MASTER OF SCIENCE BY RESEARCH

The effect of salbutamol in the heart following ischemia/reperfusion

Peter, Arun

Award date:
2011

Awarding institution:
Coventry University

THE EFFECT OF SALBUTAMOL IN THE HEART FOLLOWING ISCHEMIA/REPERFUSION

Submitted by

ARUN PETER

A thesis submitted in partial fulfilment of the University requirements

For

MSc by Research in Molecular Pharmacology 2009

Faculty of Health and Life Sciences, Coventry University, Priory Street

Coventry, United Kingdom

CONTENTS

LIST OF FIGURES

Acknowledgement

It is with the deep sense of gratitude; I wish to render my thanks to Dr.Helan Merdek, Department of Cardiovascular science, Coventry University, under whose esteemed guidance and supervision the work presented in this was carried out. I have learnt a lot from Dr.Helan, and her help has been vital for the production of this thesis

I am great full and indebt full to Dr.Afthab Husain, Research fellow, Department of Cardiovascular science, Coventry University, for his valuable support, contributions, discussions time and patience throughout the study.

I wish to express my thanks to Mr. Mark Bodycote for his help in the lab providing the animals, chemicals and reagents throughout my research

I would also like to thank all the technicians and friends who were great help throughout the laboratory testing part of this study

Finally I would like to thank my parents for all the support that they have given throughout my studies

ABSTRACT

Activation of $beta_2$ adrenoceptors with salbutamol has been reported as cardio toxic. In this study, the investigation was to find out whether the $beta_2$ agonist salbutamol protects the myocardium from ischemic reperfusion injury, when administered at reperfusion or post reperfusion.

Administration of $beta_2$ agonist salbutamol (1nM) at the onset of reoxygenation significantly decreased the live cells. Moreover, the administration of salbutamol (1nM) in the presence of $beta_2$-AR selective antagonist ICI 118,551 showed a significant decrease in the live cells through a cardio toxic manner. This study further showed that, $beta_2$ agonist salbutamol in the presence of $beta_1$-AR selective antagonist CGP 12177 decreased the live cell population.

Administration of $beta_2$ agonist salbutamol (0.01nM) throughout the reperfusion injury also decreased the population of live cells. Also, the administration of salbutamol (0.01nM) with the $beta_2$ antagonist ICI and $beta_1$ antagonist CGP showed the decrease in live cells. Besides the administration of $beta_2$ agonist salbutamol (1µM, 10nM, and 0.1nM) at the onset of reperfusion showed a significant increase in necrosis and the administration of $beta_2$ agonist salbutamol (1nM) in the presence of $beta_2$ antagonist ICI throughout the reperfusion injury exposed an increase in the necrosis.

Caspase-3 is an effector caspase. The activity of the Caspase-3 is monitored in this study to detect the apoptotic status of cell population. In this study, the intracellular staining of isolated rat myocytes with Alexa Fluor® 488 labelled caspase-3 antibodies were used. The flowcytometric analysis of caspase-3 activation in cells showed an increase in the caspase-3 level, especially with the administration of salbutamol 10nM and 0.1nM. Moreover, the administration of salbutamol (1nM, 0.1nM) in the presence and absence of β_2 antagonist ICI and the β_1 antagonist CGP showed a fold increase in the caspase level.

Collectively, this study demonstrated that, the administration of β_2 agonist salbutamol at the onset of reperfusion will not protect the ischemic reperfused rat myocardium from the ischemic reperfusion injury. Besides, it causes a toxic effect in the cardiomyocyte with the increase of apoptotic and necrotic status of cell population. Also, the decrease in live cells population showed the cardio toxic effect of β_2 agonist salbutamol.

I N T R O D U C T I O N

1. 1 Coronary Artery Disease

Coronary artery disease is one of the most chronic diseases in the world. There are many factors in the formation of acute myocardial infarction. One of the factors is cardiac ischemia, which is the lack of oxygenated blood supply to the heart. There will be some metabolic and structural changes in response to myocardial ischaemia. In order to avoid all these complications, reperfusion or restoration of myocardial blood flood flow is essential (Colins et al., 1997).

The various forms of cardiovascular diseases are coronary heart disease (heart attacks), cerebrovascular disease, raised blood pressure (hypertension), peripheral artery disease, rheumatic heart disease, congenital heart disease and heart failure. Globally, cardiovascular diseases are the main cause of death and are projected to remain so. An estimated 17.5 million people died from cardiovascular disease in 2005, representing 30 % of all global deaths. In these deaths, 7.6 millions were due to heart attacks and 5.7 million due to stroke (Rosamond et al., 2007). Moreover, coronary artery disease mainly occurs as a result of dyslipidemia, family history, diabetes and hypertension. Studies shows that the obesity rates in the people are also not differ significantly with the coronary artery disease (Kolliaki et al., 2010).

The gradual occurrences of fatty deposits on the walls of coronary arteries are the major cause of coronary heart disease. As a result, the coronary artery narrows and makes it harder for the coronary artery to supply blood and oxygen. The medical term for this condition is atherosclerosis and the fatty material is known as Atheroma.

1. 2 Atherosclerosis

Atherosclerosis is a chronic disease which occurs in the walls of the arteries. This is a very progressive disease in which plaques are (consisting of deposits of cholesterol and other lipids, calcium, and large inflammatory cells called macrophages) building up in the arteries (Catherine et al., 1999). Due to the high impact of these plaques on the arteries will make a partial or complete obstruction to the blood flow, which finally creates the sudden occlusion of artery. This condition is called as arterial thrombosis. Recent studies showed that high density of lipoproteins can make an impact on the risk of atherosclerosis. By multiple pathophysiological mechanisms, these high density lipoproteins will reduce the risk of atherosclerosis (Ragbir et al., 2010).

Figure 1.2.a : The occurrence of plaques in the arteries, which is leading to the ultimate blockage of blood ischemia **(NHLBI, Diseases and condition indexes)**

Ischemia is an absolute or relative shortage of the blood supply to an organ. The confliction of blood supply (oxygen delivery) and the blood request for adequate oxygenation of the tissue reveals the relative shortage. Ischemia of the tissue is due to the lack of oxygen and inability to meet the nutrient demand. Ultimately, this causes great damage. As the myocardium is unable to meet oxygen demand, the ischemic tissue reverts from aerobic to anaerobic respiration by means of generating energy. This process involves the conversion of glucose to lactic acid to yield energy leading to significance of lactic acid accumulation in the ischemic zone (Solani and Harris. 2005).

At the intracellular level, the lingering ischemia has physiological effects like morphological changes. (Cell swelling) (Lichtig and Brooks. 1974).Swelling of the cell membrane (sub-lethal damage) and the lethal damages (necrosis and apoptosis) are the two ways of occurring cell death (Lodish et al., 2004). During the period of cell swelling, the cells become widened and the conductance of the membrane will increase (Tseng et al., 1992). Interference of oxidative phosphorylation, adenosine tri-phosphate (ATP) depletion, collapse of the ATP dependant Na^{+}/K^{+} and Ca^{2+} pumps, decrease in intracellular pH, reversible and irreversible myocyte damage (Graham et al., 2004), expansion of the calcium and oxygen paradox (Braunwald and Kloner. 1985) and free radical mediated injury also occurred (Wall. 2000).

1.3. Cardiac ischemia

Cardiac ischemia (chest pain) is a pathological condition that is caused by the narrowing of significant coronary arteries by pathological processes such as atherosclerotic plaque or blood clot formation. In extreme case, complete blockage results in cell death in a few hours and infarction (heart attack) progression unless a revascularization procedure is performed as soon as possible. The formation of this plagues can originate the narrowing of arteries, where the amount of blood flowing is not sufficient to supply oxygen rich blood to the heart, especially during the times of physical or emotional stress. There are some physical symptoms like chest pain, pressure or discomfort, which is called as Angina. Cardiac ischemia, which occurs for a long period, can be a sign of a heart attack. Figure 1.3.a.

Figure 1.3.a. The chain of process which is leading to ischemia and finally to death (1aim.com)

1.4. Myocardial infraction

Myocardial infarction is commonly known as heart attack, which arises due to the atherosclerotic plaque. In other words, it is the rapid development of myocardial necrosis caused by a critical imbalance between oxygen supply and demand of the myocardium (Nyboe et al., 1989). This usually originates from plaque rupture with accumulation of thrombus in a coronary vessel. This will result in an acute reduction of blood supply to a portion of the myocardium. The fracturing of basement membrane results in the platelet aggregation, thrombus formation, fibrin accumulation, hemorrhage into the plaque, and varying degrees of vasospasm ends up in partial or complete occlusion of the vessel and ensuing myocardial ischemia (Thygesen et al., 2007). Total occlusion of the vessel for more than 4-6 hours results in irreversible myocardial necrosis, but reperfusion within this period can restore the myocardium and reduce morbidity and mortality (Lee et al., 2006).

1.5. Pre and Post-Conditioning

The outcome of studies on reperfusion over the years has shown the importance of implementing pharmacological and mechanical interventions at the time of reperfusion. Murry et al., 1986 were the first, who shown the significant reduction in infarct size when they introduced the concept of ischemic preconditioning (IPC). Prior to this discovery, pharmacological strategies were yielding inconsistent results and it was unknown whether anything could be done to protect the ischemic heart from cell death. This powerful endogenous form of cardio protection admits various episodes of ischemia which will results in the protection of myocardium from the ischemia. This concludes in the arrangement of cardio protective properties like reduction in infarct size, preservation of

vascular endothelial function, decrease in polymorpho nuclear neutrophil (PMN) accumulation, and reduction in the appearance of apoptosis (Han 2001). The possibility of the cardio protection of this strategy has been difficult to relay into clinical practice, because of the inability to predict the onset of ischemia. Recent studies were shown that reperfusion injury can be stopped or reduced by adapting the conditions of reperfusion (Judy et al., 1998).

There are many studies focused on preconditioning which stated a cardio protective effect of pharmacological agents. In most of these studies the pharmacological agents are giving before the onset of ischemia. But, effectively this procedure is not worth; because it is not possible to detect that somebody is going to have a heart attack. Ischemic preconditioning is an experimental technique for making the resistance to the blood supply and thereby oxygen to the tissues (Marmor et al., 2004).Previous studies proved that the selective pharmacological antagonists like nor-binaltorphimine di-hydrochloride and GNTI di-hydrochloride of the k-opioid receptors (Kappa opioid receptors) are blocking the ischemic preconditioning and the agonists of the same receptors mimic the ischemic preconditioning (Gross GJ. 2003).

There are brief episodes of myocardial ischemia will take place during reperfusion after an extended ischemic insult. This may attenuate the total ischemic reperfusion injury. This phenomenon has been termed as ischemic post conditioning (Michael et al., 2004). In other words, post conditioning is the phenomena in which the reperfusion is interrupted with a vast coronary occlusion and reperfusion sequences. It is another form of cardio protection which is similar to the pre conditioning (Zaho et al., 2003). The effectiveness of post conditioning has been suggested to be as beneficial as preconditioning in limiting infarct size and preserving post ischemic endothelial function. Besides, ischemic post conditioning has a strong anti arrhythmic effect against persistent reperfusion induced tachycardia. (Michael et al., 2004)

Ischemic post conditioning consists of repeated brief cycles of ischemia-reperfusion. This is performed immediately after reperfusion followed by a prolonged ischemic insult, which

dramatically reduces infarct size in experimental models. Research from the laboratories of Vinten-Johansen in 1993 have also suggested that, the cardio- protection offered by post conditioning may be related to the inhibition of superoxide anion generation and oxidant-mediated cellular membrane damage. Recent inventions in the pharmacological sector showed that the post conditioning effect can reduce the myocardial infarct size (Staat et al., 1998).

Previous studies have also observed the significance of relevance of reactive oxygen species (ROS) in consider with post conditioning. (Yasuo et al., 2007). These oxygen-derived free radicals generate the release of pro-inflammatory mediators, transcription factors and stimulate the surface expression of adhesion molecules on the coronary vascular endothelium. The reduction of oxygen radicals after post conditioning showed that it may decrease the formation of reactive oxygen species (ROS) (Bohuslav et al., 1999). It is also assumed that, post conditioning may affect the release of autacoids (e.g. adenosine and nitric oxide), that may sequentially produce the generation of ROS from neutrophils and other cell sources. This is regarded as an important component of the cardio protection offered by post conditioning (Rada et al., 2008).

1.6. Mechanisms of protection with pre- post-conditioning

Research conducted by Downey and colleagues (1991) were showed the additive effects of preconditioning and post conditioning on the same rabbit heart. Ischemic pre conditioning is one of the most powerful methods to protect myocardium from the ischemic injury. Before the onset of ischemia, there was a protocol of short episodes of myocardial ischemia and reperfusion which will protect the myocardium from ischemia and reperfusion (Schluz et al., 2007).Reperfusion is essential and an absolute requirement for endurance of the ischemic myocardium. This reperfusion

process is able to make some myocardial injuries. The restoration of the circulation causes the oxidative damage. When a tissue is subjected to ischemia, a sequence of chemical events is initiated, that may ultimately lead to cellular dysfunction and necrosis (Clarke et al., 2005). The events of injuries will again take place even after the ischemia which is ended by the reperfusion. Thus, the brief decrease or interlude of the blood flow is the result of both ischemic and the reperfusion injury. For shorter durations of ischemia, the indirect or reperfusion mediated damage becomes more important. For example, it has been shown that the intestinal injury induced by 3 hours of ischemia (flow reduced to 20% of normal) and one hour of reperfusion is several times greater than that observed after 4 hours of ischemia alone (Parks and Granger, 1986).This shows that reperfusion plays an important role in ischemia/reperfusion injury.

1.7. Cell Death

Cell death is still a controversial process as it is the triggers that happen during the time of ischemia or reperfusion. In order to attain further understanding of reperfusion injury and cardio protective strategies, it is also important to gain depth knowledge of cardiac myocyte death. Apoptosis and Necrosis are the two different forms of cell death. In necrosis, cell death happens by the bursting of cells, and in apoptosis, by the bordering of cells (Freeman et al., 2000).

1.8. Apoptosis and necrosis

Apoptosis is also defined as programmed cell death. A diverse range of cell signals which may arise either from extrinsic inducers or intrinsic inducers are the controlling factors of the process of

apoptosis (Andrew 2007). Bcl_2 proteins are situated in the outer membrane of mitochondria. This will involve a major role in defending the cell death. Due to the decrease in intracellular energy and the ATP depletion, there will be an absence of apoptosis occurs during ischemia. Therefore, the reoxygenation and restoration of glucose supply during reperfusion generates the ATP required for the completion of the apoptosis (Popov et al., 2002). Apoptosis and necrosis takes place during the early stages of reperfusion (Leist et al., 1997). It is now widely accepted that both necrosis and apoptosis are responsible for myocyte cell death during myocardial ischemia and reperfusion. Necrosis is characterized as a passive response, which occurs due to the external factors such as infections and toxin. It involves swelling of the cell and its organelles, disruption of mitochondria, membrane rupture and cell lysis. It is a destructive and unregulated process and can subsequently cause the death of surrounding cells through the release of its cellular content into the environment. In contrast, apoptosis does not cause much damage to surrounding tissue as the cell neatly commits suicide in what is often referred to as “programmed cell death”.(Dejean et al., 2006)

Some studies have confirmed that ischemia itself can trigger apoptosis. (Everett et al., 1999).Moreover, some studies reveal that, apoptosis has to be co-ordinated by the releasing of calcium from the endoplasmic reticulum which synchronizes the mass exodus of cytochrome c from mitochondria (Mark et al., 2003).

The absence of apoptosis during a short period of ischemia is due to the depletion of ATP, which is needed for the generation of pro-apoptotic proteins. Therefore, re- oxygenation and restoration of glucose supply during reperfusion generates the ATP required for the completion of apoptosis (Santos et al., 2000). Additional data suggests, apoptosis and necrosis occur simultaneously during the early-phase of reperfusion followed by a slower appearance of apoptosis during the later phase of reperfusion. However, many controversial theories have arisen with regards to the initiation of apoptosis. Several studies pointed out that, the release or activation of some bioactive substances is involved in the triggers in the development of apoptosis. Moreover, some studies proved that, there

is a significant co-relation between these triggers and neutrophills on ischemia and reperfusion. And this will clearly states the neutrophill activation in the triggering apoptosis (Dejean et al., 2006).

1.9. Caspase 3- Role in the execution phase of cell apoptosis

Caspases, or cysteine-aspartic acid proteases, are the family of cysteine proteases, which plays a vital role in apoptosis (programmed cell death). Caspases have variety of roles in apoptosis and in the development and most other stages of adult life. So, it is known as the executioner proteins. Failure of apoptosis is one of the main contributions to tumour development and autoimmune diseases (Harrington et al., 2008).

Mainly caspase proteins are of two types. One is initiator caspases and the other is effector. Initiator caspases (e.g. CASP2, CASP8, CASP9 and CASP10) cleave inactive pro-forms of effector caspases. Effector caspases (e.g. CASP3, CASP6, and CASP7) in turn cleave other protein substrates within the cell, to trigger the apoptotic process (Gregersen et al., 2007).

Apoptosis is a selective process for deletion of cells in various biological systems. In the pathway of apoptosis there are various groups of molecules are there. One set of mediators implicated in apoptosis are belong to the aspirate-specific cysteinyl proteases. Caspase-3 (CPP32, apopain, YAMA) has been identified as a key mediator of apoptosis of mammalian cell (Yuan et al., 2004). Kothakota *et al,* (1997) monitored the paraphrase products of a murine protein library to find the substrates that are susceptible to cleavage by caspase-3.

1.10. Apoptotic cell signalling pathway

Extrinsic and Intrinsic are the two apoptotic cell signalling pathways. Extrinsic pathways are also called as death receptor pathways. Intrinsic pathways are mediated by mitochondria, which releases intermembrane proteins such as cytochrome c, Smac/DIABLO, Endonuclease G, Omi/HtrA2 and Apoptosis Inducing Factor (AIF) (Degterev et al., 2008)

The death receptor pathway (extrinsic) involves the activation of death receptor located on the extracellular surface of the cell by apoptotic stimuli. The death receptors are closely related to the tumour necrosis factor gene super family and play divergent regulatory roles apart from regulating apoptosis (Degterev et al., 2008). Activation of the death receptor will results in the initiation of the apoptosis processes this will lead to the receptor association with the adaptor protein Fas associated death domain (FADD). FADD has a death effector domain (DED) that associates with pro-capsize 8. The formation of the FADD-pro-capsize 8 complex results in the immediate cleavage of pro-capsize 8 to active caspase-8. Activated caspase-8 can bind to pro-caspase-3 resulting in activation of caspase-3 and execution of cellular apoptosis. Caspase-3 results in the cleavage of various death substrates and leading to a range of biochemical and morphological changes characters of apoptosis (Taylor et al., 2008).Figure 1.9.a.

Figure 1.10.a. Proposed mechanism of apoptosis regulation (Gill et al., 2002).

