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Cardioprotective Role of Sphingosine 1-Phosphate Receptor Agonist Fingolimod (FTY720) in Global Ischemia-Reperfusion Models

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DECLARATIONS

This thesis represents my own work. Wherever, I have also used materials from other investigators, this is clearly stated. This thesis has not been submitted for a degree to any other academic institution. Information derived from published work by other authors has been acknowledged in the text and a references list is given.

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Dedication:

To my beloved father Pir Muhammad (Late)

ABBREVIATION

ABC	Avidin-Biotin Complex
ACLS	Advance Cardiac Life Support
Akt	Protein Kinase B
AMP	Adenosine Mono-Phosphate
ATP	Adenosine Tri-Phosphate
Bad	Bcl-2 Associated Death Promoter
Bax	Bcl-2 Associated X protein
BCA	Bicinchominic Acid
Bcl-2	B cell Lymphoma 2
BLS	Basic Life Support
BSA	Bovine Serum Albumin
CA	Cardiac Arrest
Ca++	Calcium
CABG	Coronary Arterial Bypass Graft
CK-MB	Creatinine Kinase-MB
CO	Cardiac Output
CPB	Cardiopulmonary Bypass
CPR	Cardiopulomonary Resuscitation
cTnI	Cradiac Troponin I
CVF	Collagen Volume Fraction
DMD	Disease Modifying Drug
DNA	DeoxyriboNucleic Acid
ECC	Extra Corporal Circulation
ECLS	ExtraCorporeal Life Support
ECMO	Extra Corporal Membranous Oxygenation
EF	Ejection Fraction
EMA	European Medinces Agency
ERK	Extracellular signal–Regulated Kinases
$FADH_2$	Reduced Flavin Adenine Dinucleotide
FDA	Food and Drug Administration
FFA	Free Fatty Acid
GIK	Glucose Insulin Potassium
GPCR	G protein coupled Receptor
H/E	Hemotoxylin Eosin
H_2O_2	Hydrogen Peroxide
HDL	High Density Lipoprotein
HEP	High Energy Phospahtes
HT	Heart Transplantation

I/R	Ischemia Reperfusion
ICAM-1	Intercellular Adhesion Cellular Molecule 1
IHD	Ischemic Heart Diease
IL-10	Interleukin 10
IL-1β	Interleukin 1beta
IL-6	Interleukin 6
IL-8	Interleukin 8
IPC	Ischemic Pre Conditioning
K+	Potassium
LBBB	Left Bundle Branch Block
LDL	Low Density Lipoprotein
LVAD	Left Ventricular Assisted Device
LVEDP	Left Ventricular End diastolic Pressure
LVEF	Left Ventricular Ejection Fraction
LVESP	Left Ventricular End Systolic Pressure
MI	Myocardial Infraction
Mptp	mitochondrial Permeability Transiting Pore
MRI	Magnatic Resonance Imaging
MVO_2	Myocardial Oxygen Demand
Na+	Sodium
NADH	Nicotinamide Adenine Dinucleotide
NOS	Nitric Oxide Synthase
$PaCO_2$	Aterial Carbondioxide tension
PaO_2	Arterial Content of Oxygen
PBS	Phospahte Buffer Saline
PCI	Percutaneous Coronary Intervention
PKC	Protein Kinase C
PV Loops	Pressure Volume Loops
PVDF	Polyvinyldene Floride
RISK	Reperfusion Injury Salvage Kinase
ROS	Reactive Oxygen Species
S1PR1-5	Sphingosine 1-Phosphate Receptors
SAFE	Surviving Activating Factor Enhancement
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SV	Stroke Volume
TLR9	Toll-Like Receptor 9
TNF-a	Tumor Necrosis Factor alpha
TUNEL	Terminal deoxynucleotidyl transferase dUTP Nick End Labeling
VA	Veno-Arterial
VF	Ventricular Fibrillation
VT	Ventricular Tachycardia

VV	Veno-Venous

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ABSTRACT

Background and purpose: Ischemic Heart Diseases (IHD) are the most common cause of morbidity and mortality. Incidence and prevalence is continuously growing. There is an escalating risk for revascularization or resuscitation in patients with IHD. Recently, it has been reported that sphingosine 1-phosphate receptor agonist plays anti-apoptotic and anti-inflammatory role in ischemia-reperfusion injury. The aim of our study is to investigate the cardioprotective effects of sphingosine 1-phosphate receptor agonist fingolimod (FTY720) in global ischemia-reperfusion injury related to the cardiac arrest, cardiopulmonary bypass, and heart transplantation.

Methods: In all the three experimental studies, global ischemia-reperfusion was achieved by cardiac arrest either by Ventricular fibrillation or cardioplegia (CPB HT) in anesthetized (sodium pentobarbital, 60 mg/ml/kg i.v) and ventilated male Sprague-Dawley rats (300 - 350 g). The global ischemic period lasted 10 min in the cardiac arrest and cardioplegic arrest, but in transplantation, ischemia time was 60 min while reperfusion times were maintained for either 60 min or 24 hours. Where applicable, monitoring was done using electrocardiogram and hemodynamic data recorded at baseline, 1 hour and 24 hours of reperfusion. The level of high energy phosphates was measured. Apoptotic, inflammatory and oxidative markers were analyzed. The statistical significance was considered as $p \leq 0.05$.

Experimental design: In all the above mentioned protocols, 15 animals were used in each of the control and treated groups. The first study examined the cardioprotective potential of fingolimod using the following treatment strategy, treatment administered (post ischemia) immediately at the early phase of reperfusion (fingolimod 1mg/kg i.v) compared to saline controls (0.9% saline). In next two experimental models, treatment administration was done 15 min before ischemic phase (fingolimod 1mg/kg i.v.) compared to controls (0.9% saline), followed by 10 min ischemia and 60 min reperfusion in cardioplegic arrest while 60 min ischemic phase remained from heart explantation to reperfusion, and reperfusion phase lasted for 24 hours following blood and tissue collection

Results: Three experimental models demonstrated significant myocardial protection in Fingolimod treated groups as compared to control groups. Hemodynamic assessment showed improved cardiac function at late phase. Reduced frequency of apoptotic cells and inflammatory mediators were found in the treated group. High energy phosphates were preserved in the treated as compared to control groups. Reactive Oxygen Species (ROS) were also attenuated in the fingolimod-treated group. Increased phosphorylation of Akt and Erk1/2 signaling pathways found in fingolimod treated group as compared to control, these are important part of Reperfusion Injury Salvage Kinase (RISK) and Survivor Activating Factor Enhancement(SAFE) pathways.

Conclusions: The intravenous administration of fingolimod in global ischemia-reperfusion was cardioprotective. Fingolimod cardioprotection appears to be mediated through preservation of high energy phosphates, reduction in oxidative stress, inhibition of apoptosis and inflammation leading to preserved cardiac functions.

In summary, targeting sphingosine 1-phosphate receptors modulation may offer a new potential therapeutic cardioprotective agent to attenuate myocardial damage during global myocardial ischemia and reperfusion.

SECTION-IA

1. Introduction- Myocardial Ischemia Reperfusion Injury

Cardioprotection

Cardiovascular diseases are the most common cause of mortality and an estimated 17.5 million people died last year worldwide i.e., one-third of global deaths (World Health Organization, 2016). About 2.19 million deaths in Europe every year are attributed to ischemic heart diseases (World health federation, 2016). In Europe, Italy is performing the second highest number of cardiac procedures over 100 thousand in 2014 (Eurostat, 2016). The last century has provided major advances in cardiovascular science from non-invasive procedures to interventional, Coronary Artery Bypass Graft (CABG) surgery and cardiac transplantation. In spite of all major advancements, cardiovascular diseases are still the most commonly growing cause of morbidity and mortality in developing as well as developed world. It poses a huge financial burden on the health care management system. Unfortunately, basic cardiovascular science could not achieve a goal of cardioprotection in the era of growing cardiovascular diseases with poor outcomes because of failure in potential basic research translation into clinical settings. Currently, the hot topic of research and discussion is " Myocardial ischemia-reperfusion injury" to prevent irreversible myocardial damage to protect ventricular dysfunction that is leading cause of heart failure. Indeed, recent advances like ischemic pre and post conditioning encourage and optimize toward finding discoveries such as ischemic preconditioning have opened more doors than they have closed and enhanced optimism towards finding the eventual "cardioprotective" mechanisms. The cardioprotection usually defined as "all mechanisms and means that contribute to the preservation of the heart by reducing or even preventing myocardial damage.

The research work presented in this thesis adds knowledge in cardiovascular science about cardioprotection by investigating the effect of sphingosine 1-phosphate agonist fingolimod in different global ischemia-reperfusion *in-vivo* models.

1.1. Brief overview of Myocardial Ischemia

Ischemic myocardial damage results from an inadequate supply of oxygen and nutrients to the myocardium. It is a pathological picture found in a variety of scenarios concerning the heart as in myocardial infarction in the territory, during cardiac surgery to intervene in the heart beating and heart transplant[1].

Since the myocardium is highly dependent on proper coronary perfusion, the sudden interruption of the latter rapidly leads to the anaerobic metabolism, depletion of high-energy phosphates and the onset of anaerobic glycolysis. A quick and early restoration of blood flow in the ischemic tissue is, therefore, essential in all patients that present different conditions associated with local or global interruption of blood flow, to secure recovery of cells if the damage from these immediately is reversible; Reperfusion, despite the undeniable utility, is associated with multiple adverse changes at the cellular level. Consequently, reperfused tissues may lose an additional number of cells in addition to those already damaged by ischemia. Reperfusion itself

can cause damage to the cardiomyocytes, increase the oxidative phenomena and induce apoptosis; this iatrogenic damage can go to constitute up to 50% of the total area of necrotic myocardium[2, 3].

Therefore, considering the counterproductive implications of myocardial reperfusion injury, there is absolute need to put the research to discover new pharmacological approaches aimed at reduction in the incidence of heart failure, left ventricular dysfunction as much as possible on cardiac remodelling and improve the survival of patients who suffered a myocardial ischemic event [3,4].

1.2. Circulatory Arrest

Circulatory arrest is a state of cessation of a mechanical heart activity, confirmed by no pulse, with immediate termination of the blood flow, leading to a rapid depletion of oxygen, with depression of brain function and then loss of consciousness, sleep apnea or agonal breathing in patients on the territory or in patients already hospitalized[5]. Cardiac arrest is further differentiated into two sub divisions, nonfatal and fatal cardiac arrest, by responsiveness or not, to maneuvers of Basic Life Support (BLS) or Advanced Cardiovascular Life Support (ACLS). The incidence of cardiac arrest in the hospital varies according to the type of construction, type of hospital patients and the presence of a primary care team and oscillates between 1 and 7%[6, 7] with survival that is around 20%[8-11].Mortality at a distance varies according to age, pathology and neurological status of patients after discharge from the hospital, and is higher in adults than paediatric population (27% versus 18%)[8].

The cardiac causes are the most frequent etiology for cardiac arrest that comprise 63%[12], of which 35% is contributed by myocardial infarction, 15% pulmonary causes, and 22% by other causes. Other causes includes valvular heart disease, congenital, myocarditis and cardiac tamponade[12].

Extra cardiac causes includes severe acute respiratory failure, severe hypoxemia, severe respiratory insufficiency, pulmonary edema, pulmonary embolism, dissection or rupture of the aorta, cerebral haemorrhage, marked bleeding, sepsis, serious electrolyte disturbances (hypo/hyperkalemia) and poisonings or side effects of medications or anesthetics[13, 14].

About cardiac causes, these alterations include either electrical impulse, or mechanical barriers, and in the case that the Genesis is primitively of one kind or another, you determine finally the ineffectiveness of both components. The most common electric mechanism is represented by Ventricular Fibrillation (VF), followed by asystole, pulseless electrical activity (Electromechanical dissociation,) in which the ECG detects electrical activity, but there is no effective cardiac output, or pulseless Ventricular Tachycardia (VT). The mechanical mechanisms include the rupture of the ventricle, pericardial tamponade, mechanical obstruction of a coronary

artery and the acute rupture of a large caliber vessel.The immediate consequence is the absence of systemic perfusion. The acute myocardial infarction is characterised by an evident myocardial necrosis that presents clinically as[15]:

1. Patient death preceded by a typical symptom of myocardial ischemia and electrical signs of ischemia or Left Bundle Branch Block (LBBB) not present in previous ECG, before a possible check of positivity of biochemical markers of myocardial damage.

2. Raising or reduction of fingolimod markers higher than 99 percentile of reference values, accompanied by at least one of the following:

- Symptoms related to myocardial ischemia
- Appearance of Q waves on ECG
- ECG appearance of T wave abnormalities or myocardial infarction or LBBB
- The appearance of myocardial substance loss or alterations of motility of the ventricular walls highlighted with ultrasound or other myocardial imaging tests.
- Coronary thrombosis or any possible stent, highlighted with coronary angiography or an autopsy

3. Following the intervention of coronary angioplasty or stent placement if:

Increase Troponin C (cTn) (> 5x99 percentile values) in patients with normal baseline or 2% increased of baseline values for patients with baseline values increased and simultaneous presence of symptoms related to myocardial ischemia, appearance of Q waves on the ECG, appearance of alteration of the T wave, myocardial infarction, LBBB, myocardial, ventricular dyskinesias, substance loss , evidence of coronary thrombosis and eventual stent, or in case of specific complications during the procedure.

4. Following coronary artery bypass surgery

Increase of cTn (10x99 percentile > reference values) in patients with normal basal values and simultaneous presence of presence of symptoms related to myocardial ischemia, or appearance of Q waves on the ECG, or appearance of alteration of the T wave, myocardial infarction or LBBB, or loss of myocardial ventricular dyskinesias, or substance loss or evidence of coronary angiography and possible highlighted by stent or autopsy.

In the field of cardiac surgery, in adult patients, the incidence of fatal cardiac arrest goes from 0.7 to 2.9%[16, 17]while the acute myocardial infarctions range from 2 to 15%. In pediatrics, cardiac arrest is more frequent in patients with complex congenital heart disease perioperative (6%)[18], mainly its common during cardiac catheterization (1%)[19]and is a frequent cause of death in the follow-up (22%) of deaths related to congenital heart disease [20].

1.3. Myocardial Ischemia; Ischemic heart diseases

The main conditions under which patient may experience an ischemic infarction are Ischemic heart disease and Cardiopulmonary bypass (CPB). The term "Ischemic Heart Disease*"* (IHD) refers to a series of clinical pictures all share an underlying myocardial ischemia, generated spontaneously. Ischemic heart disease is still a major cause of death and disability worldwide, and it associates more healthcare costs compared to other chronic diseases. In the United States 13 million people are affected, out of them, more than 6 million suffering from angina pectoris, and more than 7 million have already had a MI. Although this disease is increasing in lower income classe. In both Europe and the United States, mortality was reduced by providing treatment, and by better control of risk factors regarding prevention. Men constitute 70% of all patients with angina pectoris, and an even greater percentage of them are younger than 50 years [21].

The main risk factors include genetic predisposition, a high-calorie diet and high in fat, smoking and lack of physical activity, which together with high plasma levels of Low-Density Lipoprotein (LDL) cholesterol, low levels of High-Density Lipoproteins (HDL), hypertension, diabetes mellitus, age over 75 years, morbid obesity, peripheral myocardial ischemia and/or prior cerebrovascular disease and myocardial infarction, are all risk factors for coronary atherosclerosis.

The atherosclerotic disease represents the overall main cause of heart disease, being able to determine a more or less marked degree of occlusion of coronary arteries (coronary artery disease) level[22]: its starting point is an alteration of endothelial functions including the regulation of vascular tone, retaining control of adhesion and diapedesis of inflammatory cells. When these functions are lost, it is inadequate to determine vasoconstriction, thrombus formation in the lumen and an abnormal interaction by the vascular endothelium activated with erythrocytes, platelets, and monocytes. These changes in endothelial defensive capabilities lead to the formation of atherosclerotic plaque as a result of the accumulation of fat subintimal smooth muscle cells, fibroblasts, and the intercellular matrix; these collections are distributed unevenly in different portions of the epicardial coronary circulation.

The site of the obstruction influence clinical outcomes and their severity, so when it is borne by the main left coronary artery and proximal left anterior descending can be very dangerous for the patient's life. Very often, in the case of reductions in coronary patency, the creation of collateral circulation which may, at rest but not in the case of increased blood flow demand, to overcome the myocardial perfusion. Other pathological conditions such as vascular spasms (Angina Prinzmetal's variant), vasomotor physiological phenomena borne of a coronary artery stenosis, coronary arterial thrombi, emboli, abnormal vascular dilating capacity, impaired constrictions (microvascular angina), and far more rare in adult congenital anomalies, may also reduce coronary blood flow and myocardial perfusion.

In addition to inadequate adaptations of coronary blood flow, myocardial ischemia can also occur when oxygen demands are exceedingly increased. Obviously, this happens especially when coronary blood flow reduced, as in a left ventricular hypertrophy caused by aortic stenosis or hypertensive patient. An alteration in the transport capacity of oxygen by hemoglobin, for example under conditions of severe anaemia or carbon monoxide poisoning conditions, rarely can determine an ischemic event but can reduce the threshold level needed for moderate coronary obstruction manifests clinically.

1.4. Extra Corporeal Circulation

Extra Corporeal Circulation (ECC) is essential for cardiopulmonary bypass in the course of a cardiac surgery and Extra-Corporeal Membrane Oxygenation (ECMO) in the course of the Extra Corporeal Life Support (ECLS)[23].

In the course of ECC, a systemic inflammatory response develops, which contributes to the lung and kidney damage. Negative outcomes borne by these and other organs are supported by different mechanisms, such as surgical trauma, the hemodilution, endothelial damage induced by edema, ischemia-reperfusion injury borne by these organs and blood components.

In the course of surgery with ECC, myocardial ischemic damage occurs can be divided into two main phases: an antecedent ischemia, as a result of a pre-existing coronary artery disease, hypotension or ventricular fibrillation; protected ischemia, which begins with the infusion of cardioplegia and connection of cardiopulmonary bypass. Reperfusion injury sustained during intermittent infusions of cardioplegia, after removal of the aortic clamp, or after discontinuation of cardiopulmonary bypass. Reperfusion injury may itself be divided into early ($\leq$ 4 hours) and a late stage (from 4 to 6 hours).

