this was reversed upon co-administration with Tiotropium bromide. Similarly, resveratrol showed a significant decrease in apoptosis, however co-administration showed a reversal of this protective effect. Tiotropium bromide showed a decrease in genes associated with cellular anti-oxidant machinery such as glutathione S

transferase (Gstπ1) and nuclear factor like 2 (Nfe2l2) and saw a non-significant increase in lactate dehydrogenase A (Ldha).

Chapter 6 Summary – The role of Calcium signalling in Tiotropium mediated cardiotoxicity in *ex vivo* and *in vitro* Cardiac models

The effect of the L-type calcium inhibitor, nifedipine (1 nM) and the calmodulin kinase II inhibitor, KN-93 (400 nM) were investigated on Tiotropium bromide mediated cardiotoxicity. Nifedipine showed a decrease in coronary flow when co-administered with Tiotropium bromide in normoxic conditions, whereas KN-93 showed a significant increase in left ventricular developed pressure following co-administration with Tiotropium bromide in normoxic conditions. Nifedipine showed a substantial attenuation of infarct size when administered alone and combined with Tiotropium; although KN-93 abrogated infarct size, there was a greater reduction in infarct size in ischaemia/reperfusion however co-administration in both conditions showed a comparable decrease in infarct size. KN-93 showed increased Akt phosphorylation compared to normoxic controls, however this was dampened in comparison to Tiotropium mediated Akt phosphorylation. Similarly, CaMKII phosphorylation was increased with Tiotropium bromide administration however co-administration with KN-93 showed some abrogation of CaMKII activation. Interestingly, Tiotropium bromide saw an increase in Fluo-3AM fluorescence, a marker of Ca^{2+} release, however KN-93 did not show any significant change when administered alone or combined, this was also observed with apoptotic cardiomyocytes.

General Summary

Langendorff models mimic drug effects at an organ level (Bell, Mocanu and Yellon 2011, Igić 1996, Skrzypiec-Spring et al. 2007); assessing myocardial responses in the absence of confounding factors (Liao, Podesser and Lim 2012). Left ventricular pressure and contractility are often seen to be affected by drugs, therefore contractility assessment is an important aspect of toxicology (Sarazan, Kroehle and Main 2012). Drug-induced cardiotoxicity includes the inhibition of the human Ether-à-go-go-Related Gene (hERG), which results in prolonged QT syndrome, associated with Torsade de Pointes (TdP) (Mordwinkin, Burridge and Wu 2013). Doxorubicin is known to cause cardiotoxicity (Fisher et al. 2005, Ganey et al. 1991, Gharanei et al. 2013a, Gharanei et al. 2013b, Hahn, Lenihan

and Ky 2014, Hole et al. 2013); as well as the long- and short-acting β_2 receptor agonists (LABA/SABA) salmeterol and salbutamol (Prevost et al. 1997), and the short acting muscarinic receptor antagonist (SAMA), Ipratropium bromide (Harvey, Hussain and Maddock 2014). Long acting muscarinic receptor antagonists (LAMAs) have no previous link to cardiotoxicity; however, the meta-analysis conducted by Singh et al. (2008) highlighted the potential cardiovascular risks associated with the use of LAMAs such as, Tiotropium bromide (Cazzola et al. 2017a, Mathioudakis et al. 2014, Singh, Loke and Furberg 2008).

Oxidative stress and dysfunctional calcium homeostasis are known factors of drug induced cardiotoxicity (Fearnley, Roderick and Bootman 2011, Orrenius, Gogvadze and Zhivotovsky 2015). Like oxidative stress, calcium signalling functions as a switch, where a shift in the balance of intracellular calcium favours abhorrent calcium signalling, or oxidants in the case of oxidative stress (Cunningham and Gotlieb 2005, Ermak and Davies 2002). Cardioprotective strategies protect the heart from damage (Heusch 2013); including targeting mitochondria (Garlid et al. 2003, Garlid et al. 2009, Halestrap, Clarke and Javadov 2004, Oldenburg et al. 2002), survival kinases (Fujio et al. 2000, Hausenloy and Yellon 2004, Miyamoto, Murphy and Brown 2008, Yu et al. 2007) and acetylcholine mediated muscarinic signalling (Kakinuma et al. 2005, Krieg et al. 2004, Pan et al. 2012, Qin, Downey and Cohen 2003, Sun et al. 2010, Wang et al. 2012c). The current study focuses on the cardiotoxic capabilities of LAMAs, with emphasis on Tiotropium bromide mediated cardiotoxicity and the pathways involved.

7.1 Effect of long acting muscarinic receptor antagonist (LAMA) administration in an *in vitro* model of myocardial Ischaemia/Reperfusion injury

The data presented indicates the directly toxic effects of long acting muscarinic receptor antagonist (LAMA) administration in a cardiac model of ischaemia/reperfusion injury, with respect to infarct size to risk ratio. Conversely, there is no significant difference observed in haemodynamic function following the administration of LAMAs, with respect to the ischaemia/reperfusion control and between concentrations. Administration of Aclidinium, Tiotropium and Umeclidinium bromides throughout the reperfusion period in conditions of ischaemia/reperfusion show enhanced infarct to risk ratio with respect to ischaemia/reperfusion controls; this enhancement is not seen following the administration

of Glycopyrronium bromide. Aclidinium bromide (10 nM – 1 nM) administration shows that the lowest concentrations show the greatest infarct development with respect to the ischaemia/reperfusion controls. Umeclidinium bromide (1 μM – 1 nM) shows decreasing infarct development with increasing concentration and all concentrations of Tiotropium bromide (10 μM – 0.1 nM) shows similar infarct size to risk ratio.

Muscarinic receptors are thought to have a role in bladder smooth muscle contractility through stimulation, therefore use of antagonists could potentially affect aspects of cardiac contractility (Wang, Luthin and Ruggieri 1995). Activation of cardiac muscarinic receptors leads to a decrease in heart rate through slowing of the sinoatrial node (Brodde and Michel 1999, Harvey 2012). However, the present study shows very little effect on the contractile and haemodynamic function of Langendorff hearts, following LAMA administration throughout reperfusion. A study showed that electrical stimulation of vagal nerves prevented ventricular tachycardia, fibrillation and death; this protective effect was abrogated with the use of atropine (Mioni et al. 2005). Aside from the role of M_2 and M_3 receptors in cardiac contractility (Brodde and Michel 1999, Hornigold et al. 2003, Matsui et al. 2002a, Wang, Shi and Wang 2004, Wang et al. 2001), the activation of M_3 receptors is known to results in cardioprotection following ischaemia/reperfusion injury (Roy et al. 2015). Therefore, the inhibition via antagonists of this receptor subtype is likely to affect cardiomyocyte damage as it would block the cardioprotective effects.

Ischaemia is a stress factor on the heart, arterial occlusion leads to damaging consequences on the myocardium, resulting in depleted contractile function (Ibáñez et al. 2015). Animal models are a useful tool in studying the various effects of ischaemia/reperfusion injury (Bell et al. 2016), in a controlled model where the duration of ischaemia and reperfusion can be set; most studies suggest a minimum reperfusion period of 60 minutes, to observe triphenyltetrazolium (TTC) mediated infarct size (Herr, Aune and Menick 2015, Lateef, Al-Masri and Alyahya 2017, Rossello et al. 2016). Induction of ischaemia results in depressed coronary flow and LVDP, with function returned upon reperfusion (Bell, Mocanu and Yellon 2011, Herr, Aune and Menick 2015, Skrzypiec-Spring et al. 2007). Interestingly, the use of intraventricular balloons to record LVDP, may act as a preconditioning effect on the heart, through survival kinases such as JNK, ERK1/2 and p38-MAPK and have an anti-

arrhythmogenic effect due to localised inflation-induced ischaemia, which may mask experimental effects (Stensløkken et al. 2009, Wilder et al. 2016).

Ischaemia/reperfusion injury occurs following obstructive blood flow to the myocardium, resulting in ischaemic insult, which can only be minimised through reperfusion, this itself causes paradoxical injury (Dhalla and Duhamel 2007, Ibáñez et al. 2015, Murphy and Steenbergen 2008). Oxidative stress through the production of mitochondrial free radicals (Chouchani et al. 2014) and elevated calcium are essential mediators of ischaemia/reperfusion injury (Cadenas 2018), resulting in mPTP opening mediated cell death (Ibáñez et al. 2015, Ong et al. 2015c, Turer and Hill 2010). Figure 7.1.1 shows mitochondrial reactive oxygen species (ROS) generation following ischaemia/reperfusion injury. Different forms of cardiomyocyte death are thought to contribute to infarct size development following ischaemia/reperfusion injury, mediated by intracellular Ca^{2+} overload and ROS generation (Hausenloy and Yellon 2015, Hausenloy and Yellon 2013, Ibáñez et al. 2015). The abhorrent signalling of calcium and ROS during the induction of ischaemia and subsequent reperfusion are known to affect infarct size, but not necessarily haemodynamic function, which can be restored following reperfusion in animal models (Pfeffer et al. 1979), or percutaneous coronary intervention (PCI) in patients with myocardial ischaemia (Bulluck, Yellon and Hausenloy 2016, Hausenloy and Yellon 2013). The short acting muscarinic receptor antagonist, Ipratropium bromide exacerbates ischaemia/reperfusion injury in a Langendorff model (Harvey, Hussain and Maddock 2014) and causes suicidal cell death in erythrocytes via Ca^{2+} (Shaik et al. 2012). These indicate that the LAMAs which exacerbate ischaemia/reperfusion injury may also utilise pathways associated with ROS and Ca^{2+} in mediating cardiotoxicity.

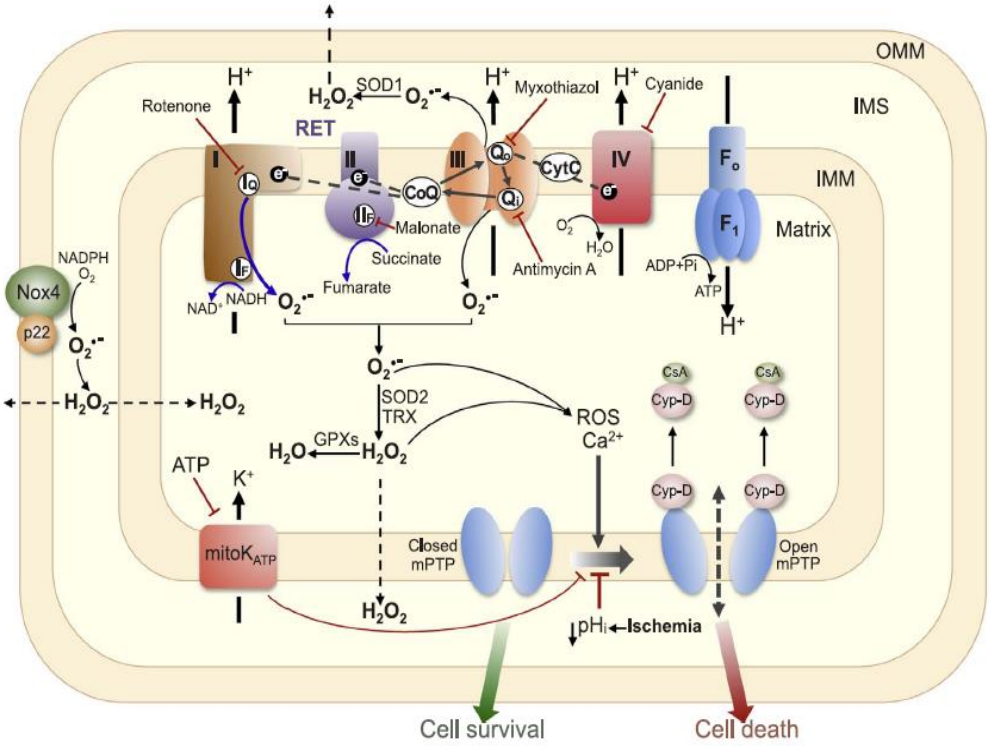

Figure 7.1.1: The role of reactive oxygen species (ROS) in mitochondria following ischaemia/reperfusion injury. *The opening of the mitochondrial permeability transition pore (mPTP) is inhibited via the acidic pH in ischaemia but initiated following reperfusion through the combined effect of Ca^{2+} overload and ROS. Succinate plays an important role in the generation of ROS in early reperfusion. CsA, cyclosporin A; CyP-D, cyclophilin D; GPX, glutathione peroxidase; IMM, inner mitochondrial membrane; IMS, intermembrane space; $mitoK_{ATP}$, mitochondrial ATP-sensitive K^+ channels; OMM, outer mitochondrial membrane; RET, reverse electron transport; SOD, superoxide dismutase; TRX, thioredoxin (Cadenas 2018).*

Although Aclidinium, Tiotropium and Umeclidinium bromides exacerbate ischaemia/reperfusion injury through infarct size to risk ratio, Glycopyrronium bromide does not show any significance in infarct size to risk ratio with respect to the control. All LAMAs carry the risk of cardiovascular adverse effects such as tachycardia, due to the presence of muscarinic receptors in the heart (Buhl and Banerji 2012); functional studies have shown Glycopyrronium bromide to exert greater safety and stability than Tiotropium bromide, with a greater therapeutic index for bradycardia and hypotension. Tiotropium

bromide also showed greater inhibition of M_2 receptors compared to Glycopyrronium bromide (Trifilieff et al. 2015); the latter is thought to affect tachycardia less and block bradycardia more effectively than atropine (Gomez, Bellido and Sanchez de la Cuesta 1995). In terms of onset of action, both Glycopyrronium and Aclidinium bromide are quicker than Tiotropium bromide and are recommended when immediate effect is desired for COPD symptoms (Cazzola and Page 2014). However, Glycopyrronium bromide has a better safety profile when compared to Tiotropium bromide (Trifilieff et al. 2015), and thus supports the observations of this study with infarct size to risk ratio.

These studies support previous clinical observations which may suggest the role of Tiotropium bromide mediated cardiac adverse effects in COPD patients (Jara, Wentworth and Lanes 2012). This propagates the need for clinical studies with emphasis on cardiac safety monitoring factors other than functional parameters, particularly the potential use of sensitive biomarker known to reflect cardiac damage which may not be observed with function alone (Ibáñez et al. 2015).

7.2 Effect of Tiotropium bromide administration in normoxic cardiac conditions on haemodynamics and infarct size

The administration of Tiotropium bromide (10 nM – 0.1 nM) in normoxic hearts results in an increase in infarct size with respect to controls, when administered following stabilisation. Although there is an increase in infarct size, the severity of cardiac damage assessed by infarct size is not as great as known cardiotoxic therapies such as anti-cancer drugs (Duran et al. 2014, Force, Krause and Van Etten 2007, Hahn, Lenihan and Ky 2014). There are also significant differences observed with coronary flow and LVDP following administration of Tiotropium bromide. This implies that in otherwise 'normal' cardiac conditions, the inhibition of muscarinic receptors results in altered cardiac function and increased cardiomyocyte damage observed as an increase in infarct size.