Cell signalling cascades are the primary factor in the apoptotic pathways. Other key regulators of apoptosis, which may be used in apoptotic suppressive strategies are stress proteins, growth factors, calcium, and oxidants The latest identification of mitochondrial factors (AIF, Endo G and Omi/HtrA2) involved in apoptosis has greater importance on unscrambling other mediators of the apoptotic program (Salvesen et al.,2008). Almost all of the caspase activations are occurring in mitochondria. The apoptotic death pathway is initiated when the mitochondrial membrane ruptures leading to the translocation of cytochrome c and other pro-apoptotic factors into the cytosol.(See Figure 1.9.a) Interventions aimed at protecting mitochondrial membrane integrity would prevent the occurrence of downstream morphological changes (Meulmeester et al., 2008).

The following diagram illustrates the mitochondrial death pathway and the proposed pro-death factors that are released from the mitochondrial intermembrane space:

Figure 1.10.b. Schematic representation of mitochondrial death decision pores (Belizario et al., 2007)

PT Pores are the large non selective pores which help in the realising of cytochrome c and other apoptogenic factors. Proteins of the BCL 2 families may act together with major mitochondrial voltage depended anion channel known as VDAC, adenylate nucleotide translocase (ANT) and cyclophilin D (Cyclo D). There is also evidence that, the release of apoptogenic factors could occur through a putative channel named as mitochondrial apoptosis-inducing channel or MAC (8) and through a large channel containing ceramide lipids, i.e., the ceramide channel.

1.11. Mitochondria and ischemic –reperfusion injury

Mitochondria have a significant role in ischemic-reperfusion injury. Cytochrome c is the heme protein in mitochondria, which has been implicated in many studies as an initiator of apoptosis in

the ischemic reperfused heart (Honda et al., 2005). Cytochrome c will disassociate from mitochondria in response to cellular stresses like ROS and calcium overload. The liberation of the cytochrome c in to cytoplasm in response to the cell death signals will results in the activation of apoptosis. This will occurred by the binding of cytochrome c to Apaf-1 (apoptotic protease activating factor 1), which leads to the pattern of apoptosome formation. These apoptosomes will binds to ATP and caspase 9 (Lundberg and Szweda. 2004). Caspase 3 can be activated by the caspase activation complex which is formatted from these apoptosmes.

In the transduction of intrinsic apoptotic signal, the Bcl-2 family of proteins plays an important role. This is mainly by incorporating the anti apoptotic proteins (Bcl-2 and Bcl-x_L) and pro-apoptotic proteins (Bax, Bak, Bid, Bad, Bnip3/Nix and others). A major role in regulating apoptotic cell death is played by Bcl-2 and Bax proteins. Regulators of apoptosis at the level of caspases have also been involved such as cFLIP and the inhibitor of apoptosis (IAP) family, which counteract the effect of caspase inhibitors. Decrease in ATP level and increase in the intracellular $ca^{2}+$ level are main factors in the derangement of mitochondrial function and these alterations will finally cause the viability of ischemic myocardium (Fabio et al., 2003).

1.12. Beta Agonists

Beta agonists are one of the most powerful medications for the bronco related diseases which mainly affects on the muscles around the airways. Some studies conducted in the cornel and Stanford University's showed that, common beta agonist inhalers causes' double death rate in COPD patients. There are mainly two types of Beta agonists. One is short acting and other is long acting. Beta-agonists can be given in several ways but the most common way is by inhalation. Pills, tablets and intravenous forms of the drugs are used but have more side-effects. Some studies showed that the effect of beta agonist that stimulates the cardiac contractility and enhance the cardiac dysfunction following myocardial ischemia (Yoo et al., 2009)

The rapidity in working of short-acting beta-agonists is best when it is compared to the long-acting one. Short-acting beta-agonists are the effective medications which gives relief very faster for the breathlessness. This breathlessness is often happens due to the frequent showering, exercise, or going out in the cold air. The combination of β_1, β_2 agonists are therapeutically effective in post myocardial infarction (Chavasse et al., 2002).

Unevenness and cramps in the hands are some side effects occurring due to the medication. Also, fast heart rate and shakiness causes anxiety (nervousness) and worsens breathlessness. Some of these types of illness may occur for a short period of time and after a few days of time it will depart by its habitual usage (Spitzer et al., 1992). Occasionally these side-effects may lasts for some period of time and have the tendency to continue. Then it should be the time to change the drug. This effects are frequently happened due to the over usage of medications which coats the mouth and it will get absorbed rather than inhaled (Ramanujan et al., 2006). The representation of the side-effects is calculated in the frequency in usage of the medications. For instance, various short-acting beta-agonists should not be taken more than every 4 hours, except or else instructed by the provider.

Types of Adrenoceptors

β_1 and β_2 adrenoceptors are the two types of adrenoceptors found in the heart. Among these the major receptor type in number and function is β_1 (Dutta et al., 2002). Sympathetic adrenergic nerves are the source of the production of nor-epinephrine in which the β_1 receptors are binded very firmly. Beta$_2$ agonists must always be used with caution in patients with cardiopathies, since it may precipitate the concomitant cardiac disease (Cazzola et al., 2005). Asthma or chronic obstructive pulmonary disease (COPD) may occur, when the muscles around the airways may tighten. Bronchodilator medicines will relax those muscles by opening the airways. Beta-agonists can be administered by inhalers or orally. They activate the beta$_2$ receptor on the muscles surrounding the airways. As a result the muscles around the airways relax and may lead to the opening of the airways Dilating airways helps to relieve the symptoms of dyspnoea (shortness of breath).It has

been shown that the action of β_2 agonists to reduce the dyspnoea in many asthma and COPD patients. The action of beta$_2$ agonists start within minutes after inhalation and lasts for about 4 hours. The commencement of action of these β_2 agonists is very fast and that will help the patients who suffer from shortness of breath symptoms. The duration of action of these are short (Haney and Hancox 2007).

1.13. G-Protein coupled receptors

The pharmaceutical research in the modern world has focused on a various types of protein families. The G-protein coupled receptors (GPCR) are one of the most assorted forms of proteins in nature. These are in the variance of the biological and pathological ways like development and proliferation, neuromodulation, angiogenesis, metabolic disorders and viral infections (Wettschureck et al., 2005).

G-protein coupled receptors have a critical role as indicators of disease with indicative and analytical potential. GPCRs are the principal signal transducers for the sense of sight and smell (Ulrik et al., 1998). G-Proteins are the basic source in the activation of adenyl cyclase. These adenyl cyclases are meant to be the factor for the formation of cAMP from ATP. cAMP is the major factor of the increased amount of calcium entry in the cells. These are mainly by the mechanism of the phosphorylation of L-type calcium channels (See Figure 1.12.a.). As a result, this will increase in the calcium entry during the action potentials .Also, this may leads to the increase in the contractility (King et al., 2003).

Figure 1.13.a. shows the ligand binding to a GPCR's extracellular region triggers changes in protein's trans-membrane region. As a result, the release of guanosine diphosphate (GDP) and the uptake of guanosine triphosphate from the G-protein occur. This will stimulate the activation of predefined signalling pathways (Filmore et al., 2004).

Moreover, the activation of these Gs-proteins may leads to the increase in the heart rate, which is by the opening of the ion channels which is mainly responsible for the pacemaker current in the sinoatrial node. The phosphorylation of protein kinase-A in the sarcoplasmic reticulum causes the enhancement of the calcium release through the ryanodine receptors. As a result, supplementary doses of calcium will be provided for the binding of the troponin-C.Due to this effect, there will be an increase in the inotrpy. At last, the myosin light chains are also be phosphorilated by the PK-A, which can also put in to the positive inotropic effect of beta adrenoreceptor stimulation. In summary, the cardiac effects of a β-agonist are increased heart rate, contractility, conduction velocity, and relaxation rate. Some studies proved that, the inhibition of GRKs to regulate GPCRs are useful in the treatment of heart failure and hypertension (Brinks et al., 2010).

1.14. Salbutamol

Salbutamol is one of the most common and very familiar drugs used in the treatment of asthma and chronic obstructive pulmonary disease. Asthma is a chronic disease affects on the lungs mainly by the inflammation of the lower airways. The airways become sensitive, swollen and inflamed while asthma occurs. Salbutamol reduces asthma symptoms by producing the relaxation of airway muscles and thus making the breathing easier. In a recent study, the large dose of salbutamol increases coronary flow. However, it may have bad effects on patients with coronary artery disease (Kochiadakis et al., 2007).

Various forms

Nebulizers: These forms deliver the drug straight to the lungs over a long period of time. The inhaled forms of salbutamol have a faster onset of action, fewer side effects, and are more effective than the syrup or tablet forms. Studies has proved that inhaled or nebulised salbutamol has no effect on myocardial ischemia, arrhythmia, and changes in heart rate in patients with CAD and COPD (Rossinen et.al., 1998).

Solution (Injections + syrups): In the case of severe asthma, salbutamol can be injected intravenenously, subcutaneously or intramuscularly

Side effects

The research studies have proved that the usage of salbutamol in hypoxic patients will cause a sudden death (Burggraaf et al., 2001).Most of the side effects include the following:

Aggression, agitation, cough, diarrhoea, dizziness, excitement, general bodily discomfort, headache, heartburn, increased appetite, increased blood pressure, indigestion, irritability, laboured breathing, light-headedness, muscle cramps, nausea, nervousness, nightmares, nosebleed, over activity, palpitations, rapid heartbeat, rash, ringing in the ears, shakiness, sleeplessness, stomach ache, stuffy nose, throat irritation, tooth discoloration, tremors, vomiting, wheezing, worsening bronchospasm (Rossi 2004).

1.15. Pharmacology of Salbutamol

Salbutamol is a selective β_2 adrenoceptor agonist. Salbutamol will directly precede on the $beta_2$ adrenoreceptors of the pulmonary bronchial muscles. The bronchodialation happens due to the action of salbutamol. Salbutamol stimulates the production of cyclic AMP. This will increases the binding of intracellular calcium to the cell membrane and endoplasmic reticulum which leads to the bronchodialation. The opening of ATPase channels will coerce the potassium from the extra cellular to the intra cellular space. As a result, there will be a decrease in the extra cellular hyperkalemia following by the increase in the intracellular potassium (Assoufi et al., 1989).

The absorption of the salbutamol will takes place in the gastrointestinal tract and the metabolism is in the liver. Half of the excretion will occur in the urine and about 30% is excreted as unchanged salbutamol. Generally, the action of the bronchodilator will starts within minimum of 3-5 minutes and at 15-20 minutes of the maximum (Barnes et al., 1983).

The possible effect of Salbutamol in Ischemic Heart

White and his colleges in 2008 have studied the effect of salbutamol in pig's heart. They concluded that, salbutamol is unlikely to increase the severity of cardiovascular diseases (White et al., 2008). Others have shown that there are some rare occurrences of myocardial ischemia associated with salbutamol (Bennett et al., 1994).

However, the pharmaceutical research has given the clear evidences which are more reliable in the study of effect of salbutamol in cardiovascular disease. There are some relevant factors which showed that salbutamol has an effect of increasing heart rate in cardiac patients. Experiments done in 9 patients with the intravenous infusion of salbutamol after cardiac surgical operations resulted with a greater increase in the heart rate, maximum acceleration of aortic blood flow, and maximum rate of change of left ventricular power (Leitch et al., 1976). But the mean falls in left atrial pressure and systemic vascular resistance were similar. These results showed that the variation would include more myocardial oxygen consumption with salbutamol. So, the combination of the salbutamol has to be controlled very hardly to demonstrate the presence of functional cardiac beta $_2$-adrenoceptors. Some studies showed that the salbutamol is a useful drug in heart failure (Bourdillon et al., 1980).

1.15. Aim and Objective

The aim of this study was to investigate the effect of beta agonist salbutamol in the heart following ischemic-reperfusion injury. The aim was to determine whether salbutamol increase the apoptosis, necrosis in cardiomyocytes subjected to Hypoxia/Reoxygenation injury. The objective of this study was to determine whether the administration of salbutamol in heart during ischemic reperfusion injury can cause an increase in infract size and whether it elicit the necrotic/apoptotic properties in isolated myocytes following Hypoxia/Reoxygenation injury. Moreover this study aims to find out the effect of beta-2 agonist (salbutamol) in the presence of inhibitors when administered throughout the reperfusion injury

2. METHODS

2.1 Langendorff heart perfusion studies

2.1.1 Introduction to Langendorff heart perfusion studies

Langendroff preparation is the traditional method in the perfusion of a heart. The normal functioning of the heart has to be maintained while the perfusion through the aorta and circulates through the coronary artery.

2.1.2. Animal use - Langendorff

The Male Sprague-Dawley rats (250 g $\pm$ 50 g body weight) were used in the experiments given by Coventry university animal house (Coventry, U.K.).These animals received human care according to the guidance on the operation of animals (Scientific procedures act 1986). The investigation conforms to the Guide for the *Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.1.3. Krebs Henseleit buffer preparation, heart extraction and isolated heart perfusion

The Krebs Henseleit buffer (in mM: NaCl 118.5, $NaHCO_3$ 25.0, Kcl 4.8, $MgSO_4$ 1.2, KH_2PO_4, 1.2, $CaCl_2$ 1.7, and glucose 12) was prepared freshly each day prior to each experiment, dissolved and gassed with 95% O_2 / 5% CO_2, at a pH of 7.4-7.5 at 37 °C.

Heart Preparation

Animals were sacrificed by cervical dislocation. The hearts were excised, placed in ice-cold buffer, and within 1min mounted in a constant-pressure Langendorff system (80mmHg). They were perfused retrogradely with a modifiedKrebs-Henseleit (KH) bicarbonate buffer containing (in mM) 118.5 NaCl, 25 NaHCO3, 4.8 KCl, 1.2 MgSO4, 1.2 KH2PO4, 1.7 CaCl2, and 12 glucose. All

solutions were gassed with 95%O2-5% CO2 (pH 7.4) and maintained at 37°C. The temperature was permanently monitored by a thermocouple inserted in the right ventricle. A latex isovolumic balloon was introduced in the left ventricle through an insertion performed in the left atrial appendage and inflated up to 5–10 mmHg.Functional monitoring was performed via a pressure transducer connected to a Lab System chart recorder. Left ventricular developed pressure, heart rate, and coronary flow were registered at regular intervals. To induce ischemia, a surgical needle was inserted under the left main coronary artery and the ends of the thread were passed through a small plastic tube to form a snare. Tightening the snare induced regional ischemia and releasing the ends of the thread allowed reperfusion to commence. At the end of reperfusion, the snare was tightened to re-occlude the coronary artery branch. A saline solution of 0.12% Evans blue was infused slowly via the aorta to delineate the non ischemic zone of the myocardium, which stained dark blue. The hearts were frozen, sliced into 1-mm-thick transverse sections, and incubate in triphenyl-tetrazolium chloride solution (1% in phosphate buffer, pH 7.4) at 37°C for 10–12 min. They were then fixed in 10% formalin for at least 4 h. Viable tissue stained red and infarcted tissue appeared pale. The risk zone and infarct areas were traced onto acetate sheets. With the use of computerized planimetry (Summa Sketch II, Summagraphics), the percentage of infarcted tissue within the volume of the myocardium at risk was calculated (I/R%).

2.1.4. Measurement of individual parameters, LVDP, HR and CF

When the coronary perfusion was performed in the heart was working properly, a latex, fluid filled is volumetric balloon was inserted in the left ventricle through the left atrial appendage and inflated to give a preload of 8 to 10 mmH g. The balloon was connected with a pressure transducer through which the LVDP and HR was monitored by the help of power lab system (ADI instruments, Mountain View, CA). It should be maintained and make sure that the pressure transducer system was calibrated every time before the experiment.

2.1.5. Treatment protocol

The treatment protocol is shown in Figure below. All hearts were allowed to stabilise for 20 minutes prior to the induction of regional ischemia for 35 minutes, followed by 120 minutes of reperfusion. Drug administration was introduced at the onset of reperfusion and throughout the reperfusion phase.

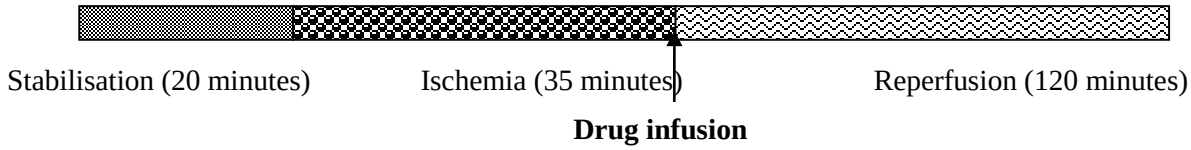


Figure 2.1.5.a. Representation of experimental protocol for infarct studies

Experimental groups

The hearts were allocated to one of the following 10 treatment groups:

Group 1: Control hearts; no drug was added at reperfusion.

Group 2, 3, 4, and 5: Drugs were added at reperfusion for 120 minutes (salbutamol 1 nm, 10nm, 100nm, ICI with salbutamol, CGP with salbutamol, ICI alone, CGP alone)

2.1.6. Infarct size analysis

After finishing the reperfusion, the surgical thread was pulled tightly and tied for the re-occlusion in the left coronary artey. Then, the saline solution of Evans blue was slowly perfused through the valve of the cannula in to the aorta. This saline solution stains in blue colour. After that, the hearts were frozen for 1-4 hrs at -20. Hearts were sliced in to 2-3 sections, and placed in freshly prepared pre heated triphenyl- tetra zolium chloride (TTC) solution for the incubation for 15-20 minutes. The

heart slices were then put in 10% formaldehyde solution for 24hrs before the observation. This step increased the contrast between the infarct and risk areas. Within each sliced heart section, the pink/red areas were identified as risk tissue and the pale white areas were identified as infarct tissue.

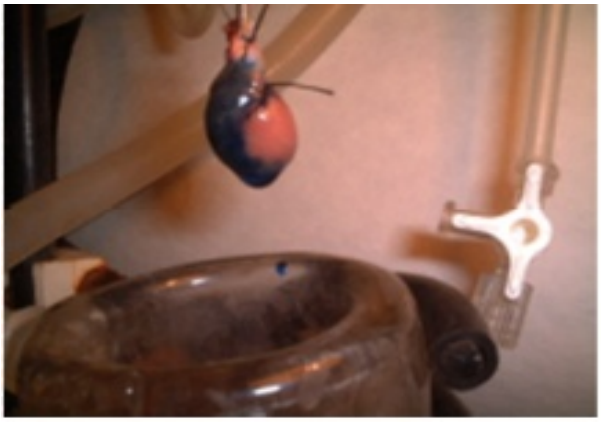


Figure 2.1.6.a. Photograph of an isolated rat heart perfused with Evans blue

2.1.7. Infarct and risk area measurements

The area measurement of the infarct and risk zone is a very wide procedure. The slices were placed between two glass plates and pressed. It was then very carefully secured with clips. An acetate sheet placed on the top of the plate in order to mark the risk and infarct areas according to the slice colours. Each of these marks were analysed by the help of computerised planimettry. (Image Tool version 3.1, Rockford, USA)

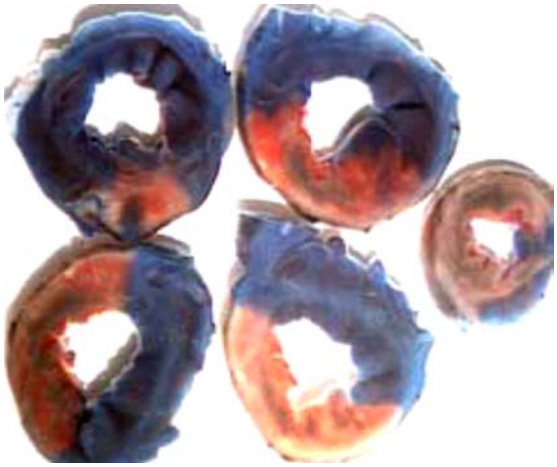


Figure 2.1.7.a. Representative photograph of an isolated rat heart slices perfused with Evans blue and TTC stained. The viable tissue is stained blue, risk tissue stained in pink and infarct tissue in pale/white.