1.5. Physiology of myocardial perfusion

At baseline, myocardial oxygen extraction almost maximum at rest and on average reaches about 75% of the arterial oxygen content [3], so any increase in the oxygen demands by cardiomyocytes can be satisfied only by a proportional increase in coronary blood flow and oxygen supply, viable firstly by vasodilatation of the coronary arterioles [24]. In addition to the coronary blood flow, oxygen supply is determined directly from the arterial content of oxygen (PaO2): it is equal to the product of the haemoglobin concentration and arterial oxygen saturation, plus a small amount of oxygen dissolved in plasma.

The basal requirement of myocardial oxygen is low (15%), and the cost of activation power is insignificant when the mechanical contraction ceases during diastolic shutdown (as in cardioplegia) and decreases during ischemia. The myocardial

oxygen demand is assessed clinically by multiplying heart rate-systolic pressure, resulting in the so-called "double" as myocardial contractility and wall stress cannot be measured in clinical practice. Normally the myocardium regulates the flow of oxygenated blood depending on the required variables in oxygen, to prevent any hypo perfusion of cardiomyocytes and subsequent ischemia to necrosis: a metabolic adjustment takes place in the course of physical activity or emotional distress, where the increased oxygen demands and energy substrates affect coronary vascular resistance; an autoregulation occurs instead of the physiological changes in blood pressure to maintain within a normal range the coronary flow.

The main determinants of myocardial oxygen consumption (myocardial oxygen demand, MVO_2) are essentially the heart rate, left ventricular contractility and the systolic pressure (or wall of the stressed myocardium). To meet the myocardial oxygen demands is in turn determined by appropriate respiratory function, levels of oxygen saturation, haemoglobin concentration by appropriate coronary circulation.The blood flow through the coronary arteries to the myocardium is influenced by the diameter and vascular tone, by the presence of collateral circulation, from the perfusion pressure determined by the pressure gradient between the aorta and coronary arteries and between arteries and capillaries endocardiac (being the direct flow from endocardium to epicardium), heart rate, which influences the duration of diastole in inverse proportion. Therefore, the heart rate is a determinant of both demand and supply of oxygen.

1.5.1. Role of coronary circulation

The blood flow in the coronary arteries is most represented during the filling phase and diastolic relaxation. The overall resistance of Coronary Vascular bed can vary considerably, with a concomitant change in the blood flow, myocardial demand, while draws a constant and high (70%) amount of oxygen from the blood. The coronary vascular resistance is primarily determined by the arterioles and intramyocardial capillaries, while large epicardial arteries are less influenced. The coronary vessels are the arteries so-called "functional end", meaning that there are anastomotic branches, but they are insufficient to establish a side circle functionally valid. Therefore, the area of coronary artery perfusion of a given place distal to the occlusion site represents the region at greater risk of MI. When coronary blood flow is reduced significantly for a time ranging between 20 and 40 minutes, myocardial infarction begins to develop from the centre of the area at risk, from the sub endocardial layer, and then spread to subepicardial layers and at the edge of the area at risk with the passage of time.

The evolution of myocardial infarction reflects the distribution of coronary blood flow pattern, stream most represented in the outer layers of the myocardium and the margins of the area at risk [25, 26]. The evolution of myocardial infarction also depends

on the species concerned and the presence or absence of efficient collateral circulation: rodents such as have a high heart rate and tend to have a rapid evolution of myocardial necrosis[26].

Among the various aspects that can influence the development of myocardial infarction, in contrast to previous knowledge, the hemodynamic conditions of the subject seem to have a vital role; only the heart rate to some extent, can influence the progression of myocardial necrosis[27].

The primates possess few collateral circulations innate but are relatively resistant to ischemic myocardial infarction; there is not any MI for a time of coronary occlusion between 40 and 60 minutes, and even after the 90 minutes the size of the infarcted area still lower than for example in that of pigs. In humans even after a period of coronary occlusion between 4 and 6 hours, from 20% to 50% of the area at risk remains vital and therefore it can be retrieved through the reperfusion. The myocardium can be "saved" even after twelve hours of clinical onset, resulting in an improvement of the prognosis of the patient[28, 29]. It is unclear at the moment what is a mechanism in human's this ability to resist blood flow deprivation for so long, if the presence of well-developed collateral circulation at the time of the stroke, or an inherent resistance to ischemic damage, or if it reflects a preconditioning ischemia-reperfusion due to previous episodes of ischemic reperfusion. Also, the use of medications such as beta-blockers, Inhibitors of Renin-angiotensin system, Statins or P2Y2 receptor antagonists, can provide a pre-existing cardioprotection and thereby reduce negative outcomes of myocardial ischemia-reperfusion injury[30].

1.6. Consequences of Myocardial Ischemia

The consequences of inadequate myocardial perfusion affecting the metabolic, mechanical and electrical aspect of the heart: is to be altered contractile function of the myocardium, determining areas of hypokinesia or Akinesia (visible by echocardiography) more or less extensive or even in severe cases protrusions of the wall, thus leading to a significant impairment of myocardial pump function. Besides the above mentioned metabolic abnormalities, myocardial ischemia also determines electrical and mechanical alterations: as for the first it basically concerns the phase of Ventricular Repolarization, whose deterioration is visible to the electrocardiogram as reversal of the T wave and, when more severe, from elevation or ST segment depression, respectively if it is transmural necrosis or subendocardial infarction. Also, the ischemic myocardial damage leads to cardiac electrical instability that would result in possible isolated premature ventricular beats, tachycardia or atrial fibrillation.

Most of the relative myocardial ischemia mortality and morbidity is still to be connected to the remodeling that follows the same; in fact, chronic systolic heart failure and an inappropriate remodeling of the left ventricle are crucial determinants

of morbidity and long-term clinical outcomes[31]. The cardiac remodeling is not to be considered solely as a late event in the post-MI but is closely related to the starting size of infarct associated with it. Therefore, an adequate remodeling following a severe ischemia-reperfusion can greatly improve patient outcomes[4].

The most typical and negative changes of left ventricle that occur after a myocardial infarction are represented by dilatation, hypertrophy, and change in spherical room: the hypertrophy is an initial compensatory mechanism that takes place in the remote area of myocardium not subject to MI, useful if limited in time, since a prolonged myocardial hypertrophy causes a concomitantly increase in the left ventricular mass. The adaptive process associated with long-term adverse clinical outcomes[32]. Another feature of left ventricular remodeling is ischemic myocardial interstitial fibrosis. From a biochemical point of view at the base of the left ventricular remodeling and its adverse effects, a fundamental role is played by the activation of Akt and Erk 1/2 that is in the long term, leads to deleterious hypertrophy of cardiomyocytes when the short term limits their apoptosis. Another of the most important markers of this counterproductive adaptation to the post myocardial infarction is the activation of a long-term neurohormonal response, in particular, the sympathetic nervous system and the Renin angiotensin aldosterone system: both the levels of aldosterone[33] both of catecholamines are in fact useful predictors of cardiovascular mortality in patients post myocardial infarction[34].

1.6.1. Ischemia-related Metabolic Alterations

The myocardial ischemia causes important alterations that usually occur in a temporal sequence defined "ischemic cascade," which first involves the metabolic aspect, then the mechanical and finally that of the myocardial electric cells. Unlike the hypoxia, where due to reduced availability of oxygen, can continue the production of glycolytic energy by anaerobic glycolysis. In ischemia, due to the reduced supply of oxygen and nutrients due to a reduced blood flow, it compromises the availability of substrates for Glycolysis. Under physiological conditions, the myocardium metabolizes fatty acids(60-90% of which constitute the total energy source), and glucose(10-40% of total energy) by transforming them into carbon dioxide and water.

The arrest of myocardial perfusion can be accidental when it takes place on the territory or iatrogenic to allow intervention, quickly leads to the arrest of aerobic metabolism. The fatty acids cannot be oxidised so that, through anaerobic glycolysis, glucose is metabolized to lactic acid resulting in the release of free fatty acids causing arrhythmia and inhibits mitochondrial K-ATP channels, who can no longer maintain mitochondrial membrane potential, and glycogenolysis is gradually slowed and inhibited the increase in NADH and FADH2 and the drop in pH [8-10]. This is followed by the formation of lactates, a decrease in intracellular pH, the

progressive reduction of the high energy phosphates such as , as well as the accumulation of several catabolites, including those resulting from the pool of Adenine nucleotide. The reduction of the reserves of ATP affects the ion exchange at the level of the sarcolemma, with increased Na^+ and K^+ intracellular reduction due to its outflow, causing the onset of cardiomyocytes death.

The increase in intracellular Na^+ results in an increase of intracellular calcium through an increased exchange of Na^+ and Ca^{2+}. The reduced availability of ATP also lowers the intake of sarcoplasmic reticulum Ca^{2+} and reduces the extrusion of Ca^{2+} from the cell. The increase in intracellular Ca^{2+} produces an overload of Ca^{2+} in mitochondria and that further decrease ATP production. Therefore Ca^{2+} is playing a central role in the vicious cycle that leads to irreversible damage of the cell in case of persistent ischemia. If hypoxia continues, the aggravation of ATP depletion causes more damage: the cytoskeleton is dispersed, resulting in the disappearance of ultrastructural characteristics as microvilli and the formation of small protrusions on the cell surface. At this stage the mitochondria are swollen to an inability to control their volume, the endothelial pattern remains dilated, and the whole cell becomes particularly voluminous, with increased water content and the concentration of sodium and potassium chlorides. Due to the high dependence of myocardial function by oxygen, severe ischemia induces loss of contractility within 60 seconds. This can lead to acute heart failure even before myocardial necrosis occurs. If the availability of oxygen is restored, all these changes are reversible; On the contrary, if ischemia persists, it reaches to irreversible damage and necrosis.

A myocardial ultrastructural alterations, cellular and mitochondrial swelling, glycogen depletion are potentially reversible. While severe ischemia lasting for at least 20-30 minutes leads to irreversible damage, with necrosis of cardiomyocytes. The ultrastructural evidence of irreversible myocardial damage occurs only after a serious and prolonged myocardial ischemia. A key characteristic of the initial phases of myocardial necrosis is the loss of the integrity of sarcoplasmic membrane, which allows the intracellular molecules moves to cardiac interstitium and then into the circulation. Necrosis is typically complete within 6 hours after the onset of severe myocardial ischemia. From the morphological point of view, the irreversible myocardial damage is associated with a serious mitochondrial swelling, an extensive cytoplasmic membrane damage with the appearance of myelin figures, and a bulge of lysosomes. In mitochondrial matrix, large dense bodies and amorphous develops. In the myocardium, these elements confirms to the irreversibility of the lesions and can be observed after 30-40 minutes of ischemia.

A fatal injury begins to be determined after 20 minutes of the coronary blood flow occulusion, and myocardial exclusion progresses as a wavefront from subendocardial to avoid the epicardium. After 60 minutes, the third most internal wall is irreversibly damaged. After 3 hours it remains only a fabric edge subepicardial, and the trans mural infarct extension is complete after 3-6 hours of

occlusion [35]. Instead, the entire experimental subendocardial infarction undergoes irreversible damage within an hour of the occlusion, and myocardial necrosis progression is 4-6 hours after widely by coronary occlusion. The apoptosis of cardiomyocytes has been identified as a fundamental process of all stages of myocardial infarction, suggesting that it might be largely responsible for the death of cardiomyocytes during the acute phase of myocardial ischemia, as well as the progressive loss of cells survived during the first subacute and chronic stages[36]. Apoptosis also seems to play a significant role in the deterioration of left ventricular function in ischemic regions [37].

This may suggest that the inhibition of apoptosis may limit the loss of cardiomyocytes induced by ischemia, particularly in chronic ischemic myocardium reducing left ventricular remodeling and improving the prognosis. The way of apoptosis is primarily triggered by the liberation of proapoptotic molecules from damaged mitochondria, called intrinsic or mitochondrial pathway. The latter is the result of increased mitochondrial permeability and release proapoptotic molecule within the cytoplasm. The release of mitochondrial proteins in the cytoplasm break the delicate balance between pro-and anti-apoptotic members of the BCL family. During normal conditions, anti-apoptotic proteins reside in the cytoplasm and in the mitochondrial membranes where they control the mitochondrial permeability and prevent the leakage of mitochondrial proteins.

The anti-apoptotic Bcl-2 and BCL-X factors are antagonized by the pro-apoptotic proteins. The combination of these events leads ultimately to the release of cytochrome into the cytoplasm; a mitochondrial protein can trigger, along with other within a structure called the apoptosome, caspase 9, the initiator of the mitochondrial pathway. A group of cysteine aspartate protease, which in normal cells reside in the cytosol as inactive form (pro-caspases). This terminates at the final phase of apoptosis, also common to the intrinsic pathway activation, characterized by activation of effector caspases, which ultimately determine the characteristic DNA cleavage, fragmentation of the nucleus and lyses of myofibrils. Apoptotic cells and their fragments are then recognized by different receptors on macrophages and finally swallowed. In the ischemic heart then we witness an alternative activation and inactivation of multiple genes and their products.

Among the events resulting from myocardial ischemia are found metabolic acidosis, reduced production of Adenosine Tri-Phosphate (ATP), the loss of sodium-potassium ATPase pump, as well as the release of chemicals that stimulate chemo- and mechanoreceptors innervated by non-myelinated nerve cells in the structure of cardiomyocytes and around the coronary arteries[38]. Among the substances which are released there are lactate, serotonin, oxygen free radicals and adenosine[39, 40]; in addition to these there are others, such as serotonin, 5-hydroxytryptamine, thromboxane A2 coming from platelets that are frequently found aggregated in correspondence at the coronary obstruction, which may be partly responsible for

myocardial ischemia and angina [41, 42]. In the ischemic heart there is the considerable production of Reactive Oxygen Species (ROS), or oxygen molecules with an excess of electrons that make it chemically very reactive: ROS, using the phenomena of lipid cell membrane constituents, can damage of cardiomyocytes and thus contribute to ischemic damage.

1.6.2. Myocardial Stunning and hibernation

The extent and nature of myocardial damage depend largely on how long and how much has been reduced coronary blood flow [43, 44]. In fact, a cessation of coronary blood flow lasting less than 20 minutes results in a reversible damage represented by the contractile dysfunction associated with a complete although slow recovery after myocardial reperfusion a phenomenon referred to as "myocardial stunning" [45, 46]. The mechanism underlying this prolonged contractile dysfunction comprises firstly an increased production of reactive oxygen species, which occurs early during reperfusion [47], and an alteration of coupling between excitation and contraction of cardiomyocytes due to oxidative modification of Sarcoplasmic reticulum and contractile proteins. Another phenomenon which follows repeated brief episodes of coronary artery occlusion or to the reduction of moderate coronary blood flow but more prolonged is the so-called "hybernating myocardium" which consists of a reduced contractile function of the myocardium which remains vital and subject to a possible recovery following the reperfusion(Fig. 1.1). The hibernating myocardium presents both aspects of the damage, such as loss of contractile proteins, fibrosis and abnormal donut-shaped mitochondria, both signs of adaptation which has met, such as altered expression of mitochondrial proteins, and proteins related to cardioprotection [48, 49].

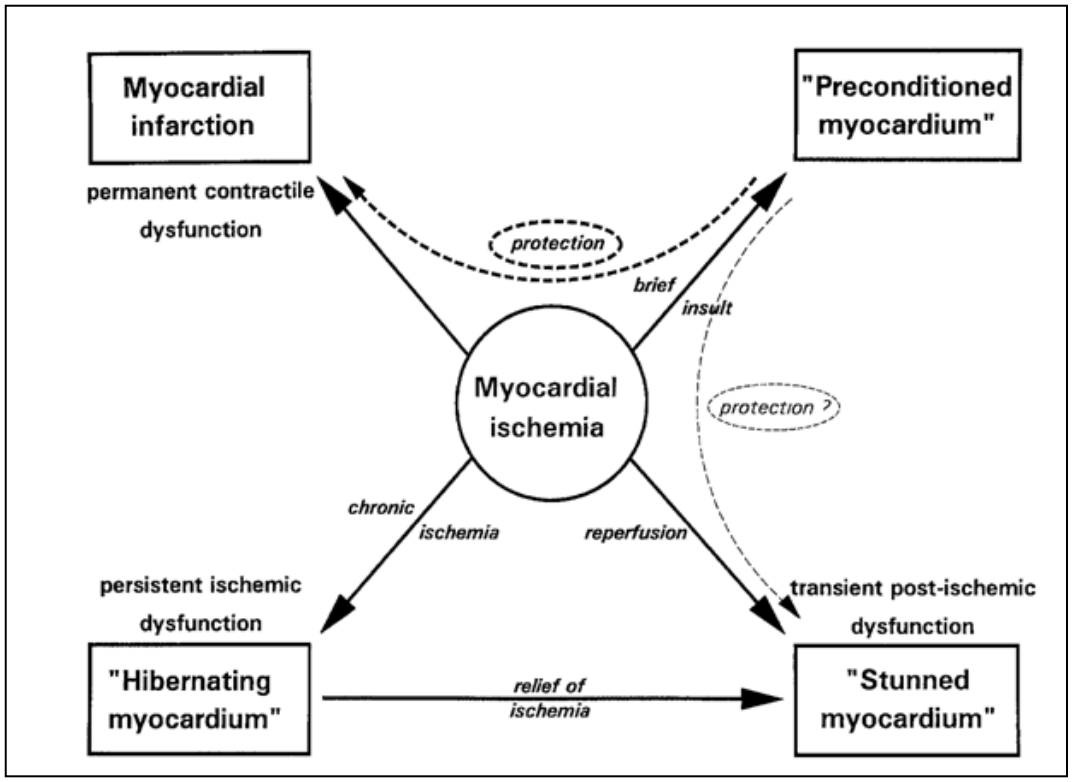


Figure 0.1. *Myocardial Ischemia may present as reversible myocardial dysfunction in association with reversible electrocardiographic changes and angina pectoris. Additional consequences of myocardial ischemia include: (1) myocardial infarction with permanent contractile dysfunction caused by severe long lasting ischemia, (2) hibernating myocardium as a result of chronic hypoperfusion, (3) stunned myocardium after reperfusion of ischemic myocardial tissue; and (4) preconditioned myocardium after brief ischemic insults. The preconditioned myocardium may in turn offer protection during a subsequent ischemic bout and has been demonstrated to reduce infarct size in animal models. It is not clear at present weather the preconditioned myocardium is capable of having the recovery of stunned myocardium. Adapted from (Margreeth B et. Al. 1996)*[50].