Muscarinic signalling is known to play a role in cardiac function through the parasympathetic nervous system (Brodde and Michel 1999). Cardiac muscarinic receptors are involved in the chronotropic regulation of the heart, specifically via the M_2 receptor subtype, however M_3 receptors are also thought to play a role in cardiac function (Gordan,

Gwathmey and Xie 2015). Parasympathetic innervation modulates the cardiac pacemaker function through regulation of the G protein gated inwardly rectifying potassium channel (GIRK) $I_{K\,ACh}$ and through the cardiac 'funny' current I_f (Baruscotti, Bucchi and DiFrancesco 2005, Dhein, Van Koppen and Brodde 2001). M_2 receptors are the predominant subtype associated with the heart and control of heart rate, with some role in atrioventricular node conduction (Harvey 2012). In bladder smooth muscle however, the M_3 receptor mediates contractility through the G_i and $G_{q/11}$ sub-families (Wang, Luthin and Ruggieri 1995). Muscarinic agonists increase cardiac contractility through protein kinase C (PKC) and PKA mediated mechanisms (Bers 2002, Harvey and Belevych 2003, Patane 2014). Figure 7.2.1 shows a schematic representing adrenergic and muscarinic receptor mediated excitation coupling. It is thought that stimulation of cardiac muscarinic receptors inhibits the 'funny' current via the pertussis sensitive $G_{\alpha i/0}$ protein, resulting in inhibition of adenylyl cyclase (Baruscotti, Bucchi and DiFrancesco 2005, Brodde and Michel 1999, Dhein, Van Koppen and Brodde 2001).

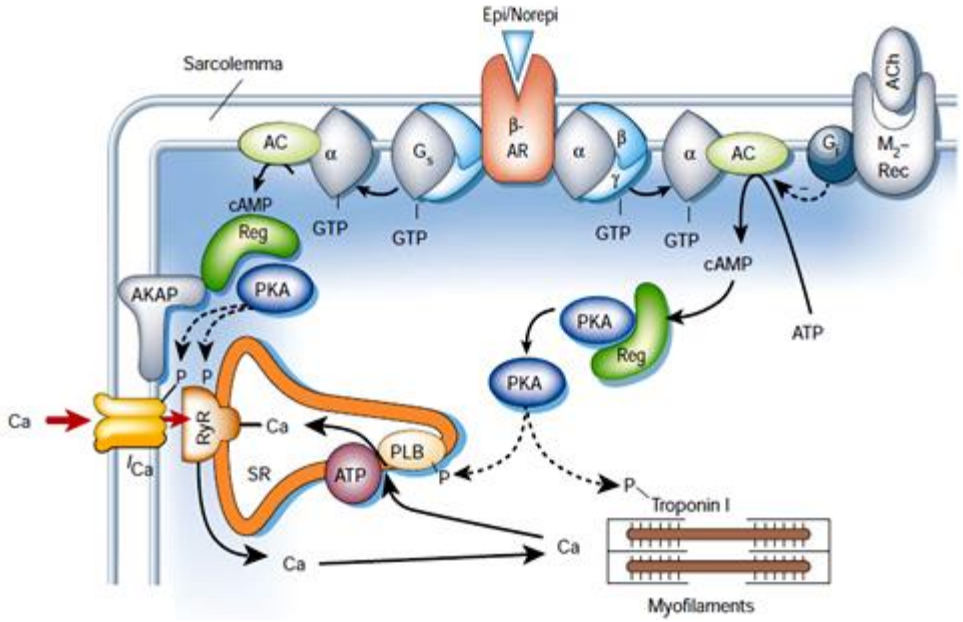

Figure 7.2.1: Agonist binding to β adrenergic receptors increases contraction and results in a decrease in intracellular Ca^{2+} concentrations through cAMP*. M_2 receptors can also contribute to cAMP increase or decrease depending on $β_1$ or $β_2$ adrenergic receptor co-stimulation (Bers 2002). AC – adenylyl cyclase, PKA – protein kinase A, Reg – PKA regulatory subunit, cAMP – cyclic adenosine monophosphate, RyR – ryanodine receptors, SR – sarcoplasmic reticulum, ATP – adenosine trisphosphate, PLB – phospholambans, Ca – calcium, GTP – guanosine trisphosphate, β AR – β adrenergic receptor, M_2 rec – muscarinic M_2 receptor, AKAP – A kinase anchoring protein.*

The cardiac 'funny' current (I_f) generated by cAMP-activated ion channel 4 (Hyperpolarisation-activated cyclic nucleotide gated channel 4 – HCN4) is essential for the heart's pacemaker activity; it is regulated by cAMP activity and therefore can be inhibited by M_2 receptor stimulation (Perde et al. 2015, Ravagli et al. 2016). The current is responsible for pacemaker depolarisation (Accili et al. 2002, DiFrancesco 2010, Perde et al. 2015), leading to a change in heart rate. Antagonism at the muscarinic receptor could potentially reverse the effect of the I_f current, which is normally inhibited through a decrease in cAMP from M_2 stimulation (Baruscotti, Bucchi and DiFrancesco 2005). Thus, the I_f current presents an opportunity to understand the effect of Tiotropium bromide on cardiac function.

M_3 receptors are coupled to G_q leading to phospholipase C mediated production of diacylglycerol (DAG) and inositol trisphosphate (IP_3), leading to calcium release and activation of PKC (Hornigold et al. 2003, Luo, Busillo and Benovic 2008). Whilst M_2 receptors dominate in the heart, M_3 receptors are known to influence atrial inotropy and are also instrumental in cardioprotection following ischaemia/reperfusion injury (Pan et al. 2012, Roy et al. 2015, Wang et al. 2012c, Zhao et al. 2010a), and in stress responses particularly in M_2 KO mice (Tomankova et al. 2015). A key mediator for GPCR signalling, is the G protein coupled receptor kinase (GRK) (Luo, Busillo and Benovic 2008), these activate following agonist occupied or stimulated GPCRs; GRK2 is ubiquitously expressed including the heart and couples to $\beta_{1/2}$ adrenoreceptors as well as $M_{2/3}$ receptors (Bünemann and Hosey 1999, Luo, Busillo and Benovic 2008, Rockman, Koch and Lefkowitz 2002). GPCRs undergo sensitisation involving the action of GRKs and β arrestins, which lead to GPCR phosphorylation following agonist binding, hindering GPCR function; other kinases such as PKA, PKC and c-Src phosphorylate GRKs; interestingly Ca^{2+} bound calmodulin inhibits GRK2 activity (Kohout and Lefkowitz 2003). Figure 7.2.2 depicts the signalling of muscarinic receptors with GRK interaction.

Agonist mediated activation of M_2 receptors leads to activation of G protein gated K^+ channels in atrial cardiomyocytes, this response occurs through the βγ subunit leading to receptor de-sensitisation; other G_i coupled receptors are also de-sensitised as a result (Bünemann and Hosey 1999). Carbachol is a muscarinic receptor agonist which shows the involvement of multiple mediators in downstream signalling, following activation of G_q leading to PLC mediated breakdown of PIP_2 into DAG and IP_3. IP_3 then binds to ER based IP_3R to result in an increase in cytosolic Ca^{2+}, whereas DAG recruits PKC and activates the Ras-Raf-MEK-ERK1/2 pathway (Luo, Busillo and Benovic 2008). GRKs and arrestins modulate this signalling, specifically GRK6 which is regulated more sensitively by Ca^{2+}-calmodulin (Bünemann and Hosey 1999). GRK6 mediated M_3 receptor phosphorylation leads to the recruitment of arrestins and therefore terminates subsequent GPCR activation. Arrestins recruit diacylglycerol kinase (DGK) to terminate PKC mediated signalling; GRKs and arrestins inhibit both arms of muscarinic signalling (Luo, Busillo and Benovic 2008). The involvement of GRKs and arrestins presents another facet to agonist bound muscarinic signalling, particularly in the absence of external stress factors such as

ischaemia/reperfusion injury (Johnson, Scott and Pitcher 2004), without GRK signalling there is an alteration in GPCR response (Walker et al. 1999); the binding of antagonists may also modulate GPCR activity through alternative GRK/arrestin mechanisms. This presents a novel explanation for the effects observed following Tiotropium bromide binding at cardiac muscarinic receptors.

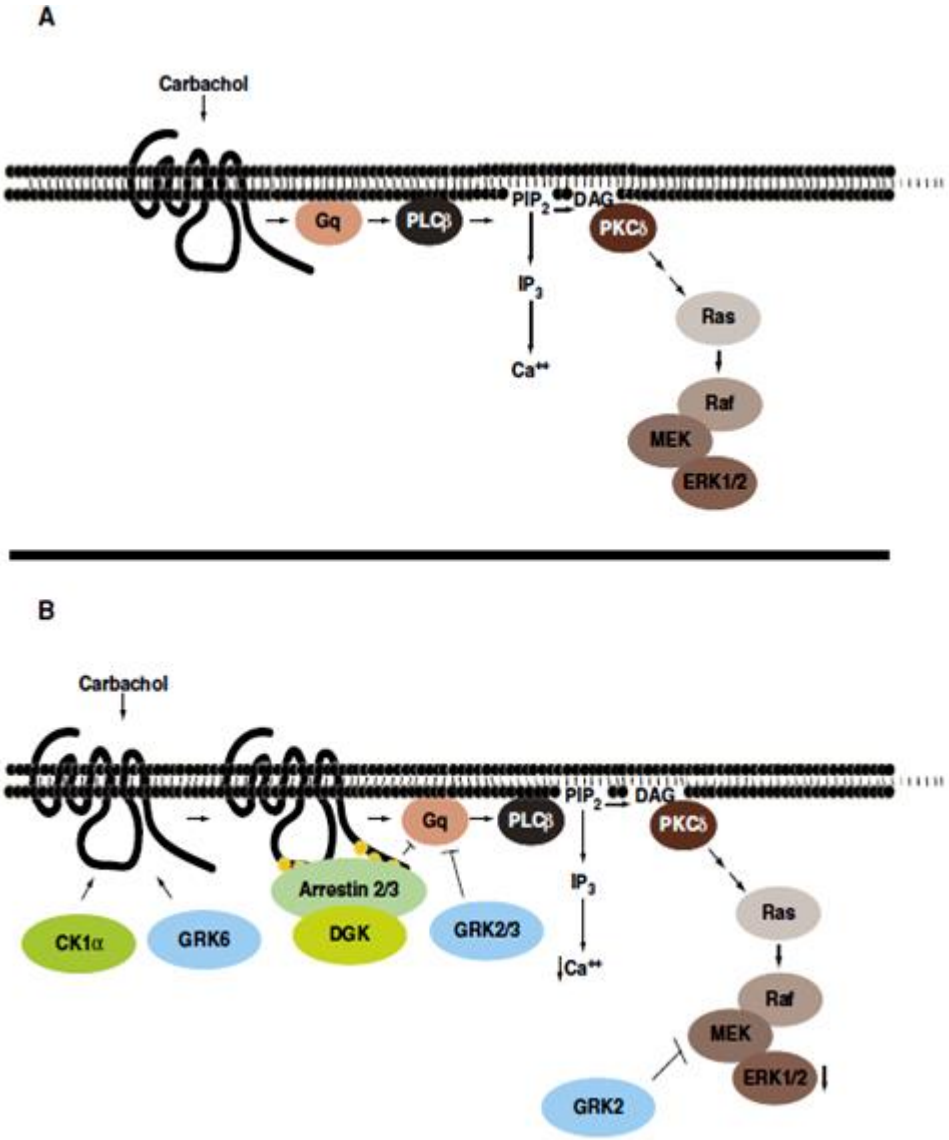

Figure 7.2.2: Carbachol binding to muscarinic receptors may result in the activation of two different signalling pathways, via arrestin mediated signalling. *The schematic above depicts the mechanism of muscarinic receptors following carbachol binding in A) interaction of GPCR to G_q subunit mediated signalling and B) GRK and arrestin mediated signalling (Luo, Busillo and Benovic 2008).*

It should be noted, that whilst Tiotropium bromide is often referred to as an antagonist of muscarinic receptors (with higher specificity for M_3 receptors), studies also refer to the drug as an inverse agonist (Casarosa et al. 2010, Kruse et al. 2012, Kruse et al. 2014a, Kruse et al. 2014b). This definition of Tiotropium bromide would add considerable weight to the studies observing a signalling effect following administration of this antagonist. Antagonists are generally thought to be neutral in their effect with no capability of signalling (Bond and IJzerman 2006, Khilnani and Khilnani 2011, Rosenbaum, Rasmussen and Kobilka 2009); however inverse agonists are described as showing negative intrinsic activity (Milligan, Bond and Lee 1995), the definition for inverse agonists changes when describing structural stability of the receptor (Bokoch et al. 2010). Figure 7.2.3 shows a schematic describing the various efficacies of different receptor binding intermediates. Nearly all anti-histamines are inverse agonists, despite often being thought of as antagonists (Khilnani and Khilnani 2011). Tiotropium bromide is an orthosteric ligand for M_3 receptors; however, it also binds to the allosteric site transiently (Gosens et al. 2017, Kruse et al. 2012), which may be sufficient to show variation in binding activity can dictate reclassification (Cooke et al. 2015). In the last decade, many GPCR ligands have been structurally identified when bound to their receptors using X-ray crystallography, this has shown the potential for many ligands to have functional selectivity based on allosteric binding (Cooke et al. 2015, Stewart, Sexton and Christopoulos 2010). Tiotropium bromide as an inverse agonist may result in a signalling cascade which opposes acetylcholine or agonist-mediated stimulation; this may even involve an arm of the β arrestin signalling pathway (Azzi et al. 2003).

The present studies indicate the potential role of Tiotropium bromide in cardiomyocyte signalling; this may occur as a result of 'inverse agonist' binding potential and may also include β arrestin signalling (Azzi et al. 2003, Kruse et al. 2014a), accounting for the observed effects on haemodynamic function and infarct size via cardiomyocyte cell death pathways. In order to investigate this phenomenon, further studies focused on specific signalling pathways following receptor binding are needed to elucidate the role of Tiotropium bromide in cardiomyocyte signalling.