2.2 Myocyte Isolation

2.2.1. Introduction to Myocyte Isolation

The perfusion of the rat heart was performed in a modified langendroff system. In myocyte isolation, the rat heart was cannulated in a retrospective way with three different buffers.

2.2.2. Preparation of buffers and reagents

Krebs-Henseleit buffer was prepared with the following components (in mM); NaCl 116, KCl 5.4, $MgSO_4.7.H_20$ 0.4, Glucose 10, Taurine 20, Pyruvate 5, $Na_2HPO_4.12H_20$ 0.9 dissolved in HPLC grade H_20. The solution was stirred and oxygenated with 95% O_2/5% CO_2 for 30 minutes at room temperature. After 30 minutes, $NaHCO_3$ (25mM) was added. The solution was incubated in a 37^0C water bath. Once the desired temperature of 37^0C was reached (~10 minutes), the pH was adjusted to 7.4 with NaOH. The remaining buffers were freshly prepared. Krebs Buffer with addition of $CaCl_2$ (0.75mM), Collagenase (Krebs buffer containing 0.5% bovine serum albumin (Roche Biochemicals) and 0.075% Collagenase (Type II) (Worthington Biochemicals)), and

Restoration Buffer (Krebs buffer containing 1% bovine serum albumin, 5mM Creatine, 2mM Carnitine, 50µM $CaCl_2$ and 1% Penicillin-Streptomycin).

2.2.3. Isolation of adult rat cardiomyocytes

The hearts were quickly excised, mounted on a Langendorff apparatus, and perfused with modified ADS control buffer containing(in mM) 137 NaCl, 3.8 KCl, 0.49 MgCl2, 4 HEPES, 10glucose, and 10 2,3-butanedione monoxime (pH 7.4). The perfusate was bubbled with 95% O2-5% CO2 and maintained at 37°C. After 5 min, the hearts were switched to a modified tyrode solution containing 1.0 mg/ml collagenase (Worthington type II) and 50 _M calcium for 10–15 min. They were then perfused for 5 min with ADS buffer containing 50 M calcium alone. The hearts were removed from the perfusion apparatus, and the atria were trimmed away. The ventricles were minced and incubated in a shaking bath for 5 min in collagenase containing solution. Cells were then filtered through nylon mesh and washed with restoration buffer [containing (in mM) 137 NaCl, 3.8 KCl, 0.49 MgCl2, 4 HEPES, 10 glucose, 102,3-butanedione monoxime, 2 carnitine, 5 creatine, 5 taurine,and 5 Na-pyruvate (pH 7.4)]. The calcium concentration was gradually brought back to 1.25 mM.

2.2.4. Hypoxia / Reoxygenation

The cardiac myocytes were exposed to lethal simulated ischemia as follows: the normal restoration medium was replaced with 2 ml Esumi ischemic buffer(5) [containing (in mM) 137 NaCl, 12 KCl, 0.49 MgCl2, 0.9CaCl2 _H2O, 4 HEPES, 20 Na-lactate, 10 deoxyglucose, and10 KCN (pH 6.5)], and the cells were incubated at 37°C for 45min in the hypoxic chamber in an atmosphere of 0% O2-5%CO2 balanced with argon (BOC gases). For control conditions with or without drug treatments, myocytes were cultured with 2 ml modified Esumi control buffer for 2.75 h at 37°C in an atmosphere of 21% O2-5% CO2 balanced with N2 (normoxicenvironment). To investigate whether salbutamol protected adult cardiac myocytes from reoxygenation injury, the cells were incubated with different concentrations of salbutamol (1µM, 0.1µM, 10nM, 1nM, 0.1nM, 0.01nM) for 2 h at

the point of reoxygenation and in the presence and absence of the selective beta antagonists CGP 12177 and ICI-118551

2.2.5. Flow Cytometry Analysis

The fluorescence assimilated cell sorter (FACS, Becton Dickinson) was used for analysis of myocytes treated +/- drugs after hypoxia/reoxygenation and normoxic controls. Two different experimental models were used. 1) Vibrant ® Apoptosis Assay Kit #10. It was used for measurement of live, necrotic and apoptotic cell populations. 2) Intracellular staining for Caspase-3 using Alexa Fluor® 488 conjugate. It was used to detect cleaved Caspase-3 protein expression levels by Flow Cytometry Analysis

2.2.6. Measurement of Apoptosis and Necrosis

The cells were washed with 1 ml of cold PBS (pH 7.4; 140m M NaCl, 5m McCall, and 1.8m M CaCl2). They were then incubated for 10min in the dark at room temperature in annexin V-FITC solution (1:50 in annexin binding buffer). Propidium iodide(PI; 100 mg/ml) was added to the myocytes loaded with annexin V. Samples were analyzed immediately by flow cytometry using a Partec flow cytometer (Partec; Mu¨ nster,Germany) equipped with a 488-nm argon laser, with settings optimized for detection of fluoresce in and PI. Annexin V has been shown previously to detect the early stages of apoptosis by binding to the phosphatidyl serine (PS) residues, which are trans-located on the external face of the cell membrane. Translocation of PS occurs early in apoptosis while the cell membrane is still intact. Cellular necrosis was determined using PI. The assay is based on the vital binding of PI to the nuclei of cells whose plasma membranes have become permeable due to cell damage. Results are expressed as the percent annexin V-positive/PI-negative (early apoptotic) and annexin-positive/PI-positive (necrotic) total numbers of cells.

2.2.7. Cell Death

1 x Annexin V buffer was prepared from 5x stock by diluting 4 ml 5X Annexin V (50 mM HEPES, 700mM NaCl, 12.5 mM $CaCl_2$, pH 7.4) with 16 ml ddH_20. 50 µM C_{12} resazurin (component B) was prepared by adding l µl of 1 mM C_{12} resazurin stock into 19 µl ddH_20. 1 µM Sytox Green stain (component C) was prepared by adding 5 µl of 10 µM Sytox green stain into 45µl 1X Annexin V buffer.

The measurement of apoptotic myocytes were done by Annexin V allophycocyanin, which has a higher affinity for Sytox green dye. Apoptotic myocytes were measured using Annexin V allophycocyanin that has high affinity for phosphatidyl serine (PS).

The cell suspensions from the 24 well plates were transferred to the respective labelled eppendroffs. Then, these eppendroffs were undergone a centrifugation at 500 rpm for 5 minutes. The supernatant was removed and the 300µl of annexin V buffer was added. Then, these cells were meant to be centrifuged at 500 rpm for 5 minutes. The supernatant was removed and add 100µl of annexin V. The pellets were resuspended by the addition of 1 µl component B, 1 µl component C and 5µl component A. After that, these eppendroffs were wrapped with a foil and placed in room temperature for 15 minutes. Finally, 400 µl Annexin V buffer was added and analyse on the FACS Calibur flow cytometer.

2.2.8. Caspase-3 Intracellular Staining

Cells from the 24 well plates were transferred to the labelled eppendroffs.These eppendroffs were centrifuged at 1200 rpm for 2 minutes. After the centrifugation, the supernatant was removed and

cell pellets were washed with 2.5ml of phosphate buffered saline (PBS).Then, 3% formaldehyde was added and incubated at 37^0C for 10min. After that, they were chilled in ice for 1 min. Then, it was centrifuged at 1200 rpm for 2 minutes. The supernatant was removed and the cells were permiabilised with 90%ice cold methanol, and incubated for 30 minutes. The cells were centrifuged at 1200 rpm for 2 min. After removing the supernatant, the cells were washed twice in 200ml incubation buffer (0.5% bovine serum albumin in PBS). Cells were placed at room temperature in 1000µl incubation buffer for 10 min. After that, the cells were incubated for 1 hour at room temperature in 1:100 dilution of Caspase-3 cleaved. Cells were centrifuged at 1200 rpm for 2 min and washed with 2X in antibody dilution buffer. Then, the cell pellets were resuspended in 200µl of secondary antibody, (Alexa Fluor 488).The cells were centrifuged at 1200 rpm for 2 minutes and washed in antibody dilution buffer and resuspended in 500µl of PBS.

3. RESULTS

3.1 Hemodynamic data analysis

The haemodynamic data consists of the measurement of left ventricular developed pressure, heart rate and coronary flow. Assessment of Left ventricular pressure, Heart rate, Coronary flow in the presence of different concentrations of β_2 agonist Salbutamol has been identified that, there was no significant effect on the haemodynamics when the data's analysed statistically. Figure 3.1.a.b.c.

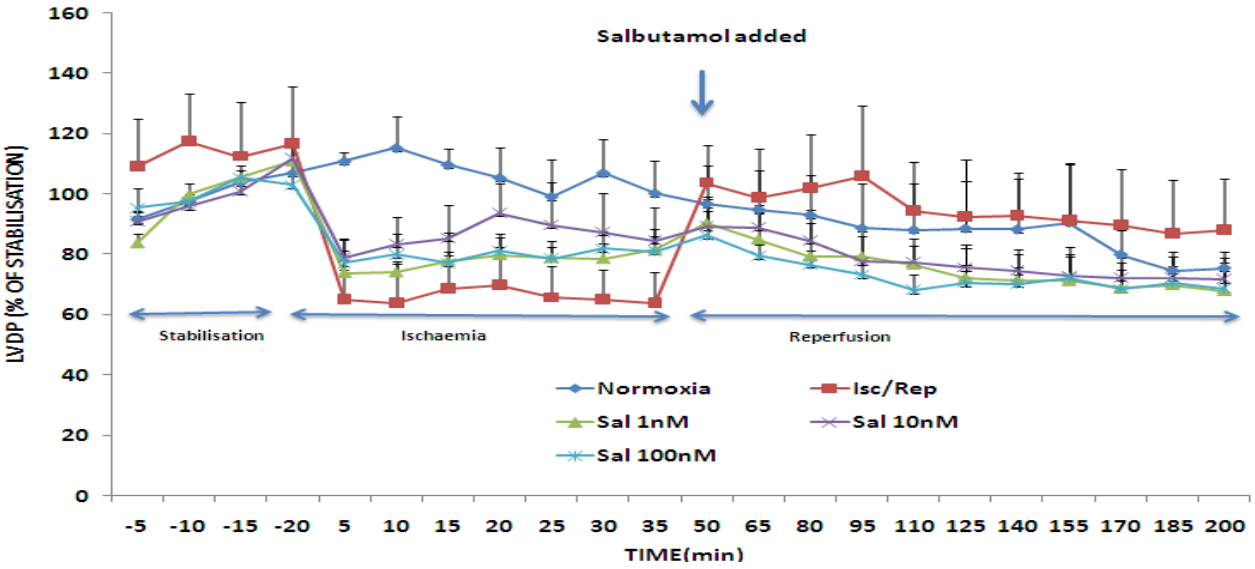
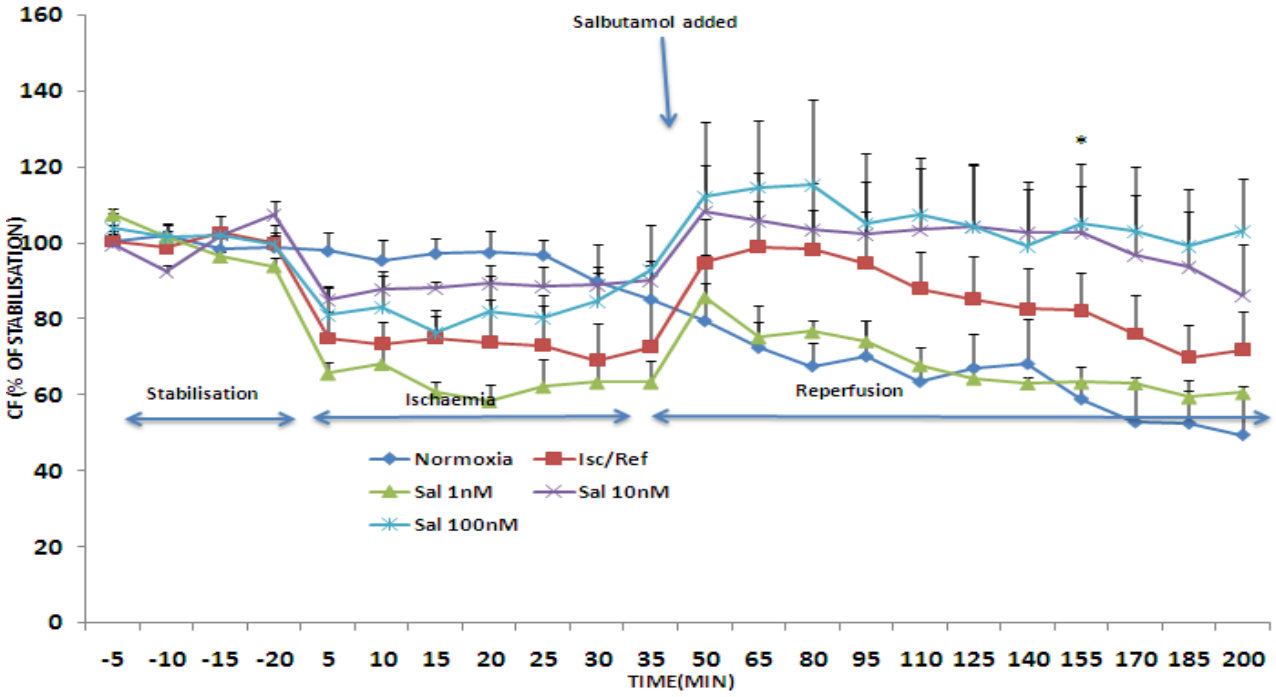
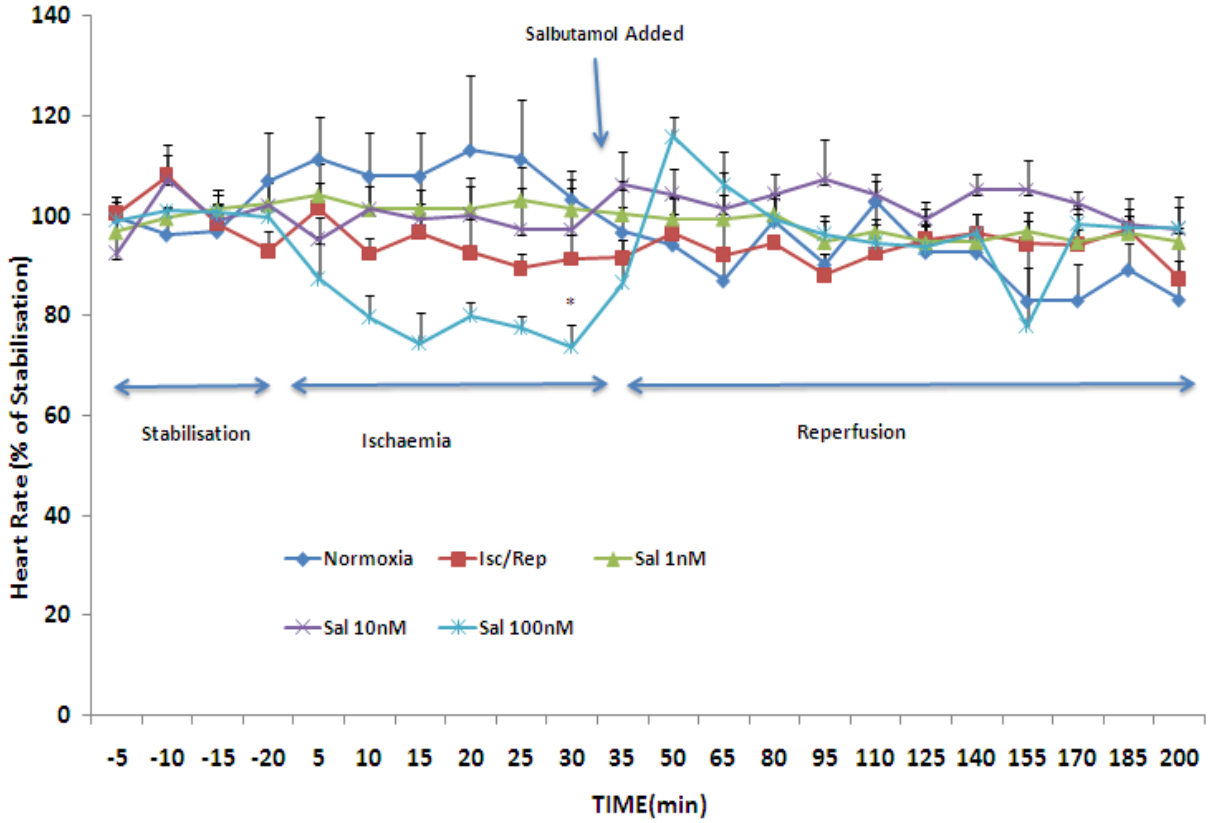


Figure 3.1.a. The chart shows the changes in left ventricular developed pressure in isolated rat heart subjected 35 minutes of ischemia and 165 minutes of reperfusion. Different concentrations of the β_2 agonist salbutamol were administered at reperfusion. Results are expressed as mean of the stabilisation period. MEAN + SEM. n=6



***p<0.05 Salbutamol 100nM vs. Ischaemia/Reperfusion.**

Figure 3.1.b.The chart shows the changes in Coronary flow in isolated rat hearts subjected to 35 minutes of ischaemia and 165 minutes of reperfusion. The β_2 agonist Salbutamol was administered at reperfusion. Results are expressed as the mean of stabilisation period MEAN+SEM. n=6.