1.7. Treatment option of myocardial ischemia

Although a timely reperfusion of the myocardium at risk represents the most effective way to restore homeostasis of myocardial cell, it is also important to maintain an optimal balance between supply and demands of oxygen, so as to save the widest possible area of myocardium at risk around the areas most damaged by ischemia. The progression of irreversible damage is in fact accelerated by factors that increase myocardial oxygen consumption as tachycardia or reduce its contribution as in arterial hypotension. Potential mechanisms and measures to counteract the lack of oxygen at myocardial cells include firstly the reduction of myocardial oxygen consumption: this will lead to a prolonged survival by saving energy on the part of the myocardial cells and to a reduction in the catabolites

production.Since your heart rate is the main determining factor in myocardial consumption of oxygen (which is halved with halving the number of heartbeats), its reduction is a crucial mode of protection from myocardial ischemia[51]by keeping the patient at rest, possibly using a light sedation and through the use of beta-blockers medications, which reduce the myocardial work, also reducing blood pressure (after load) and contractility.

Although there are not always unique data about the deleterious effects of Free Fatty Acids (FFA)[52] is considered. It is believed that this metabolic pathway in ischemic conditions presents several disadvantages to the myocardial cells. Indeed, in addition to a higher oxygen consumption, also involves the inhibition of glucose oxidation and an increase in the production of lactate and protons, which further depresses myocardial function in ischemic areas. Furthermore, the FFA alter cellular ionic homeostasis leading to an increase of arrhythmic substrate. Therefore, therapeutic interventions to partially inhibit the uptake and oxidation of FFA and to promote the use of glucose utilization during ischemia should have favorable effects on myocardial cells [53].

It has been long used a solution of Glucose-Insulin-Potassium (GIK) with the aim of increasing Glycolysis and reduce the uptake of FFA and metabolism of myocardial cells during Acute Myocardial Infraction (AMI), and even the administration of insulin alone was proposed with a similar goal. The effects of GIK solution (or insulin alone) in preserving the integrity of cardiomyocytes in this context, however, remain controversial. In fact, some studies have shown favourable effects on survival with this approach, both in diabetic patients and in non-diabetics, but other studies have failed to confirm these findings[53].

1.7.1. Pre and Post conditioning

The 2-5 minute typically transient ischemia, it becomes metabolically more resistant to any possible subsequent more prolonged ischemia; this phenomenon is called ischemic preconditioning. It provides protection against ischemia occurring in 2 hours after transient ischemic preconditioning (early preconditioning), but also against a late-occurring after 24 hours and ischemia for up to 72 hours after the episode of ischemic preconditioning (delayed or chronic preconditioning) [54]. This reduces the size of myocardial necrosis by reducing the infarcted area of 40-75%and protects the heart from ischemia-induced damage. A key role is carried out in particular by the Protein Kinase C (PKC), and specifically its isoform PKCε, it seems like a central mediator of the IPC (Fig. 1.2). In fact, various substances that accumulate in the interstitium during myocardial ischemia and that are involved in the IPC have in common the ability to activate PKC through the activation of Phospholipase C (such as bradykinin, adenosine, norepinephrine, opioid) [55, 56]or

through other routes, such as the activation of protein kinase G (as in the case of nitric oxide) [57].

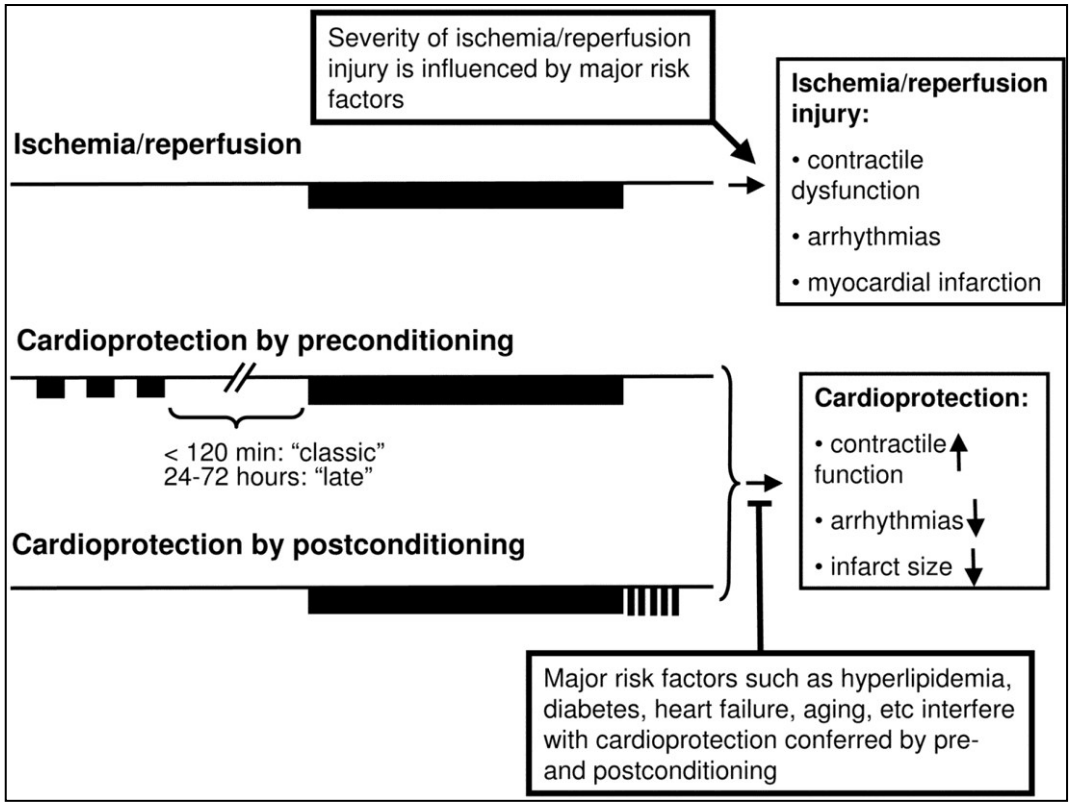


Figure 0.2 *The graphical representation of ischemia reperfusion injury and cardioprotection by pre- and postconditioning (adapted from Ferdinandy P. et al. 2007)*[58].

The key event finals of the IPC, resulting in the activation of PKC, seems to be the opening of ATP-sensitive potassium channels (KATP channels) and the cytoplasmic membrane of mitochondria is opening which is mediated by phosphorylation of proteins channel by precisely PKC and that causes a reduction of the calcium influx and cellular energy expenditure. The IPC can be blocked by glibenclamide an antagonist of K-ATP channels[59].

The formation of low amounts of ROS after the brief period of ischemia, although insufficient to cause cellular damage, may be able to activate cellular mechanisms involved in the Cardioprotection. Low concentrations of ROS seems to activate certain enzymatic pathways involved in IPC (Guanylate Cyclase, cyclooxygenase, tissue factor, tyrosine) [60]. Moreover, some substances capable of generating ROS (acetylcholine, bradykinin, opioids, anaesthetics) are also known as preconditioning reagents[61].

Finally, recent data suggest that preconditioning effect against ischemic myocardial damage can be achieved by transient episodes of ischemia induced in other areas of the body (for example, lower limbs)[62], a phenomenon termed remote

preconditioning.A final protective mechanism, the post-conditioning myocardial infarction, is the ability to provide pharmacological agonists administered by throwing an intermittent ischemia or heart at the time of reperfusion; this protection mechanism by myocardial ischemic injury has the greatest potential to influence the irreversible damage because it can be induced after myocardial ischemia is established, rather than requiring pre-treatment[63].

1.7.2. Reperfusion

The dependence of myocardial recovery from treatment time applies particularly to the patients treated with fibrinolysis or PCI[64]. This time dependence can be especially critical for fibrinolysis due to decreased efficacy of fibrinolytic drugs with the progressive organization of coronary thrombi over time. The study conducted by De Luca et al.[64] demonstrated how every minute of delay at the beginning of the treatment, such as performing a primary angioplasty, engrave on mortality to one year, even after considering the basic conditions of the patient more precisely it has been seen as the one-year mortality risk is increased by 7.5% for every 30 minutes of delay in initiation of treatment(Fig. 1.3).

The cornerstone of myocardial ischemia treatment consists of procedures enabling a rapid restoration of coronary blood flow in the area of ischemic myocardium[65]. This is a concept not older than 40 years reported by Ross and Associates[66, 67] who first said that 180 minutes of reperfusion after coronary occlusion reduces the infarct size in dogs. These findings were quickly transferred to patients suffering from acute myocardial infarction who underwent Percutaneous Coronary Intervention (PCI) or thrombolysis to restore blood flow to the myocardium[44, 68].

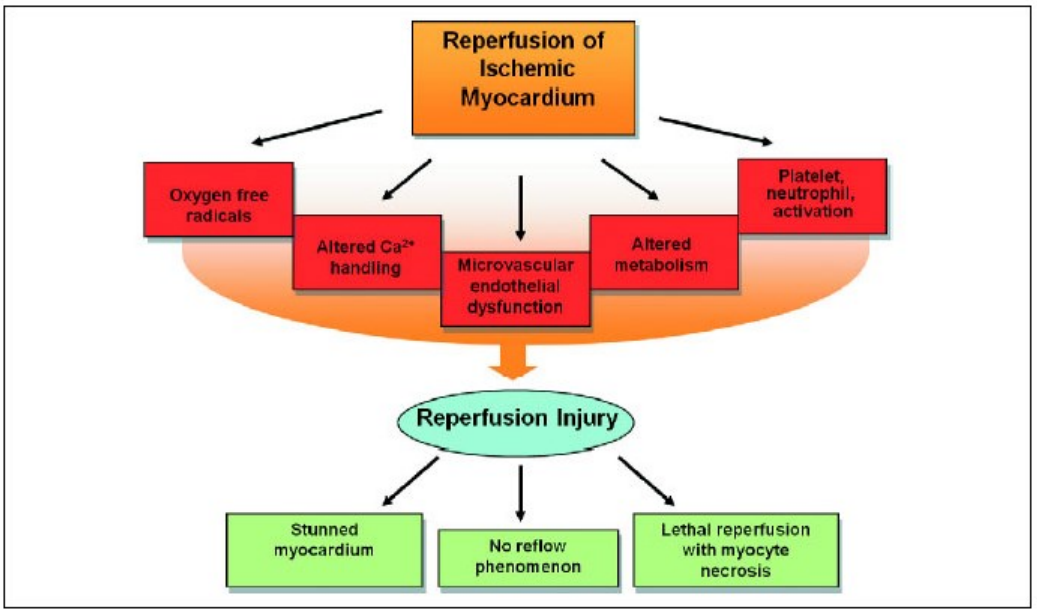


Figure 0.3. *Mediators in myocardial reperfusion injury Reperfusion injury results from several complex and interdependent mechanisms that involve the production of ROS, alterations in intracellular calcium handling, microvascular and endothelial cell dysfunction, altered myocardial metabolism, and activation of neutrophils and platelets. Reperfusion injury is manifested as stunned myocardium, reversible microvascular injury, and lethal myocyte necrosis(adapted from M. Zubrzycki et al. 2013).*

The prevention of cell death by restoring blood flow depends on the severity and duration of pre-existing ischemia. When more rapidly blood flow is restored, the greater will be the recovery of left ventricular systolic function improving diastolic function and the reduction in overall mortality.

The benefits of coronary reperfusion were already established, Krug et al. [69] and then Kloner et al. [70] showed the drawback in their studies conducted on dogs, the so-called "coronary no-reflow phenomenon", which is the absence of reperfusion of the ischemic area with terminal coronary occlusion. According to these studies, while after 40 minutes, long enough to cause irreversible damage to most cardiomyocytes, you are to determine little or no change in the pattern of perfusion of large areas of previously ischemic tissue. After 90 minutes of transient ischemia following instead of a reduced or absent perfusion of large areas of ischemic tissue. The no-reflow phenomenon [71]is found in approximately 35% of patients undergoing reperfusion treatments, and its incidence increases with increasing delay in the start of reperfusion and is associated with a poor prognosis in patients with acute myocardial infarction undergoing reperfusion therapy [72]. Also to note how the Interventional or surgical revascularization may actually, in turn induce a myocardial infarction collateral to the same procedure.

Despite considerable improvements in cardioprotection intraoperative cardioplegic arrest (cessation transient contractile activity of the heart, produced by infusion in

coronary circulation hypothermic conditions solutions based on potassium chloride), which allows you heart surgery while protecting the muscle from ischemic damage due to the interruption of blood flow in the coronary arteries, hypothermia and many more advanced surgical techniques. It is not always possible to use surgical reperfusion through CABG promptly. Although primary angioplasty compared with thrombolysis is able to get a higher rate of reperfusion in patients "late" with respect to the appropriate time to attack the coronary occlusion, it cannot avoid or prevent the same myocardial necrosis, which is closely related to the duration of the occlusion especially in high-risk patients[73, 74]. The size of infarcted area turns out to be the main determinant of the long-term mortality of chronic heart failure, and therefore, the ability to limit the extent of the myocardial damage remains one of the main objectives[44].

1.8. Ischemia Reperfusion Injury

Although the primary purpose is the timely restoration of coronary blood flow to get a reduction in the infarct size and an improvement in ventricular function, the same reperfusion may result in tissue damage, lethal and a series of cellular events that are defined as "myocardial ischemia-reperfusion damage"[75]. Among the first experimental studies by Reimer et al.[25], [26] demonstrated in numerous studies conducted on dogs, as the sign of irreversible myocardial damage such as rupture of the sarcolemma were particularly evident during reperfusion; at that time still we did not know clearly if the irreversible damage caused to the myocardium was attributable to reperfusion or if only became more manifest with it(Fig. 1.4). So, it opened new debate about the identity of this lethal reperfusion myocardial damage[76], after a long debate finally an epilogue found by Vinten-Johansen et al.[77], in which he identified the so-called "ischemic post conditioning phenomenon" repeated episodes of coronary occlusion during early reperfusion proved to be able to reduce the size of infarcted area in dogs. This discovery was later confirmed in patients with acute myocardial infarction undergoing coronary reperfusion[78]. These studies once again emphasized what had already been partially mentioned in previous studies on reperfusion, or that modified reperfusion procedures were able to attenuate the myocardial damage otherwise irreversible [79], reducing the signs of damage at both morphological and functional level [80].

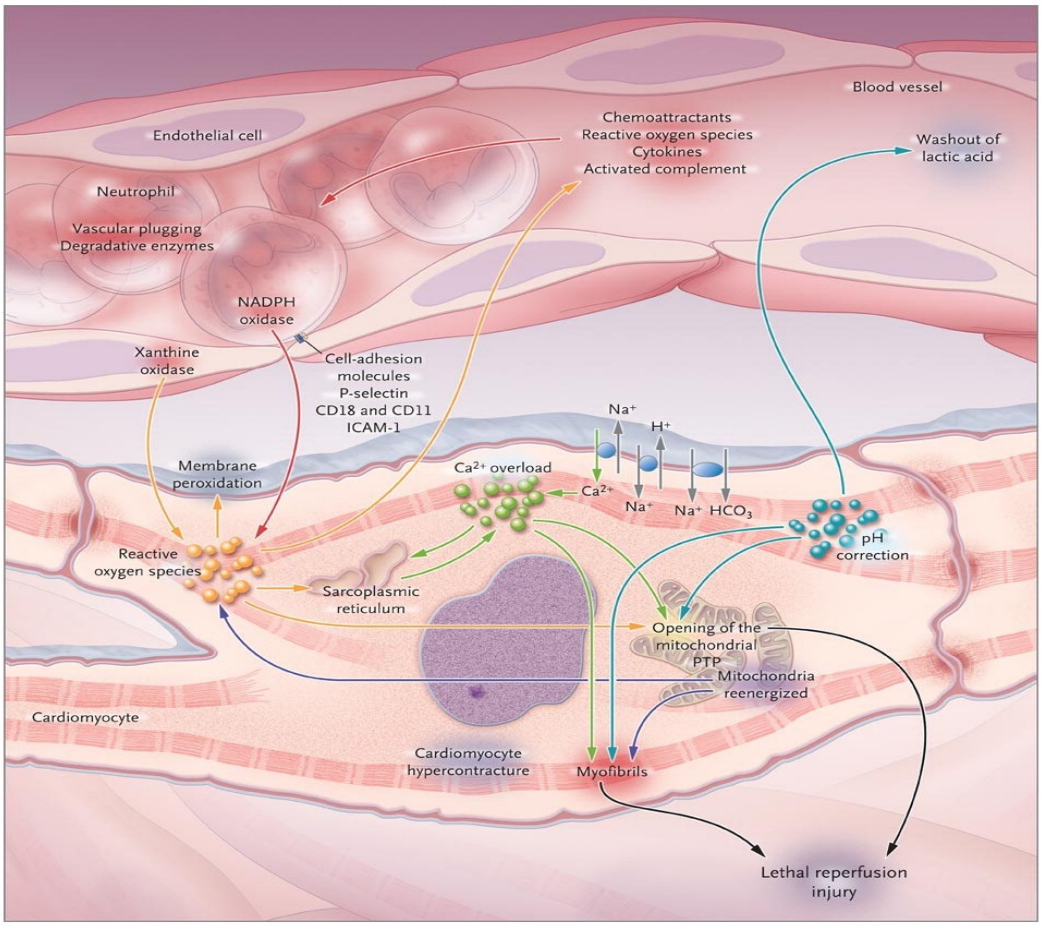


Figure 0.4. *Major mediator of lethal reperfusion injury. Ischemia-reperfusion injury in the heart. Cellular changes during reperfusion of myocardium include mitochondrial re-energization, generation of ROS, intracellular Ca2+ overload, rapid restoration of physiologic pH, and inflammation, which all contribute to cardiomyocyte death via an opening of the mitochondrial permeability transition pore.(adapted from Ernest Levy 2010).*

1.8.1.Oxygen free Radicals

A key role in ischemia-reperfusion injury is done by the release of reactive oxygen species[81, 82]. While during ischemia increases there is an increase of purines derived from the catabolism of ATP. The conversion of Xanthine dehydrogenase in Xanthine oxidase by Ca^{2+}, enzyme which converts Xanthine to uric acid, during reperfusion the oxygen supply enables to Xanthine oxidase to form uric acid from purine accumulated as a byproduct of the reaction produce reactive oxygen species.