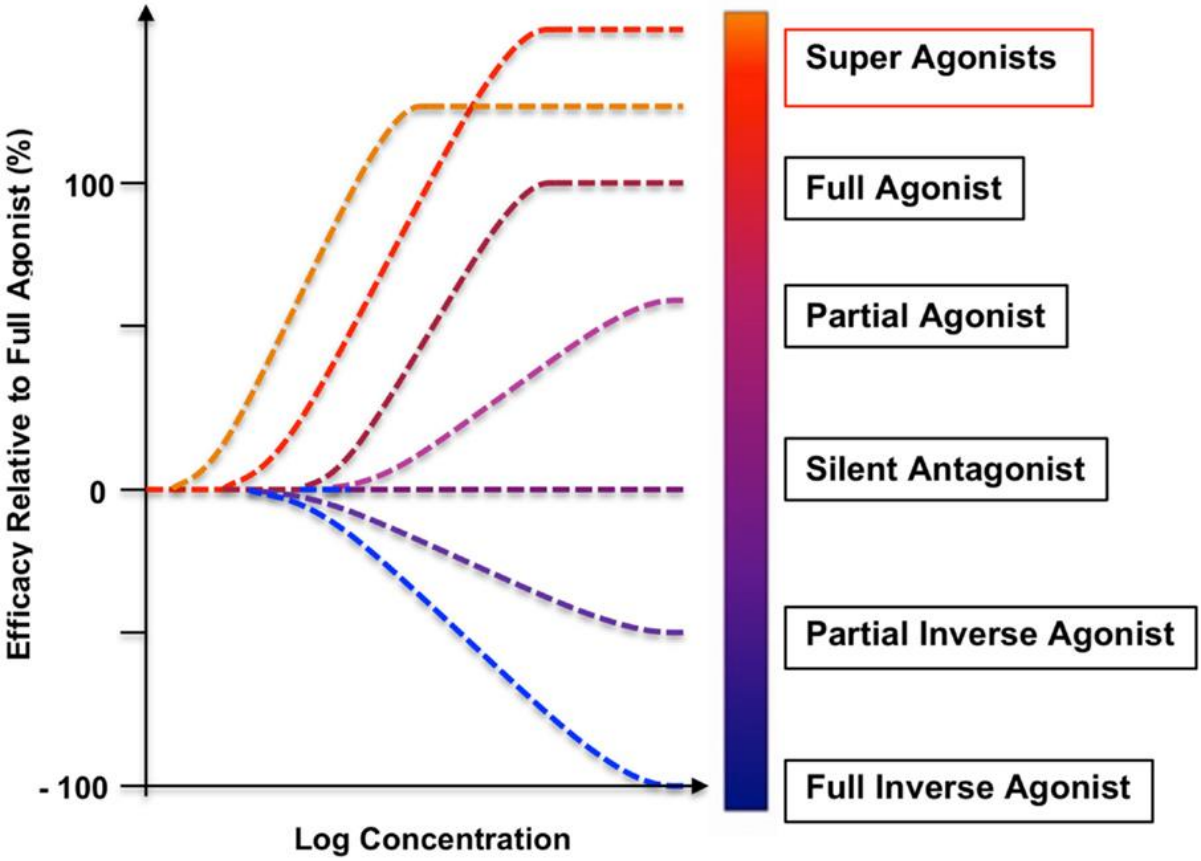

Figure 7.2.3: The model representing levels of agonist and antagonist activity following receptor binding. *Conventionally an agonist elicits a signalling response following receptor binding, and an antagonist results in cessation of signalling activity. However, studies have shown that 'antagonist' can result in negative activity and have therefore been termed inverse agonists (Szkudlinski 2015).*

7.3 Oxidative stress and Calcium signalling in Tiotropium bromide mediated cardiotoxicity

Cardiotoxicity is any toxic effect on the heart including myocardial infarction and heart failure (Scherrer-Crosbie 2016). In ischaemia/reperfusion injury, cell death such as apoptosis occurs as a result of oxidative stress or Ca^{2+} overload and opening of the mitochondrial permeability transition pore (mPTP) (Halestrap and Richardson 2015, Kinnally et al. 2011, Ong et al. 2015c), these mechanisms mediate cardiotoxicity (Angsutararux, Luanpitpong and Issaragrisil 2015, Braña, Zamora and Tabernero 2013, Díaz-Muñoz et al. 2006).

Oxidative stress and reactive oxygen species (ROS) contribute to cell death, particularly in myocardial injury, both as a preconditioning agent and a mediator of cell damage (Cohen et al. 2001, Ray, Huang and Tsuji 2012, Xu et al. 2014). Drug induced oxidative stress is a cause of cardiotoxicity (Deavall et al. 2012, Šimůnek et al. 2009); ROS in the heart is generated via mitochondrial oxidative phosphorylation from aerobic metabolism, xanthine oxidase, NAD(P)H oxidases, cytochrome P450 or nitric oxide synthase (NOS) uncoupling (Giordano 2005). Excess ROS triggers oxidative stress via direct or in-direct interaction with proteins, lipids and DNA (Circu and Aw 2010, Ray, Huang and Tsuji 2012). In addition to enzymatic antioxidants (Birben et al. 2012), dietary antioxidants such as resveratrol and vitamin E improve diseased states (Devasagayam et al. 2004).

Resveratrol is vasodilatory and cardioprotective, via an NO- and adenosine-dependent mechanism (Bradamante et al. 2003, Dernek et al. 2004, Orallo et al. 2002, Ray et al. 1999). This study shows that resveratrol (10 µM) increases coronary flow in normoxic conditions, this increase is significant when co-administered with Tiotropium bromide (1 nM). The increase in coronary flow following co-administration suggests that vasodilation occurs independently of Tiotropium bromide. The lack of change in coronary flow following ischaemia/reperfusion is observed with hearts undergoing global ischaemia (Ray et al. 1999). However, resveratrol following ischaemia, shows observational recovery of cardiac function which can be abrogated with the use of the non-selective NO inhibitor, L-NAME (30 µM) (Bradamante et al. 2003); this implies the involvement of the nitric oxide pathway in the recovery of cardiac function.

Resveratrol affects cell death by reducing infarct size in ischaemia/reperfusion (Lin et al. 2008, Ray et al. 1999, Tsai et al. 2007), and is protective in the diabetic myocardium (Csiszar 2011, Thirunavukkarasu et al. 2007). In both ischaemia/reperfusion and normoxic conditions, resveratrol showed decreased infarct sizes compared to Tiotropium bromide and attenuated ischaemia/reperfusion injury; co-administration also abrogated Tiotropium bromide mediated infarct size. Resveratrol has been shown to reduce infarct size in an NO dependent manner; co-administration of L-NAME blocks the protective effect of resveratrol following ischaemia (Hung, Su and Chen 2004, Thirunavukkarasu et al. 2007). Nitric oxide has been shown to be increased during ischaemia and early reperfusion, via endothelial nitric oxide synthase (eNOS), through activation of PI3K and Akt (Gao et al.

2002, Schulz, Kelm and Heusch 2004). Xanthine oxidase also produces NO in hypoxia and facilitates cardioprotection via vasodilation mediated contractile function and relaxation (Schulz, Kelm and Heusch 2004). Resveratrol also indirectly affects vasodilation by inhibiting endothelin mediated vasoconstriction (Andriantsitohaina et al. 2012, Mokni et al. 2013).

Resveratrol inhibits mRNA for atrial natriuretic peptide (ANP) and transforming growth factor beta 1 (TGF β1) (Lin et al. 2008). ANP correlates with enhanced mortality in myocardial infarction, showing prognostic value (Omland et al. 1993, Takashio et al. 2016). TGF β1 is detrimental in myocardial infarction, suppresses inflammation mediated infarct healing (Bujak and Frangogiannis 2007, Frangogiannis, Smith and Entman 2002) and tissue remodelling (Asano et al. 2010). Figure 7.3.1 depicts resveratrol mediated signalling (Mokni et al. 2013). Resveratrol is also cytoprotective via NF-E2 related factor 2 (Nrf2), coordinating expression of antioxidant mechanisms, including heme oxygenase 1 (Csiszar 2011).

Resveratrol has antioxidant activity greater than vitamin E (Dernek et al. 2004, Hung, Su and Chen 2004, King, Bomser and Min 2006, Magyar et al. 2012, Mokni et al. 2013) through inhibition of LDL oxidation (Chan et al. 2008, Mokni et al. 2013, Wu and Hsieh 2011). However, resveratrol is most effective through recruitment of internal antioxidant machinery and protective pathways (Bradamante et al. 2003, Li, Xia and Förstermann 2012), such as PI3K/Akt (Davidson et al. 2006). Western blot showed Tiotropium bromide (1 nM) and resveratrol (10 μM) significantly increase p-Akt $_{(Ser473)}$, however the latter to a lesser extent. Co-administration of Tiotropium bromide with resveratrol attenuates the Tiotropium bromide mediated p-Akt $_{(Ser473)}$ increase. PI3K/Akt activation is involved in the preconditioning-like effect of resveratrol through the adenosine A_1 receptor leading to increased Bcl-2 activity (Carrizzo et al. 2013, Das and Maulik 2006, Li, Xia and Förstermann 2012). Resveratrol increases Akt phosphorylation in cardiomyocytes, contributing to improved left ventricular diastolic function (Magyar et al. 2012). Resveratrol phosphorylates Akt via SIRT1 mediated FoxO1 activity (Ni et al. 2007) and affects Akt differently under different conditions. In myotubules and hepatocytes, resveratrol decreases Akt phosphorylation through inhibition of PI3K (Pirola and Fröjdö 2008). However, in endothelial progenitor cells, resveratrol induces an increase in telomerase

activity and Akt phosphorylation (Xia et al. 2008). It is evident from the data in this study that resveratrol exerts protective effects and affects Akt phosphorylation.

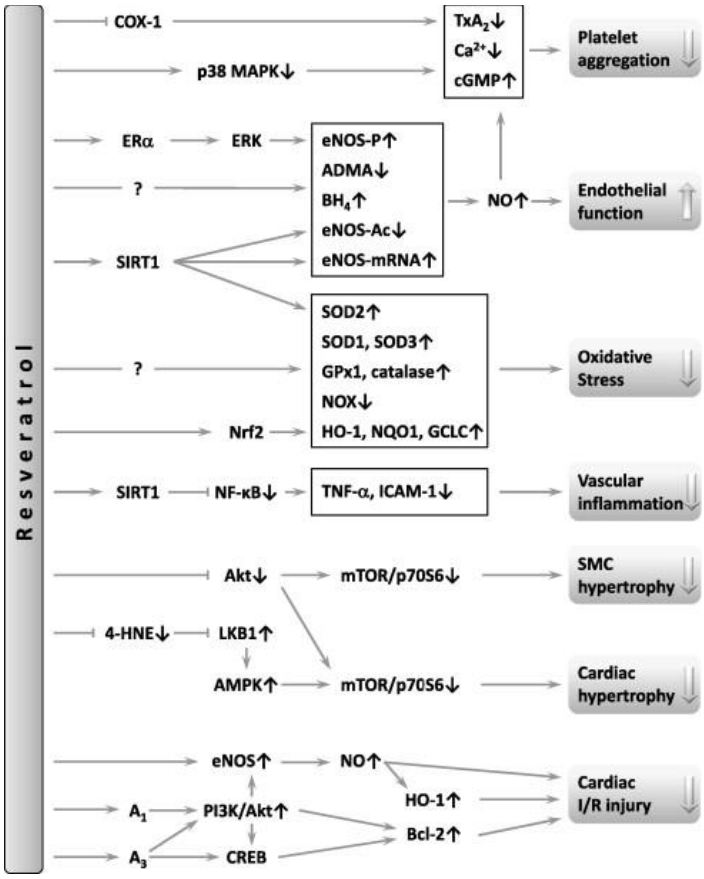

Figure 7.3.1: The various interactions of resveratrol involved in conferring protection. *A schematic depicting the different interactions of resveratrol with cellular mediators, leading to several cellular effects ranging from decreasing cardiac ischaemia/reperfusion injury to increasing endothelial function (Li, Xia and Förstermann 2012).*

Figure 7.3.2 depicts drug induced oxidative stress. Tiotropium bromide (1 nM) significantly downregulates protective genes associated with oxidative stress. Nuclear factor (erythroid derived 2) like 2 (Nfe2l2), a transcription factor responsible for activating antioxidant genes (Deavall et al. 2012) and Akt1, associated with cell survival were downregulated with Tiotropium bromide (Häggblad Sahlberg et al. 2017, Nogueira et al. 2008). Gluthatione S-transferase pi 1 (Gstp1), protein kinase c ζ (Prkcz), tumour protein 53 (Tp53), cytochrome

b-245 light chain (Cyba), activating transcription factor 2 (Atf2), lipocalin 2 (Lcn2) and protein kinase AMP activated alpha 1 catalytic subunit (Prkaa1) were also downregulated. Lactate dehydrogenase A (Ldha) and hypoxanthine guanine phosphoribosyltransferase (Hprt1) showed an observable increase. LDH from damaged membranes catalyses lactate oxidation to pyruvate and reduction of NAD^+; increased LDH is observed with doxorubicin and acts as a marker of cellular damage (Šimůnek et al. 2009, Yang et al. 2009), therefore implying that Tiotropium bromide may elicit a comparable mechanism of cell damage.