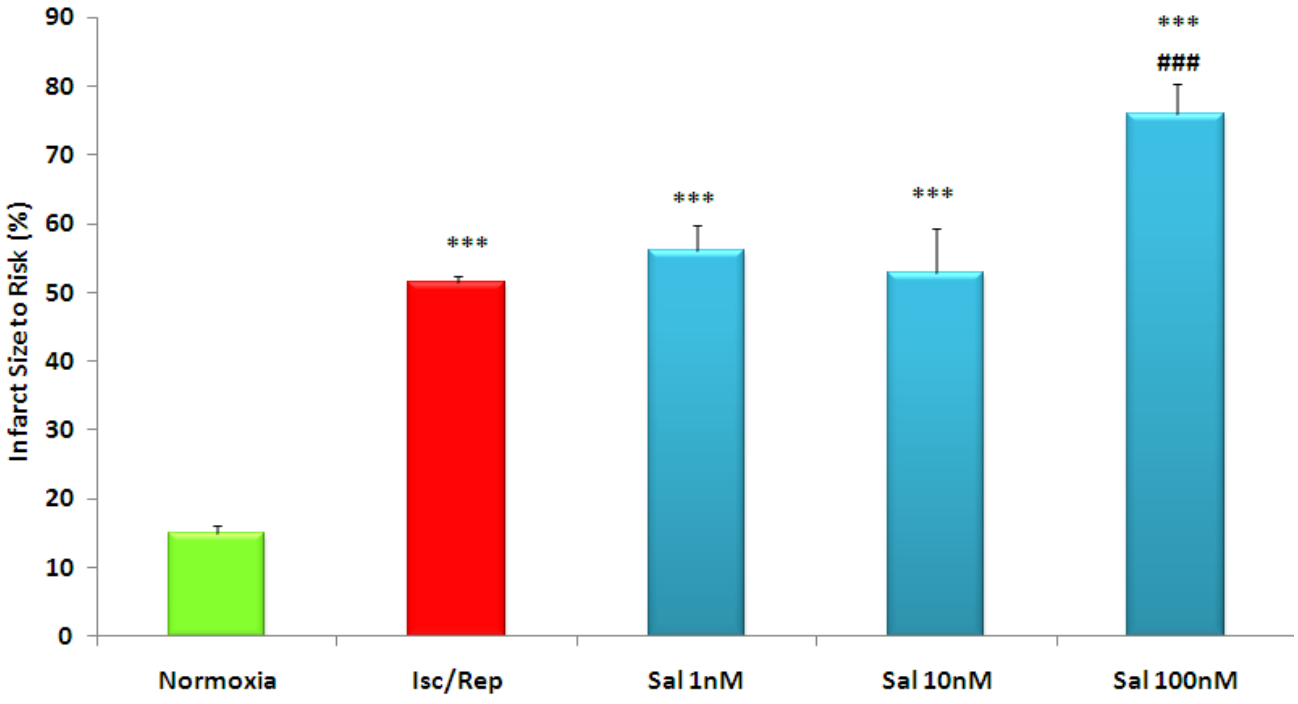
#p<0.05 Salbutamol 100nM vs. Ischaemia/Reperfusion

Figure 3.1.c. The chart shows the changes in heart rate in isolated rat hearts subjected to 35 minutes of ischaemia and 165 minutes of reperfusion. The β_2 agonist Salbutamol was administered at reperfusion. Results are expressed as mean of the stabilisation period MEAN+- SEM. n=6

3.2 Infarct Size to Risk Ratio Analysis

3.2.1. Effect of β_2 adrenoceptor agonist Salbutamol when administered at reperfusion in the rat myocardial model of ischaemia reperfusion injury.

The main focus of this study was to investigate whether the β_2 agonist Salbutamol has a toxic effect on the rat heart when administered throughout reperfusion. Three different concentrations (1nM, 10nM, 100nM) of Salbutamol were used in this experiment. Isolated perfused heart underwent 35 minutes of ischaemia and 165 minutes of reperfusion. β_2 agonist Salbutamol (1nM, 10nM, 100nM) was administered throughout the reperfusion period. The study showed that the administration of $\beta_2$agonist Salbutamol (100nM) throughout the reperfusion period significantly increases infarct size to risk ratio compared to the non-treated control groups. ($P<0.001$ vs. non treated controls, Fig 3.2.1).Administration of salbutamol (1nM or 10nM,) throughout the reperfusion had no significant effect on infarct size to risk ratio compared to non-treated control hearts ($P>0.05$ vs. non-treated controls, Fig 3.2.1.).



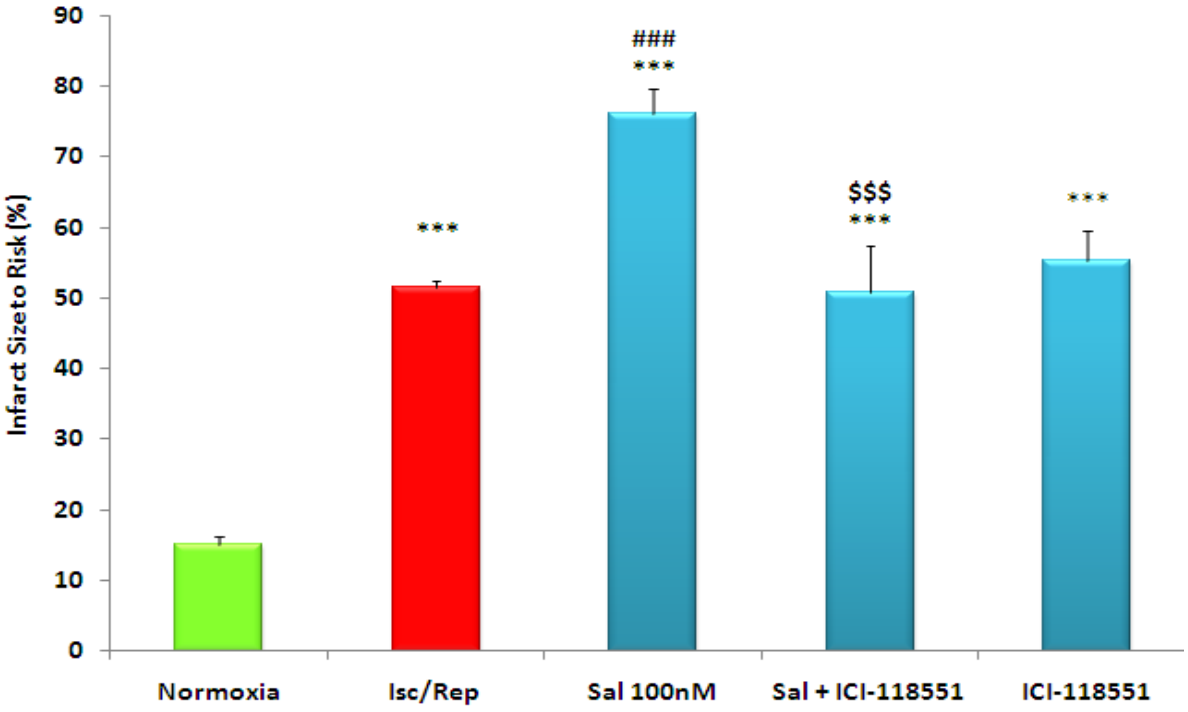
*****P<0.001 vs. Normoxia, ### P<0.001 vs. Isc/Rep**

Figure.3.2.1. Infarct size to risk ratio (%) in non-treated control and salbutamol treated ischaemic reperfused hearts. Isolated perfused hearts were subjected to 35 minutes of ischaemia and 165 minutes of reperfusion where the β_2 adrenoreceptor Salbutamol in different concentrations was administered throughout reperfusion. Results are shown as MEAN +/- SEM from 4-5 individual experiments.

3.2.2. Role of $\beta_2$agonist salbutamol (100nM) in the presence and absence of beta$_2$ antagonist ICI-118551 when administered at the reperfusion in the rat myocardial model of ischaemia reperfusion injury.

Administration of Salbutamol (100nM) throughout the reperfusion period significantly increased infarct size to risk ratio (%) in hearts subjected to 35 minutes of ischaemia and 165 minutes of reperfusion. To determine whether the cardio toxic effect of Salbutamol was due activation of β_2 adrenoceptors, we used the β_2 adrenoceptors antagonist ICI.

Administration of salbutamol in the presence of the β_2 adrenoceptor antagonist ICI decreased the infarct size to risk ratio. Administration of ICI alone throughout reperfusion has no significant effect on infarct size to risk ratio (%) compared to non-treated ischaemic reperfused hearts (P>0.05 vs. non-treated control, Fig 3.2.2.).



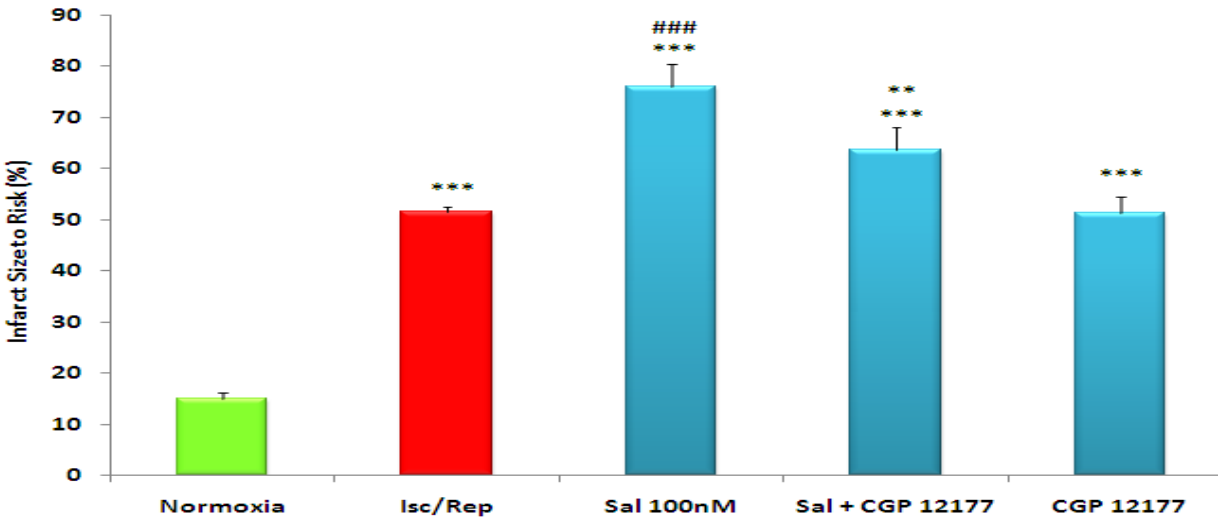
*****P<0.001 vs. Normoxia ### P<0.001 vs. Isc/Rep $$$ P<0.001 vs. Sal 100nM**

Figure3.2.2. Infarct size to risk ratio (%) in non-treated control and salbutamol treated ischaemic reperfused hearts. Isolated perfused hearts were subjected to 35 minutes of ischaemia and 165 minutes of reperfusion where the β_2 adrenoreceptor salbutamol (100nM) in the presence and absence of β_2 antagonists ICI-118551 was administered throughout reperfusion. Results are shown as mean + SEM from 4-5 individual experiments.

3.2.3. Role of $\beta_2$agonist salbutamol (100nM) in the presence and absence of beta$_1$ antagonist CGP-12177 when administered at the reperfusion in the rat myocardial model of ischaemia reperfusion injury.

The administration of salbutamol (100nM) in the presence and absence of β_1 antagonist CGP - 12177 throughout the reperfusion significantly reversed the cardio-toxic effects of salbutamol (P<0.001 vs. Salbutamol, Fig 3.2.3).

Administration of CGP-12177 alone, throughout reperfusion has no significant effect on infarct size to risk ratio (%), compared to non-treated ischaemic/reperfused hearts (P>0.05 vs. Isc/Rep, Fig 3.2.3.



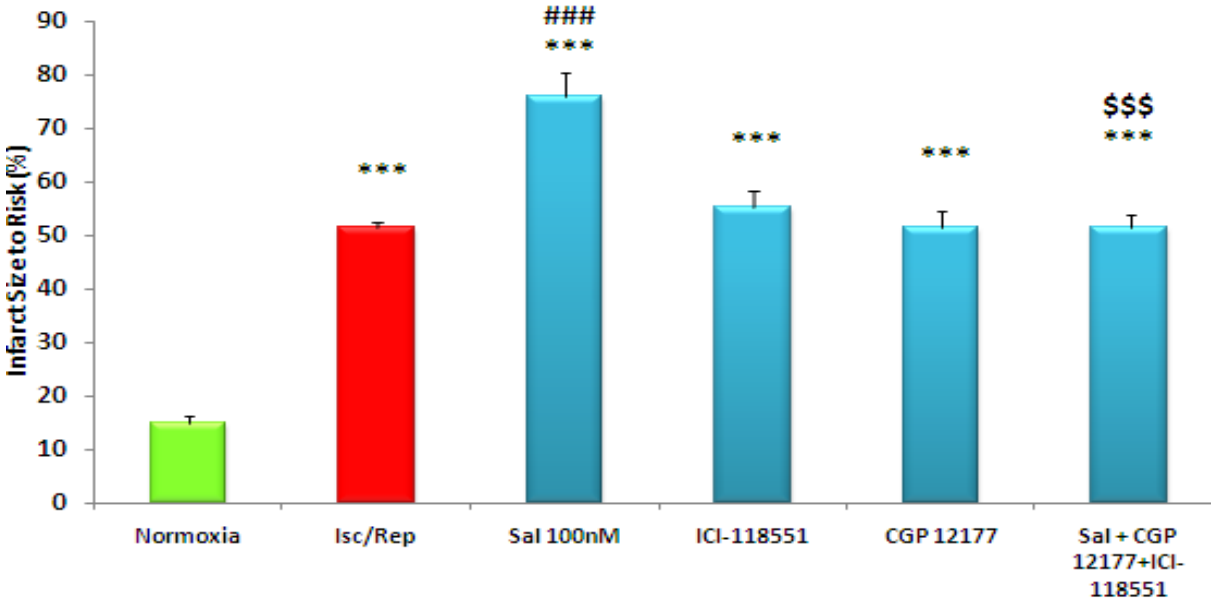
*****P<0.001 vs. Normoxia ### P<0.001 vs. Isch/Rep **P<0.01 vs. Sal 100nM.**

Figure3.2.3. Infarct size to risk ratio (%) in non-treated control and salbutamol treated ischaemic reperfused hearts. Isolated perfused hearts were subjected to 35 minutes of ischaemia and 165 minutes of reperfusion where the β_2 adrenoreceptor Salbutamol (100nM) in the presence and absence of β1 antagonists CGP 12177 was administered throughout reperfusion. Results are shown as mean + SEM from 4-5 individual experiments.

3.2.4. Role of $\beta_2$agonist salbutamol (100nM) in the presence and absence of β_1 antagonist CGP-12177 and β_2 antagonist ICI-118551 when administered at the reperfusion in the rat myocardial model of ischaemia reperfusion injury.

To determine whether the cardio toxicity afforded by salbutamol (100nM), we administered salbutamol in the presence and absence of β_1 antagonist CGP and β_2 antagonist ICI.

The administration of salbutamol (100nM) in the presence of the β_1 antagonist CGP-12177 and beta$_2$ antagonist ICI significantly reversed the cardio toxic effect of Salbutamol in isolated perfused rat hearts subjected to 35 minutes of ischaemia and 165 minutes of reperfusion (P<0.001 vs. Salbutamol, Fig 3.2.4).



*****P<0.001 vs. Normoxia ### P<0.001 vs. Isch/Rep $$$ P<0.001 vs. Sal 100nM**

Figure.3.2.4. Infarct size to risk ratio (%) in non-treated control and salbutamol treated ischaemic reperfused hearts. Isolated perfused hearts were subjected to 35 minutes of ischaemia and 165 minutes of reperfusion where the β_2 adrenoreceptor Salbutamol (100nM) in the presence and absence of β_1 antagonists CGP 12177 and β2 antagonist ICI 118551 were administered throughout reperfusion. Results are shown as mean + SEM from 4-5 individual experiments.

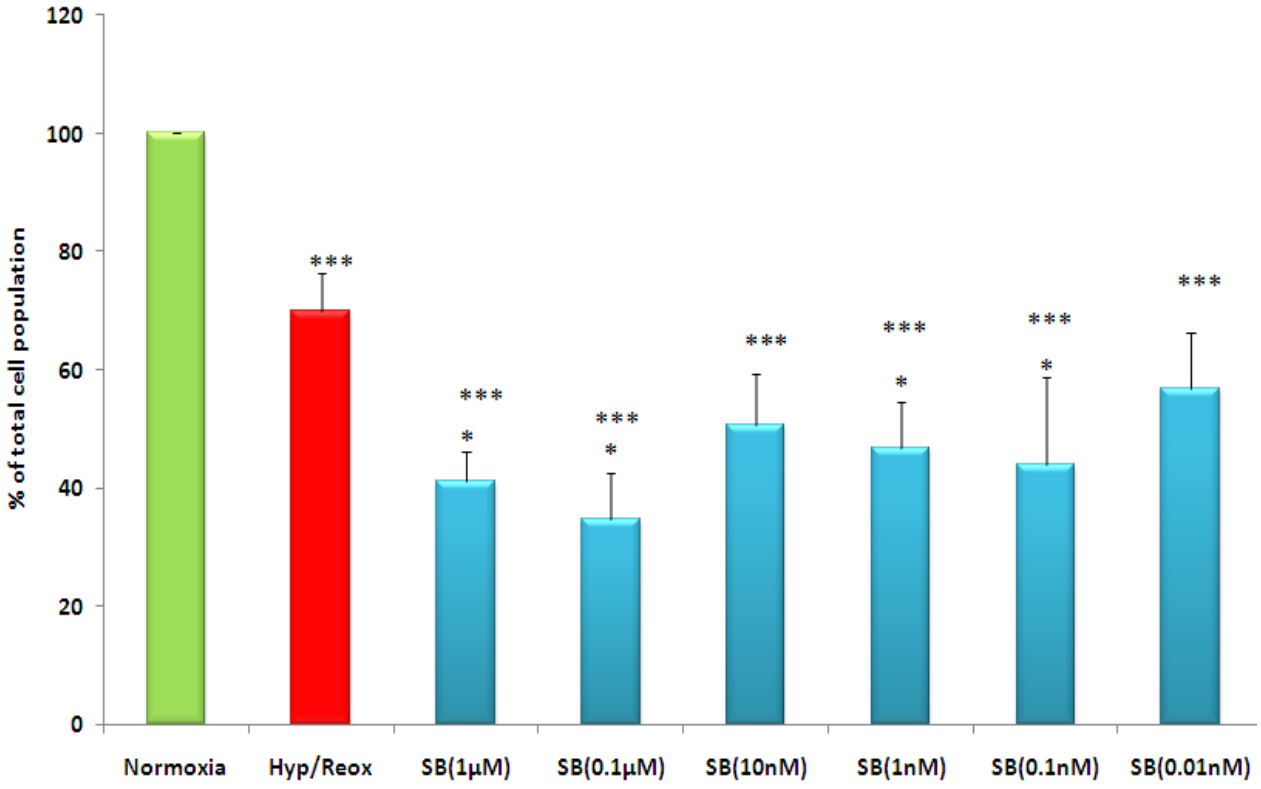
3.3 Role of β_2 agonist salbutamol in the live cell population of rat cardio myocytes administered at reperfusion in the isolated perfused heart.

The β_2 agonist salbutamol at different concentrations was administered throughout the reoxygenation period. After reoxygenation the cells were stained with Fluorochromes specific for

apoptotic, necrotic and live cells and analysed on the flow cytometer.Isolated rat cardiomyocytes subjected to 6 hours of hypoxia followed by 18 hours of reoxygenation resulted in significant decrease in the number of live cells when compared to normoxic group ($P<0.001$ vs. Normoxia, Fig 3.3.0).

To find out the effect of the β_2 agonist salbutamol, rat cardiomyocytes were subjected to 6 hours of hypoxia followed by 18 hours of reoxygenation. β_2 agonist Salbutamol (1μM, 0.01μM, 10nM, 1nM, 0.01nM) was administered throughout the reoxygenation period.

Administration of Salbutamol (1μM, 0.1μM, 1nM, 0.1nM) throughout reoxygenation significantly decreased the population of live cells compared to non-treated cells ($P<0.05$ vs. Hyp/Reox, Fig 3.3.0).