Reactive Oxygen Species (ROS) such as the superoxide anion (O^{-}_{2}), the hydrogen peroxide(H_2O_2)and the hydroxyl radical(OH), play a key role in both mitochondrial and cytosolic levels as well as for normal functions of the mitochondria. An imbalance between production and removal of free oxygen radicals results in so-called "oxidative stress", involved in various pathological conditions as well as in

determining macromolecular damage[83]. In the evolution of damage by inducing endothelial cell surface alterations occur adhesion and activation of circulating neutrophils. The neutrophils release ROS and hydrolytic enzymes that damage cells in ischemia. The ischemia-reperfusion also activates the enzyme Nitric Oxide Synthase (NOS), which leads to the production of Nitric Oxide (NO) which reacts with the ROS forming toxic reactive species (peroxynitrite). Reactive oxygen species generated during reperfusion phase by various oxidative reactions, involving among other are, the NADPH oxidase and myeloperoxidase leukocyte[84], participating in the endothelial dysfunction and subsequent altered vasomotor capacity of coronary artery[85].

In addition to these actions, reactive oxygen species can produce deleterious effects on myocardial cells resulting in a structural and enzymatic proteins and lipid peroxidation of cell membranes. Their importance in reperfusion injury is suggested by experimental studies that showed the ability of treatment with antioxidants, such as superoxide dismutase, to improve cardiac function in ischemia-reperfusion [86]. However, it lacks the evidence that antioxidant treatment improves myocardial damage associated with reperfusion in humans and some study has not been able to confirm the induction of irreversible damage by free radicals in patients undergoing cardioplegic arrest and reperfusion during coronary artery bypass graft surgery [87].

In some pathological conditions, the overproduction of reactive oxygen species (ROS) can be stimulated by an overload of calcium (Ca^{2+}), in spite of calcium itself has numerous positive functions at the mitochondrial level [81]. This malicious calcium overload can be determined by the loss of adenosine triphosphate (ATP), loss in turn determined by an altered function of the Na^{+} /Ca^{2+} exchanger.

1.8.2. Inflammation and activation of complement system

The inflammatory damage in the reperfusion is primarily determined by a massive circulating neutrophils invoked in the reperfused area by cytokines and adhesive molecules expressed by endothelial and parenchymal cells. Above all release by the cytosolic components from necrotic cells. This inflammation causes, in turn, further tissue damage. The damage triggered by reperfusion is primarily associated with the release of several inflammatory molecules such as cytokines including IL-1β, IL-6, IL-8, IL-10, complement activation, the release of polymorphonuclear leukocytes as well as its actual cellular damage[88, 89].

To demonstrate a state of inflammation in the blood boosted survivors following a cardiac arrest reveals a level of IL-6 twenty times higher than patients who survived[90], or about fifty times exceeding the levels under physiological conditions[91]. This state of inflammation triggers very quickly as soon as coronary blood flow is re-established [92, 93].The extent of the damage at the cellular level depends on some factors, including the duration of absence blood flow level

particularly in myocardial tissues, the degree of response to the more or less optimal treatment as well as the probability of individual patient survival [94].

To emphasize how many patients with early hemodynamic dysfunction following a cardiopulmonary resuscitation for cardiac arrest have favorable neurological outcome[95], therefore the number of patients who escapes the clinical outcomes associated with ischemia-reperfusion injury could be significantly reduced by all those treatments to reduce premature mortality and damage associated with inflammation and trauma present in the chain of ischemia-reperfusion events. The IL-6 , TNF-α and endothelin that cause vasoconstriction, increasing the adhesion of neutrophils and platelets to endothelium, chemotaxis of neutrophils, and effects with systemic abnormalities of vascular function. Although, the complement system is capable of contributing to ischemia-reperfusion injury[96]: some IgM antibodies for unknown reasons tend to be deposited in ischemic tissues; when blood flow is restored, the complement fractions bind to these antibodies are activated and induce a further cellular damage and increased inflammatory reaction[97].

1.8.3. Increased permeability of mPTP

Under hypoxic conditions, cellular metabolism switches from a state to an oxidative glycolytic state, the pH is reduced leading to a state of acidosis, and Na^+/H^+ exchanger tries to re-establish a physiological cellular pH. The sodium gradient thus increased driving the counter current by Na^+/Ca^{2+} exchange, but the latter cannot act same, because the lack of ATP prevents their activation. Consequently, it is to determine an excess which eventually leads both to an overproduction of reactive oxygen species and the opening of the mPTP. Specifically, overstressing of calcium induced by calcium, combined with certain pathological conditions, results in a persistent state of the opening of these mitochondrial pores. Due to this ischemic tissue, the pathological condition characterized by a reduced value for Adenosine Mono-Phosphate/Adenosine Tri-Phosphate (ATP/AMP) and the depletion of adenylic nucleotides.

Once the mPTP opens, mitochondrial membrane potential is no longer maintained, leading to a major influx of water, mitochondrial swelling and rupture[81]. Specifically, the opening of mitochondrial transition pore determines a cell membrane depolarization and a bulge in the array. This in turn, leads to the outer mitochondrial membrane rupture and release of proteins such as cytochrome C from intermembranous space to the cytosol. The latter cellular event plays an important role in cell death both in neuronal and myocardial level[98, 99].

The continuing opening of mPTP not only makes the mitochondrion unable to produce ATP through oxidative phosphorylation but also determines the breakdown of ATP molecules produced through glycolysis in an attempt to restore the physiological cellular pH and concentration gradient normally present across the

mitochondrial membrane. During prolonged periods of ischemia that determine acidosis exerts an inhibition on the mitochondrial transition pore preventing you from opening; Once blood flow is restored and re-established adequate partial oxygen tension, the cell reverts to aerobic metabolism and the physiological pH increases, resulting in the opening of mPTP and an increased release of reactive oxygen species. Although as just seen low pH levels can inhibit the opening of mPTP, normalizing pH alone cannot prevent tissue damage that ensues. Nevertheless, it is still important for the purpose of inhibiting the opening of these mitochondrial pores and ultimately reduces ischemia-reperfusion injury, maintain proper mitochondrial function and prevent pathological conditions predisposing tissue damage related to mitochondria.

1.8.4. Role of mitochondrial DNA

During the development of ischemia-reperfusion injury following the rupture of mitochondria, mitochondrial DNA is released. Just considering this process, mitochondrial DNA has been recently evaluated as a possible marker of myocardial infarction[100, 101]. In fact, in addition to apoptosis induced by the rupture of mitochondria, the same circulating mitochondrial DNA seems to be able to contribute to the death of cardiomyocytes [101].

When it increase a tissue damage, mitochondrial DNA that is released into the circulation as a result of disruption of mitochondria is recognised by TLR9 as foreign material, as very similar to bacterial DNA, as a result, the body develops a systemic inflammatory response[102]. In support of this, recent clinical studies have used the increased circulating levels of mitochondrial DNA and overexpression of TLR9 as predictors of mortality in patients hospitalised in ICU [103]. Furthermore, urinary mitochondrial DNA has been used as a marker of mitochondrial dysfunction in acute kidney injury [104]. Thus circulating mitochondrial DNA could constitute an important means for evaluating myocardial damage, specifically in the transplant ischemia induced by the sequence of events and reperfusion.

In a recent study of CABG, an intervention that temporarily blocks blood flow to the myocardium, was seen as being induced the release of massive amounts of mitochondrial DNA free in the circulation[105] strengthens the hypothesis that this same DNA, can be used as marker of ischemia-reperfusion injury.

1.8.5. Myocardial Cellular Death

Ischemia-reperfusion may cause different forms of cell death, such as programmed cell death, necrosis and apoptosis. In mammalian cells, there are two most important pathways in the activation of the apoptotic cascade, although there are other lesser known pathways: intrinsic and extrinsic. The "intrinsic" is triggered by

ischemia/reperfusion, hypoxia and oxidative stress, and is mediated by the damaged mitochondria, which releases substances that activate the caspases cascade and translocation in the nucleus where they induce, directly and indirectly, DNA fragmentation.

1.9. Myocardial Protection related to Ischemia-Reperfusion Injury

In recent decades, numerous studies have shown that the myocardial cells possess several coping mechanisms that aim to limit the damage of ischemia/reperfusion. The cellular mechanisms underlying certain phenomena of myocardial cell protection from ischemia/reperfusion (as the pre and post conditioning) remain to be clarified in an appropriate way, but they are likely to be numerous. It remains to be proven whether pharmacological interventions can improve myocardial cellular metabolism towards a more efficient use of oxygen and energy stocks can help protect the myocardium under ischemic conditions. Also, the option of using drugs to mimic the phenomenon of preconditioning or Post conditioning *in vivo* application of ischemic episodes post conditioning and the ability to prevent, with pharmacological, cell death by apoptosis, are other fascinating possibilities still developing security assessment.

It should also be noted that other possible treatment options proposed to prevent the reperfusion injury based on the physiopathological basis (including Na+/H+ exchanger inhibitors, regional hypothermia, Anti-inflammatory and reperfusion gradual), none has been tested successfully in patients with AMI.

1.9.1. Anti-apoptosis

Inhibition of apoptosis can limit the loss of myocardial cells induced by programmed cell death. It has been shown how different drugs known to have favourable effects in ischemic cardiomyopathy, including angiotensin converting enzyme inhibitors, Angiotensin II antagonists and beta blockers, have anti-apoptotic effects in animal models, through inhibition of the Renin-angiotensin system and sympathetic nervous system effectors that, under certain conditions can trigger apoptosis [106]. Antioxidant agents can act as anti-apoptotic substances, because oxidative stress and the generation of ROS may trigger the "intrinsic" apoptosis. In a mouse model of ischemia-reperfusion, in fact, the antioxidant was able to prevent the over-expression of various pro-apoptotic molecules [107]. Specific target potential to prevent apoptosis include caspases and endonuclease. Inhibitors of these enzymes are capable of reducing infarct and left ventricular remodelling in experimental models of ischemia-reperfusion damage [107].

Finally, the insulin-like growth factor can improve heart function in animal models of cardiomyopathy through an anti-apoptotic effect mediated by inhibition of

Caspase-3. However, they cannot be overlooked some boosts in application limits of therapeutic strategies aimed at inhibiting the apoptosis in clinical practice, especially about the carcinogenic potential of such intervention. Furthermore, while in animal models the time and doses of anti-apoptotic drugs are well controlled, there is no well defined by referring to their application in clinical practice.

1.9.2. Mechanical Assistance

Among the mechanical assistance systems that can be used in conditions of myocardial ischemia, infarction and post cardiac arrest is Extracorporeal Membrane Oxygenator (ECMO). It is a closed loop system that replaces the heart and lungs of the patient when these are not able to perform its task, providing a valid cardiopulmonary support when this is necessary for an extended period and allowing a functional recovery of heart and lungs. It has no therapeutic action, but allows you to implement a medical treatment which would exclude from the cardiac and pulmonary functionality.

There are mainly two types of Veno-Arterial (VA) ECMO: and Continuous Veno-Venous (VV)*(Fig.1.5)*. And both types provide respiratory support, but only that hemodynamic support VA also provides a cardio-circulatory function.

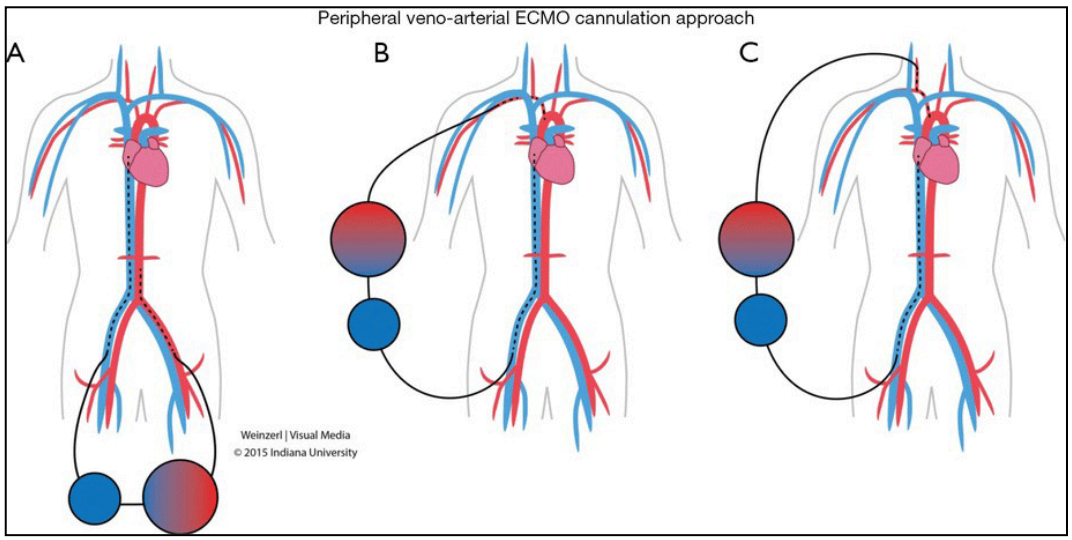


Figure 0.5. *Peripheral venou-arterial ECMO cannulation approach femoral vein (for drainage), (A) femoral, (B) axillary, (C) carotid, artery are used for perfusion. ECMO, Extra Corporeal Membrane Oxygenation.(Adapted from George Makdisi 2015)*[108]

In the first type, the blood is drained from the venous access is then conveyed to the arterial access, while in the second case the blood is drained and re-infused directly in vein. When the patient is connected to the ECMO, blood is taken from the patient's cardiovascular system through a cannula, conveyed towards the membrane oxygenator tanks to pressure exerted by the centrifugal pump. The later gives to the

blood a circular motion, and convey it in a vortex that, creating a vacuum allows the recall of blood from the patient and on the other hand a kinetic energy that pushes the blood through the spiral towards the membrane oxygenator.

The pump then generates the flow, and the number of turns determines the share of ejection fraction and, therefore, the scope of the system. The pump types that are generally used are centrifugal pumps and magnetic levitation pumps. The flow that is produced is one of the linear types, and not pulsed. At the level of the output line from the centrifugal pump is positioned a flow detector. The Oxygenator consists of a continuous membrane, consisting of hollow fibers, in which the mixture of air and oxygen flows countercurrent to the blood, which flows perpendicularly to it; It creates a wide exchange surface between air and blood with a minimum thickness, the diffusion exchange of oxygen and carbon dioxide. The oxygenation is determined by the flow rate, while the elimination of carbon dioxide can be controlled by adjusting the speed of the flow upstream within the oxygenator. There is also a controller of the spray, which can change the flow rate of the mixture and the oxygen fraction in the mixture itself. The heat exchanger maintains a physiological body temperature or relatively hypothermic; this is necessary because the continuous passage of the entire cardiac output through the pipes, pumps and oxygenator positioned outside the patient determines a rapid heat loss resulting in hypothermia.

Before making the connection with the ECMO and during use, the patient should be on anti-coagulation, usually with unfractionated heparin, and then proceeds with the cannulation and connection to the circuit of ECMO. Heparinized coating has been developed for various circuit components, so as to reduce the inflammatory response that coagulation associated with extracorporeal circulation. [109]. The Cannulation can be performed using various techniques, surgical or percutaneous: when you need a cardiopulmonary resuscitation, straws can be positioned with percutaneous technique Seldinger technique to accelerate the procedure. Otherwise, they prefer to implement a surgical isolation of the vessels. Cannulation can also be central or peripheral[110].
Following a cardiac surgery, the site of cannulation "Central", aorta and right atrium, used for cardiopulmonary bypass, translates to "serial" jugular femoral-femoral. Alternatively the same cannula can be detached from the cardiopulmonary bypass machine and connect to the circuit of ECMO.

Cannulation can be central or peripheral blood that can be made to the common femoral artery, axillary artery or common carotid artery, while the venous cannula is placed at the level of the common femoral vein or internal jugular vein; subsequently, the cannula correctly positioned, are fixed with wire stitches to the patient's skin[111]. Specifically, in VV ECMO the venous cannula are usually placed to the left or right common femoral vein to drain and the right internal jugular vein for the infusion. The VA ECMO a venous cannula is placed in the inferior vena cava or

right atrium, and an arterial cannula is positioned in the right femoral artery. Occasionally the femoral vessels may not be available to cannulate the ECMO; in those cases, we can use the left common carotid artery or subclavian artery[112].

After cannulation, the patient is connected to the ECMO circuit, and the blood flow is increased to obtain satisfactory hemodynamic and respiratory parameters. Assuming the brain function of a post-cardiac arrest patient is reduced minimally or not assessable, ECMO VA can provide a valid cardiopulmonary support: in these cases the device is used until the patient can recover independently or otherwise prior to implantation of a Ventricular assist device, or *LVAD* can be used as a bridge therapy to cardiac transplantation.

Among the indications for the use of ECMO themselves, in addition to cardiac arrest, even the heart failure secondary to myocardial infarction, the refractory cardiogenic shock, the impossibility to weaning from cardiopulmonary bypass following cardiac surgery, and as an aid in conventional cardiopulmonary resuscitation[113]. In two observational studies, the use of ECMO in patients with cardiac arrest has been associated with an increased survival compared to single conventional cardiopulmonary resuscitation[114, 115].

The only absolute contraindication to the use of ECMO is a pre-existing condition of severe neurological damage or impossible recovery, such as a severe neurological impairment or end-stage cancer. Relative contraindications, which must be assessed on a case by case basis include uncontrollable bleeding and patients with a poor prognosis very strict criteria need to initiate. The main complications related to the use of ECMO system includes bleeding, the most common complication, due to the extended period of coagulation, Thrombo-embolic complications, infrequent but can be devastating, especially in ECMO VA; these complications can be prevented by maintaining an adequate anticoagulation and observing the circuit looking for signs of a blood clot forming Neurological damage, Heparin-induced thrombocytopenia and platelet transfusion becomes necessary, Systemic inflammation, Multi-Organ Failure (MOF).

The consumption of coagulation factors and thrombocytopenia often needed blood transfusions, which can cause adverse reactions and secondary damage to the lungs. Systemic inflammation contributes to various clinical outcomes, including kidney, heart and lung damage[116]. Among the main mechanisms underlying includes surgical trauma, the hemodilution, endothelial damage induced by oedema, the damage from ischemia-reperfusion injury of various organs, the contact activation of blood components during extracorporeal circulation[117]. In determining the leukocyte activation and systemic inflammation, it was recently demonstrated in an experimental model of extracorporeal circulation, like the oxygenator has a role in this [118]. Due to these numerous side effects on hemodynamic support with ECMO can be maintained only for short periods and should be removed as soon as there is a

recovery of cardiac and respiratory function or after applying the necessary treatment.