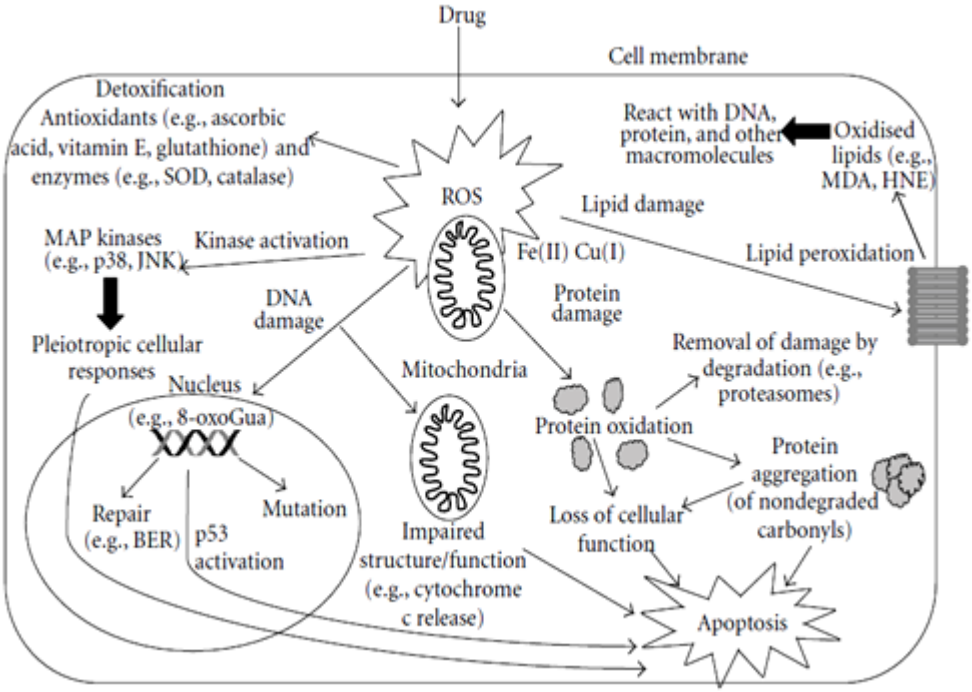

Figure 7.3.2: The multiple effects that reactive oxygen species (ROS) have on cellular processes. *These include lipid damage, DNA damage, activation of antioxidants, protein oxidation essentially all leading to apoptotic cell death (Deavall et al. 2012). SOD – superoxide dismutase, JNK – c-Jun N-terminal Kinase, 8-oxoGua – 8 oxoguanine, BER – base excision repair, MDA – malondialdehyde, HNE – 4-hydroxynonenal.*

Tiotropium bromide induced a small increase of ROS in cardiomyocytes compared to controls; resveratrol alone saw a significant reduction in ROS with respect to the control and Tiotropium alone. Drugs known to cause cardiotoxicity show increased ROS as a

marker of oxidative stress (Burridge et al. 2016, Olsen et al. 2013, Yokoyama et al. 2017). Similarly, the observation with resveratrol supports its action as an antioxidant and ROS scavenger (Carrizzo et al. 2013, Das and Maulik 2006). The effect of resveratrol was also assessed in apoptotic cells. Tiotropium bromide (1 nM) showed a non-significant increase in apoptotic cells; however, resveratrol showed a decrease in apoptotic cells, this was sustained with the co-administration of Tiotropium bromide. Resveratrol has previously been known to abrogate the toxicity of doxorubicin (Hamlaoui et al. 2012). This study shows that resveratrol protects against Tiotropium bromide induced cardiotoxicity and implies ROS may mediate the cardiotoxicity of Tiotropium bromide.

Calcium overload is also associated with regulating cell death (Fearnley, Roderick and Bootman 2011, Orrenius, Gogvadze and Zhivotovsky 2015), a shift in the balance of intracellular calcium results in abhorrent calcium signalling (Ermak and Davies 2002, Kristian and Siesjo 1998, Pinton et al. 2008, Salas et al. 2010). Defects in calcium handling are observed in heart failure (Gorski, Ceholski and Hajjar 2015), cardiac hypertrophy and tachyarrhythmias (Kang and Lebeche 2013). Ca^{2+} overload results in reversal of Na^{+}/Ca^{2+} exchanger (NCX) function; leading to increased intracellular Ca^{2+} (Garcia-Dorado et al. 2012, Krebs, Agellon and Michalak 2015). L-type Ca^{2+} channels mediate cellular calcium entry during excitation-coupling (Murgia and Rizzuto 2014), leading to calcium induced calcium release (CICR) which can contribute to Ca^{2+} overload (Shen, Jiang and Pappano 2000). Doxorubicin impairs Ca^{2+} sequestration resulting in increased intracellular Ca^{2+} (Hahn, Lenihan and Ky 2014). Ipratropium bromide directly results in erythrocyte death via Ca^{2+} (Shaik et al. 2012). The role of Ca^{2+} signalling in cardiomyocyte death means it can be involved in drug cardiotoxicity and targeted pharmacologically.

Nifedipine is an L-type Ca^{2+} channel inhibitor used for hypertension (Brown et al. 2000); studies in isolated hearts have found nifedipine to be cardioprotective (Okuda et al. 2005, Pijl et al. 1993). Nifedipine reduces LDH leakage in myocardial ischaemia and reduces neutrophil accumulation in the myocardium following ischaemia/reperfusion (Huang et al. 2009). In this study, nifedipine (1 nM) significantly decreased coronary flow with Tiotropium bromide (1 nM) co-administration in normoxic conditions but did not affect LVDP or heart rate. Nifedipine has negative inotropic and chronotropic effects but increases coronary flow at low concentrations (Kumar et al. 2012). The data suggests that

muscarinic inhibition in addition to L type Ca^{2+} channels may result in vasoconstriction. Studies observing the effect of M_3 receptor and L type Ca^{2+} channel inhibition in rainbow trout, showed decreased cholinergic induced gut contractility (Aronsson and Holmgren 2000). Nifedipine is a cardiodepressant (Kumar et al. 2012) but associated with cardioprotection (Huang et al. 2009, Kitakaze et al. 2000, Kloner and Przyklenk 1990, Okuda et al. 2005); however, nifedipine can be toxic by enhancing the cardiodepressant effect of other compounds such as magnesium sulfate (Thorp et al. 1990). Nifedipine significantly decreases infarct size, compared to Tiotropium bromide; co-administration abrogates Tiotropium bromide mediated infarct size. This may be linked to NO production which is enhanced following nifedipine administration (Huang et al. 2009, Kitakaze et al. 2000). This study suggests that whilst nifedipine administration may dampen the effect of Tiotropium bromide on infarct size, it does not fully reverse it, implying the involvement of other mediators aside from L-type Ca^{2+} channels.

Calcium concentrations are regulated by mediators including ion channels, kinases and phosphatases (Bootman 2012, Fearnley, Roderick and Bootman 2011, Harada et al. 2014). Ca^{2+}/calmodulin dependent protein kinase II (CaMKII) phosphorylates many proteins, transcription factors and ion channels (Zhang, Miyamoto and Brown 2004). In heart failure and arrhythmias (Fearnley, Roderick and Bootman 2011, Said et al. 2011), there is a marked increase in CaMKII activity and abnormal Ca^{2+} release (Cheng et al. 2012, Fischer et al. 2013, Sossalla et al. 2010, Zhang, Miyamoto and Brown 2004), which is proapoptotic (Harada et al. 2014). CaMKII inhibition is protective against ischaemia/reperfusion injury (Vila-Petroff et al. 2007). M_1, M_3 and M_5 receptors increase endoplasmic reticulum Ca^{2+} via phospholipase C (PLC), which activate calmodulin-dependent mechanisms involving CaMKII, depicted by figure 7.3.3 (Harvey 2012). CaMKII in ischaemia/reperfusion and myocardial infarction promotes cell death through mPTP opening and phosphorylation of ryanodine receptors (Di Carlo et al. 2014, Joiner et al. 2012). Ischaemic preconditioning alters cardiac function through the Ca^{2+} paradox, where CaMKII mediated phosphorylation of phospholamban protects against Ca^{2+} overload (Kawabata et al. 2000, Piper 2000, Valverde et al. 2006). KN-93 inhibits CaMKII, preventing calmodulin-CaMKII interaction (Anderson et al. 1998) and inhibits increases in sarcoplasmic reticulum (SR) Ca^{2+} leading to

decreased excitation coupling, implying a role in contractility (Zhang, Miyamoto and Brown 2004).

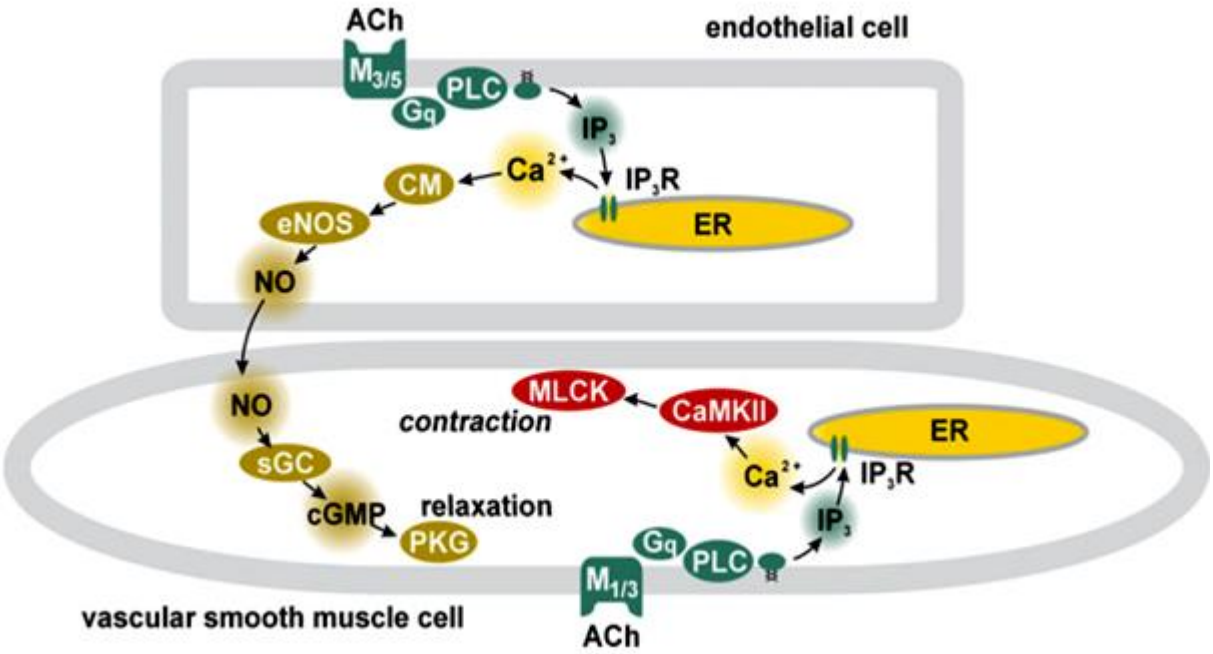

Figure 7.3.3: Muscarinic receptors have an important role in vascular tissue contractility*. M_3 receptor activation in endothelial cells leads to phospholipase C (PLC) activation; leading to inositol triphosphate (IP_3) mediated release of Ca^{2+}. Nitric oxide (NO) is released via calmodulin-dependent (CM) activation of endothelial nitric oxide synthase (eNOS). NO diffuses into adjacent cells to stimulate cGMP production via soluble guanylyl cyclase (sGC), resulting in protein kinase G (PKG) mediated relaxation. M_3 receptor activation in vascular smooth muscle cells, results in calcium mediated activation of Ca^{2+}/calmodulin dependent kinase II (CaMKII) leading to activation of myosin light chain kinase (MLCK) (Harvey 2012).*

KN-93 (400 nM) showed significantly increased LVDP in normoxic conditions when co-administered with Tiotropium bromide (1 nM); no other parameters showed any significance in ischaemia/reperfusion or normoxic conditions. KN-93 pre-treatment has been shown to prevent LVDP elevation following clofilium treatment, an anti-arrhythmic agent (Anderson et al. 1998). CaMKII inhibition by KN-93 in the failing myocardium

improves contractility following ischaemia (Vila-Petroff et al. 2007); however, this is not observed in non-failing hearts (Sossalla et al. 2010). LVDP is a measure of contractility (Sarazan, Kroehle and Main 2012, Valverde et al. 2006) indirectly relating to intracellular Ca^{2+} concentrations (Lou, Janardhan and Efimov 2012). Co-administration of KN-93 with Tiotropium bromide in normoxic conditions may mimic conditions observed in heart failure therefore, inhibition of CaMKII may be beneficial to LVDP. KN-93 significantly decreased infarct size with respect to Tiotropium bromide in ischaemia/reperfusion and normoxic conditions; co-administration with Tiotropium bromide abrogated Tiotropium bromide mediated infarct size. Constitutive activation of ryanodine receptors by CaMKII is known to increase ischaemia/reperfusion injury (Di Carlo et al. 2014); inhibition of CaMKII decreases infarct size however these studies used KN-93 at 2.5 µM (Di Carlo et al. 2014, Salas et al. 2010, Vila-Petroff et al. 2007). In the context of ischaemic- and desaflurane induced preconditioning, KN-93 abolishes cardioprotection (Lange et al. 2008, Osada et al. 2000) implying a role for CaMKII. This study suggests CaMKII activation may play a role in Tiotropium bromide mediated cardiac damage.

CaMKII activation by β-adrenergic receptors is well documented (Anderson, Brown and Bers 2011, Fischer et al. 2013, Grimm, Ling and Brown 2011, Zhang, Miyamoto and Brown 2004), however the role of muscarinic receptors in CaMKII signalling is less clear. A study in adipocytes, found that M_3 receptor stimulation induces Ca^{2+} oscillation via PI3K which then activates CaMKII (Turovsky et al. 2013). Overexpression of CaMKII is known to phosphorylate Akt in human breast cancer (Chi et al. 2016), in prostate cancer (Rokhlin et al. 2007) and in heart failure (Harada et al. 2014) where Akt is highly phosphorylated. However, restoration of SERCA2a activity improves cardiac function in an Akt/FoxO3A dependent manner (Kumarswamy et al. 2012). Akt is normally associated with cardioprotection, however overactivation can be detrimental (Castino et al. 2008, Chaanine and Hajjar 2011, Harada et al. 2014, Kumarswamy et al. 2012, Los et al. 2009, Nogueira et al. 2008, O'Neill and Abel 2005). Tiotropium bromide increases p-Akt $_{(Ser473)}$, co-administration of KN-93 dampens Tiotropium bromide mediated Akt phosphorylation. This study implies a role for CaMKII in Tiotropium bromide induced Akt phosphorylation. In heart failure, Akt phosphorylates FoxO3a which translocates to the cytosol (Kumarswamy et al. 2012), FoxO3a is a transcription factor involved in the regulation of

pre-microRNA 1, which in the mature form affects NCX activity (Harada et al. 2014) and regulates ROS formation (Afanas'ev 2011). Akt can inhibit ROS scavengers downstream of FoxO, leading to apoptosis (Los et al. 2009, Nogueira et al. 2008). Figure 7.3.4 depicts the 'two sided' nature of Akt, which can be cardioprotective, but also pathological.