***P<0.05 vs. Hyp/Reox, ***p<0.01 vs. Normoxia**

Figure.3.3.0. Assessment of live cells in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. The β_2 agonists salbutamol (1μM, 0.1μM, 10nM, 1nM, 0.1nM, 0.01nM) were added at the onset of reoxygenation. Results are shown as Mean+SEM n=6-8 experiments.

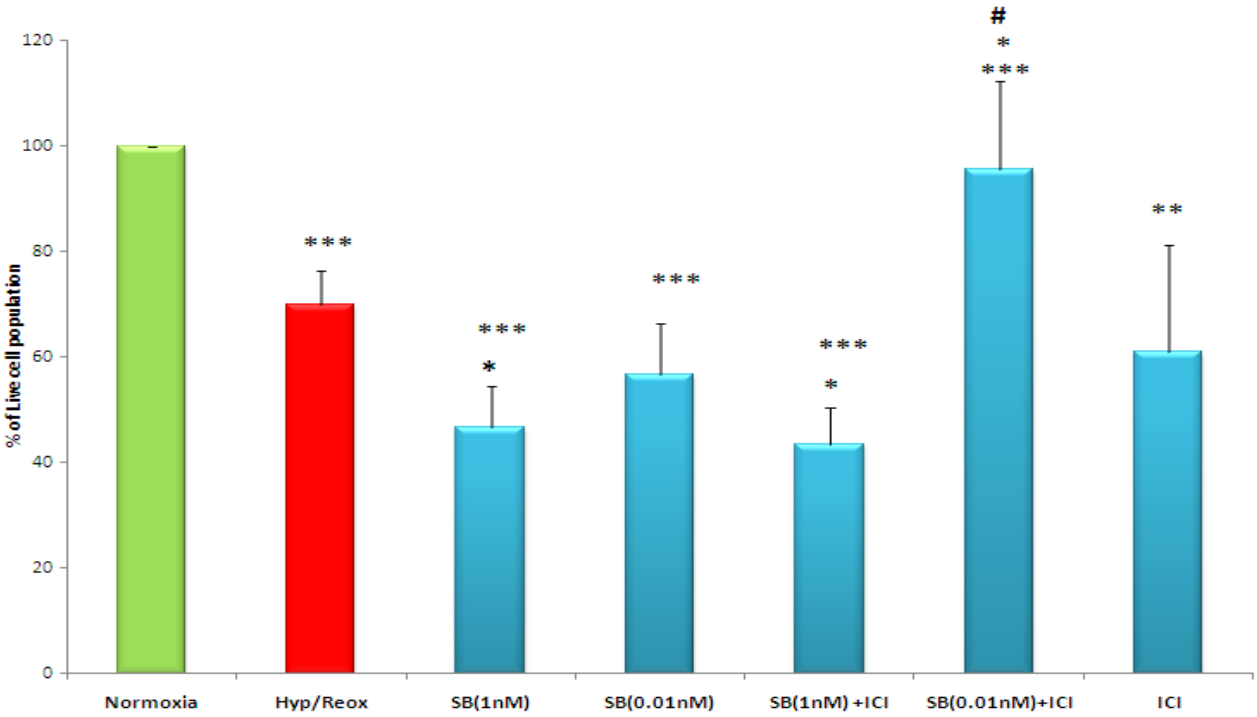
3.3.1. Role of β_2 agonist salbutamol in the live cell population of rat cardiomyocytes in the presence and absence of β_2 antagonist IC1-118551 when administered throughout reoxygenation

Administration of β_2 agonist salbutamol in rat cardiomyocytes in the presence and absence of β_2 antagonist ICI was performed to find out whether the presence of β_2 antagonist can significantly reverse the cardio-toxic effects of salbutamol.

Isolated rat cardiomyocytes were subjected to 6 hours of hypoxia followed by 18 hours of reoxygenation. The β_2 agonist Salbutamol (1nM, 0,01nM) was administered throughout the reoxygenation period in the presence of the β_2 antagonist ICI.

The administration of β_2 agonist Salbutamol (1nM) in the presence of β_2 antagonist ICI in rat cardiomyocytes throughout reoxygenation was failed to reverse the cardio toxic effect of salbutamol (P>0.05 vs. Salbutamol, Fig 3.3.1). Administration of Salbutamol (0.01nM) in the presence of β_2 antagonist ICI was significantly abolished the cardio toxicity.

Administration of ICI alone throughout reoxygenation had no significant effect on live cell population compared to non-treated cells, subjected to hypoxia/reoxygenation injury (P>0.05 vs. Hyp/Reox, Fig 3.2.1)

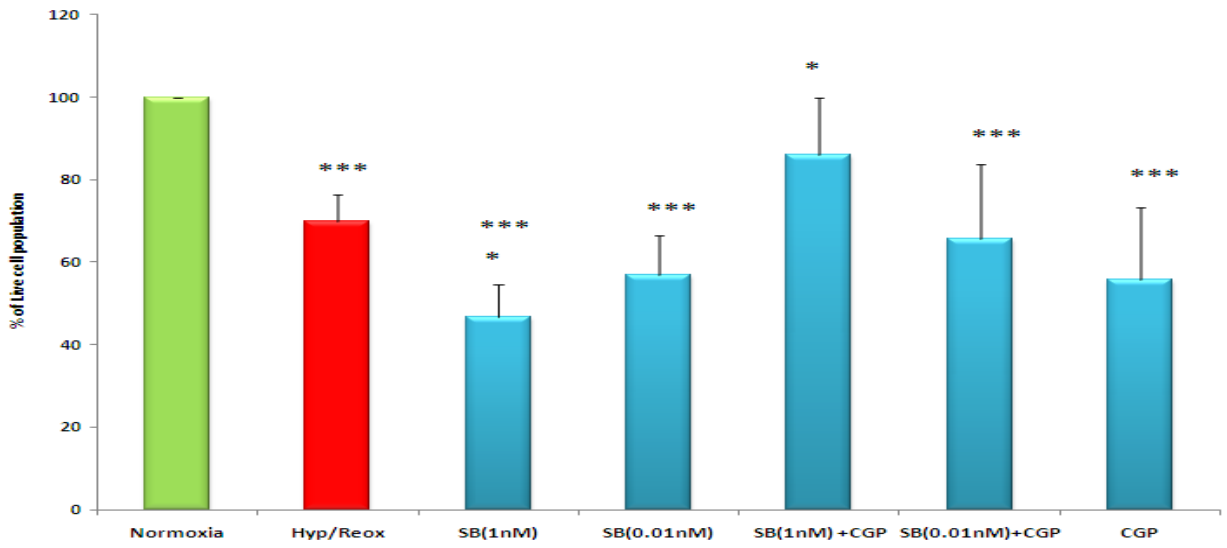


***p<0.01 vs. Hypo/Reox, ***p<0.001 vs. Normoxia, #p<0.05 vs. Salbutamol 0.01nM.**

Figure.3.3.1. Assessment of live cells in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. The β_2 agonist salbutamol (1nM, 0.01nM) was added in the presence and absence of β_2 antagonist ICI-118557 at the onset of reoxygenation. Results are shown as Mean+ SEM.

3.3.2. Role of β_2 agonist salbutamol in the live cell population of rat cardiomyocytes in the presence and absence of β_1 antagonist CGP-12177 when administered at reperfusion in isolated perfused heart.

The β_2 agonist salbutamol (1nM, 0.01nM) was administered in the presence and absence of β_1 antagonist CGP throughout the reoxygenation period. Administration of salbutamol (1nM or 0.01nM) significantly reduced the population of live cells compared to non-treated control cells (P<0.001 vs. Hyp/Reox, Fig 3.3.2.). Co-administration of salbutamol (1nM) in the presence of the β_1 antagonist CGP significantly reversed the decrease in live cells when Salbutamol (1nM) was administered alone throughout reoxygenation.

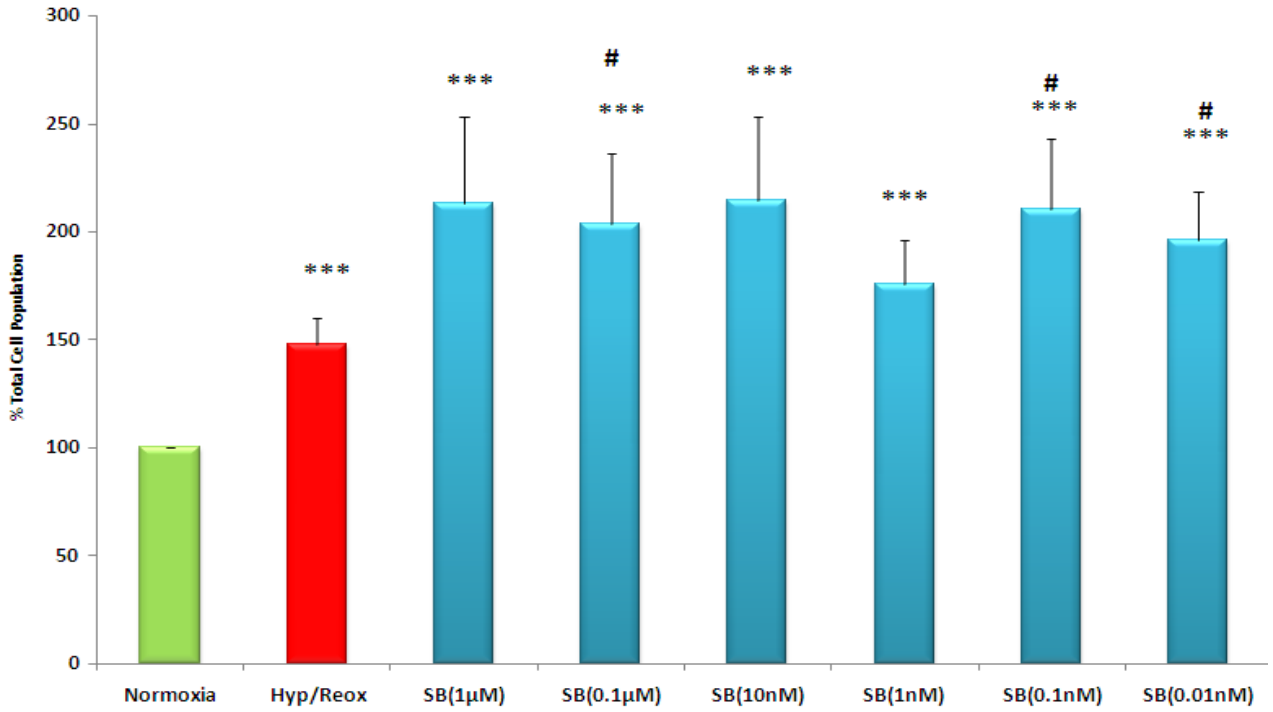


*****p<0.001 vs. Normoxia,*p<0.05 vs. Hypo/Reox. #p<0.05 vs. Sal 1nM**

Figure.3.3.2. Assessment of live cells in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. The β_2 agonist Salbutamol (1nM, 0.01nM) was added in the presence and absence of β_1 antagonist CGP-12177 at the onset of reoxygenation. Results are shown as Mean+ SEM.

3.4 The effect of the β_2 agonist Salbutamol necrotic cell death in rat cardiomyocytes subjected to hypoxia / reoxygenation injury.

The percentage of the necrotic cells was significantly increased when the β_2 agonist salbutamol (0.1µM, 0.1nM, 0.01nM) was administered throughout the reperfusion when it was compared to non-treated cells subjected to 6 hours of hypoxia and 18 hours of reoxygenation (P<0.05 vs. Hyp/Reox, Figure 3.4.0.).

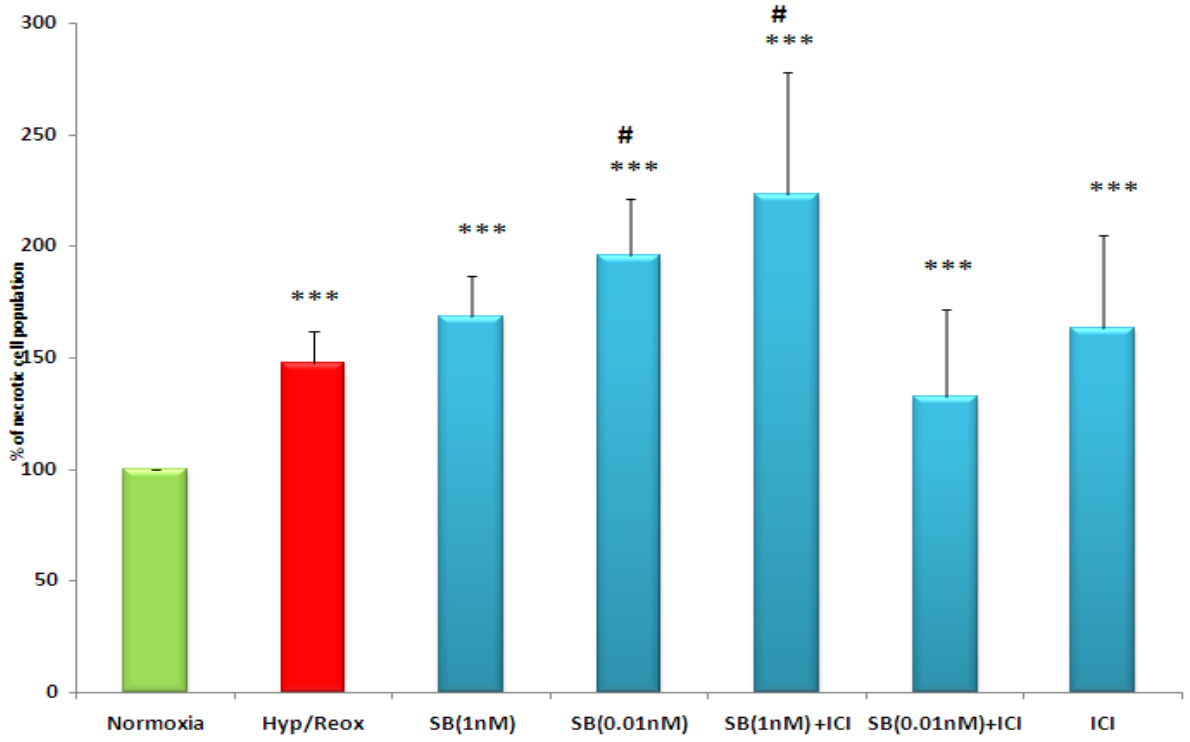


*****p<0.05 vs. Normoxia, #p<0.05 vs. Hypo/Reox.**

Figure.3.4.0. Assessment of necrotic cells in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. The β_2 agonist salbutamol (1µM, 0.01µM, 10nM, 1nM, 0.1nM, 0.01nM) was added at the onset of reoxygenation. Results are shown as Mean+SEM

3.4.1. Role of β_2 agonist salbutamol (1nm,and 0.01nm) when administered in the presence and absence of β_2 antagonist ICI-118551 in necrotic cells of adult rat cardiomyocytes subjected to 6h hypoxia and 18h reoxygenation.

Administration of salbutamol (0.01nM) throughout reoxygenation resulted in a significant increase in necrotic cardiomyocytes (P<0.05 vs. Hyp/Reox, Fig 3.4.1). Administration of salbutamol in the presence of the β_2 AR antagonist ICI reversed the pro-necrotic effect of salbutamol, but did not reach statistical significance (Fig 3.4.1). Administration of ICI alone throughout reoxygenation had no significant effect of cardiac myocyte necrosis (Fig 3.4.1.).

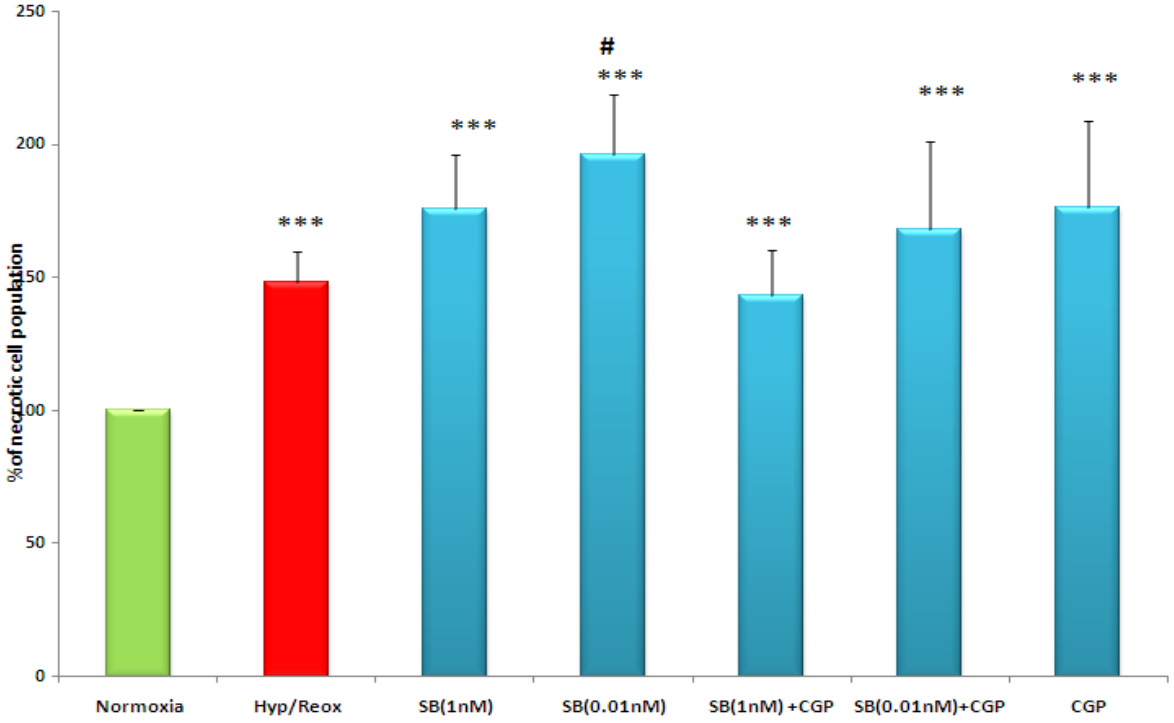


*****p<0.001 vs. Normoxia. #p<0.05 vs. Hypo/Reox.**

Figure 3.4.1. Assessment of necrotic cells in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. The β_2 agonist salbutamol (1µM, 0.01nM) was added in the presence and absence of β_2 antagonist ICI-118551 at the onset of reoxygenation. Results are shown as Mean+ SEM.

3.4.2. Assessment of the administration of β_2 agonist salbutamol (1nM, 0.01nM) in the presence and absence of β_1 antagonist CGP-12177 in necrotic cells of adult rat cardiomyocytes subjected to 6h hypoxia and 18h reoxygenation.

Administration of Salbutamol (0.01nM) throughout reoxygenation resulted in a significant increase in necrotic cardiomyocytes (P<0.05 vs. Hyp/Reox, Fig 3.4.2). Administration of salbutamol in the presence of the β_1 AR antagonist CGP failed to reverse the pro-necrotic effect of salbutamol. Administration of CGP alone throughout reoxygenation had no significant effect of cardiac myocyte necrosis (Fig 3.4.2.).