1.10. Pharmacological targets for known ischemia-reperfusion injury mechanisms

During last two decades, considerable basic research has been performed to target ischemia-reperfusion injury mechanisms. Main targets focused were cellular receptors including adenosine, opioids, adrenergic and muscarinic receptors studied by Lasley and Mentzer, 1998[119]; Vinten-Johansen et al., 1999[120], effect of ion exchangers in ischemia reperfusion demonstrated by Avkiran, 2001[121] (e.g., Na^+/H^+, Na^+/Ca^{2+}), nitric oxide pathways also reported by Ronson R et al.[122] and intracellular signaling pathways activation e.g., PKC, tyrosine protein kinase, guanylate cyclase and MAP kinases have been investigated to understand cardioprotective mechanism of different drugs in ischemia-reperfusion injury[58]. Similarly, in this thesis, Sphingosine 1-phosphate receptors were studied because of its well-established effect on inflammation, apoptosis and oxidative stress.

SECTION -IB

2. Introduction to Fingolimod (FTY720)

Sphingosine-1-phosphate

Sphingosine-1-phosphate present in the plasma is mainly produced by endothelial cells, from erythrocytes, platelets and hepatocytes; is a bioactive lysophospholipid deriving from sphingomyelin, ubiquitous lipid cell membranes[123]. Sphingosine is formed by the enzyme sphingosine kinase. (Figure 2.1).

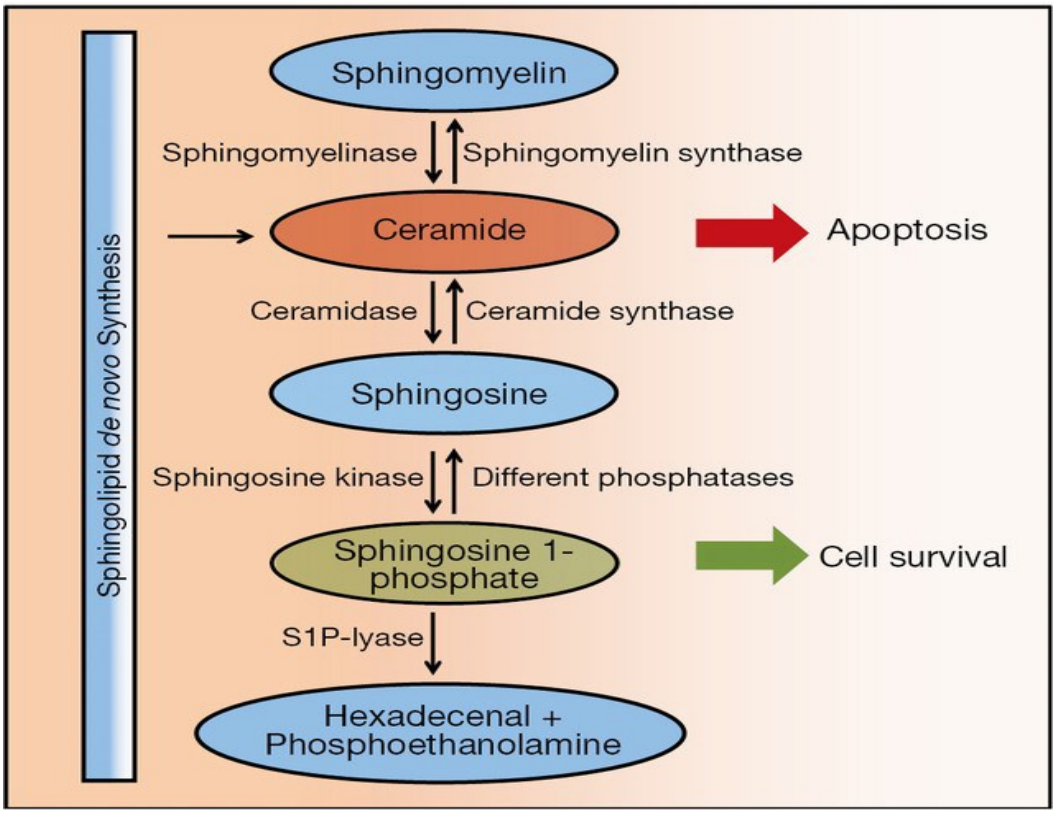


Figure 2.1. *The sphingolipid metabolism. The degradation of sphingosine-1-phosphate (S1P) by S1P-lyase is the only irreversible step in this pathway. Ceramide and S1P have antagonizing functions, which makes their balance essential for cell fate (adapted from Christina-Maria Reimann et al. 2015).*

The new therapeutic perspectives of Fingolimod result from increasing knowledge on sphingosine-1-phosphate (S1P) [123] including cytoprotective, antioxidants, immunosuppressive [124], and its possible role in reducing ischemia-reperfusion injury[125]. The S1P mediate different physiological functions [126], including cell proliferation, differentiation and survival, as well as the reorganization of the cytoskeleton, the formation of cytoplasmic extensions, cell motility and chemotaxis, intercellular adhesion and formation of the junctions between cells. It is therefore involved in many physiological aspects of the body, such as immunity, maintaining the tone and pulmonary vascular smooth muscle, endothelial barrier integrity,

feature morphogenesis for the cardiovascular and the central nervous system. Although S1P is derived primarily from erythrocytes, other sources include the platelets, mast cells, endothelial cells, fibroblasts, and central nervous system[123, 127-129]. Sphingosine-1-phosphate shall perform its functions through 5 subtypes including G-protein receptors S1P1, S1P2, S1P3, S1P4, S1P5 [130].

Recent studies have shown that sphingosine-1-phosphate reduces ischemia-reperfusion injury in liver[131], in the kidney[132], and in the brain[133, 134]. Sphingosine 1-phosphate as sphingosine are also able to increase the survival of cardiomyocytes during episodes of hypoxia; evidence emerged from in vitro studies [135, 136], it can also reduce the size of the infarcted area in productions of isolated hearts ex vivo [137, 138].

2.1. Fingolimod

The drug Fingolimod (2-amino-2-[2-(4-Octyl-phenyl) ethyl]-1.3-propanediol hydrochloride), is a synthetic structural analogue of sphingosine that acts as a receptor agonist of sphingosine-1-phosphate, and represents a new frontier in the treatment of ischemia-reperfusion injury.

The Fingolimod hydrochloride is a powder that is freely soluble in water, alcohol and propylene glycol. Currently, fingolimod represents as a unique sphingosine-1-phosphate receptor agonist approved by U.S. Government agency "the Food and Drug Administration (FDA) for clinical use in human [139].

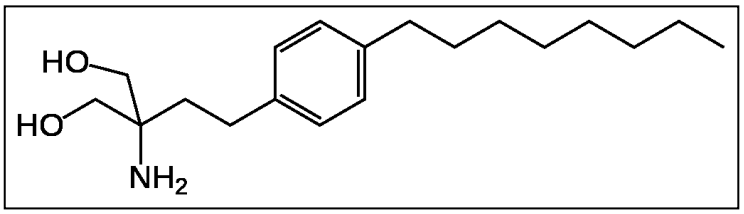


Figure 2.2. *Molecular structure of fingolimod*

2.1.1 History of Fingolimod

The molecule of Fingolimod, also known as FTY720, was synthesized for the first time in 1995. Fingolimod is derived from chemical modification of a natural product, myriocin derived from Isaria sinclairii[140 141]. The active ingredient of natural origin for immunosuppressive agents found in 1992 by a group of Japanese researchers. On September 22, 2010, the Food and Drug Administration (FDA) had approved its use as first-line therapy for relapsing-remitting multiple sclerosis, and March 17, 2011, the European Commission has issued an authorization for placing commercially valid throughout the European Union. On November 22, 2011, fingolimod was approved commercially in Italy.

2.1.2 Use of Fingolimod in clinical setting

Fingolimod was defined by the European Medicines Agency (EMA) for first-line therapy in patients with severe and rapidly progressing form MS, or as second-line therapy in case of failure with the use of Interferon-β (IFN-β). In European Union, Fingolimod is approved for use as monotherapy, as Disease-Modifying Drug (DMD), in selected patients with a form highly active relapsing-remitting multiple sclerosis. The Efficacy of Fingolimod consists not only in reducing recurrence rates of MS, also improvement suggested by MRI monitoring for active disease, both compared with placebo than with INF-β and also in reducing the progression of disability compared to placebo [142, 143]

Fingolimod is different from existing therapies available for multiple sclerosis as primarily represents the first oral treatment in 0.5 mg capsules. For the treatment of MS, in the last two decades, first considerable progress regarding the effective control of the disease, especially for control of relapse[144]. Multiple sclerosis is a chronic inflammatory disease of the central nervous system, caused by an abnormal functioning of immune system. Several disease-modifying drugs (disease-modifying drugs, DMD) were approved but new molecules, such as Fingolimod, is the best treatment option for this condition. The DMD are molecules that Act on the causes of the disease, and currently, the most common DMDs are immunosuppressive agents, which work by reducing the action of the immune system and thus hindering the destruction of myelin, and immunomodulating agents that alter the delicate balance of the immune system, decrease its action, with the objective of limiting the attack on myelin[145].

Among the DMDs there is a mechanism of innovative action of fingolimod: its active metabolite "fingolimod-phosphate" resulting from the action of enzyme sphingosine kinases[139], acts as a Sphingosine-1-phosphate receptor agonist receptor on S1PR1, S1PR3, S1PR4 and S1PR5[146]. Fingolimod-phosphate binding to its receptor agonist effect initially, but in the long term, it leads to internalization of receptors and consequent degradation to proteasome level, then in receptor inhibition[147, 148].

Fingolimod modulates receptor of sphingosine-1-phosphate and induces a lymphocyte surface down-regulation, making memory T lymphocytes, and T naive remain sequestered within lymph nodes, this mechanism has the potential to reduce the traffic of these cells "pathogenesis" in the central nervous system [149, 150]. Since this is highly lipophilic, it can cross the blood-brain barrier and penetrate within the central nervous system [151] there is also increasing evidence; about a direct action on S1P receptors oligodendrocytes, astrocytes and neurons level [152].

2.1.3 Effects of Fingolimod

Regarding the safety profile of the drug Fingolimod has generally proved safe and well tolerated, presenting medium grade adverse events including Sinus bradycardia, atrioventricular block, infections, increased liver enzymes, hypertension, and macular edema. The cardiovascular effects still constitute a major source of concern in the clinical setting, especially after the first dose of medication administered[144].

The cardiovascular effects of Fingolimod have been assessed through four clinical trials phase IV: *TRANSFORMS, FREEDOMS, FREEDOMS II, and FIRST*[142, 143, 153-156]. In these studies is seen as reducing the heart rate is similar in patients with and without cardiovascular risk factors, a transient reduction and dose-dependence. Reducing the heart rate is observed only after the first dose, in long term therapy, it returns to baseline heart rate. Associated symptoms are rare, transient and usually without clinical consequences. A recent multicenter phase IV study called "The Evaluate Patient outcomes" (EPOC) Study confirmed these findings. These studies also suggested about the effect of Fingolimod by inducing a delay in atrioventricular conduction is uncommon and, when present, is transient, and usually recover without intervention.

Additional features of this drug are that it does not change the duration of the QRS complex nor prolongs the QT interval [157] it also does not show significant effects on platelets function and counts. About long term treatment, in 4 years, trial extended to FREEDOMS, the incidence of cardiovascular adverse events remained at the same level observed in the first two years of the study, indicating that long-term treatment has no impact on cardiovascular disease. This result was later confirmed in the trial, also extended to 4.5 years TRANSFORMS. And the effects on heart rate and atrioventricular conduction may recur when treatment is resumed after more than two weeks of suspension.

2.2. The basic mechanisms of the cardiovascular effects of Fingolimod

Cardiovascular effects encountered derive first by the presence of S1P receptors in the heart and blood vessels [158, 159] ; specifically, the receptor S1p1 predominates at the sinoatrial node, atrioventricular node, cardiomyocytes and endothelial cells, whereas the S1PR2 receptor is found primarily in arterial smooth muscle cells, cells which are at the same concentrations S1p1 and S1PR2 [160-163]. In the heart, S1P receptor activation causes the G protein receptor associated dissociation, followed by the activation of potassium channels called "inwardly-rectifying potassium G protein-coupled channels (GPCR) [164] potassium efflux; that follows goes to hyperpolarize the cell membrane, inhibiting depolarization and reducing the excitability and robotics [165]. The result is, therefore, a reduction in heart rate.

The S1P1 receptor internalisation is responsible for negative chronotropic effects of impermanence and dromotropic (respectively reducing the heart rate and speed of conduction of electrical impulses in the heart) induced by Fingolimod. Both atropine, a muscarinic receptor antagonist, isoprenaline, a beta-1 receptor agonist and beta-2 reverse the effects of fingolimod[166, 167]. In endothelial cells, S1P1 receptor activation leads to phosphorylation of protein kinase B and the activation of endothelial Nitric Oxide Synthase (eNOS), events that increase the production of nitric oxide (NO)[168-170].

The S1P1 receptor in the smooth muscle cells of arteries lead to the release of calcium from intracellular reserves, increasing its concentration within the cells themselves and causing the contraction of smooth muscle cells and arterial vasoconstriction; Consequently the pressure does not change significantly in the acute phase following administration of Fingolimod for this balance between vasodilatation effect and vasoconstriction from it.

Always smooth muscle level, as a result of the internalization of S1P1 (functional antagonism), S1PR2 and S1PR3 receptor binding is favored, which leads to the opening of calcium channels increasing intracellular concentration, causing smooth muscle cell contraction; these effects are responsible for the slight increase in blood pressure, about 1-2 mmHg, observed after two months of treatment with fingolimod 0.5 mg/day reaching the peak pressure at six months and then remained stable [142, 143].

The reorganization of the cytoskeleton and cell geometry and stabilization of intercellular junctions are further effects of activation of endothelial cell level S1P1 [171]; these effects strengthen the endothelial barrier and diminish the permeability. As in the arterial smooth muscle receptor agonism is transitory, even the endothelium due to internalization of receptors, followed by activation of S1PR2 and S1PR3, which leads to increased permeability of the endothelial barrier for breaking the intercellular junctions.

2.3 Recommendations for safe use of Fingolimod

For safe use of Fingolimod, with particular reference to cardiovascular adverse events, the European Medicines Agency (EMA) recommends an observation period of six hours after the first dose of the drug in all patients, to assess the signs and symptoms of bradycardia. Heart rate and blood pressure should be measured and recorded either before administration of the first dose every hour throughout the observation period; an electrocardiogram should be performed both before the first dose, then six hours later. During those six hours of continuous ECG monitoring is recommended viewing real time. The observation period should be extended for at least another two hours if the heart rate reached lower at after six hours; in the latter case, you should monitor until your heart rate does not increase.

The conditions that involve an extension of the period of observation until at least the next day, or until their resolution is:

- Heart rate less than 45 bpm upto first six hours
- QT interval ≥ 500 ms
- Second-degree atrioventricular block of new onset within six hours
- Third-degree atrioventricular block, regardless of the time of onset
- Need for drug therapy during the observation period. In this case, monitoring should be conducted for the night with medical assistance and should be repeated after the second dose of medication.

2.4. Contraindication of fingolimod

1. Patients predisposed to developing cardiac arrhythmia as those with heart block Mobitz II, third-degree heart block, sick sinus syndrome, or with a history of symptomatic bradycardia or recurrent syncope.

2. patients such as those with little explanation tolerant ischemic heart disease, cerebrovascular disease, heart failure, patients with high blood pressure controlled, serious little sleep apnea just check, or a history of myocardial infarction or cardiac arrest .

3. Patients being treated with class Ia antiarrhythmic drugs or class III.

4. Patients taking heart rate lowering medications like beta-blockers and non-dihydropyridine calcium channel blockers.

Fingolimod is not absolutely contraindicated in such cases and may be administered when the benefits outweigh the risks. Monitoring should be extended to at least the first night in the first, second and fourth of the above patient's group of patients if they cannot suspend rate lowering drug therapy.

Since the effects of Fingolimod on heart rate and atrioventricular conduction can occur at the time of resumption of the drug, also depending on the duration of the suspension, it is recommended to repeat monitoring after the first dose when therapy is interrupted for one or more days during the first two weeks of treatment, for more than seven days during the third and fourth week, or for two or more weeks if after a month or more of therapy.

2.5. Mechanisms of Cardioprotection

Sphingosine-1-phosphate on cardiomyocytes, binds to three G-protein coupled receptors: S1p1, S1PR2, and S1PR3, leading to activation of numerous signal

transduction pathways involved in cardioprotective action "intracellular" potentially, S1p1, in particular, is the main receptor of sphingosine-1-phosphate in cardiomyocytes, and typically activates downstream signal transduction pathways RISK and SAFE [125, 172]. Cardioprotective effects of Fingolimod are mediated by activation of RISK and SAFE, because both the antiapoptotic action, antioxidant action that is reducing the size of the MI, are reversed using concomitantly monoamine pathways RISK and SAFE. The main molecular Cascades can inhibit mitochondrial transition pore openings are the RISK, and SAFE pathways [3, 173, 174], and previous in vitro studies claim that S1P can activate these pathways [138, 175, 176].

The downstream pathway S1P receptor-R is a potential therapeutic target peri-infarct. Evidence arising from previous preclinical studies suggest that sphingosine-1-phosphate represents a very promising pharmacological target for mitigating the damage from myocardial ischemia-reperfusion. In ventricular cardiomyocytes of rats, both adults and babies [135, 136]sphingosine 1-phosphate, increased cardiomyocyte survival-during episodes of hypoxia. The S1P also induces resistance to ischemia-reperfusion injury in rat hearts ex vivo studies of wild-type [137] and rat[138]. By these findings, the hearts of mice lacking the enzyme sphingosine kinase, an enzyme necessary for the synthesis of sphingosine-1-phosphate, develop upper ischemia-reperfusion myocardial damage compared with controls [177, 178]. Always confirming the role of S1P in size the myocardial damage, S1P receptor knockout mice-R show an area of larger infarct compared to controls [179, 180].

The Metabolism of sphingosine also seems to be a key mediator in Preconditioning and Post conditioning, recognized strategies Cardioprotection [2]. In fact, both the pre and postconditioning can reduce the size of the MI, which does not take place in the hearts of sphingosine kinase or missing S1P receptor(Fig. 2.3) [138, 177, 178, 180]. The mitochondrial transition pore opening represents the final step that leads to apoptosis of cardiomyocytes in ischemia-reperfusion injury and is able to trigger oxidative stress that characterizes it; therefore, preventing the mitochondrial transition pore opening you can reduce infarct size [181, 182].