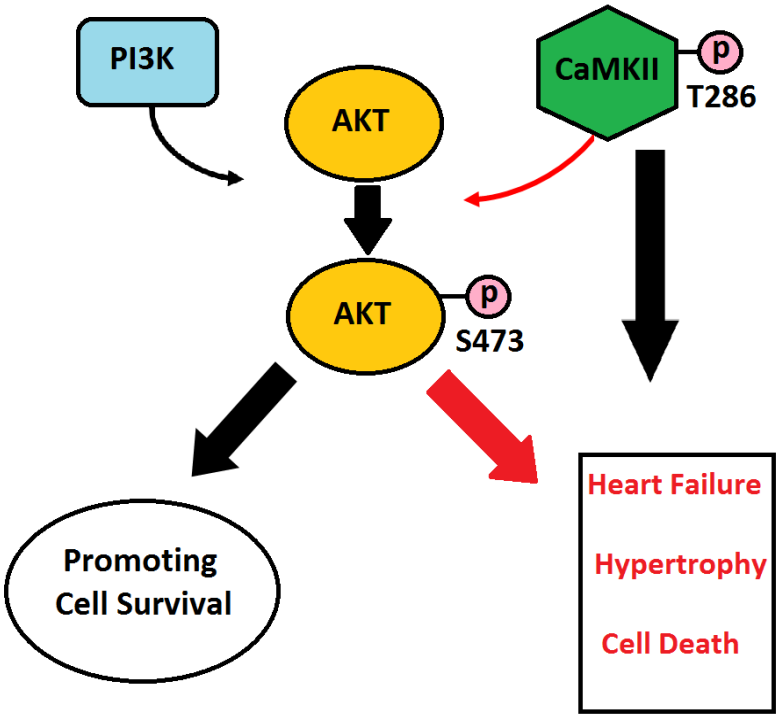

***Figure 7.3.4: The role of Akt phosphorylation in promoting cell survival and leading to cell death**. Ordinarily, Akt phosphorylation as a result of PI3K activity leads to cardioprotective effects in the heart, however studies have shown that in heart failure the over-activation of CaMKII also leads to Akt activation, which has a role in cell death.*

Tiotropium bromide increased phosphorylated CaMKII $_{(Thr286)}$ with respect to normoxic controls; co-administration with KN-93 attenuated this increase. The IC_{50} for KN-93 is approximately 370 nM to compete with the calmodulin binding site (Gao, Blair and Marshall 2006, Yung-Chi and Prusoff 1973), however, KN-93 does not inhibit the catalytic activity of auto-phosphorylated CaMKII (Pellicena and Schulman 2014, Sumi et al. 1991, Tokumitsu et al. 1990). KN-93 is good for assessing the function of CaMKII, however due to

its competitive nature with calmodulin, autonomous activity can't be inhibited, additionally, it also affects voltage gated K^+ and Ca^{2+} channels (Ledoux, Chartier and Leblanc 1999, Vest et al. 2007). This study suggests that Tiotropium bromide increases CaMKII phosphorylation, which is attenuated by KN-93. Chronic CaMKII activation follows an increase in autonomous activity; this may be due to CaMKII oxidation (Luczak and Anderson 2014) and may lead to atrial and ventricular dysfunction (Khoo et al. 2006).

In heart failure, abnormal Ca^{2+} release from CaMKII phosphorylated ryanodine receptors leads to arrhythmia and contractile dysfunction (Ai et al. 2005), therefore measurement of Ca^{2+} is a useful tool (Ai et al. 2005, Farber 1990, Zhang et al. 2009). Fluo-3AM is a cell permeable Ca^{2+} indicator used in the study of cardiomyocytes (Loughrey et al. 2003, Petroff et al. 2001). Tiotropium bromide (1 nM) increased intracellular Ca^{2+} with respect to controls, co-administration with KN-93 (400 nM) showed no significant difference. Protein kinase A (PKA) and PKG are also involved in ryanodine receptor phosphorylation and Ca^{2+} movement in addition to CaMKII (Ai et al. 2005, Berridge, Bootman and Roderick 2003, Garcia-Dorado et al. 2012). KN-93 preconditioning elicits the most effective response in dampening CaMKII activity (Lange et al. 2008, Osada et al. 2000), which may explain why the effect of KN-93 was not prominent in this study. Tiotropium bromide non-significantly increased apoptotic cardiomyocytes, however no significant change was observed following KN-93. The effect of KN-93 may not manifest here, as a lower concentration of KN-93 was used compared to other studies. Additionally, mitochondrial Ca^{2+} may be more significant in dysfunctional Ca^{2+} signalling than SR Ca^{2+}, as the mPTP can lead to cell death following calcium overload (Joiner et al. 2012), this is particularly true for ischaemia/reperfusion injury (Di Carlo et al. 2014). These studies indicate that Tiotropium bromide induced cardiotoxicity may involve CaMKII mediated Ca^{2+} dysregulation through abhorrent Akt phosphorylation but warrants further investigation with regards to mitochondrial Ca^{2+}.

Caspase-3 mediates apoptosis; caspase-3 is an effector caspase and is responsible for some of the characteristic features associated with apoptosis (Lakhani et al. 2006, Porter and Jänicke 1999). The use of the caspase-3 inhibitor, Z-DEVD-FMK (Ekert, Silke and Vaux 1999, Putinski et al. 2013, Xu et al. 2014) shows increased coronary flow in normoxic conditions; no other effect is observed on haemodynamic function in ischaemia/reperfusion and

normoxic conditions. Z-DEVD-FMK significantly decreased infarct size to risk ratio in ischaemia/reperfusion and attenuated Tiotropium bromide mediated infarct size in both conditions. Tiotropium bromide also increased cleaved caspase-3 with respect to normoxic controls, Z-DEVD-FMK reversed this increase upon co-administration. Inhibition of caspase-3 has previously been found to be cardioprotective against ischaemia/reperfusion injury (Harvey, Hussain and Maddock 2014, Kudelova et al. 2015). Tiotropium bromide (10 µM – 0.1 nM) showed a concentration-dependent relationship on apoptosis with respect to normoxic controls, however only the highest concentration showed a significant increase; Z-DEVD-FMK (70 nM) significantly attenuated Tiotropium bromide (1 nM) mediated apoptosis. Caspase-3 is largely associated with initiating apoptosis, particularly during reperfusion (Kang et al. 2003, Porter and Jänicke 1999, Ueno et al. 2006), and is important in cardiac hypertrophy, both *in vivo* and *in vitro* (Balakumar and Singh 2006, Putinski et al. 2013). Caspase-3 along with caspase-8 mediate ceramide induced cardiomyocyte death (Wang et al. 2000). Muscarinic receptors can inhibit caspase-3 resulting in protection, therefore antagonism could attenuate this protection indirectly leading to cell damage (Resende and Adhikari 2009). Apoptosis is the transitioning factor from left ventricular hypertrophy to heart failure (Li et al. 2009a), parasympathetic signalling is known to be involved in heart failure and diabetes (LaCroix et al. 2008, Pathak et al. 2005), but is protective in congestive heart failure (Communal et al. 1998). These studies suggest that caspase-3 activation may be involved in Tiotropium bromide mediated cardiomyocyte death.

Myocardial infarction is a manifestation of ischaemic heart disease (IHD); occuring following atheroma mediated obstruction in blood flow (Hansson 2005, Marzilli et al. 2012). Cardiac comorbidity in COPD patients is common, due shared risk factors such as COPD-mediated arterial stiffening, inflammation and endothelial dysfunction (Cazzola et al. 2017a, Mills et al. 2008, Patel et al. 2012). The expression of genes involved in myocardial infarction was assessed following Tiotropium bromide (1 nM). Casp3 expression showed a non-significant increase, indicating that Tiotropium bromide may induce an upregulation of caspase-3, which corroborates with the study observing increased cleaved caspase-3 activation.

Ppargca1 and Bax showed significant downregulation following Tiotropium bromide administration. Ppargca1 (PGC-1α) regulates mitochondrial biogenesis and oxidative stress, in heart failure it is downregulated, (Madrazo and Kelly 2008, Sun et al. 2007, Ventura-Clapier, Garnier and Veksler 2008), however PGC-1α activity is upregulated in patients with ST elevated acute myocardial infarction (Fabregat-Andrés et al. 2011). The differential expression of PGC-1α in heart failure and ischaemia/reperfusion suggests other factors may contribute to mitochondrial biogenesis (Fabregat-Andrés et al. 2011, Ventura-Clapier, Garnier and Veksler 2008). In heart failure, there is an observed increase in Akt (Haq et al. 2001), which can downregulate PGC-1α (Ventura-Clapier, Garnier and Veksler 2008). This suggests that the pattern of expression observed with Tiotropium bromide may be indicative of a heart failure phenotype, where PGC-1α is also downregulated. Additionally, PGC-1α expression is involved in glucose homeostasis and mitochondrial activity and has been shown to play an important role in metabolic diseases and is activated with resveratrol administration (Borriello et al. 2010). This suggests that by downregulating PGC-1α, Tiotropium bromide administration may inhibit the protective properties associated with PGC-1α activation, and therefore lead to indirect cell toxicity.

Bax is a pro-apoptotic protein, however in this study this is significantly downregulated following Tiotropium bromide administration despite Tiotropium bromide showing an increase in infarct size. Ischaemia/reperfusion injury and heart failure show upregulation of Bax in canine models of heart failure and in patients with dilated cardiomyopathy or ischaemic heart disease (Cardin et al. 2003, Latif et al. 2000, Li et al. 2009a, McCully et al. 2004). Bax induces cell death without DNA fragmentation through caspase-independent release of cytochrome *c* (Porter and Jänicke 1999, Yellon and Baxter 2000). Cell death can occur without Bax, through drug induced autophagy such as with etoposide in murine embryonic fibroblasts (Shimizu et al. 2004). The study indicates that cell death following Tiotropium bromide administration may occur independently of Bax at a gene level but may still involve the activation of caspase-3 as observed; it would appear that the administration of Tiotropium bromide induces a state similar to heart failure, due to downregulation of the Ppargca1 gene and activated Akt protein, which are observed in heart failure. This level of gene expression also corresponds with the data observing the effect of Tiotropium bromide on apoptosis, as there is no significant difference observed

with Tiotropium bromide (1 nM) with respect to control groups ($p<0.05$), despite an increase in caspase-3 activity. This corresponds to the observations that Bax induces apoptosis via caspase-independent mechanisms, therefore in the case of this study the activation of caspase-3 may itself downregulate Bax activity.

Stat3 and VEGF-A show non-significant downregulation following Tiotropium bromide; Stat3 is involved in signalling via the Jak-Stat pathway leading to cytoprotective signalling, therefore downregulation of this may be indicative of cytotoxic signalling (Heusch et al. 2011, Negoro et al. 2000, Oshima et al. 2005). Rapamycin induces cardioprotection against ischaemia/reperfusion injury through Jak-Stat signalling; the downregulation of Stat3 attenuates this cardioprotection in Langendorff models (Das et al. 2012). The observation of downregulated Stat3, suggests that Tiotropium bromide administration inhibits cardioprotective pathways such as Jak-Stat and may indirectly contribute to cytotoxicity. VEGF-A is involved in the initation of angiogenesis, leading to the formation of new vessels (Hao et al. 2007). Studies have shown that there is an increase in VEGF-A mRNA following myocardial infarction (MI) in patients with stable angina and Sprague-Dawley rat models (Kranz et al. 2000, Zhao et al. 2010b). Hypoxic conditions are strong stimuli for VEGF signalling (Hayashi et al. 2016, Narasimhan et al. 2009) due to the need for oxygenation, which can be fulfilled with new vessel formation, therefore circulating levels of VEGF-A following myocardial infarction are a potential biomarker of cardiac damage (Kranz et al. 2000). In dilated cardiomyopathy patients, VEGF-A mRNA is downregulated, with a decrease in vascular density (Abraham et al. 2000); this is also observed in patients with progressive kidney disease, despite an increase in hypoxia inducible factors (HIF) (Rudnicki et al. 2009).

These studies suggest that although hypoxic conditions are known to induce VEGF-A expression for angiogenesis, expression is also affected by other variables aside from hypoxia. Angiogenesis increases the formation of new blood vessels and therefore increases blood supply to hypoxic or ischaemic areas, which in turn can improve cardiac function as a result of myocardial infarction (Nakamura et al. 2012). The downregulation of VEGF-A and Stat3 in the current study offers a suggestion that Tiotropium bromide does not induce the formation of new blood vessels, which is observed as a protective strategy, this combined with downregulation in the protective transcription factor, Stat3, shows

impaired cytoprotective signalling, which may contribute to the observed toxicity with Tiotropium bromide.

7.4 Cardioprotective strategies on Tiotropium bromide mediated cardiotoxicity

Parasympathetic muscarinic signalling in the heart is mediated via M_2 receptors (Harvey 2012), involved in controlling heart rate (Dhein, Van Koppen and Brodde 2001). However, M_3 receptors play a role in cardioprotection (Pan et al. 2012, Wang, Lu and Wang 2007, Wang et al. 2012c). M_3 receptor signalling is involved in cardioprotection through the reduction in reactive oxygen species (ROS) (Pan et al. 2012, Sun et al. 2010, Wang, Lu and Wang 2007, Yao et al. 1999) and decreasing Ca^{2+} overload (Dhein, Van Koppen and Brodde 2001). Cardiac M_3 receptors induce cardioprotection upon stimulation through the up-regulation of cyclooxygenase-2 and inhibit ischaemia mediated connexin-43 dephosphorylation (Ando et al. 2005, Zhao et al. 2010a). Parasympathetic innervation protects against oxidative stress through enhanced antioxidant activity via AMPK leading to PKC mediated NAPDH oxidase (Nox) inactivation, atropine abolished this effect (Kong et al. 2012). L-NAME mediated nitric oxide synthase (NOS) inhibition attenuates protection conferred by acetylcholine and inhibits ischaemia induced ROS, whilst inhibition of PKC also abrogates the protective effect of ACh it does not affect ROS formation (Liu et al. 2001). Figure 7.4.1 depicts the various interactions of M_3 receptors.

Acetylcholine mediated muscarinic signalling is necessary in conditions of stress, resulting in heart rate variability, from non-neuronal sources of acetylcholine (Roy et al. 2016, Schulte et al. 2014). Cardioprotective kinases are involved in phosphorylating key downstream mediators to result in changes at a gene level (Hausenloy and Yellon 2004, Hausenloy and Yellon 2006, Hu et al. 2004, Lee et al. 2006). Cardioprotection can also involve mitochondrial signalling (Garlid et al. 2003, Garlid et al. 2009, Halestrap, Clarke and Javadov 2004, Oldenburg et al. 2002). Akt and ERK1/2 confer protection against oxidative stress (Dai and Rabinovitch 2009), loss of these kinases is associated with decreased tolerance to oxidative stress. Moderate Akt overexpression promotes cardiac growth (Condorelli et al. 2002), however fully activated forms of Akt1/3 result in pathological consequences and cardiac dysfunction (Matsui et al. 2002b, Wessells and Bodmer 2007). Cardioprotective strategies are a growing field, aimed to protect the heart, notably from ischaemia/reperfusion injury (Heusch 2013).