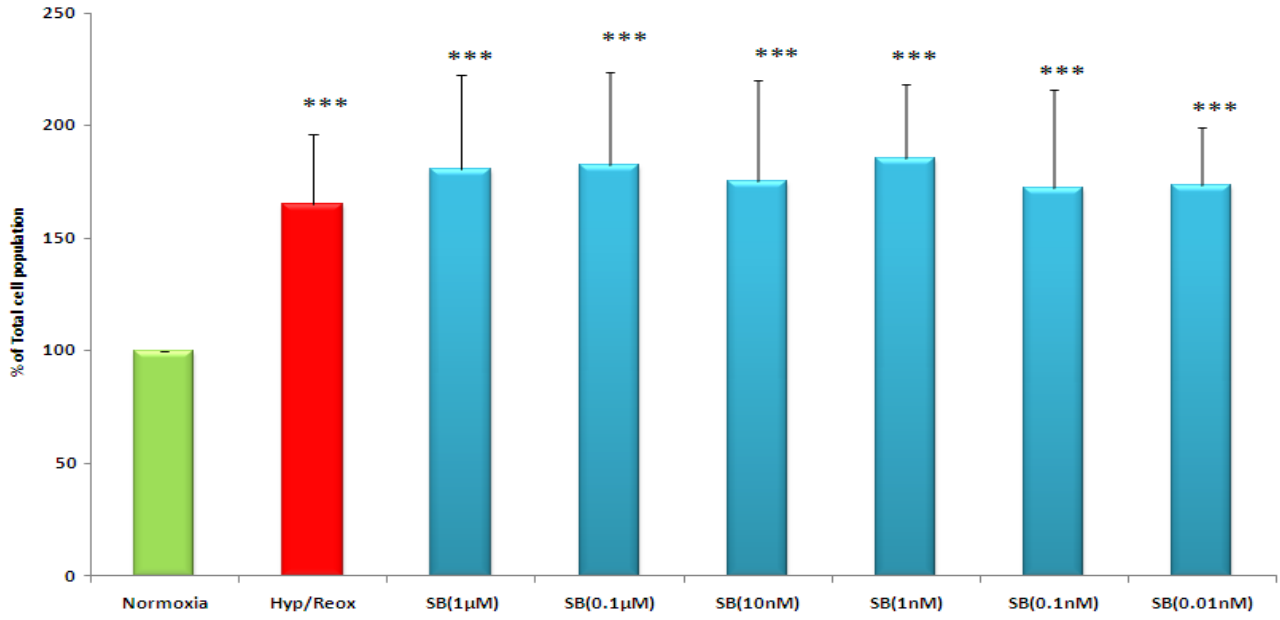


*****p<0.001 vs. Normoxia. **p<0.05 vs. Hypo/Reox**

Figure.3.4.2. Assessment of necrotic cells in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. The β_2 agonist salbutamol (1µM, 0.01nm) was added in the presence and absence of β_2 antagonist CGP-12177 at the onset of reoxygenation. Results are shown as Mean+ SEM.

3.5 Role of β_2 agonist salbutamol in the apoptotic cell population of rat cardio myocytes when administered at reperfusion

Rat cardiac myocytes subjected to 6 hours of hypoxia and 18 hours of reoxygenation resulted in a significant increase in apoptosis compared to naive non-stressed cardiac myocytes (P<0.001 vs. Normoxia, Fig 3.5.0).Administration of salbutamol ((1μM-0.01nM) had no significant effect on cardiac myocyte apoptosis compared to non-stressed cardiac myocyte.(P>.5 vs. Normoxia Fig 3.5.0)



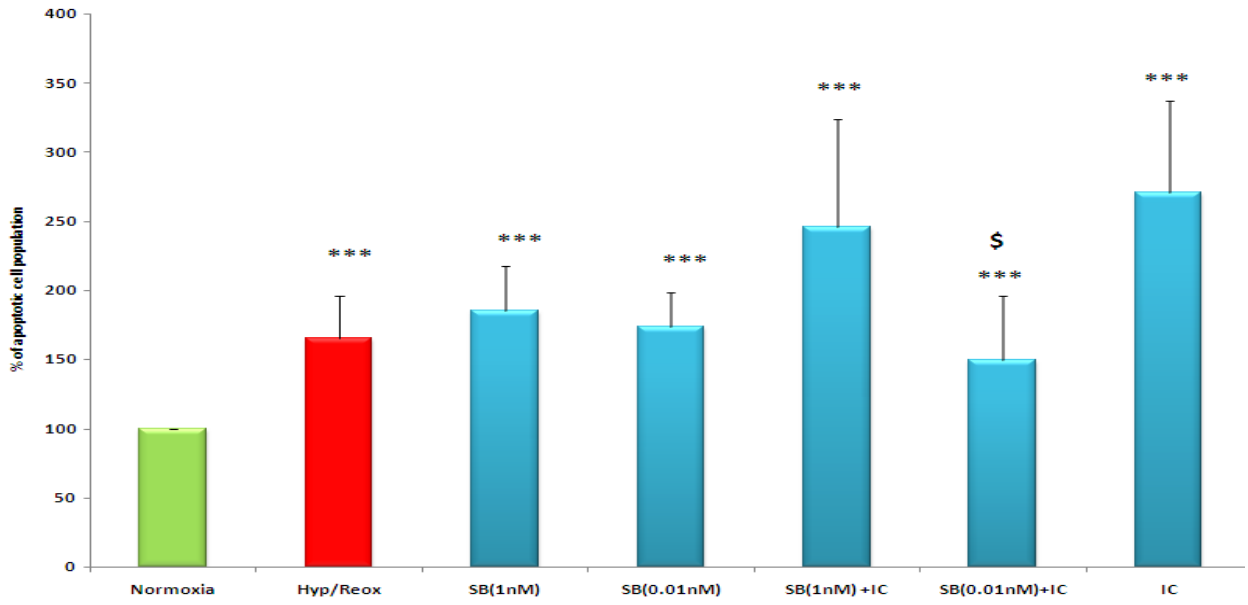
*****p<0.01 vs. Normoxia**

Figure 3.5.0. Assessment of apoptotic cells in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. The β_2 agonist salbutamol (1μM, 0.01nM) was added at the onset of reoxygenation. Results are shown as Mean+SEM.

3.5.1. Role of β_2 agonist salbutamol (1nM,and 0.01nM) when administered in the presence and absence of β_2 antagonist ICI-118551 in apoptotic cells of adult rat cardiomyocytes subjected to 6h hypoxia and 18h reoxygenation.

Isolated cells were exposed to 6 hours of hypoxia and 18 hours of reoxygenation in the presence of β_2 agonist salbutamol in the presence and absence of β_2 antagonist ICI. Administration of Salbutamol in the presence of the β_2 adrenoceptor antagonist increased cell death by apoptosis but did not reach statistical significance (Fig 3.5.1.).

Administration of ICI alone throughout reoxygenation also increased the cardiac myocyte death by apoptosis, but failed to reach statistical significance (Fig 3.5.1.).



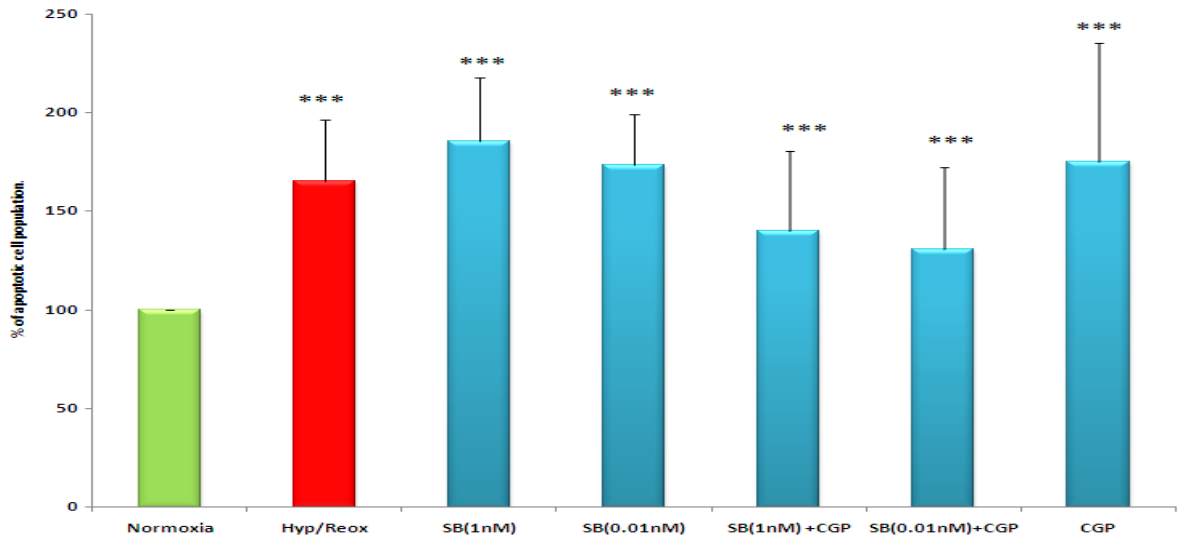
*****p<0.05 vs. Normoxia**

Figure.3.5.1. Assessment of apoptotic cells in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. The β_2 agonist salbutamol (1µM, 0.01nM) was added in the presence and absence of β_2 antagonist IC-118551 at the onset of reoxygenation. Results are shown as Mean+ SEM.

3.5.2. Role of β_2 agonist salbutamol (1nM,and 0.01nM) when administered in the presence and absence of β_1 antagonist CGP-12177 in apoptotic cells of adult rat cardiomyocytes subjected to 6h hypoxia and 18h reoxygenation.

The β_2 adrenoceptor agonist salbutamol was administered in the presence and absence of β_1 adrenoceptor blocker CGP in adult rat cardiomyocytes subjected to hypoxia/reoxygenation injury. Administration of Salbutamol (1nM, 0.01nM) throughout reoxygenation had no significant effect of cardiac myocyte apoptosis compared to non-treated cardiac myocytes subjected to hypoxia/reoxygenation injury.

Administration of the β_1 antagonist CGP alone throughout reoxygenation had no significant effect of cardiac myocyte apoptosis (Fig 3.5.2.).



*****p<0.05 vs. Normoxia**

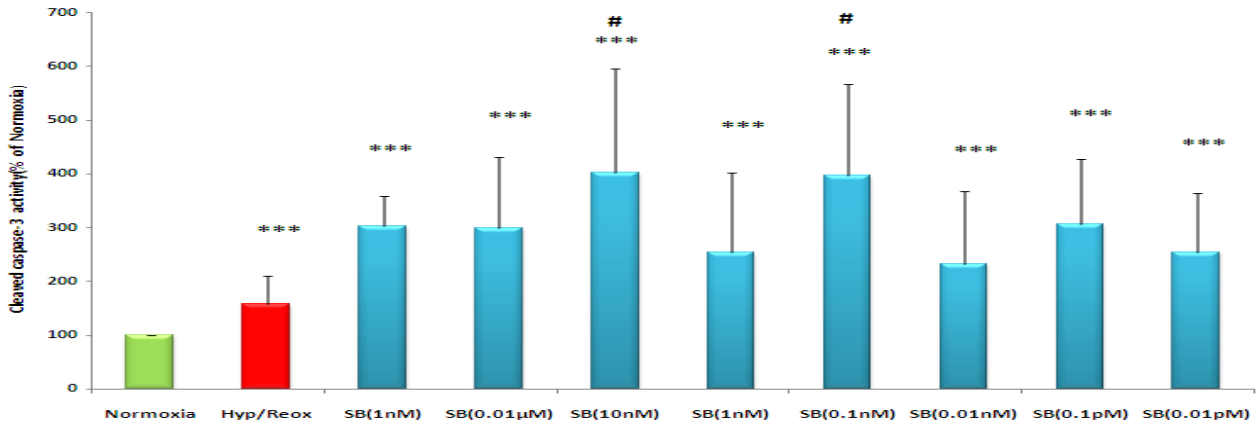
Figure.3.5.2. Assessment of apoptotic cells in isolated adult rat cardiomyocytes subjected to 6 hours hypoxia and 18 hours of reoxygenation. The β_2 agonist salbutamol (1µM, 0.01nM) was added in the presence and absence of β1 antagonist CGP-12177 at the onset of reoxygenation. Results are shown as Mean+ SEM.

3.6 Intracellular Caspase-3 levels in cardiomyocytes as analysed by flowcytometry after the administration of salbutamol at reoxygenation.

Caspase-3 protein has a pivotal role in the execution phase of apoptosis. The β_2 adrenoceptor agonist salbutamol was administered throughout reoxygenation in isolated adult rat cardiac myocytes subjected to 6 hours of hypoxia and 18 hours of reoxygenation.

Cardiac myocytes subjected to 6 hours of hypoxia and 18 hours of reoxygenation resulted in a significant increase in cleaved caspase 3 activity (P<0.01 vs. Normoxia, Fig 3.6.0).

Administration of salbutamol(1µM, 10nM, and 0.1nM) throughout reoxygenation significantly increased cleaved caspase-3 activity in isolated adult rat cardiac myocytes subjected to 6 hours of hypoxia and 18 hours of reoxygenation compared to non-treated Hyp/Reo group (P<0.05 vs. Hyp/Reox,Figure.3.6.0.).



*****p<0.01 vs. Normoxia. #p<0.05 vs. Hypo/Reox.**

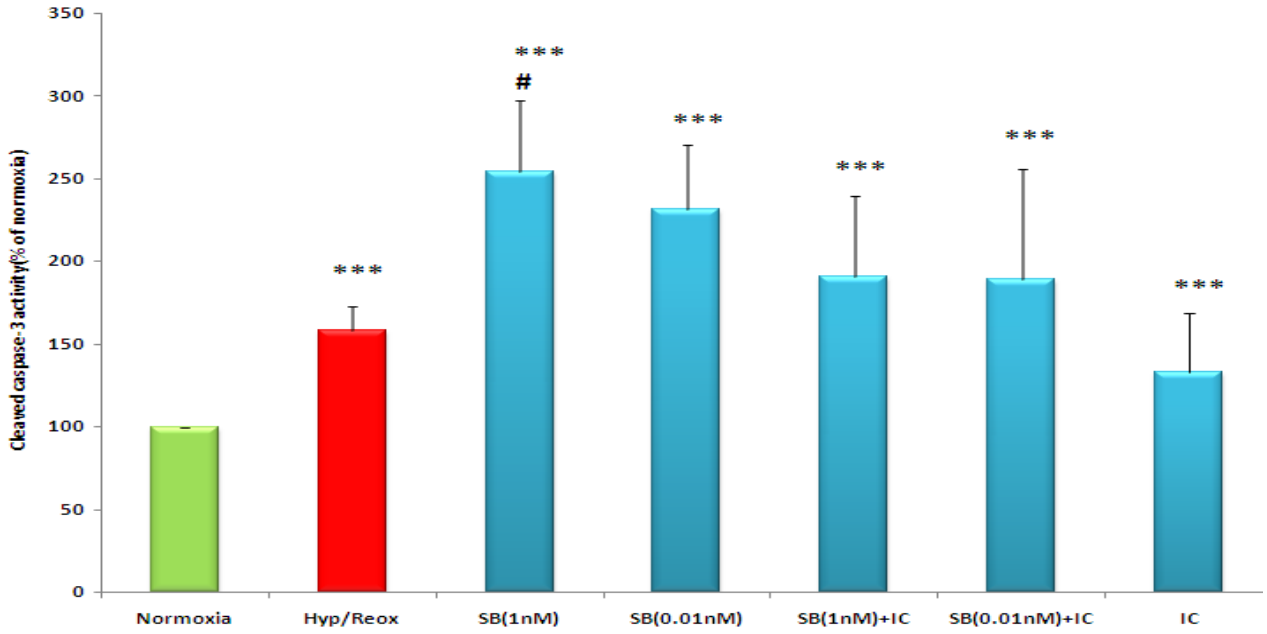
Figure.3.6.0. Cleaved-caspase3 activity in isolated adult rat cardiomyocytes subjected to 6 hours of hypoxia followed by 18 hours of reoxygenation. The β_2 agonist salbutamol (1µM, 0.01µM, 1nM, 0.1nM, 0.1pM, 0.01pM, 10nM.0.01nM) was administered at the onset of reoxygenation. Mean + SEM of 5 individual experiments.

3.6.1. Intracellular caspase-3 levels in the cardiomyocytes after the administration of salbutamol (1nM, 0.01nM) in the presence and absence of β_2 antagonist ICI-118551 along at reoxygenation.

To determine whether the β_2 antagonist ICI make any impact on the caspase3 activity in the presence of salbutamol (1nM and 0.1nM), salbutamol was administered with the presence and absence of the β_2 antagonist ICI in the isolated rat cardiomyocytes.

Administration of salbutamol (1nM and 0.1nM) alone increased caspase 3 activity in the isolated adult rat cardiomyocytes at the onset of reperfusion, subjected to 6 hours of hypoxia and 18 hours of reoxygenation.

Administration of salbutamol (1nM,0.1nM) throughout the reperfusion in the presence of β_2 antagonist ICI showed no significant effect compared to non-treated cardiac myocytes ($P>0.05$ vs. Hyp/Reox, Figure3.6.1.). Administration of ICI throughout reoxygenation alone had no significant effect on caspase 3 activity compared to non-treated control myocytes ($P>0.05$ vs. Hyp/Reox, Figure3.6.1.)



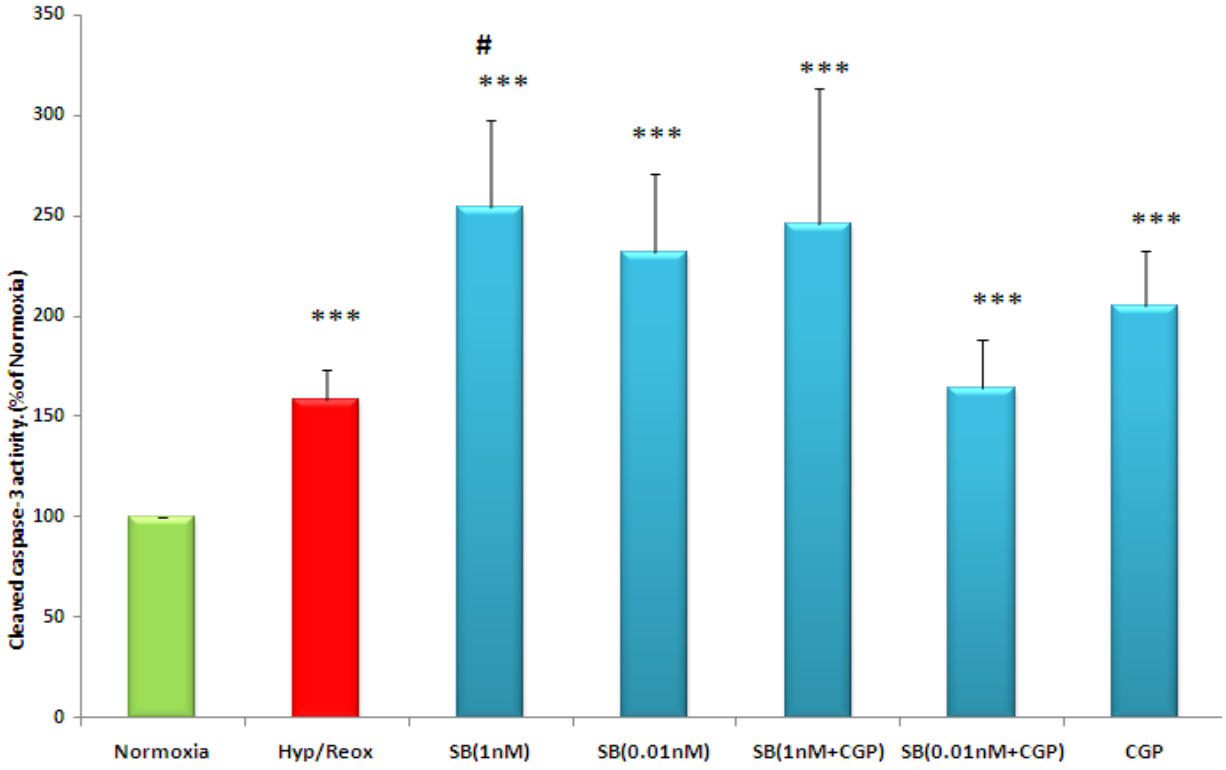
#p<0.05 Salbutamol (1nM) vs. Hypo/Reox.*p<0.001 vs.Normoxia**

Figure.3.6.1. Cleaved-caspase 3 activity in isolated adult rat cardiac myocytes subjected to 6 hours of hypoxia followed by 18 hours of reoxygenation. The β_2 agonist salbutamol (1nM, 0.1nM) was administered in the presence and absence of β_2 antagonist ICI-118551 at the onset of reperfusion. Mean + SEM of 5 individual experiments.