Previous studies have shown great benefits in immunosuppression of the prevention of ischemia-reperfusion injury[183]. Fingolimod is a drug which, among other effects, as already discussed about its use in MS, also exerts an immunomodulatory action able to regulate the traffic of lymphocytes from peripheral blood and tissues to lymph nodes and reduces the output of lymphocytes from lymph nodes themselves [123, 139].

In a recent study of 2014 conducted by Wang G et al. [155] using a mouse model of spontaneous coronary atherosclerotic occlusion based, saw itself as in mice treated with Fingolimod you got a reduction in the size of the infarcted area (ex vivo) as well as lower mortality. In these same mice is there has been a reduced count of CD4 and CD8 T cells and an increased number of T regulatory cells, suggesting how

the immunosuppressive effects of Fingolimod contribute to its cardioprotective properties.

Together these findings emphasize as sphingosine-1-phosphate and synthetic analogue Fingolimod promising may have a role in the prevention of ischemia-reperfusion. Several evidence claims that average Fingolimod cardioprotective effects: preliminary in vitro studies have shown that S1P receptor activation mediated by this drug armies antiapoptotic effects, reducing the loss of cardiomyocytes in hypoxic conditions[125, 175, 184]. Studies on preparations of mouse and rat isolated hearts have shown that Fingolimod is also able to reduce ischemia-reperfusion injury and to improve myocardial function[184]. Despite these different confirmations about various cardioprotective effects, they have not yet been studied in large animals models.

As reported in the study by Santos-Gallego et al in a model of myocardial ischemia-reperfusion conducted on pigs, according to a short Protocol term (Administration of Fingolimod 15 minutes before reperfusion or saline in controls) and a long-term Protocol (Administration of Fingolimod 15 minutes before reperfusion or saline in controls; the same treatment then once a day for three days) [185] S1P receptor activation , mediated by Fingolimod, given before the myocardial reperfusion injury, resulting in a significant reduction of cardiomyocyte apoptosis in the outskirts of ischemic myocardium, quantifiable by Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL); this technique allows to detect fragmented DNA through the labeling of nucleic acid Terminal residue. It is commonly used to detect fragmented DNA resulting from the cascade of activation of apoptosis and relies on the presence of breaks in the DNA sequence that can be made evident through the use of terminal deoxynucleotidyl transferase (TdT), an enzyme that catalyses the addition of dUTP, subsequently detected by a marker.

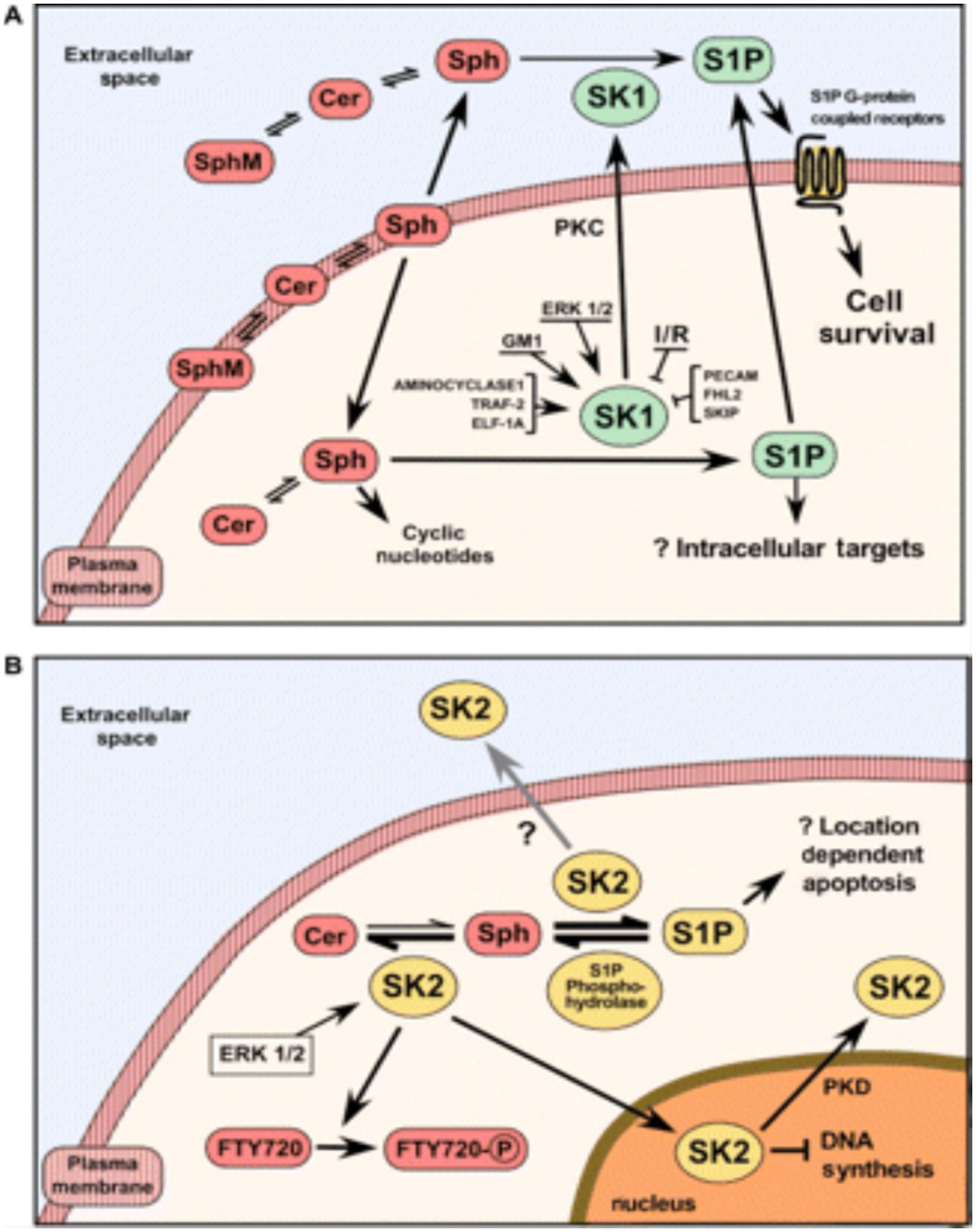


Figure 2.3. *Regulation and function of S1P (A) The diagram depicts some of the negative and positive regulators of SK1 activity and the synthesis and location of the SK1/S1P pathway either present in the heart or derived experimentally. (B) A second isoform of SK (SK2) is also responsible for the S1P generation. The location of S1P generation within the cell may determine its effect, but the outcomes of such location-based effects have not been determined(Adapted from Karliner J et al. 2008)*[186].

2.5.1. Anti-apoptotic action

To reduce cardiomyocyte apoptosis, Fingolimod mediated through the activation of signal transduction pathways "Reperfusion Injury Salvage Kinase (RISK) and

Surviving Activating Factor Enhancement (SAFE); to confirm this, the simultaneous administration of Wortmannin, an inhibitor of pathway RISK (including Akt/ERK/GSK-3 β), or AG490, SAFE pathway inhibitor (Janus kinase/STAT3), delete the antiapoptotic effect towards the cardiomyocytes. As further data in favour of activation by Fingolimod, pathways that regulate the protection of cardiomyocytes ischemia-reperfusion damage, RISK and SAFE [3, 174], it has been shown that Akt, GSK-3β, ERK1 1/β and phosphorylated STAT3 are markedly on the edge of the ischemic myocardium in pigs treated with Fingolimod after 1 day after myocardial infarction, reflecting the cardioprotective effect. In contrast, Akt and ERK1/2 signalling pathways are less remote myocardial infarcted phosphorylated after one month after myocardial infarction, to witness a minor remodelling. So the downstream signalling cascades of S1P-R mediate the activation of anti-apoptotic pathways, in the acute phase, and in the later stages reduce remodelling. Santos-Gallego et al. [185] demonstrated how Fingolimod induces significant Akt phosphorylation, ERK1/2, STAT3-β, and 24-hour GSK3 after a myocardial infarction, increased compared with controls. Therefore, in a porcine model of ischemia-reperfusion, Fingolimod has proved able to activate both RISK and SAFE in the acute phase of myocardial infarction.

The signal transduction pathways, including Akt and ERK1/2, are important stimuli for growth and survival of cardiomyocytes; although their short-term activation results in decreased apoptosis, in the long run, leads to cardiomyocyte hypertrophy. Long-term activation is one of the key molecular features of left ventricular remodelling, negative, in the post- infarction[187, 188]. The evidence of the antiapoptotic action of Fingolimod is further supported by detection from one side of a reduced activation of proapoptotic proteins Bax and Caspase-3, the other to an anti-apoptotic protein such as Bcl-2 expression increased and the kinase C-ε in pigs treated with FTY720. As a result of anti-apoptotic effects of Fingolimod, this medication can significantly reduce the size of the infarcted area.

2.5.2. Antioxidant action

Cardioprotective effects attributable to Fingolimod, the antioxidant is demonstrated by reduced myocardial ischemic area exists at the edge of oxidative stress in pigs treated with this medication, associated with a concentration of 8-hydroxydeoxyguanosine, markers of oxidative stress; always in support of this aspect of Fingolimod, antioxidant superoxide dismutase enzyme activity is increased in animals treated with FTY720.

2.5.3. Attenuation of ischemic cardiomyopathy

Fingolimod actions to attenuate myocardial damage includes, reducing the extent of myocardial infarcted mass acting on both the absolute and the percentage of the

infarcted left ventricular myocardium. As evidenced by the Santos-Gallego et al, Fingolimod can increase the protection for infarction of approximately five times compared to controls that have not been given the drug[185].

Besides the reduction of the infarcted area, Fingolimod has proved capable of improving left ventricular systolic function, as evidenced by increased Left Ventricular Ejection Fraction (LVEF), assessed by cardiac magnetic resonance, performed for a week to a month after myocardial infarction; also cases treated with Fingolimod have a greater contractile reserve compared with controls. These findings are important because of both improved LVEF that the presence of proper contractile reserve, are associated with more favourable outcomes [189, 190].

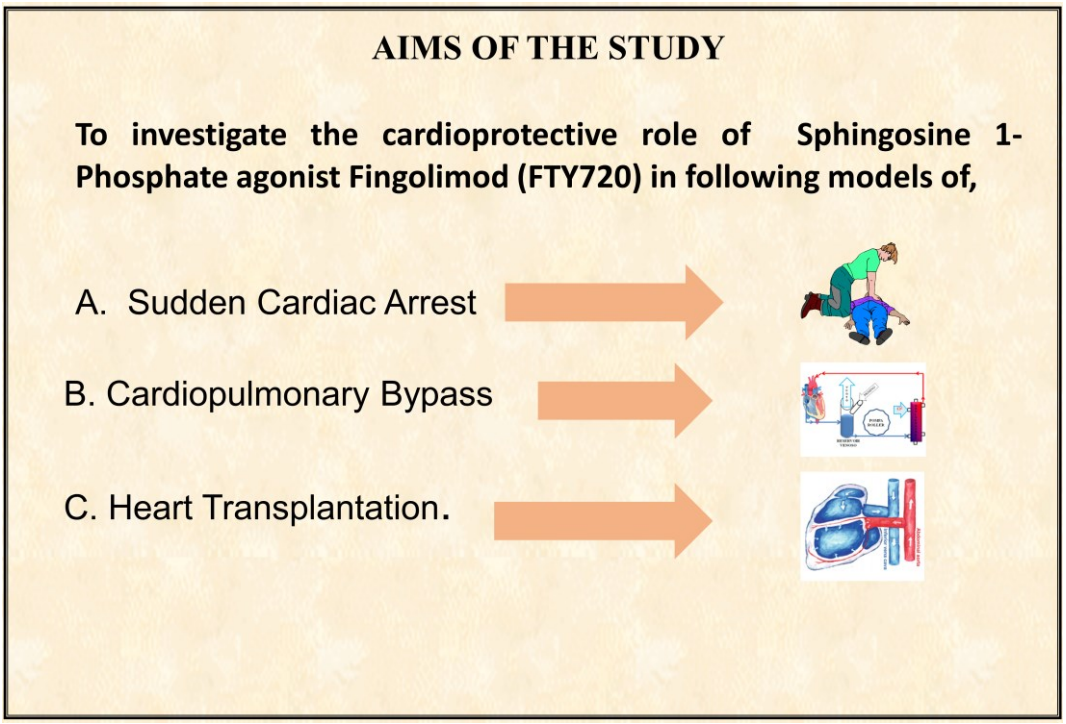
One of the worse complication of myocardial infarction is definitely the structural remodeling that characterized by, dilation, compensatory hypertrophy and changes in left ventricular sphericity. Fingolimod reduces the extent of left ventricular remodeling, visible as reduced left ventricular mass or to one week to one month after myocardial infarction; In addition, compensatory hypertrophy of the wall, the wall thickness index calculated by echocardiography, is lower than in the Group of pigs treated with Fingolimod. Santos-Gallego et al. was the first to report that Fingolimod not only reduces ischemia-reperfusion injury and the size of the infarcted area but also resizes the development of ischemic cardiomyopathy.

The effects on remodelling operated by minor evidence of fingolimod are therefore caused by structural changes in the left ventricular load, including a reduced dilation of the heart confirmed by echocardiography and the MRI both. The anti-remodeling is also visible at histological level, as demonstrated by the reduced deposition of collagen in the myocardial interstitium. Fingolimod treated group; also have small cardiomyocytes, confirming as Fingolimod go to alleviate post-infarct remodeling by microscope. The attenuation of the anatomical remodelling is supported by lower neurohormonal activation, supported by reduced levels of Aldosterone and blood Metanephrines; These findings emphasise as Fingolimod group in harmony with the best anatomical left ventricle-level outcomes in cases treated with this drug.

2.5.4. Role of sphingosine-1-phosphate and HDL in Cardioprotection

About 60% of the plasma is carried by HDL S1P [65]. More than 30 years ago it was shown that cholesterol carried by High-Density Lipoproteins (HDL) is strongly and in inverse proportion linked to coronary artery disease. Recent studies assigning HDL properties that go far beyond the capacity to promote cholesterol efflux: these represent the largest lipoproteins transport mediated by binding to S1P, transporter apoprotein M associated with HDL (apoM), and S1P is active in various HDL-associated signal transduction cascades through activation of its receptors on various cell types [172, 191].

HDL protect against CAD properties have been attributed to the ability of S1P to preserve endothelial function and inhibit proinflammatory leukocyte level signalling [192]. This aspect is important because, in the evolution of myocardial infarction, accumulation of inflammatory cells in the infarcted area occurs rapidly after initiation of the procedure of reperfusion and reach at peak level of neutrophils infiltration usually after 24 hours [193].



HYPOTHESIS

We hypothesized that the injury induced by myocardial ischemia reperfusion can be attenuated by sphingosine 1-phosphate activation via the activation of Akt/Erk1/2 pathways, inhibition of apoptosis and inflammation, reduction of oxidative and nitrative stress.

SECTION II

3. Materials and Method

3. Introduction

This thesis consists of experimental studies; all were *in vivo* rat model of global myocardial ischemia-reperfusion injury. The surgical preparation and techniques for particular experimental models have been mentioned in the following section. For all experimental studies, tissue and blood were collected to analyze for further analysis to understand the mechanism of cardioprotection in our experimental models with fingolimod.

3.1. Animals

The experimental study was held at C.I.R.S.A.L. (Interdepartmental Research Centre for Laboratory Animals) of the Biological Institutes, University of Verona, Verona, Italy. The housing, handling and sacrifice of animals were carried out according to the regulations (Declaration of Helsinki and "Guide for the Care and Use of Laboratory Animals" - Institute of Laboratory Animal Resources - National Institutes of Health) after approval of the Ethics Committee and the Ministry of Health. We used healthy Sprague-Dawley (SD) male rats weighing 300-350 grams kept in rooms with controlled temperature and humidity with typical light-dark cycle fed with standard feed and water.

3.2. Cardiac arrest by inducing VF

Cardiac arrest is one of the leading causes of morbidity and mortality in Western countries. Despite initiatives for emergency medical services, and better techniques of defibrillation, the rate of survival following cardiac arrest (CA) is still very low. In fact, of the 0.7 million patients per year undergo resuscitation after CA, out of them survival rate is 3-10%[194]. Ventricular fibrillation (VF) is the most common cause of sudden cardiac death[195]. The incidence of VF-induced sudden CA is over 10 per 100,000[196]. In this field of cardiovascular science, on developing CA models that encompass most cardiovascular variables observed in routine human cases and that may play additional important role to study different effect of CA in heart and

brain. Presently, many models are available to induce CA in rats, including CA induction by a rapid intra-atrial injection of potassium chloride, delivering current to the right ventricular endocardium, trans-oesophageal cardiac pacing, transthoracal electrical fibrillation, , chest compression, compression of the heart vessels against the chest wall by use of a microsurgical instruments, asphyxia and simultaneous aortic occlusion and right atrial occlusion created by an arterial and venous balloon catheter respectively. All of these models have a few advantages and disadvantages. Although the VF induced model mimics the "square wave" type of myocardial insult (rapid loss of pulse and pressure) commonly seen in adult humans at the onset of CA. Here we will use a modified, simple, and reliable VF technique induced in rats as a model of CA that could be useful in studying the mechanisms of myocardial ischemia-induced injury and effect of fingolimod on it.

3.2.1. Anesthesia Induction

Rats were anesthetized using 5% isoflurane in 50% O_2 in a 5 liter plastic induction box. After induction, oro-tracheal intubation was done with a 14G cannula, and the rats were ventilated by a mechanical device for small animals (Harvard Model 687, Harvard Apparatus, Holliston, MA). The tidal volume was 6 ml/kg, and the breathing rate was 50–60 breaths/min with an air and oxygen mixture/FiO_2 = 0.5). Arterial blood gases were assessed to adjust ventilation to maintain arterial CO_2 tension ($PaCO_2$) of 35-45 mmHg. Anesthesia was maintained with 2.5% isoflurane, and intraperitoneally pancuronium-bromide (2 mg/kg) was used to maintain muscle relaxation. Rats were secured in supine position on a heating board. Throughout the experiment, ECG was monitored by using limb leads. The right femoral artery was isolated and inserted a miniaturized catheter of 2-Fr diameter (model SPR 838, Millar Instruments, Houston, TX) for the monitoring of systemic blood pressure. To administered treatment, femoral vein cannulation was done with 24G cannula.