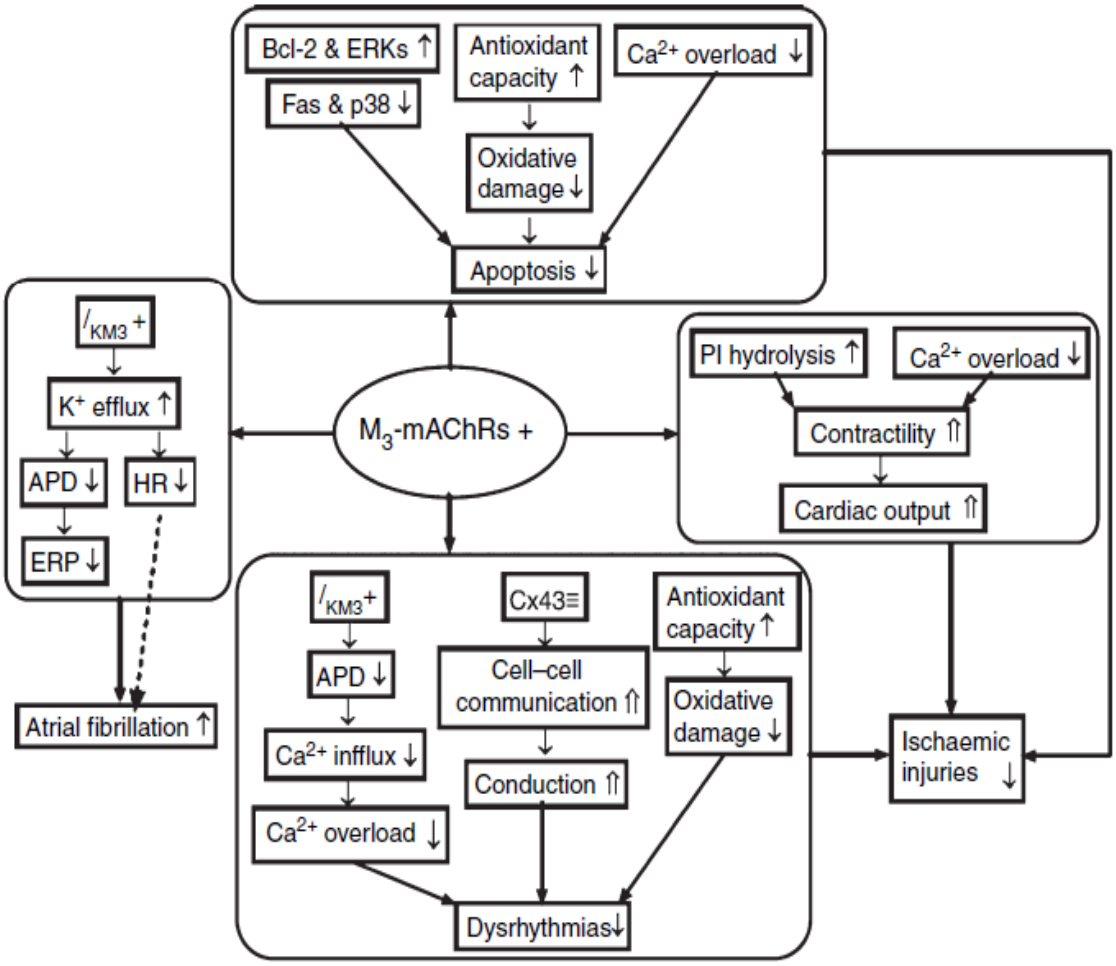

Figure 7.4.1: M_3 receptors are thought to increase atrial fibrillation, decrease dysrhythmias and ischaemic injury as well as decreasing apoptotic cell death. *(Wang, Lu and Wang 2007). ERKs – extra cellular regulated kinases, Cx43 – connexin 43, HR – heart rate, APD – action potential decrease, ERP – effective refractory period, I_{KM3} – M_3 activated delayed rectifying K^+ current, PI – phosphoinositide, black upwards arrow – increase, black downwards arrow – decrease, unfilled upwards arrow – improved activity.*

In this study, acetylcholine (ACh 100 nM) was administered in ischaemia/reperfusion and normoxic conditions. Co-administration of Tiotropium bromide (1 nM) and acetylcholine shows a significant decrease in coronary flow with respect to Tiotropium bromide and acetylcholine alone. No other significant changes were observed in haemodynamic

parameters. Coronary flow is used to measure vascular resistance, therefore decreases in coronary flow can be observed as an increase in vascular resistance, or vasoconstriction (Skrzypiec-Spring et al. 2007). Acetylcholine increases nitric oxide (NO) production, involved in the protective effects of preconditioning (Liu et al. 2001). NO results in cardioprotective effects through vasodilation (Ferdinandy and Schulz 2003, Gao et al. 2002). Antagonism at the M_3 receptor which is responsible for mediating the protective effects of ACh, may specifically result in decreased coronary flow.

Ischaemia/reperfusion injury normally depresses contractile function (Li et al. 2010); in streptozocin induced diabetic rats, acetylcholine following ischaemia/reperfusion injury results in an increase in contractile function (Knezl et al. 2006). This correlates with the current study which shows observable recovery of cardiac function mediated by ACh. Acetylcholine is normally associated with a decrease in contractility and heart rate, through slowing down of sinoatrial node action potentials and decreased conductance at the atrioventricular node (Florea and Cohn 2014). However, the current study shows no significance observed with heart rate following acetylcholine administration. Atropine increases heart rate through parasympathethic inhibition (Brodde and Michel 1999); in heart failure, there is a diminished response and depressed baroreceptor reflex following atropine administration, implying a role for muscarinic receptors (Florea and Cohn 2014). A decrease in heart rate following ischaemia/reperfusion injury can have a protective effect (Viviana et al. 2015) and may account for the cytoprotection afforded by ACh co-administration, as a decrease in heart rate in observed with the co-administration group. Therefore, a decrease in heart rate can preserve cardiac function through decreased expenditure (Viviana et al. 2015). However greater concentrations of ACh show a paradoxical increase in these parameters, displaying positive inotropy (Buccino et al. 1966, Krieg et al. 2004, Wang, Shi and Wang 2004), suggesting a dual function of muscarinic receptor stimulation on cardiac function.

Infarct size is a measure of cardiomyocyte death, therefore interventions to reduce infarct size are crucial (Bulluck, Yellon and Hausenloy 2016, Candilio 2016, Hausenloy 2016, Kloner and Braunwald 2016, Pfeffer et al. 1979). Muscarinic receptor agonists reduce cardiac infarct size, which can be attenuated with the specific M_3 receptor antagonist, 4-DAMP (Pan et al. 2012, Yang et al. 2005). ACh (100 nM) protects against ischaemia/reperfusion

injury by decreasing infarct size; co-administration with Tiotropium bromide (1 nM) shows abrogation of Tiotropium bromide mediated infarct size in ischaemia/reperfusion and normoxic conditions. This study corroborates the role of M_3 receptors in cardioprotection (Wang, Shi and Wang 2004); in this study, ACh affords cardioprotection via M_3 receptors, as protection is partially affected by Tiotropium bromide administration.

Akt and phosphatidyl-inositol 3 kinase (PI3K) are also involved in conferring acetylcholine mediated cardioprotection as observed in human salivary gland cells and Sprague-Dawley rat ventricular myocytes (Kajiya et al. 2012, Sun et al. 2010). Akt is critical in cardioprotection via the reperfusion injury salvage kinase pathway (RISK) (Chen et al. 2017b, Davidson et al. 2006, Filippone et al. 2017, Hausenloy and Yellon 2006, Thomas et al. 2015). The RISK pathway involves the activation of multiple pro-survival kinases, such as ERK1/2 and Akt at the time of reperfusion, following ischaemia (Mockridge, Marber and Heads 2000, Rossello and Yellon 2018). Wortmannin inhibits PI3K activity (Nagoshi et al. 2005, Yu et al. 2007), PI3K inhibition in this study does not show any significant changes in haemodynamic function in ischaemia/reperfusion and normoxic conditions. In salivary glands, the use of an M_3 receptor antagonist and PI3K inhibitor showed a decrease in the cytoprotective effect observed with carbachol administration (Kajiya et al. 2012).

Wortmannin shows no protective effect on ischaemia/reperfusion injury, nor reversal of Tiotropium bromide induced infarct size when co-administered in ischaemia/reperfusion or normoxic conditions. In normoxic conditions, wortmannin alone shows a significant decrease in infarct size compared to Tiotropium bromide. Figure 7.4.2 depicts PI3K/Akt signalling. PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP_2) generating PIP_3; both recruit phosphoinositide dependent kinase 1 (PDK1) and Akt (Nagoshi et al. 2005). Phosphorylated Akt inhibits Forkhead transcription factor (FOXO) and Bad (Häggblad Sahlberg et al. 2017, Sussman et al. 2011) resulting in nitric oxide production (O'Neill and Abel 2005). PI3K/Akt has antiapoptotic effects (Ravingerová et al. 2007) therefore inhibition of this pathway could inhibit cytoprotection. However, overexpression of Akt is also shown to result in an increase in infarcts (Nagoshi et al. 2005). The current study implies that inhibition of PI3K abrogates cardiomyocyte survival.

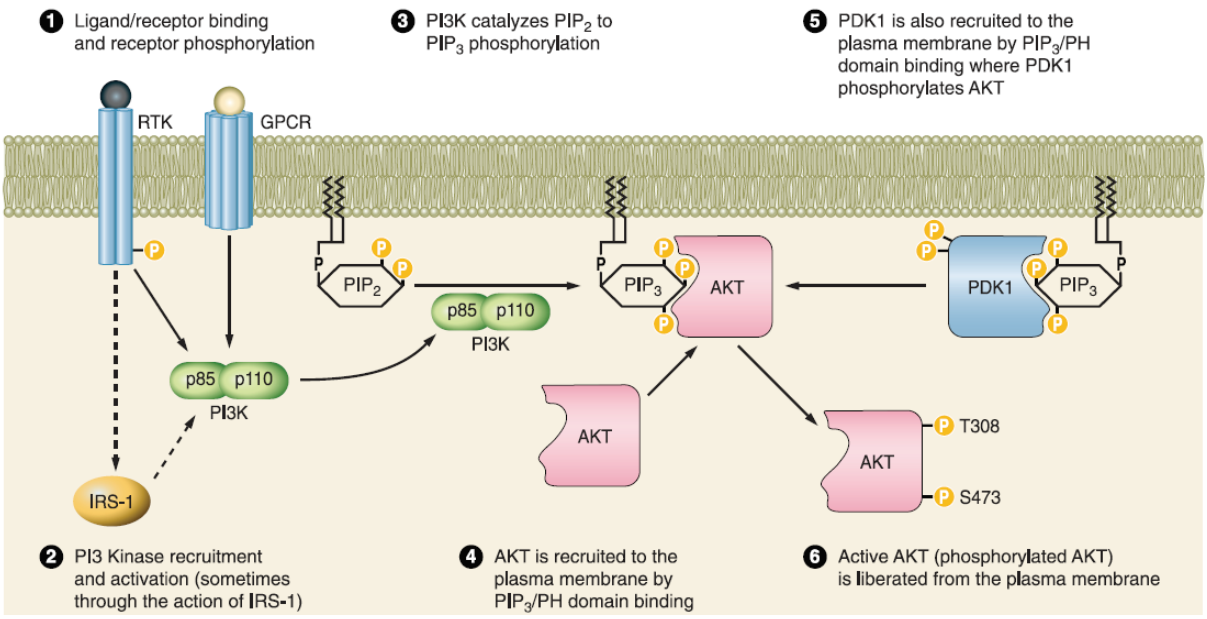

Figure 7.4.2: PI3K activation following tyrosine kinase receptor stimulation*. Akt is phosphorylated by PDK1 and PIP_3. Akt activation leads to activation/inhibition of several proteins involved in cell survival (Sussman et al. 2011).*

The current study showed an increase in phosphorylated Akt $_{(Ser473)}$ following administration of Tiotropium bromide (1 nM). Co-administration with acetylcholine (100 nM) or wortmannin (100 nM) abrogates this, with wortmannin showing very little or no phosphorylation. Acetylcholine can activate the PI3K/Akt/eNOS pathway (Turovsky et al. 2013) involved in cardioprotection (Krieg et al. 2004). However, PI3K/Akt activity can also be induced via reactive oxygen species (Giordano 2005, Griendling et al. 2000, Nogueira et al. 2008). PI3K activation occurs following activation of epidermal growth factor receptor (EGFR) by muscarinic stimulation (Kajiya et al. 2012, Krieg et al. 2002, Wang 2016); M_3 receptor induced cytoprotection is attenuated following inhibition of the EGFR coupled proto oncogene kinase Src (Kajiya et al. 2012). Short term Akt activation enhances mTOR mediated VEGF, preventing apoptosis in myocardial ischaemia/reperfusion (O'Neill and Abel 2005). Figure 7.4.3 depicts the PI3K/Akt pathway following activation by insulin receptor substrate (IRS).

Evidence suggests that Akt may also contribute to cell death (Liu et al. 2014). Constitutive activation of Akt is common in tumours (Los et al. 2009) and failing hearts (Chaanine and

Hajjar 2011, Haq et al. 2001), leading to severe cardiac dysfunction with no regression in hypertrophy, and decreased IRS-1/PI3K activation (O'Neill and Abel 2005). Several mediators can result in an increase in Akt, including H_2O_2, implying oxidative stress in Akt activation leading to apoptosis (Griendling et al. 2000, Nogueira et al. 2008, Ushio-Fukai et al. 1999). This study implies the involvement of muscarinic receptors and PI3K in Tiotropium bromide mediated Akt phosphorylation, which may contribute to cardiotoxicity. The study also assessed the effects of acetylcholine (100 nM) and wortmannin (100 nM) on Tiotropium bromide (1 nM) mediated apoptosis. Acetylcholine and wortmannin showed significant decreases in apoptosis with respect to Tiotropium bromide. For acetylcholine, this was reversed with Tiotropium bromide co-administration. Interestingly, the decrease with wortmannin was sustained and significant upon co-administration with respect to Tiotropium bromide. Acetylcholine mediated cardioprotection is abrogated with Tiotropium bromide, indicating that M_3 receptors are involved (Luo, Busillo and Benovic 2008, Yang et al. 2005, Zhao et al. 2010a). Wortmannin enhances apoptosis (Das et al. 2016, Nesterov et al. 2001, Zhao et al. 2014), however in this study, the inhibition of PI3K shows decreased apoptosis, indicating that PI3K may be involved in Tiotropium bromide mediated apoptosis (Li et al. 2009b). Akt may regulate cardiomyocyte survival by delaying the opening of the mitochondrial permeability transition pore (mPTP) (Ong et al. 2015b).