3.6.2. Intracellular caspase3 levels in cardiomyocytes as analysed flowcytometry after the administration of salbutamol (1nM, 0.1nM) in the presence and absence of β_1 antagonist CGP-12177 at the onset of reoxygenation.

Administration of salbutamol (1nM) caused a significant increase in caspase 3 activity compared to non-treated cardiac myocytes (P<0.05 vs. Hyp/Reox, Fig 3.6.2.). This increase in caspase 3 activity

was not reversed in the presence of the β_1 adrenoceptor antagonist CGP P>0.05, Fig 3.6.2.). Administration of CGP alone throughout reoxygenation had no significant effect on caspase 3 activity compared to non-treated cardiac myocytes (P>0.05, Fig 3.6.2).



*****p<0.05 vs. Normoxia, #p<0.05 vs.Hyp/Reox.**

Figure.3.6.2. Cleaved-caspase 3 activity in isolated adult rat cardiac myocytes subjected to 6 hours of hypoxia followed by 18 hours of reoxygenation. The β_2 agonist salbutamol (1nM, 0.1nM) was administered in the presence and absence of β_1 antagonist CGP-12177 at the onset of reperfusion. Mean +/- SEM of 5 individual experiments.

4. GENERAL DISCUSSION

Ischemic heart disease is the most important factor in the progression of myocardial infarction in the world today. Many studies have tried to identify the major factors to reduce the burden of 4. General discussion

ischemic heart disease related events in human. For reducing the post ischaemic infarct size, patients undergo treatments like thrombolysis, percutaneous angioplasty, and primary bypass surgery. There remains an unscored need for novel therapies and strategies to ameliorate to detrimental effects of ischaemic heart disease and myocardial infarction.

It is unclear that whether the use of β_2 agonists makes an increase risk of heart failiure.β_2 agonists are often used in the short term treatment of enhancement of heart contractility due to its improving cardiac performances in patients. But, the data from the Acute decompensate heart failure national registry emergency module showed that 14% of the people presently with dysponea are treated from COPD. The use of inhaled β_2 agonist in these heart failure patients will make COPD associated with a worse outecome.They also suggested that the mortality rate is also very high in these patients who received the β_2 agonists (Maria et al., 2009).

The recent discovery by Vinten-Johnsen et al. of ischaemic post conditioning made a huge impact on appealing the pharmacological interventions to be applied at the onset of reperfusion.There are

mainly two types of treatments of ischaemic heart diseases conventional and alternative treatments. Conventional treatment is one which includes the treatment by the usage of drugs.

Cardio toxicity is a well-known side effect of several drugs which ultimately cause the long term morbidity. These cytotoxic drugs involve in the formation of free radicals and cause the oxidative stress. This leads to apoptosis of cardiac myocytes (Schimmel et al., 2004). The standard method for identifying the cardio-toxic compounds involves the histopathilogical analysis of tissue section (Brad et al., 2008). A large number of studies have shown endogenenous and exogenous agents to arbitrate ischaemia reperfusion injury. Some of them having cardio-toxic effects and others having cardio-protective abilities (Hausenloy and Yellon.2004).

The aim of this study was to investigate the cardio toxicity of the beta 2 adrenoceptor agonist salbutamol in the isolated perfused rat heart model of ischaemia reperfusion injury and the adult rat cardiac myocyte model of hypoxia/reoxygenation injury. Previous studies have proven that the beta agonist salbutamol will make severe problems in the heart. Barilan et al., 2010 has done a recent study on asthma patients who were using salbutamol .The result of that study was the usage of salbutamol will finally ends with myocardial infarction. In the present study, salbutamol was administered at the time of reperfusion in the infarct heart model and isolated cardiomyocytes. Some studies have implicated the activation of salbutamol on coronary circulation. George et.al., (2006) discussed about the effect of salbutamol on coronary circulation. George and his colleagues revealed a fact that the high dose of salbutamol will increase the coronary flow but not in proportion to the myocardium and decrease coronary flow reserve and they showed that this will cause many consequences in patients with coronary artery diseases causing or worsening myocardial infarction. In this present study the administration of salbutamol 100nM, throughout the reperfusion of cardiomyocytes gives a significant increase in the coronary flow compared to isch/rep. These findings cardiotoxicity of salbutamol were supported by the findings of George et al., (2006). Kochiadakis et al., (2007) researchers have shown the tolerability and safety of

salbutamol in COPD patients. Bernd et al., (2006) suggested that the large dose of salbutamol in patients with chronic obstructive pulmonary diseases will cause a comparable tolerability than other beta agonists such as formoterol. Besides, a study has done by Wijesinghe and his colleagues in 2008 have made a significant research in the study of long acting beta agonists in asthma mortality. They have suggested that the regular use of beta agonists may have the chance to make increase in asthma mortality.

Some previous studies implicated significant results in the effect of beta adrenoceptors in the heart during ischaemia/reperfusion. Jatin et al., (2004) showed the adverse effects of beta agonists in heart during ischaemia. They have suggested that while the administration of beta agonists in high doses may cause cardio toxic effects by increase in the apoptotic and necrotic cell populations. Moreover, the studies done by Heather et al., (1999) showed some effective results with the use of beta agonist in heart during ischaemia. They have also concluded with the adverse effects of the use of beta agonists in the heart. In this present study, the administration of salbutamol on cardiomyocytes at reperfusion significantly increased the infarct size. The results indicate the cardio-toxic effect of salbutamol in heart. Especially, when salbutamol 100nM was administered throughout the reperfusion significantly revealed the cardio toxicity of the drug. Moreover, the beta antagonists ICI and CGP were administered along with salbutamol 100nM at the onset of reperfusion. Although the administration of the beta antagonists ICI and CGP abrogated the toxicity afforded by the salbutamol, these were not much significant to the control. The coordination of beta antagonists ICI and CGP with salbutamol 100nm administered at reperfusion was significantly decreased the infarct size compared to the salbutamol 100nm alone. These results are in accordance with the finding of Donald et al., (1999) who showed the cardio toxicity caused by the regular use of Salbutamol. Klaus et al., (2003) has done a study on the role of β_2 adrenoceptors in the rat cardiomyocytes. They have found out the cardio toxic effect of beta$_2$ agonists, and showed the effect of beta antagonists ICI and CGP, which resulted that these antagonists also induced the apoptosis and necrosis in rat cardiomyocytes. Also, this present study supported by the findings of

studies conducted by Ruth et al., (2000).They showed the effects of beta antagonists in cardiac ischaemic patients. They were suggesting the significant reduction of cardiac death in patients with contractile symptoms.

The present study suggested the cardio toxicity of salbutamol, when administered in cardiomyocytes throughout the reperfusion. Previous studies have shown the consequences of salbutamol in heart. Administration of salbutamol in isolated cardiomyocytes induces a number of cardio-toxic properties which includes the significant decrease in the population of live cells, apoptosis, and necrosis. Carley et al., (2007) has been showed that it is very hard to explain the cardiac benefits of β_2 agonist treatment in the heart failiure.In this present study the administration of β_2 agonist salbutamol in isolated rat cardiomyocytes throughout the reperfusion significantly decrease the live cell population and proved its cardio-toxic effect. The Previous research has shown the properties of beta antagonists in the ischaemic heart diseases. Salbutamol was administered along with the beta antagonists ICI and CGP caused a cardio-toxic effect on the cardiomyocytes by decreasing the live cells of cardiomyocytes. Although, the administration of beta antagonists ICI and CGP along with salbutamol (0.1nM and 1nM respectively) abrogated the toxicity caused by salbutamol alone, these were not significantly increase the live cells compared to non hypoxia group. This concept of this study is supported by the findings of Jenne (1998) and Carbol (2001).They showed the positive effects of beta antagonists with salbutamol.

In this study, flow cytometry analysis of isolated myocytes subjected to hypoxia/reoxygenation demonstrated that there was a significant increase in the apoptotic cell population when compared to normoxic controls. In order to elucidate the mechanisms by which the toxic effect were observed there was a comparison put together on the apoptotic effect of salbutamol in coordination with the beta antagonists ICI and CGP. The administration of beta antagonists ICI and CGP along with salbutamol (0.1nM, 1nM) at the onset of reperfusion also showed the cardio toxicity of salbutamol. More importantly, the administration of β2 antagonists ICI with salbutamol (0.1nM) abrogated the

toxicity afforded by salbutamol (1nM) and ICI alone. Besides the administration of β1 antagonist CGP when administered along with salbutamol showed the cardio-toxic effect by increasing the apoptotic cell population. These findings of this present study was supported by the findings of Milton parker (2003) who has done clinical studies on beta agonists activity on hearts and he suggested that the survival in heart failure by β blocker is not likely to related in the magnitude or duration of β blockade produced by the agonists.

The study by Maak et al., (2008) in beta agonist's activity in heart failure showed a list of adverse effect in the treatment. In accordance with those findings Cates et al., (2009) found that the regular treatment with salbutamol in chronic asthma will cause serious adverse effects in heart. The present study revealed the cardio toxicity of the beta agonist salbutamol by increasing the necrotic cell population, when salbutamol administered in cardiomyocytes throughout reperfusion. The administration of salbutamol (1nM) with the beta antagonist ICI and CGP also didn't make any alteration in the toxicity afforded by the administration of salbutamol alone.

Aihua et al (2006) have done a study on the role of β_2 adrenoceptors in the heart following ischaemia/reperfusion. They have suggested that the Capase 3 activity in the cardiomyocytes induced apoptosis the cleaved caspase3 activity with the beta agonist salbutamol has been discussed in this present study. The increase in the caspase 3 level in the administration of different doses of salbutamol proved the cardio toxic effect of the beta agonists. Moreover, the administration of beta antagonist ICI and CGP has not made any abrogation in the toxicity afforded by salbutamol. These findings were supported by the concept of Vandenabeele et al., (2006) who found the progression in cell death pathways by the usage of caspase 3 inhibitor.

5. CONCLUSION AND FUTURE DIRECTION

To conclude, this present study novel demonstrating that the activation of β_2 agonist salbutamol at reperfusion did not protect the ischaemic reperfused rat heart. In the langendroff model, the activation of salbutamol (1nM, 10nM) didn't show any significant effect on the isolated perfused hearts on the onset of reperfusion. But, the administration of salbutamol (100nM) in the isolated rat cardiomyocytes of langendroff model significantly decrease the heart rate compared to the isch/rep. The cardio toxic effect of the beta agonist salbutamol was first showed by this study with langendroff model.

In isolated perfused heart, by increasing the infarct size at the reperfusion the β_2 agonist salbutamol showed the cardio toxicity of the drug. Administration of salbutamol (1nm, 10nm, 100nm) throughout the reperfusion significantly increased the infarct size. To determine whether this cardio-toxic effect of beta agonist was abolished by the beta antagonists, ICI118557, and CGP12177 was administered along with salbutamol 100nm on isolated perfused rat cardiomyocytes. This study also showed the cardio-toxic effect in isolated rat cardiomyocytes. However, the administration ICI and CGP alone at the onset of reperfusion was significantly decreased the infarct size compare to the isch/rep group. Administration of salbutamol in isolated rat cardiac myocytes 30 minutes after the onset of reperfusion significantly increased the cleaved caspase3 activity. The coordination of beta antagonists ICI and CGP with salbutamol was also significantly increased the cleaved capase3 activity in the cardiac myocyte model of hypoxia/reoxygenation. As a final point, the increase in the Isc/Rep injury by the administration of salbutamol, it should be very cautious in the usage of β_2 agonists till further studies are carried out.

The present study was carried out in an in vitro model. Further studies are required in whole animal in vivo model. Besides, the administration of salbutamol (100nM) along with the presence of beta antagonists ICI and CGP showed a significant decrease in the infarct size to risk ratio. Therefore, further studies are required to briefly explain whether t higher concentrations of salbutamol with the beta antagonists can block the toxicity. Furthermore, the administration of salbutamol (0.1nM) with the beta antagonist ICI at the onset of reperfusion abolished the cardio-toxicity by increasing the live cell population further studies are required to determine whether cardio protective agents can block the toxicity. Also, the administration of salbutamol (1nM) with the beta antagonist CGP reversed the decrease in the live cell population when administered throughout the reperfusion. Therefore, further studies are required to find out whether the higher concentration of salbutamol with the beta antagonist CGP will be cardio toxic.

7. References

Ahmet.I., Morrell.C, Lakatta.E.G., Talan.M.I., Chavasse.R.J.P.G., Seddon.P., Bara.A., Mckean.M.C. Short acting β_2 agonists for recurrent wheeze in children under two years of age. (2002). *Cochrane database of systematic reviews.Issue no:3*

Aihua .Hu.., Xiangying Jiao., Erhe. Gao., Walter .J. Said Sharifi-Azad., Zvi .Grunwald.,

Xin .L., Jian-Zhong. Sun. Chronic _-Adrenergic Receptor Stimulation Induces Cardiac Apoptosis andAggravatesMyocardialIschemia/ReperfusionInjurybyProvokingInducibleNitricOxideSynthase-MediatedNitrative Stress.(2006)

Al-Rajaibi.H., Maddock.H. Myocardial injury is reduced by administration of caspase inhibitors at various time points during reperfusion. (2006). *Heart*. 92:5

Andrew.H.W. Lecture Abstract .Agency for Science, Technology and Research. (2007).

Antonsson.B., Montessuit.S., Lauper.S., Eskes.R., Martinou.J.C. Bax oligomerisation is required for channel forming activity in liposomes and to trigger cytochrome c release from mitochondria. (2000). *Biochem.J*. 345:207-215.

Appel.E., Kazimirsky.G., Ashkenzai.E., Kim.S.G., Jacobson.K.A., Brodie.C. Roles of BCL-2 and caspase 3 in adenosine receptor induced apoptosis. (2001). *J Mol Neurosci.* 17:285-292.

Assoufi. B.K., Hodson. M.E. High dose salbutamol in chronic airflow obstruction: comparison of nebulizer with Rotacaps.(1989). *Respiratory Medicine.83:415-20.*

Baines. C.P., Molkentin .J. Stress signalling pathways that modulate cardiac myocyte apoptosis. (2005). *J Mol Cell Cardiology* (38) 47-62.

Barilan.A., Aizikovich.A., Somin.M., Beilinson.N., Basevitch.A., Goland.S., Malnick.S., Myocardial infarction following parenteral beta agonist for asthma in two patients with normal coronary arteries-A call of auction.(2010).Internet journal of internal medicine. 8(1) 0

Barnes.P.J., Pride. N.B. Dose response curves to inhaled -adrenoceptor agonist in normal and asthmatic subjects.(1983). British Journal of Clinical Pharmacology. 15:617-82.

Belevych.A.E., Harvey.R.D. Muscarinic inhibitory and stimulatory regulation of the L-type Ca^{2+} current is not altered in cardiac ventricular myocytes from mice lacking endothelial nitric oxide synthase. (200).*J. Physiol. (London)* 528: 279-289.

Belevych.A.E., Sims.C., Harvey. ACh-induced rebound stimulation of L-type Ca^{2+} current in guinea-pig ventricular myocytes, mediated by $G_{\beta\gamma}$-dependent activation of adenylyl cyclise. (2001).*J. Physiol. (London)* 536: 677-692.

Bennett.J.A., Smyth.E.T., Pavord.I.D., Wilding.P.J., Tattersfield.A.E., Systemic effects of salbutamol and salmeterol in patients with asthma.(1994). *Thorax* ;49:771-774 doi:10.1136/thx.49.8.771

Bognar. I.T., Beinhauer..B, Kann. P., Fuder.H. Different muscarinic receptors mediate auto inhibition of acetylcholine release and vagally-induced vasoconstriction in the rat isolated perfused heart. (1990). *Pharmacol.;341(4):279-87*

Bohulay.O., Frantisek.K. Cardiac ischemia: from injury to protection. (1999).

Braunwald. E., Kloner. R.A. Myocardial reperfusion: a double-edged sword. (1985). *J Clin Invest* (76) 1713-1719

Brad.H., Bart.A., Jesen.O.I., Ann.P., Thomas.M., Gregory.J.S. Toxicogenomic analysis of cardio toxicity in rats.(2008).

Brinks.H.L., Eckhart.A.D. (2010). Regulation of GPCR signalling in Hypertension. Biochim Biophys Acta.

Brodde.O.E., Konschak.U., Becker.K., Rüter.F., Poller.U., Jakubetz.J., Radke.J., Zerkowski.H.R. Cardiac muscarinic receptors decrease with age. In vitro and in vivo studies.(1998). *J Clin Invest; 101(2): 471–478*

Buckberg .G.D., Studies of controlled reperfusion after ischemia. When is cardiac muscle damaged irreversibly.(1986). *J Thorac Cardiovasc Surg* (92) 483-487

Budde. J.M., Morris.C.D., Velez .D.A., Muraki. S., Wang. N.P., Guyton. R.A., Zhao. Z.Q.

Reduction of infarct size and preservation of endothelial function bymultidose intravenous adenosine during extended reperfusion. (2004). *Journal ofSurgical Research* (116) 104-115

Burggraaf.J., Westendorp.R., Schoemaker.R., Sterk.P.(2001).Cardiovascular side effects of inhaled salbutamol in hypoxic asthmatic patients Thorax *2001;56:567-569 doi:10.1136/thorax.56.7.567*

Catherine .G., Fabricant.M.S., Julius.F. Atherosclerosis induced by infection with marke's disease herpes virus in chickens. (1997). American heart association. *Circulation.* ;95:2037-2043.)

Chavasse. R.J.P.G., Seddon. P., Bara. A., McKean M.C. Short acting beta2-agonists for recurrent wheeze in children under two years of age. (2002). *Cochrane Database of Systematic Reviews Issue 3. Art. No.: CD002873. DOI: 10.1002/14651858.CD002873*

Chen. J., Devivo.M., Dingus.J., Harry. A., Li. J., Sui.J. ,Carty.D.J.,Blank.J.L., Exton.J.H., Stoffel.R.H.,Inglese.J., Lefkowitz.R.J., Logothetis. A region of adenylyl cyclise 2 critical for regulation by G protein $\beta\gamma$subunits. (1995). *Science* 268 1166-1169.