3.2.2. Experimental model for Cardiac arrest and Extra Corporeal Resuscitation in rat

Access to the heart was achieved through a median sternotomy followed by the opening of the chest with a retractor. In order to speed up and simplify the procedures after the cardiac arrest has arranged for the preparation of animals for assistance with extracorporeal device cannulation. After recording, the skin and the subcutaneous planes in the midline of the neck have been identified as the trachea and immediately to the right of neurovascular bundle. A venous cannula (a modified version with 4 holes of a caliber of 5 French catheter) was advanced through the right external jugular vein to the right atrium allowing an excellent Venous drainage. The left common carotid artery was cannulated with a 24 G

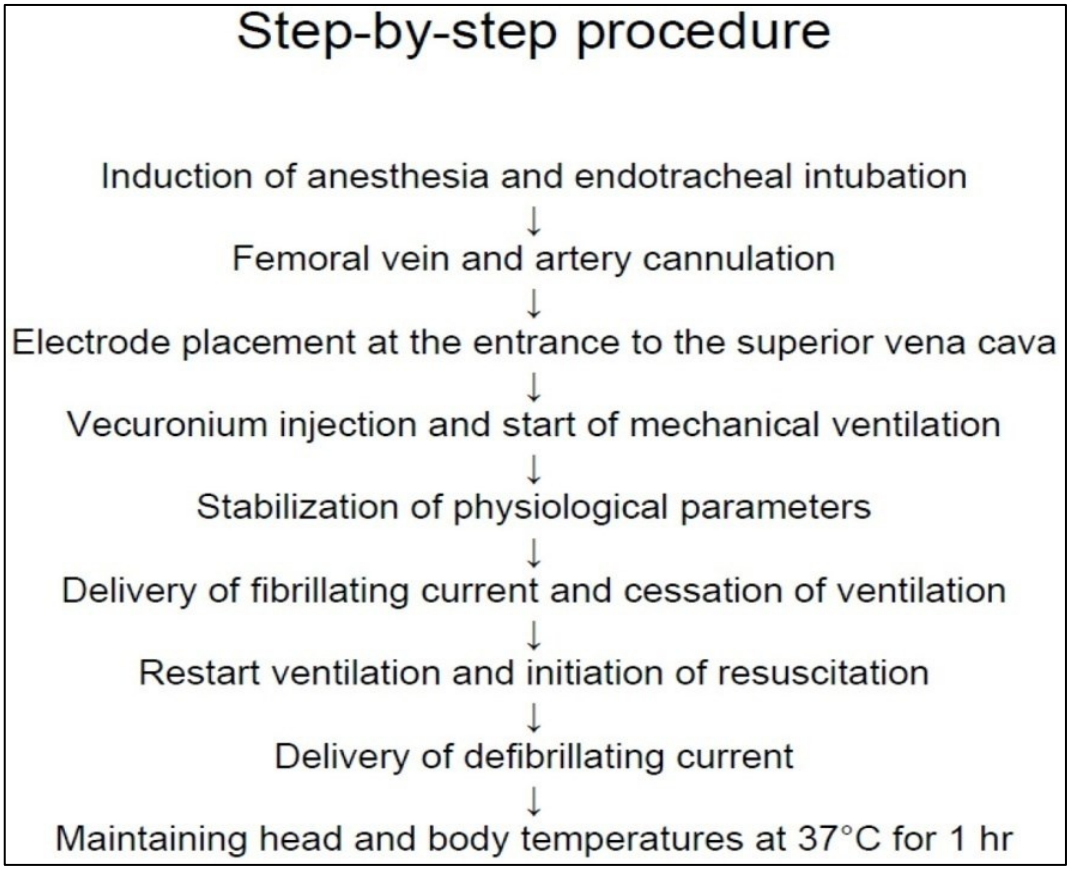


Figure 3.1. *Schematic diagram of procedural steps for induction of ventricular fibrillation-induced cardiac arrest(adapted from Kunjan R et al. 2013)*[197].

catheter, advanced to the aortic arch and connected to the arterial perfusion of the circuit as explained in flowchart (Fig. 3.2) Full heparinization (heparin 500 IU /kg)

was performed immediately before the start of extracorporeal circulation. After opening the pericardium it proceeded induction cardiac arrest which was obtained by ventricular fibrillation using a defibrillator disbursing a 3.5 mA current at 60 Hz released at the level of left ventricular epicardial region (Fig. 3.2). The current flow was maintained for 3 minutes in order to prevent a spontaneous depolarization and cardiac arrest was maintained for10 minutes, after which reperfusion occurred through extracorporeal circulation. Mechanical ventilation was stopped during induction of VF.

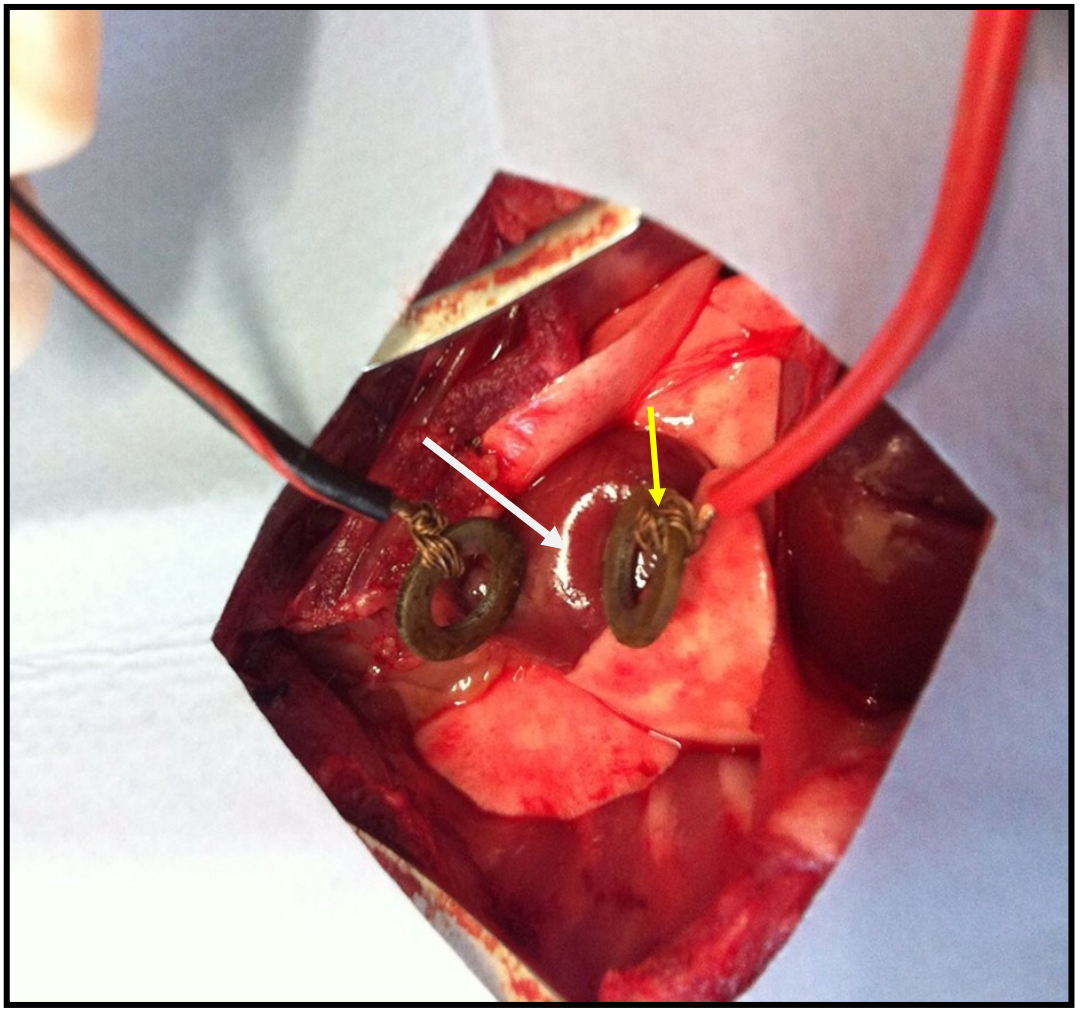


Figure 3.2. *Electrical induction of VF induced CA model, Yellow arrow indicating source for electrically induced VF, White arrow indicating left ventricle.*

3.3. In Vivo experimental rat model of Cardioplegic arrest

3.3.1. Animal preparation

After a pre-anaesthesia with vapours of diethyl ether, rats were orotracheal intubated with an atraumatic tube constituted by a venous cannula of 14 G. The rats were then ventilated with a mechanical respirator for rodents (Inhale, Harvard Apparatus, Holliston MA) with a mixture of oxygen and anaesthetic Sevorane 2% (Abbot Laboratories, Queenborough, UK) that guaranteed anaesthesia for the duration of the procedure, with a fraction of inspired oxygen (FiO2) of 90%, a tidal volume of 10 ml/kg and a frequency of 70 breaths per minute, 0.1 mg/kg of vecuronium bromide were administered to obtain complete muscular relaxation and repeated if needed. During the surgical procedure, room temperature was maintained between 23° and 25° C and a layer of isolating material (cork) was placed between the animal and the operating table.

Rats were placed in supine position, thoracic area, ventral surface of the neck and hind legs were shaved, and skin was disinfected with chlorhexidine. A thermocouple microprobe was inserted into the rectum to monitor animal temperature during the experiment. Electrocardiogram (ECG) electrodes were attached on both front limbs and left hind limb. The right femoral artery was isolated, and a miniaturised catheter with a diameter of 2-Fr (model SPR 838, Millar Instruments, Houston, TX) for continuous monitoring of systemic blood pressure was inserted. Subsequently, it was also cannulated the femoral vein with a 24-G canula (Delta Med S.p.A. Viadana (MN) Italy), followed by administration of 500UI / kg heparin to ensure patency and to be ready for CPB.

3.3.2. Surgical procedure

Access to the heart was achieved through a median sternotomy; chest was kept open with a retractor. After incision of skin and subcutaneous planes in the midline of the neck, trachea was identified. A venous cannula (a modified 4-hole catheter calibre of 5 French) was advanced through the external jugular vein to right atrium allowing an excellent venous drainage. The left common carotid artery was cannulated with a 24 G (Delta Med S.p.A. Viadana (MN) Italy) catheter that was advanced to the aortic arch and connected to the line of arterial perfusion circuit. Also, 0.2 mg pancuronium was administered before CPB. Muscle relaxation was used to prevent spontaneous ventilation that often interferes with the venous return due to movement of the mediastinal structures about the venous outflow cannula.

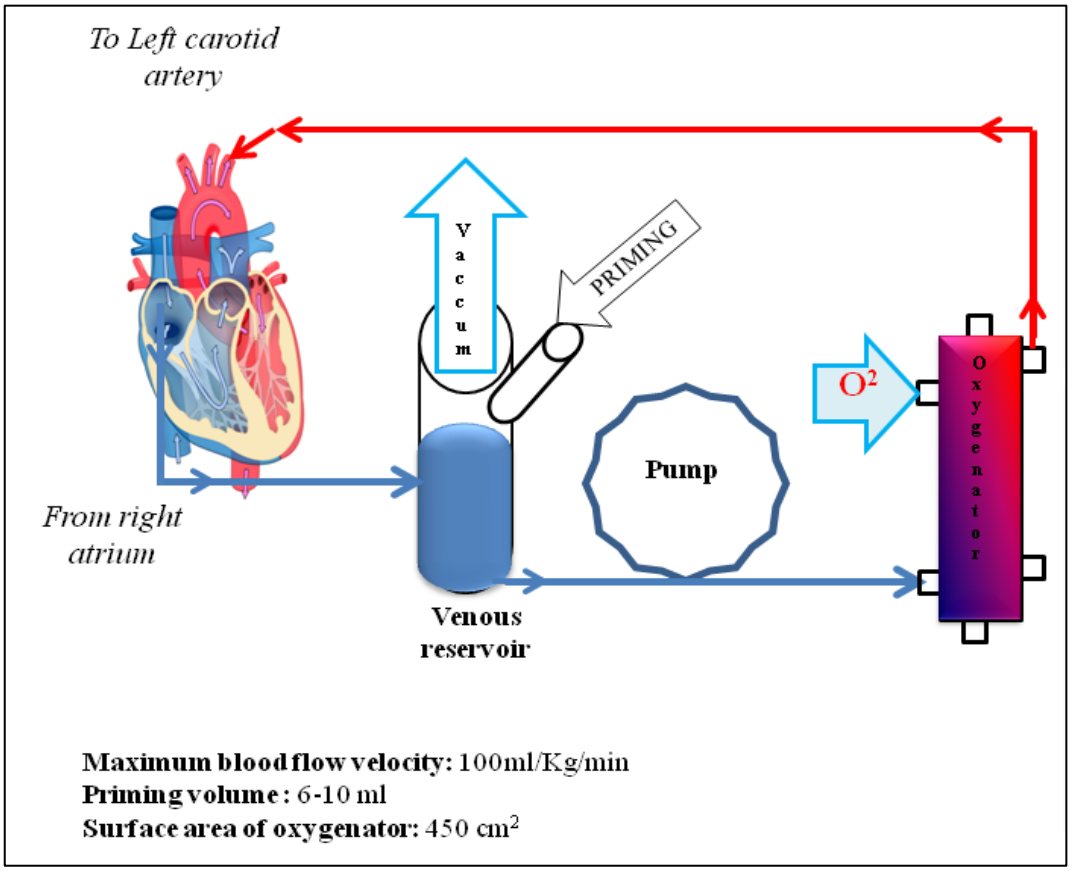


Figure 3.3. ***Schematic view of extracorporeal life support***

3.3.2. Perfusion circuit

The extracorporeal circulation circuit was constituted by a roller pump (Stockert SIII, Sorin, Germany), an hollow fiber oxygenator (Sorin, Mirandola, MO, Italy), a venous reservoir connected to a vacuum with a pressure regulator -30 mmH2O to facilitate venous drainage, all connected by plastic tubing with 1.6 mm inside diameter. The total volume of filling of the extracorporeal circuit including the oxygenator was 6 ml and constituted by colloid solution and Ringer's lactate solution. The exchange surface of the gas was 450 cm_2 and the heat exchange surface area of 15.8 cm_2. Once prepared venous and arterial accesses rat was connected to the circuit of CPB and maintained with a flow rate of 80-100ml / kg/min and a range of mean arterial pressure of 70-90 mmHg. After ensuring the adequate venous drainage and appropriate hemodynamic stability mechanical ventilation was suspended.

3.3.3. Cardioplegic Arrest

After 10 minutes of CPB ascending aorta was clamped with a vascular clamp and cardiac arrest was induced by administration of 2 ml cardioplegia (St Thomas solution) in ascending aorta. The CPB flow rate was adjusted as needed to maintain a constant venous reservoir blood level. After 10 min of cardiac cardioplegic arrest aortic clamp was removed, after few minutes heart usually started beating spontaneously, CPB was maintained for another 60 min. During the entire procedure, mean arterial pressure remained higher than 45 mmHg. Subsequently, this was suspended, and weaning from CPB was completed with reinfusion of the blood in the circuit before rat sacrifice and removal of brain and heart.

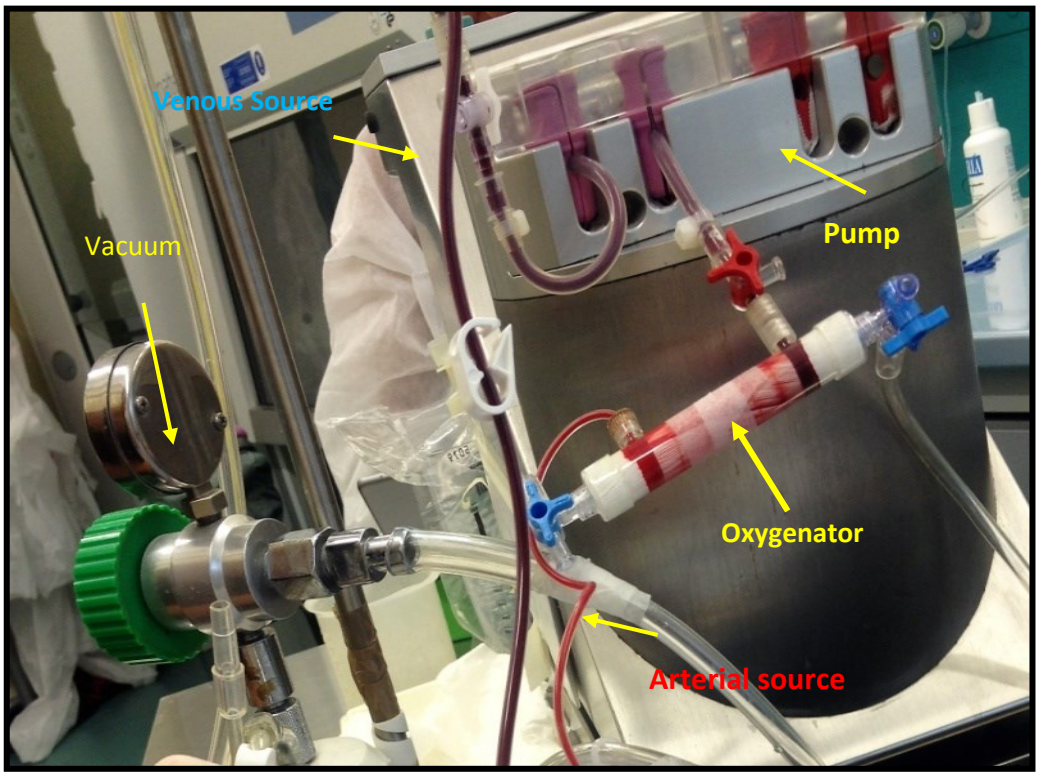


Figure 3.4. Roller pump and Oxygenator during extracorporeal circulation

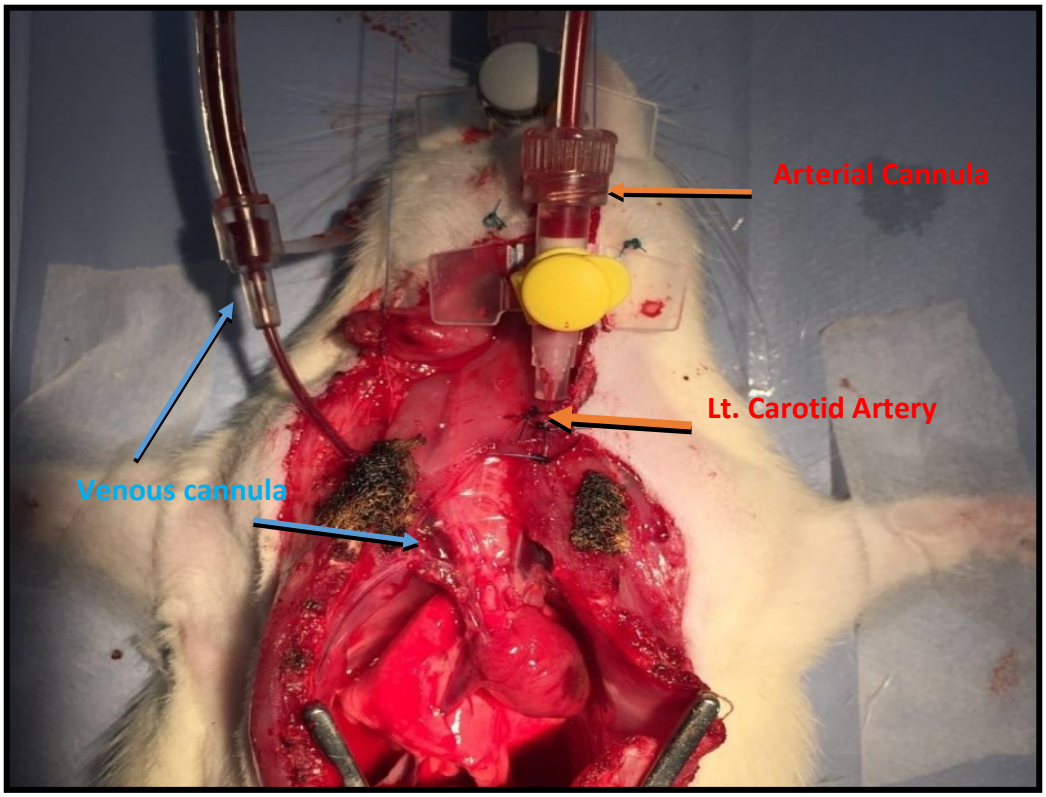


Figure 3.5. Veno-Arterial Cannulation during Extra Corporeal Circulation

3.4. Heterotopic Cardiac Transplantation Model of Rats

A technique for a heart transplant in small animals was first described by Abbot et al[198, 199]. And then by Ono and Lindsey who made some changes[200, 201]. Apart from these few have described the surgical techniques and anaesthetic management of heterotopic cardiac transplantation in rodents[202].