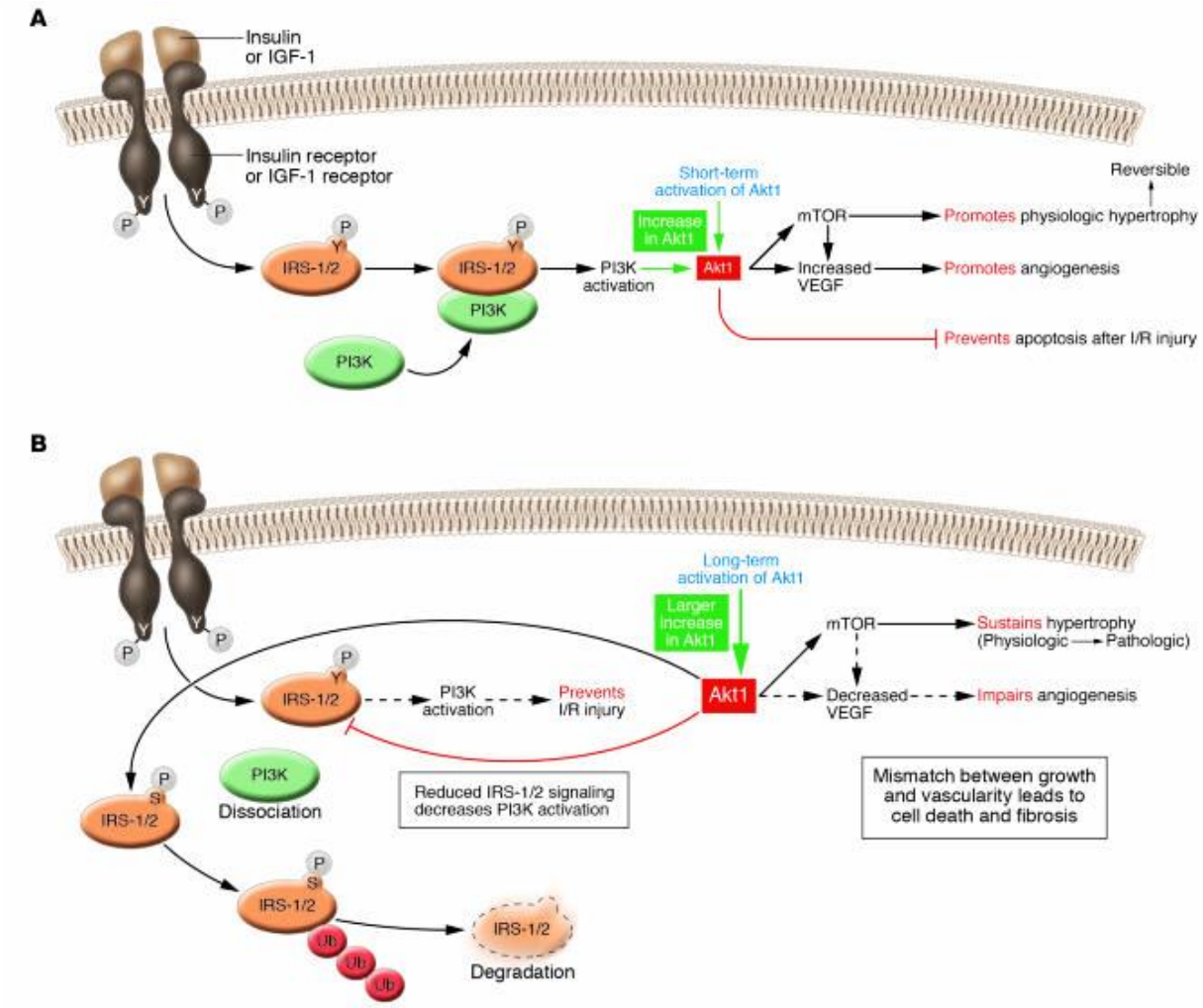

Figure 7.4.3: Insulin receptor signalling activates PI3K/Akt activation. *Short term activation of Akt1 via insulin results in physiological hypertrophy and increased VEGF which protects against ischaemia/reperfusion injury. Long term activation of Akt1 is detrimental and inhibits IRS mediated signalling, leading to IRS degradation, there is sustained hypertrophy and decreased VEGF leading to cell death and fibrosis (O'Neill and Abel 2005).*

mPTP opening is important in understanding cell death. The intrinsic and extrinsic pathways mediate apoptosis (Baig et al. 2017, Brentnall et al. 2013). The intrinsic pathway is triggered by mitochondrial outer membrane permeabilisation (MOMP); whereas the extrinsic pathway is initiated by death receptor signalling, caspase 3 and Bcl2 proteins are involved in both pathways (Brentnall et al. 2013, Gill, Mestril and Samali 2002, Shamas-Din et al. 2011). Figure 7.4.4 illustrates the signalling cascades involved in both pathways, which ultimately converge to caspase mediated apoptosis.

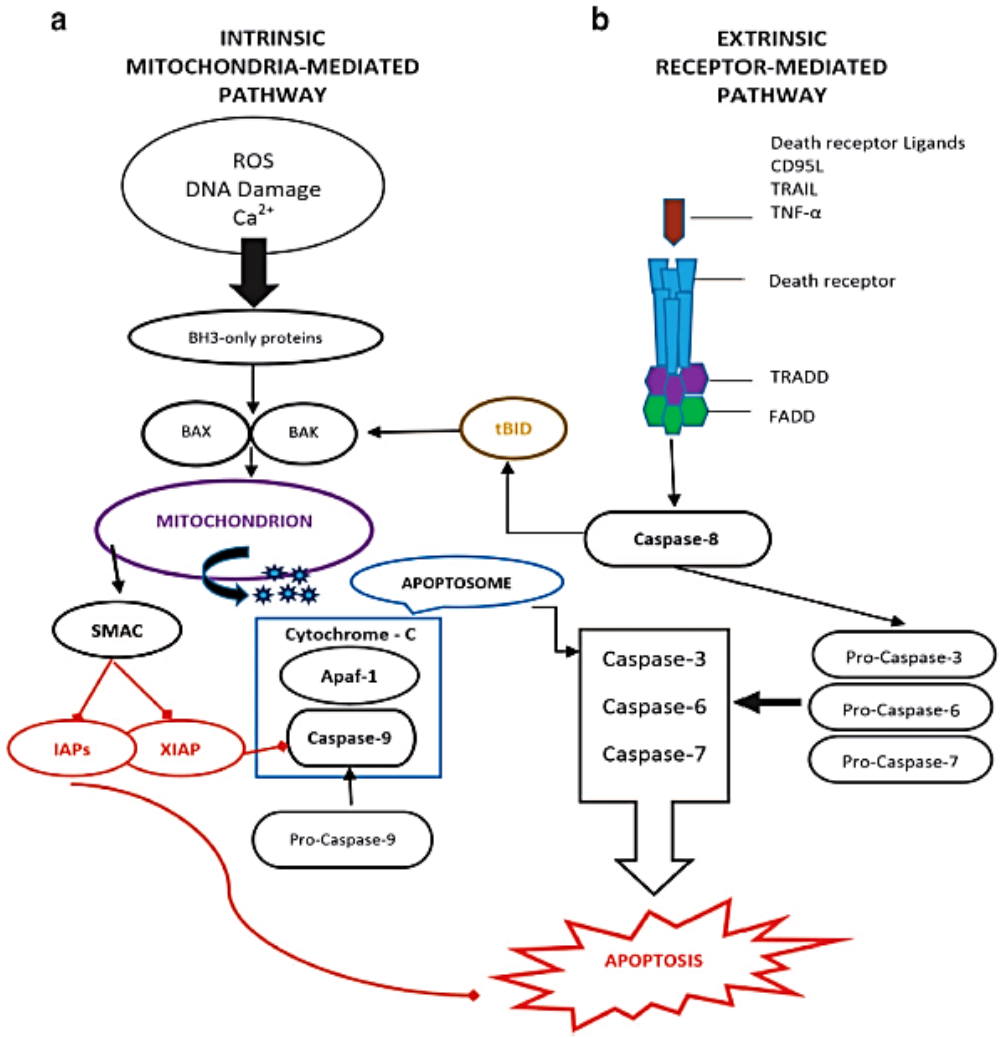

Figure 7.4.4: The two pathways involved in initiation of apoptosis. *The mitochondria are instrumental in the intrinsic pathway (a), whereas extracellular death receptors initiate the extrinsic pathway (b). ROS – reactive oxygen species, BH3 – Bcl-2 family of initiator proteins, BAX – Bcl-2 associated X protein, BAK – Bcl-2 homologous antagonist killer, tBID – truncated BH3 interacting-domain death agonist, SMAC – Diablo homolog, IAPs – inhibitor of apoptosis, XIAP – X-linked inhibitor of apoptosis, Apaf-1 – apoptotic protease activating factor 1, TRAIL - TNF-related apoptosis-inducing ligand, CD95L – Fas ligand, TNF α – Tumour necrosis factor α, TRADD – Tumour Necrosis Factor Receptor 1-associated death domain protein, FADD – Fas associated death domain (Baig et al. 2017).*

Opening of the mitochondrial permeability transition pore (mPTP) (Jeong and Seol 2008, Weiss et al. 2003), leads to depolarisation-dependent ATP hydrolysis (Kinnally et al. 2011).

Cyclosporin A (CsA) inhibits mPTP opening (Ong et al. 2015a) via cyclophillin D, which regulates pore opening (Crompton 1999, Fakharnia et al. 2017, Mott et al. 2004). Pre-clinical studies have found CsA mediated mPTP inhibition (Gyulkhandanyan et al. 2015) to confer protection (Halestrap and Pasdois 2009, Kinnally et al. 2011). However, CsA has failed in patient trials (Muehlschlegel 2014, Piot et al. 2008), particularly in ischaemia/reperfusion injury (Cung et al. 2015, Hausenloy and Yellon 2015, Song, Wang and Qi 2015).

Cyclosporin A (CsA 200 nM) significantly increases LVDP in ischaemia/reperfusion; no other significant differences were observed in haemodynamic function in ischaemia/reperfusion or normoxic conditions. Previous studies showed CsA did not affect coronary flow and heart rate (Chauhan et al. 1994, Greenberg et al. 1985, Sudhir et al. 1994). Studies observing cardiac hypertrophy found that CsA inhibits pressure overload induced hypertrophy (Meguro et al. 1999). However, in ischaemia/reperfusion, CsA restores ventricular function (Clarke, McStay and Halestrap 2002, Griffiths and Halestrap 1995) with pre-treatment and acute administration and recovers heart rate in isolated Langendorff hearts (Mirica et al. 2010). In this study, CsA administration in ischaemia/reperfusion and normoxic conditions significantly reduced infarct size compared to controls and Tiotropium bromide (1 nM), even with Tiotropium bromide co-administration. CsA administration attenuates acetylcholine induced vasodilation in the coronary circulation (Nasser et al. 2014, Sudhir et al. 1994, Vecchiati et al. 2014), linking muscarinic signalling with mPTP modulation. These studies show that Tiotropium bromide mediated infarct size can be attenuated through inhibition of mPTP opening.

Adjunctive therapies attenuate Tiotropium bromide mediated cardiotoxicty, however these are not currently a viable clinical intervention. Additionally, there is a need to correctly identify whether the effects observed in animal models are representative of patients. Studies suggest that genetic predisposition to adverse cardiovascular events in COPD patients may be an underlying cause as well as new use of LAMAs, where a nested control study showed a significant increase in risk (Matera et al. 2018, Liou et al. 2018, Wang et al. 2018).

Chapter 8: General Conclusion

Long acting muscarinic receptor antagonists (LAMAs) have no previous association with cardiotoxicity in chronic obstructive pulmonary disease (COPD), unlike Ipratropium bromide (Harvey, Hussain and Maddock 2014) and salmeterol (Proskocil and Fryer 2005, Salpeter, Ormiston and Salpeter 2004, Sears 2002). However, the potential cardiovascular risks associated with anti-cholinergics including the LAMA, Tiotropium bromide, have been highlighted (Jara, Wentworth and Lanes 2012, Singh, Loke and Furberg 2008). These findings motivated the current study, to assess the adverse cardiovascular outcomes of LAMAs in a whole heart model, including ischaemia/reperfusion injury, and the signalling pathways involved in cardiotoxicity.

8.1 Key Findings of this Study

The results from this study suggest that Aclidinium, Tiotropium and Umeclidinium bromides exacerbate cardiac injury in terms of infarct size to risk ratio in models of ischaemia/reperfusion injury. Furthermore, Tiotropium bromide in normoxic conditions also shows increased infarct size in Langendorff hearts, and an increase in caspase-3 activation in adult isolated ventricular cardiomyocytes. Conversely, an increase in phosphorylation of the cardioprotective kinase, Akt $_{(Ser473)}$ is observed with Tiotropium bromide in normoxic conditions, and increases in intracellular [Ca^{2+}] and phosphorylation of CaMKII $_{(Thr286)}$, indicating dysfunctional calcium homeostasis, a marker of cardiac damage.

8.1.1 Findings from Ischaemia Reperfusion models

The ischaemia/reperfusion models show that regional ischaemia affects haemodynamics, with recovery largely at reperfusion. LAMAs exert a tachycardic effect therefore can increase heart rate; however, in the model of ischaemia/reperfusion injury, the administration of LAMAs throughout reperfusion has no significant effect on haemodynamic function versus controls. This is in contrast to infarct size to risk ratios, which show exacerbation of ischaemia/reperfusion injury with LAMA administration, apart from Glycopyrronium bromide.

8.1.2 Assessment of Tiotropium bromide in Normoxic conditions

In contrast to ischaemia/reperfusion, Tiotropium bromide (10 – 0.1 nM) significantly affects cardiac function in normoxic conditions, as observed with changes in coronary flow and LVDP, with little effect on heart rate but increased infarct size. Cardiac muscarinic receptors are involved in parasympathetic control of cardiac function, resulting in modulation of pacemaker activity through K^+ channels and atrio-ventricular conduction (Dhein, Van Koppen and Brodde 2001), therefore inhibition may affect cardiac function. Tiotropium bromide mediated increases in infarct size are modest with respect to Doxorubicin (Gharanei et al. 2013a, Gharanei et al. 2013b) and Sunitinib (Cooper et al. 2018) or in ischaemia/reperfusion, with the short acting muscarinic antagonist, Ipratropium bromide (Harvey, Hussain and Maddock 2014). However, the increase in infarct size with LAMAs is indicative of a greater concern due to long term exposure in COPD.