Clark., Wayne .M. Reperfusion injury in stroke.(2005). *eMedicine. WebMD. Retrieved 2006-08-09.*

Claudia.F., Ingeid.H., Peter.H., Krammer., Klaus., Michael.D. (1996) .Involvement of the CD95 (APO−1/Fas) receptor/ligand system in drug−induced apoptosis in leukaemia cells *Nature Medicine* 2, 574 - 577 (1996) doi:10.1038/nm0596-574

Colecrafth.M., Egamio.J.P., Sharma.V.K., Sheu.S.S. Signalling mechanisms underlying muscarinic receptor-mediated increase in contraction rate in cultured heart cells. (1998) .*J. Biol. Chem.* 273: 32158-32166.

Collins.R., Clarke.R., Frost.C. (1997). The Treatment of *Coronary Heart Disease*: New Cardiovascular Risk .

Dutta.E.J., Li.J.T. Beta agonists.Med Clin North Am. 2002 86(5):991-1008.

Degterev .A., Yuan .J. Expansion and evolution of cell death programmes. (2008). *Nat. Rev. Mol. Cell Biol.* 9(5), 378–9De

Dheins.S., Vankoppen.C.J., Brodde.O.E. Muscarinic receptors in the mammalian heart. (2001).*Pharmacol. Res.* 44: 161-182.

Dejean .L.M., Martinez-Caballero.S., Manon.S., Kinnally .K.W. Regulation of the mitochondrial apoptosis-induced channel, MAC, by BCL-2 family proteins. (2006). *Biochim. Biophys. Acta* 1762 (2): 191–201. doi:10.1016/j.bbadis.2005.07.002. PMID 16055309.

Downey J.M Cohen M.V., Yang X.M. (1991). The pH hypothesis of postconditioning:in perfused rat liver: evidence for a pH paradox. FASEB ;5:207-210.

Endoh.M., Maruyama.M.,Iijima.T. Attenuation of muscarinic cholinergic inhibition by islet-activating protein in the heart. (1985). *Am. J. Physiol.* 249 H309-H320.

Everett.H., Mcfadden.G. Apoptosis ; an innate immune response to virus infection. (1999).

Trends Microbiol 7 (4): 160–5.

Freeman.T.B., Clichetti.F., Hauser.R.A., Deacon.T.W., Li.X.J., Hersch.S.M., Nauert.G.M. (2000); Causing neuronal death within the HD striatum, implanted fetal neural cells lacking the mutant HD . *97(25):13877-82.*

Freude. B., Masters. T.N., Robicsek. F., Fokin. A., Kostin.S., Zimmermann.R., Ullmann.C., Lorenz-Meyer .S., Schaper. J. Apoptosis is initiated by myocardial ischemia and executed during reperfusion. (2000). *J Mol Cell Cardiol* (32) 197-208

Galagudza .M., Kurapeev. D., Minasian.S., Valen .G., Vaage. J., Ischemic postconditioning: brief ischemia during reperfusion converts persistent ventricular fibrillation into regular rhythm. (2004). *European Journal of Cardio-thoracic Surgery* (25) 1006-1010

Gill.C., Mestril. R., Samali.A. 'Losing Heart: The role of apoptosis in heart disease-a novel therapeutic

target?' (2002) *FASEB J* (16) 135-146

Gottlieb .R.A., Burleson .K.O., Kloner .R.A., Babior. B.M., Engler.R.L. Reperfusion injury induces apoptosis in rabbit cardiomyocytes. (1994). *J Clin Invest* (94) 1621-1628

Gregersen. P..K. Modern genetics, ancient defences, and potential therapies. (2007). *N Engl J Med.* 356 (12): 1263–6. doi:10.1056/NEJMe078017.PMID 17377166.[PMID 17377166]

Gross .G.J. (2003). "Role of opioids in acute and delayed preconditioning". *J. Mol. Cell. Cardiol.* 35 (7): 709–18.

Gupta.R.C., Neumann.J., Boknik.P., Watanabe.A.M. M_2-specific muscarinic cholinergic receptor-mediated inhibition of cardiac regulatory protein phosphorylation.(1994). *Am. J. Physiol.* 266: H1138-H1144.

Han.H. (2001). Oxidative Preconditioning and Apoptosis in L-cells — *JBC*

Harrington .H.A., Ho.K.L., Ghosh .S., Tung .K.C. Construction and analysis of a modular model of caspase activation in apoptosis.(2008). *Theor Biol Med Model* 5: 26. doi:10.1186/1742-4682-5-26. PMID 19077196

Harris.G.F.D.A. Panel Votes to Ban Asthma Drugs. The New York Times. (2008). Available at: http://www.nytimes.com/2008/12/12/health/policy/12fda.html?ref=health. Accessed January 19, 2009.

Hartzell.H.C. Regulation of cardiac ion channels by catecholamines, acetylcholine and second messenger systems. (1988). *Prog. Biophys. Mol. Biol.* 52: 165-247

Hausenloy. D., Tsang.A., Yellon.D. The reperfusion injury salvagekinase pathway: a common target for both ischemic preconditioning and postconditioning. (2005). *Trends Cardovasc Med* (15) 69-75

Hazeki.O., Ui.M. Modification by islet-activating protein of receptor-mediated regulation of cyclic AMP accumulation in isolated rat heart cells. (1981). *Biol. Chem.*256: 2856-2862

Heather.R.C., Charles.S., Roberet.J.L., Walter.J.K., Elizabeth.M. Over expression of the cardiac β2 adrenergic receptor and expression of a beta adrenergic receptor kinase-1 inhibitor both increase the myocardial contractility but have differential effects on susceptibility to ischaemic injury. (1999). *Circ. Res.* 1999;85;1077-1084

Herzig.S., Meier.A., Pfeiffer.M., Neumann.J. Stimulation of protein phosphatases as a mechanism of the muscarinic-receptor-mediated inhibition of cardiac L-type Ca^{2+} channels. (1995). *Pflügers Arch.* 429: 531-538.

Jacob.L., Henning.K., Niels.V., Erik.J., Steffen.H., Kri.S., Peter.C., Lene.H., Marek.T., Jan.S.J., Thomas.E. Cardioprotective Effects of Ischemic Postconditioning in Patients Treated With Primary Percutaneous Coronary Intervention, Evaluated by Magnetic Resonance. (2010).

Jatin .G., Burniston.I., Lip-Bun Tan., David. F. β2-Adrenergic receptor stimulation in vivo induces apoptosis in the rat heartand soleus muscle (2005). *J Appl Physiol* 98: 1379–1386,

Jeck .D., Lindmar .R., Löffelholz .K., Wanke.M. Subtypes of muscarinic receptor on cholinergic nerves and atrial cells of chicken and guinea-pig hearts .(1988). *Br J Pharmacol. Feb;93(2):357-66.*

Jordan J.E., Thourani.V.H., Auchampach. J.A., Robinson .J.A., Wang.N.P., Vinten-Johansen.J. A3 adenosine receptor activation attenuates neutrophil function and neutrophil-mediated reperfusion injury. (1999). *Am J Physil HeartCirc Physiology* (277) H1895-H1905

Kelly.R.A., Balligand.J.L., Smith.T.W. Nitric oxide and cardiac function. (1996) .*Circ. Res. 79: 363-380.*

Kerr J.F., Wyllie A.H, Currie. A.R, (1972). "Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics". *Br. J. Cancer* 26 (4): 239–57.

Kim G.T., Chun.Y.S., Park. J.W., Kim .M.S. Role of apoptosis-inducing factor in myocardial cell death by ischemia-reperfusion. (2003).*Biochemical and Biophysical Research Communications* (309) 619-624

Kin.H., Zhao. Z.Q., Sun.H.Y, Wang. N.P, Corvera. J., Halkos.M., Kerendi.F., Guyton.R.A., Vinten-Johansen .J. 'Postconditioning attenuates myocardialischemia-reperfusion injury by inhibiting events in the early minutes of reperfusion. (2004). *Cardiovascular Research* (62) 74-85

King.N., Hittinger .C.T, Carroll.S.B. Evolution of key cell signaling and adhesion protein families predates animal origins. (2003). *Science* 301 (5631): 361–3. doi:10.1126/science.1083853.PMID 12869759.

Klaus. Ponicke ., Ingrid Heinroth-Hoffmann ., Otto-Erich Brodde. Role of β1- and β2-adrenoceptors in hypertrophic and apoptotic effectsof noradrenalin and adrenaline in adult rat ventricular cardiomyocytes (2003). *Naunyn-Schmiedeberg's Arch Pharmacol (2003) 367 : 592–599DOI 10.1007/s00210-003-0754-z*

Kothakota.S., Toshifumi.A., Christoph.R., Anke.K., Jay.T., Thomas.J., (1997).Caspase-3-Generated Fragment of Gelsolin: Effector of Morphological Change in Apoptosis

Lee.D., Kulick.D., Marks.J. (2006). D Lee, D Kulick, J Marks. Heart Attack (Myocardial Infarction) *by MedicineNet.com* .

Leist.M., Single.B., Castoldi.A.F., Kuhnle.P. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. (1997).

Leitch.A.g., Clancy.L.J., Costello.J.F., Flenley.D.C. (1976). Effect of intravenous infusion of salbutamol on ventilatory response to carbon dioxide and hypoxia and on heart rate and plasma potassium in normal men. *Br Med J ;1:365-367 doi:10.1136/bmj.1.6006.365*

Lodish.H., Berk.A., Matsudaira.P., Kaiser.C.A., Krieger.M., Scott.M.P., Zipursky.S.L., Darnell.J. Microbiology of cell. (2004).

Loffelholz.K.,Pappano.A.J. The parasympathetic neuroeffector junction of the heart. (1985). *Pharmacol Rev.* 37: 1-24

Maak.C.A., Tabas.J.A., Mcclintock.D.E. Should acute treatment with inhaled beta agonists be wiyhheld from patients with dyspnea who may have heart failiure? (2008).*J Emerg Med.*

Maddock.H.L., Gardner.N.M., Khandoudi .N., Bril.A., Broadley. K.J.Protection from myocardial stunning by ischaemia and hypoxia with theadenosine A3 receptor agonist, IB-MECA. (2003) *European Journal ofPharmacology* (477) 235-245

Maddock .H.L., Mocanu.M., Yellon.D., Adenosine A3 receptoractivation protects the myocardium from reperfusion/reoxygenation injury. (2002). *Am J Physiol Heart Circ Physiol* (283) H1307-H1313

Maria .G., Eugenio .M., Mario.C. Pharmacological modulation of b-adrenoceptor function in patients withcoexisting chronic obstructive pulmonary disease and chronic heart failure (2009).

Marmor.M., Penn.A., Widmer.K., Levin.R.I., Maslansky. Coronary artery disease and opioid use .(2004). *Am. J. Cardiol.* 93 (10): 1295–7. doi:10.1016/j.amjcard.2004.01.072.PMID 15135709.

Michal.G., Dmitry.K., Sarkis.M., Guro.V., Jarle.V. (2004). Ischemic postconditioning: brief ischemia during reperfusion converts persistent ventricular fibrillation into regular rhythm *Eur J Cardiothorac Surg* ;25:1006-1010

Murad.F., Chi.Y.M., Rall.T.W., Sutherland.E.M. Adrenal cyclise III. The effect of catecholamines and chorine esters on the formation of adenosine3':5'phosphate by preparations from cardiac muscle and liver's. (1962). *Biol. Chem.* 237: 1233-1238.pathophysiological process.' *International Journal of Cardiology* (100) 179-190

Murry.C.E., Hennings.R.B., Reimer.K.A. Preconditioning withischemia: a delay of lethal cell injury in ischemic myocardium. (1986). *Circulation*

(74) 1124-1136

Nyboe.J., Jensen.G., Appleyard.M. (1989). Risk factors for acute myocardial infarction in Copenhagen. I: Hereditary, educational and socioeconomic factors.*Page 1. European Heart Journal 10, 910-916*

Poller.U., Nedelka.G., Radke.J., Pönicke.K., Brodde.O.E. Age-dependent changes in cardiac muscarinic receptor function in healthy volunteers. (1997). J Am Coll Cardiol.;29(1):187-93

Popov .S.G., Villasmil. R., Bernardi .J. "Lethal toxin of Bacillus anthracis causes apoptosis of macrophages".(April 2002). *Biochem. Biophys. Res. Commun.* 293 (1): 349–55.

Rada.B., Leto.T.L. Oxidative innate immune defenses by Nox/Duox family NADPH oxidases. (2008). *Contrib Microbiol* 15: 164–87. doi:10.1159/000136357

Ragbir.S., Farmer.J.A. (2010).Current atherosclerosis Rep. Dysfunctional high-density lipoprotein.

Ramanujan.K. Common beta-agonist inhalers more than double death rate in COPD patients, CornelandStanfordscientistsassert. (2009).Availableat: *http://www.news.cornell.edu/stories/June06/ Salpeter.COPD.kr.html.*

Reeve .J., Duffy.A., O'Brien .R, Samali.A. Don't lose heart –therapeutic value of apoptosis prevention in the treatment of cardiovascular disease. (2005) *J Cell Mol Med* (9) 609-622

Rosamond.W., Flegal.K., Friday.G. Heart disease and stroke statistics: A report from the American heart association committee and stroke statistics subcommittee. (2007). *Circulation* 115 (5): e69–171.

Rossi.S. Australian Medicine hand book. (2004. *ISBN 0-9578521-4-2.*

Rossinen., Partanen., Stenius.,Nieminen. (1998). Salbutamol inhalation has no effect on myocardial ischaemia, arrhythmias and heart-rate variability in patients with coronary artery disease plus asthma or chronic obstructive pulmonary disease

Salvesen.G.S., Riedl.S.J .Caspase mechanisms. (2008). *Adv. Exp. Med. Biol.* 615, 13–23.

Sato.H, Jordan J.E., Zhao.Z.Q., Sarvotham.S.S, Vinten-Johansen.J. 'Gradual reperfusion reduces infarct size and endothelial injury butaugments neutrophil accumulation. (1997). *Ann Thorac Surg* (64) 1099-1107

Santos .A., Susin., Daugas.E., Ravagnan.L.,Samejima.K., Zamzami.N., Loeffler.M., Costantini. P.,Ferri.K.F. Two Distinct Pathways Leading to Nuclear Apoptosis .(2000). *Journal of Experimental Medicine* 192 (4): 571–80. doi:10.1073/pnas.191208598v1 (inactive 2009-11-14). PMID 10952727. PMC 2193229

Schimmel. K.J., Richel.D.J., Vandenbrink.R.B., Gucheiaar.H.J., Cardiotoxicity of cytotoxic drugs.(2004).Cancer treat rev(2):181-91.

Schulz.R., Boengler.K., Totzeck.A., Luo.Y. (2007) Connexin 43 in ischemic pre- and postconditioning. .*Pharmacol Rev 59: 418-58,*

Shi.H., Wang.H., Lu.Y., Yang.B., Wang.Z. Choline modulates cardiac membrane repolarization by activating an M_3 muscarinic receptor and its coupled K^+ channel. (1999).*J. Membr. Biol.* 169: 55-64.

Shi.H., Wang.H., Wang.Z. Identification and characterization of multiple subtypes of muscarinic acetylcholine receptors and their physiological functions in canine hearts. (1999). *Mol. Pharmacol.* 55: 497-507.

Shen.J.B., Pappano.A.J. On the role of phosphatase in regulation of cardiac L-type calcium current by cyclic GMP. (2002). *J. Pharmacol. Exp. Ther.* 301: 501-506.

Solani.A., Harriss.M.L. (2005). Investigationoftheeffectsofcranberryfractionson atherosclerosis in mice on control of early blight

Spitzer.W., Suissa.S., Ernst.P., Horwitz.R., Habbick.B., Cockcroft.D., Boivin.J., Buist.A., Rebuck.A.The use of beta agonist and the risk of death and near death from asthma. (1992).

Department of Epidemiology and Biostatistics, Montreal General Hospital, Canada.1992

Stemmer.P.M., Ledyard.T.H., Watanabe.A.M. Protein dephosphorylation rates in myocytes after isoproterenol withdrawal. (2000). *Biochem. Pharmacol.* 59: 1513-1519.

Thygesen.K., Alpert.J.S., White.H.D. Universal definition of myocardial infarction. (2007). *Eur. Heart J.* 28 (20): 2525–38.

Vandenabeele.P., Vandenberghe.T., Festiens.N. Caspase inhibitors promote alternative cell death pathways. (2006). *Sci STKE.* (358):pe44.

Vinten-Johansen.J., Kin.H., Sun.H.Y. 'Post-Conditioning reduces myocardial injury by inhibiting reactive oxygen species during reperfusion. (2003).*Circulation* (108) suppl IV 219

Wang.Y.G., Rechenmacher.C.E., Lipsius.S.L. Nitric oxide signalling mediates stimulation of L-type Ca^{2+} current elicited by withdrawal of acetylcholine in cat atrial myocytes. (1998). *J. Gen. Ph*

Wettschureck.N., Offermanns.SMammalian G proteins and their cell type specific functions.

Physiol . (2005).85(4):1159-204.*ysiol.* 111: 113-125

Wijesinghe.M., Perrin.K., Harwood.M., Weatherall.M., Beasley.R. The risk of asthma mortality with inhaled long actingb-agonists.(2008)

Yang. L, Mashima .T., Sato .S .(2003). "Predominant suppression of apoptosome by inhibitor of apoptosis protein in non-small cell lung cancer H460 cells: therapeutic effect of a novel polyarginine-conjugated Smac peptide"

Yasuo.M., Tsutsumi., Takaakira.Y., Yousuke.H., David.M., Hemal.H. (2007).Reactive oxygen species trigger ischemic and pharmacological ...

Yuan.J., Horvitz.H.R. A First Insight into the Molecular Mechanisms of Apoptosis. (2004). *Cell* 116: 53–56. doi:10.1016/S0092-8674(04)00028-5. Retrieved 2006-11-06.

Yoo. M., Kim .W.B, Song. I.S., Kim .C.Y (2009) Effect of DA-6034, Cancer Prevention. Direct Agonist/Antagonist Functions of Dehydroepiandrosterone ... A selective estrogen receptor-{beta} agonist causes lesion regression in . 2(11): 922 - 930.

Zhang.J.Z., Macleod.K.M. Dissociation of negative inotropic effect of carbachol from changes in cAMP and PKA in perfused rat hearts. (1996). *Am. J. Physiology* 271: H404-H409.

Zakharov.S.I., Harvey.R.D. Rebound stimulation of the Camp regulatedCl-current by acetylcholine in guinea-pig ventricular myocytes. (1997). *J. Physiol. (London)* 499: 105-120.

Zhao.Z.A., Sun.H.Y., Wang.N.P., Kerendi.F., Guyton.R.A., Vinten-Johansen.J. Hypoxic post-conditioning reduces cardiomyocyte loss by inhibiting reactive oxygen species-triggered mitochondrial calcium overload. (2004). *Circulation* (108) suppl IV 174

Zhao.ZQ., Vinten Johansen .J. Myocardial apoptosis and ischemic preconditioning. (2002). *Cardiovascular Research* (55) 438-455