8.1.3 Oxidative stress and Calcium in Tiotropium bromide induced cardiotoxicity

Resveratrol (10 µM) shows a significant increase in coronary flow in normoxic conditions with no other effect on haemodynamic function and dampens Tiotropium bromide (1 nM) mediated infarct size in ischaemia/reperfusion and normoxic conditions, whilst abrogating ischaemia/reperfusion injury. Resveratrol also dampens Tiotropium bromide mediated increased p-Akt $_{(Ser473)}$. Interestingly, Tiotropium bromide has no significant effect on ROS production in cardiomyocytes; however, resveratrol is protective by inhibiting basal ROS production and apoptotic cardiomyocytes. Nifedipine (1 nM) in normoxic conditions shows a decrease in coronary flow when co-administered with Tiotropium bromide with no other effect on haemodynamic function, but significantly decreases infarct size when co-administered with Tiotropium bromide. KN-93 (400 nM) only shows an increase in left ventricular developed pressure (LVDP) under normoxic conditions when co-administered with Tiotropium bromide and decreases Tiotropium bromide mediated infarct size in normoxic conditions and in ischaemia/reperfusion. KN-93 also abrogates Tiotropium bromide mediated p-Akt $_{(Ser473)}$. Tiotropium bromide shows an increase in p-CaMKII $_{(Thr286)}$, which is abrogated with KN-93. Tiotropium bromide also shows an increase in intracellular Ca^{2+} release, however the use of KN-93 does not affect this; similarly, there is no effect on apoptotic cardiomyocytes with the use of KN-93 or combined with Tiotropium bromide. It

is clear from this study that calcium and ROS contribute in part to the Tiotropium bromide mediated cardiotoxicity.

8.1.4 Effect of cardioprotective strategies on Tiotropium induced cardiotoxicity

Acetylcholine (100 nM) shows no significant effect on haemodynamic function, but a protective effect is observed with infarct size in ischaemia/reperfusion and normoxic conditions, where co-administration with Tiotropium bromide (1 nM), abrogates Tiotropium bromide mediated increase. Similarly, acetylcholine administration abrogates Tiotropium bromide mediated p-Akt $_{(Ser473)}$, but with little effect on total apoptotic cells. Wortmannin (100 nM) administration did not affect haemodynamic function or infarct size in either model, but abolished Tiotropium bromide mediated p-Akt $_{(Ser473)}$, and reversed Tiotropium bromide mediated apoptosis. Z-DEVD-FMK (70/140 nM) shows no effect on haemodynamic function in ischaemia/reperfusion, however in normoxic conditions there is an increase in coronary flow and a protective effect on infarct size in both models; additionally, there is a protective effect on apoptosis in cardiomyocytes. Tiotropium bromide (1 nM) in cardiomyocytes shows an increase in cleaved caspase-3 $_{(Asp175)}$, which is abolished following co-administration with Z-DEVD-FMK. Cyclosporin A (CsA 200 nM) also shows no effect on haemodynamic function in normoxic conditions, and only increases LVDP at the onset of reperfusion in ischaemia/reperfusion. However, CsA significantly decreases infarct size under both conditions, abrogating Tiotropium bromide mediated infarct size, and reversing infarct size to risk ratio due to ischaemia/reperfusion injury. This implies a greater role of the mitochondrial pore in Tiotropium bromide mediated cardiotoxicity.

8.1.5 Implications of this study

This study suggests that Tiotropium bromide may result in cardiovascular damage in an infarct size dependent manner, in normoxic conditions and ischaemia/reperfusion injury. Reversal of Tiotropium bromide mediated cardiac damage via acetylcholine, implies that muscarinic receptors mediate damage. Tiotropium bromide mediated cardiotoxicity in normoxic conditions may predominantly involve calcium signalling, through intracellular Ca^{2+} release, aberrant CaMKII and Akt phosphorylation. Although resveratrol shows a cardioprotective effect, Tiotropium bromide does not significantly affect reactive oxygen species formation, therefore resveratrol may enhance protective cellular strategies, which

are down-regulated following Tiotropium bromide. Tiotropium bromide administration sees increased cleaved caspase-3 and caspase-3 mRNA, however there is no significant increase in apoptosis, which suggests that caspase-3 alone in not sufficient for the induction of apoptosis in this study. The effect of cyclosporin A on Tiotropium bromide mediated cardiotoxicity suggests mitochondrial involvement, specifically the mPTP, which warrants further investigation.

This study highlights the importance of devising preclinical studies which can acutely assess the cardiotoxic capabilities of drugs, without solely depending on functional parameters as a measure of cardiovascular adverse effects. The current study also highlights that where there is an already heightened risk of cardiovascular complications, such as in COPD, there should be added care when devising a therapeutic intervention strategy due to the risks associated with exacerbating exisiting cardiovascular complications. Additionally, whilst this study shows that the use of several inhibitors exert protective effects on Tiotropium bromide induced cardiotoxicity, there is no scope of the use of these in a clinical setting, urging the development of cardioprotective strategies to shield patients against adverse cardiotoxic effects. This study has emphasised the importance of the preclinical testing of drugs, as well as support the studies which may observe adverse cardiovascular events with the use of LAMAs such as Tiotropium bromide. The mechanism of cardiotoxicity is important in understanding how drugs can be developed to reduce adverse effects and develop interventions which can have protective effects.

8.2 Further Investigations

Whilst Langendorff models provide the ability to observe the direct effects of drug compounds on the function of the heart, the results observed may not be mimicked *in vivo*, due to multiple interactions from other organs and limitations of the model itself (Aksentijević, Lewis and Shattock 2016, Bell, Mocanu and Yellon 2011), and may explain the discrepancies observed between multiple studies. However, there is no doubt that the cardiovascular safety of numerous drugs, including Tiotropium bromide has been challenged (Suissa, Dell'Aniello and Ernst 2017a, Suissa, Dell'Aniello and Ernst 2017b, Zou et al. 2018). It is thought that newer users of long acting bronchodilators may be subjected

to a greater risk of cardiovascular adverse effects (Wang et al. 2018), and the risks calculated in randomised controlled trials cannot be compared for different trials and different drugs due to the complexities of the selection criteria (Matera et al. 2018). There is a push and greater emphasis on combinatorial therapy, which is seen to reduce the risk of off target effects (Matera et al. 2018).

Although this study evaluates multiple facets of cardiotoxicity including calcium signalling and oxidative stress, a significant mediator of cardiomyocyte death is the mitochondrial permeability transition pore (mPTP) (Halestrap and Richardson 2015, Ong et al. 2015c); which has not been examined in detail for the purposes of this study. Mitochondria are one of the largest contributors to oxidative stress and Ca^{2+} overload and are well known to be involved in mediating cell death (Gyulkhandanyan et al. 2015, Jaeschke, McGill and Ramachandran 2012, Joiner et al. 2012, Ong et al. 2015b, Orrenius, Gogvadze and Zhivotovsky 2015, Pasdois et al. 2011). Mitochondrial activity has also been implicated in Ipratropium bromide and doxorubicin mediated cardiotoxicity (Deavall et al. 2012, Gharanei et al. 2013a, Gharanei et al. 2013b, Harvey, Hussain and Maddock 2014). These studies as well as the present study indicate that mitochondrial activity may have an instrumental role in mediating cardiomyocyte death in drug induced cardiotoxicity. This would justify further studies observing mitochondrial activity such as opening of the mPTP (Izem-Meziane et al. 2011), to measure hypercontracture in response to drug aggravation or measuring ATP in response to stress (Marroquin et al. 2007).

Muscarinic signalling is known to affect downstream mediators such as cyclic AMP (cAMP) (Harvey 2012), specifically associated with M_2 receptor signalling, which decrease cAMP production through inhibition of adenylyl cyclase (Harvey and Belevych 2003), particularly following β-adrenergic receptor mediated stimulation (Harvey 2012). It is thought that β_1 receptor mediated death signalling may occur via cAMP, involving PKA and Ca^{2+} regulation (Shin et al. 2014). cAMP has important functions in modulating pacemaker channel permeability to K^+ and Na^+ (Accili et al. 2002), the cardiac funny current (Baruscotti, Bucchi and DiFrancesco 2005) and mediating contractility via protein kinase A (PKA) (Salazar, Chen and Rockman 2007). PKA phosphorylates several important mediators of Ca^{2+} signalling, notably phospholamban, ryanodine receptors and L-type Ca^{2+} channels (Salazar, Chen and Rockman 2007). PKA phosphorylation of phospholamban occurs at a different site to

CaMKII mediated phosphorylation; whilst both sites are independent to each other, CaMKII mediated phosphorylation of phospholamban is difficult to achieve in the absence of high intracellular cAMP concentrations (Mattiazzi et al. 2005). This suggests that cAMP may be involved in high CaMKII activity, as suggested by this study. The effect on cAMP following Tiotropium bromide would elucidate the cardiac signalling which occurs, and potentially reveal any off-target receptor signalling.

Tiotropium bromide has been described as an inverse agonist (Kruse et al. 2012) and an antagonist (Casarosa et al. 2010, Cooke et al. 2015). Whilst these terms have been used interchangeably, inverse agonists are associated with a negative response which is opposing to an agonist, whereas antagonists are thought to elicit no response (Rosenbaum, Rasmussen and Kobilka 2009, Szkudlinski 2015). This study suggests that Tiotropium bromide may exert an effect as an inverse agonist, due to the cellular changes which occur such as increased intracellular Ca^{2+} following Tiotropium bromide. However, further investigations are necessary to determine the role of Tiotropium bromide as an inverse agonist capable of eliciting a signalling effect. As well as a potential role as an inverse agonist, GPCR ligands may also initiate signalling via β arrestins (Azzi et al. 2003). This requires recruitment of GPCR serine/threonine kinases (GRK) to the plasma membrane by the Gβγ subunit (Kohout and Lefkowitz 2003), to phosphorylate the receptor and recruit β arrestins. Previously β arrestins were only thought to be involved in receptor internalisation (Johnson, Scott and Pitcher 2004), however this has been challenged; this type of signalling is supplementary to normal Gα mediated signalling and introduces an alternative mediator into the potential effect of inverse agonists (Azzi et al. 2003). Another interesting mediator is known as activator of G protein signalling 8 (AGS8) or fibronectin domain III containing 1 (FNDC1) protein, which interacts with the Gβγ subunit. AGS8 has been implicated in mediating hypoxia induced apoptosis in cardiomyocytes via connexin-43 (Ishikawa et al. 2012, Sato et al. 2009), independent of G protein dissociation (Sato et al. 2006, Yuan et al. 2007). AGS8 signalling has also been implicated in VEGF mediated angiogenesis (Hayashi et al. 2016). These suggest an important role for receptor independent activation via AGS8 in mediating cellular consequences such as apoptosis and may be of interest in understanding Tiotropium bromide mediated cardiotoxicity.

Biomarkers for cardiac injury have widely been used to assess cardiotoxicity and cardiac damage, such as cardiac troponin for acute myocardial infarction. Although troponin has high negative risk prediction, it has issues with positive risk identification, and may simply be due to the lack of reference material to standardise assays (Cardinale et al. 2010). Other biomarkers include elevated levels of N-terminal pro-brain natriuretic peptide (NT-pro BNP) which correlate to increased wall stress in heart failure, preceding most anatomical and functional changes (Cardinale et al. 2017, Cross et al. 2015). However, these biomarkers, may differ from person to person due to multiple other factors which are involved. Instead, non-coding RNAs have been proposed as biomarkers for cardiac damage; nc-RNAs in particular are stable and can be detected in human fluids and are shown to be considerably different in healthy volunteers and patients, and present profiles which correlate to different diseases (Ruggeri et al. 2018). MicroRNA-1 is significantly upregulated in acute myocardial infarction and thus may present as a predictor of disease (Gupta, Bang and Thum 2010, Viereck and Thum 2017). However, there are limitations to these due to inconsistencies in reported findings, which must be resolved before taking any further action (Gupta, Bang and Thum 2010, Ruggeri et al. 2018, Viereck and Thum 2017).

Recently, vascular biomarkers have been introduced in identifying at risk populations amongst COPD patients, including aortic pulse wave velocity and intima media thickening which have been found to show that COPD is an independent risk factor for cardiovascular disease (Fisk et al. 2018). The importance of translatable pre-clinical research cannot be stressed, and it is vital to ensure that the effects observed in animal models are realistic of *in vivo* signalling processes. This study has shown the effects of Tiotropium bromide both in the context of myocardial ischaemia/reperfusion injury and in normoxic conditions finding detectable changes in cardiac damage, which has been debated in human trials. These discrepancies raise the question of ensuring that appropriate biomarkers for disease can be screened, as well as understanding that genetic predisposition may underlie cardiovascular response to LAMA administration (Cazzola et al. 2017b, Matera et al. 2018). These push the field of drug induced cardiotoxicity towards adopting a pharmacogenomics-based approach to drug toxicity (Madonna 2017). This demonstrates the need for the development of screening assays which can incorporate multiple measures of toxicity, not solely relying on the functional capacity of the heart; and also

emphasises that heading towards a genomics-based approach for therapy can prevent adverse cardiovascular effects in patients who may respond negatively to treatment, and tailor treatment specifically for individuals.

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Appendix

***Appendix 1**: The data shown below represents the parameters recording from a Langendorff model, accounting for the coronary flow (CF), left ventricular developed pressure (LVDP) and heart rate (HR) from a single experiment (n = 1). The raw values (column headings highlighted in green) are then normalised. The coronary flow is firstly normalised by the weight of the heart and then by the average stabilisation period (the mean of the first 20 minutes of the experimental protocol), the LVDP and HR are also normalised according to the average stabilisation period for each individual parameter.*

Ischaemia/Reperfusion - Tiotropium bromide (1nM) with Acetylcholine (100nM)								
	Time	**CF**	**ml/g**	**%**	**LVDP**	**%**	**HR**	**%**
Drug	**5**	20	9.09	97.56	67.41	77.19439	300	96.77419
	10	18	8.18	87.80	76.08	87.12282	290	93.54839
	15	22	10.00	107.32	107	122.5308	320	103.2258
	20	22	10.00	107.32	98.81	113.152	330	106.4516
Ischaemia	**25**	12	5.45	58.54	47.21	54.06241	300	96.77419
	30	12	5.45	58.54	47.66	54.57773	300	96.77419
	35	12	5.45	58.54	53.87	61.68909	290	93.54839
	40	12	5.45	58.54	47.78	54.71514	290	93.54839
	45	12	5.45	58.54	65.45	74.9499	270	87.09677
	50	12	5.45	58.54	62.61	71.69768	280	90.32258
	55	12	5.45	58.54	63.75	73.00315	290	93.54839
Reperfusion	**70**	20	9.09	97.56	93.47	107.0369	280	90.32258
	85	22	10.00	107.32	96.47	110.4724	290	93.54839
	100	21	9.55	102.44	92.46	105.8803	300	96.77419
	115	18	8.18	87.80	82.54	94.52047	290	93.54839
	130	19	8.64	92.68	86.04	98.52849	290	93.54839
	145	18	8.18	87.80	75.93	86.95104	290	93.54839
	160	18	8.18	87.80	76.19	87.24878	300	96.77419
	175	18	8.18	87.80	80.95	92.69969	290	93.54839
Heart Weight (g)		2.2						
Average Stabilisation		9.318182			87.325		310	