3.4.1. Description of model

For each experiment planned using 2 male rats weighing about 350-400 g. One rat was used as a receiver and the other as a donor.

3.4.2. Animal Preparation

The donor's rat chest was opened through an incision with Mayo curved scissors, to prevent injury to the major blood vessels and lungs. The cardiac exposure was done by removal of the pericardial sac. The heart was perfused with St-Thomas at 4 °C temperature, with the composition of St-Thomas nine mL and of 1 mL heparin sodium, 1000 U / mL. A 6-0 proline tie was used around the superior vena cava (SVC) and inferior vena cava (IVC). Heart perfusion was obtained by inserting a 24-

G needle into IVC. Five serial injections of 2 mL volume were delivered within 4 to 5 minutes, to achieve an equal volume of blood drainage after each 2mL infusion. After infusing first 2-mL injection, the SVC was tied with 6-0 proline. Later, after infusion of the remaining solution, IVC was also tied. Perfusion is recommended at the rate of slow speed to prevent coronary vascular damage. The transverse sinus was found under the pulmonary artery and ascending aorta with 45-degrees curved forceps, carefully to avoid injury to the left atrium with the tip of forceps. The pulmonary artery and ascending aorta were cut together by using a 60-degree sharp angled scissors least 4 to 5 mm above the origin (Fig 1A). The SVC was dissected over the knot, and the IVC was dissected below the knot. The heart was lifted with a 4-0 proline suture placed under it, then moved downward, and also the suture was tied around the pulmonary veins. The donor's heart was preserved in St-Thomas solution at 4 ° C on the ice while preparation of the recipient rat.

3.4.4. Graft Implantation

The animal was positioned with head toward the left side of the surgeon. The recipient rat abdomen was opened through a midline incision with Mayo curve scissors. The intestines have been moved to the left side and covered with moistened gauze with normal saline. The major vessels (IVC to the right of midline and abdominal aorta left side of the midline) were dissected and separated from the surrounding tissue gently with a cotton tip, using mini forceps only when necessary. The small iliolumbar veins were exposed and tied with 6-0 silk sutures or cauterised. Vascular mini-clamps with delicate teeth distally entered first, then proximally, abdominal aorta and IVC, isolation a 1.2-cm segment of the vessels between the jaws. The aorta was incised near the distal terminals using a 24-G needle to drain the blood. Extra-fine scissors were used to perform an arteriotomy of about 4 mm, with special care not to damage the posterior wall. The lumen was gently washed with a heparinized St-Thomas solution. The donor's heart was placed in the field in preparation for end-to-side anastomosis. The organ was positioned with the apex towards the tail and the aorta above the pulmonary artery. The aortic anastomosis was performed by inserting a point of anchoring the proximal end of the arteriotomy (out-in) the abdominal aorta, and then a point on the aorta graft-out, and was set with a triple knot . A second (out-in) of the suture anchor has been placed on the opposite side of the first point-out the distal end of the arteriotomy. After positioning the second suture anchor, a continuous suture (5- 6 points) was performed on the front wall from the proximal end towards the distal end of the arteriotomy. The heart graft has been repositioned by turning it over to expose the back wall. The anastomosis was completed with a continuous

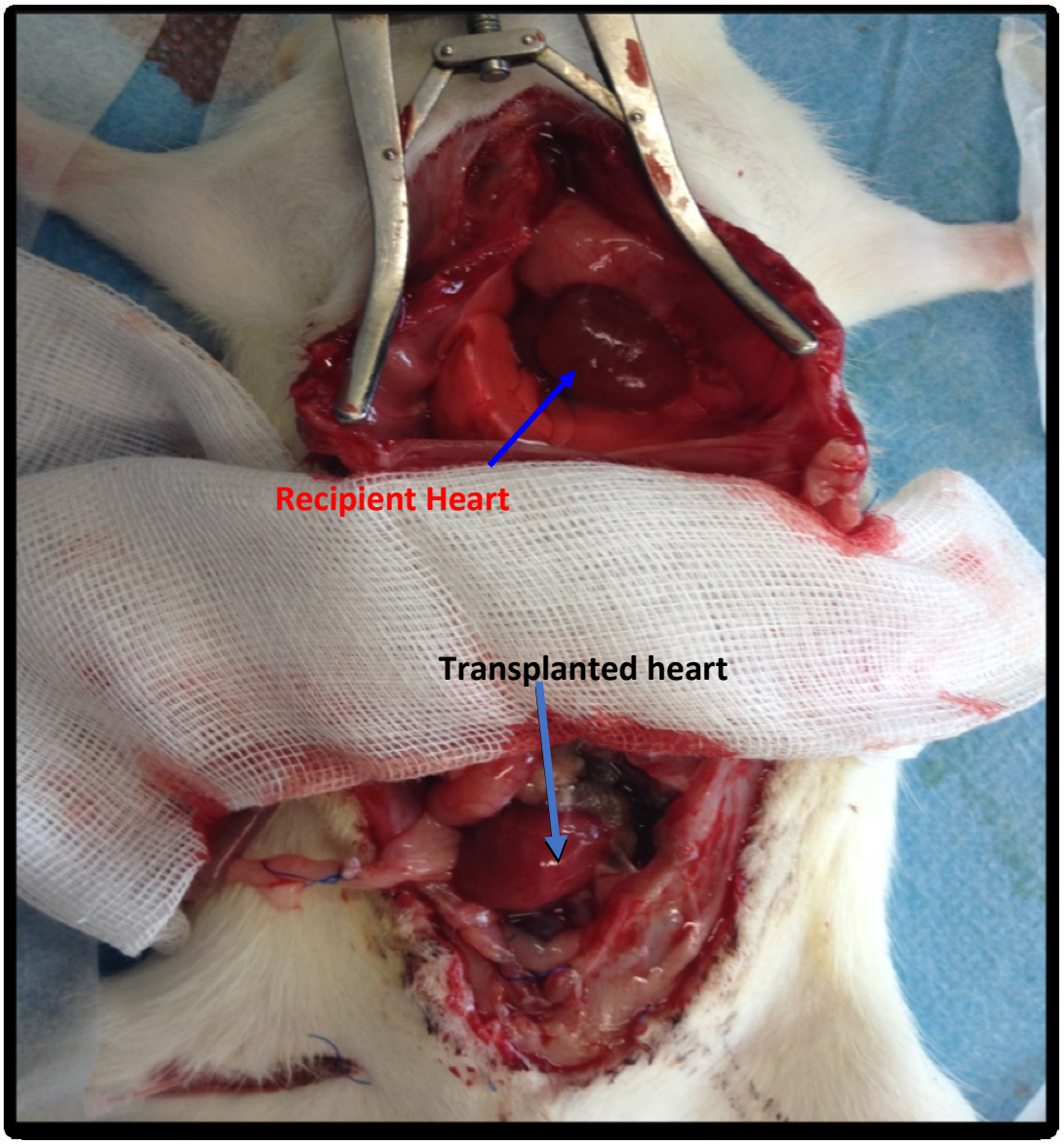


Figure 3.6. Image showing recipient and transplanted heart

running suture. The graft has been turned back to expose the anterior wall of the graft where the pulmonary artery had collapsed over the aorta. An opening 5 to 7 mm in length was made in the receiving IVC with scissors, and the lumen was flushed with the heparinized St-Thomas solution to remove any thrombus. The IVC opening should be more (5 to 6 mm in length) than the aortic opening to anastomose the pulmonary artery appropriately. An out-in stitch was kept on the distal part of the pulmonary artery and in-out at the distal part of the IVC opening. A continuous suturing was performed first a longer posterior wall and then at the anterior part of pulmonary artery-IVC . Before releasing micro vessel clamps, the suture line has been thoroughly checked for any expected leakage point. First, the distal clamp was released, and then after 30 seconds the proximal clamp. Anastomosis was checked

throughout suture point, and if there was some bleeding point, that could be controlled using cotton tips and oxidised cellulose (Tabotamp, Ethicon, and Neuchatel, Switzerland). Within few seconds after reperfusion, spontaneous heart contractions were observed in grafted heart. The total ischemia time in a successful operation ranged from 40 to 60 minutes. The abdominal structures were replaced with care to avoid torsion of intestines. The incision was closed with a non-absorbable continuous suture (4-0 proline) that included the fascia, muscles and separate suturing was done for skin. The rat was kept under the heat lamp for 15 mins to achieve normothermia. And rats were kept in a warm and oxygen rich room until sacrifice or other measurements.

Removing the weight, a constant ligation pressure could be applied and easily released. Leads were implanted subcutaneously in a lead II electrocardiogram (ECG) configuration. Animals were then positioned in a specially designed Plexiglas cradle fitted to the occlusion device. Rats stabilized for 15-20 minutes before arrest. Ischemia was visually confirmed by regional cyanosis downstream of the occlusion or changes in the ECG. The heterotopic transplanted heart *in vivo* rat model shown in (Fig. 3.6).

3.5. Hemodynamic monitoring and measurements

After recording, the skin and the subcutaneous planes in the midline of the neck were identified as the trachea immediately to the right of neurovascular bundle. The right carotid artery was isolated, linked cranial and caudal clamped. After making a small cut with microsurgical scissors and inserted miniaturized catheter of 2-Fr diameter (model SPR 838, Millar Instruments, Houston, TX) into the carotid artery (Figure 3.7).

Figure 3.7. Conductance catheter for monitoring of pressures and *volumes in the left ventricle (Millar Instruments, Texas, USA).*

This catheter was then connected to the relevant transducer and then to the unit Power-Lab (AD Instruments, Colorado Springs, CO). The latter, through the USB

port, has been connected to a computer for real-time display of pressure-volume curves and data logging using the Chart software (AD Instruments). With this set-up the catheter to conductance was introduced into the carotid artery and advanced to the left ventricle, the correct position is reached by following the trend of the pressure curve.

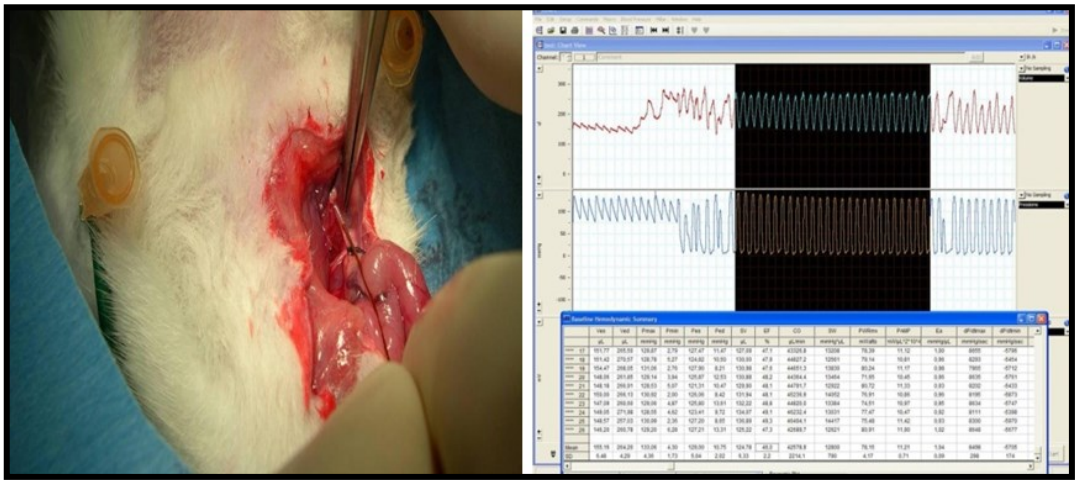


***Figure 3.8**. Carotid Catheterization(a) and registration through theChart software that shows the transition from the aorta to the left ventricle (b).*

The signal is recorded continuously with a sampling rate of 1,000 / s thus being able to monitor the hemodynamic changes during the whole duration of the intervention. The parameters considered were Left Ventricular End-Systolic Pressure (LVESP), Left Ventricular End-Diastolic Pressure (LVEDP), Ejection Fraction (EF), Stroke Volume (SV), maximum increase in systolic blood pressure (+ dP / dt) and maximum decrease in diastolic blood pressure (- dP / dt) , Maximal Power (max PWR), Adjusted Preload Maximal Power (PAMP) and tau-Weiss constant time. The calibration of the conductance system was obtained as previously described by Pacher and colleagues. Briefly, nine cylindrical holes of known diameter variable between 2 and 11 mm inserted into a 1 cm high block were filled with the heparinized whole blood of rats. In this calibration, the volume conductance-linear regression of the absolute volume contained in each cylinder compared with the raw signal obtained through the catheter in conductance was used for the volume calibration formula.

3.6. Tissue Analysis

3.6.1. Paraffin processing and embedding

Following fixation myocardial tissue was done in formalin. Myocardial tissue was placed into labelled plastic embedding cassettes and washed in running water for 30 minutes before being dehydrated through a series of alcohols and liquid paraffin (Table 3.1). Following processing, each 3mm slice was embedded in fresh paraffin and left to set before cutting.

3.6.2. Cutting

Sections (3μm) were cut using microtome from paraffin blocks. Sections were floated in the water bath with maintained temperature of 40 °C to avoid any crease, and then sections were placed on a polarised microscopic slide for immune staining and green for histology. Sections were exposed for 1 hour at 60°C to let them fix on the slide and stored until required.

3.6.3.Histopathology

Hematoxylin and eosin (H&E) staining was used, to assess the quality of sections, proper tissue fixation, and processing. H/E was also used to see morphological changes in the myocardial cell in different experimental groups.

3.6.3.1. Hematoxylin and Eosin staining

Fresh sections mounted on slides were kept in an oven at 60°C for 60 mins then de-deparaffinized in xylene for 20 mins following serial rehydration through a 100% (1 x 5 mins), 90% (5 mins) > 75% (5 mins), and rinsing with tap water (3 mins). All sections on each slide were immersed in a hematoxylin solution (Thermo-Scientific, Loughborough, U.K.) for 30 seconds and then washed in flowing tap water. For differentiation, acid alcohol solution (1%HCl in 75% ethanol) was used to immerse for 10 seconds. Sections were rinsed in tap water for 2-3 minutes before ‘bluing' in tap water solution Scott's using (2% $MgSO^4$).

Table 3-1 *The sequence of solutions heart tissue was processed through before paraffin embeddin.*

Stage	Solution	Temperature	Time in Solution
1	Running water	~ 15-20°C	30 mins
2	70% Ethanol	Room Temp.	30 mins.
3	70% Ethanol	Room Temp.	30 mins.
4	90% Ethanol	Room Temp.	30 mins.
5	90% Ethanol	Room Temp.	30 mins.
6	100% Ethanol	Room Temp.	30 mins.
7	100% Ethanol	Room Temp.	30 mins.
8	Xylene	Room Temp.	30 mins.
9	Xylene	Room Temp.	30 mins.
10	Liquid paraffin	65°C	30 mins.
11	Liquid paraffin	65°C	30 mins.
12	Liquid paraffin	65°C	30 mins.
13	Liquid paraffin	65°C	Overnight

and 0.35% $NaHCO^3$) for next 2 minutes and then another 2 minutes washed in tap water. Slides were checked under the light microscope to assess the quality of nuclear hematoxylin staining. If nuclear staining was too dark, acidic alcohol solution was used to light hematoxylin and then sections were immersed in alcoholic eosin Y solution (Surgipath., Cambridge, UK) for 3 mins following serial use of ethanol 75% (5 mins) > 85% (5 mins) > 100% (5 mins (2times)) and xylene (20 mins) for dehydration, prior to Mount cover slips using polyvinyl.

3.6.3.2. Counterstaining with hematoxylin

The immunostained sections were counterstained with hematoxylin to aid delineation of anatomical features following DAB visualisation; sections were rinsed for ten mins and then immersed in hematoxylin, differentiated and ‘blued,' then dehydrated through alcohols and xylene and mounted with cover slips using DPX as above.

3.7. Immunohistochemistry

All sections used for immunostaining were adjacent to those who had undergone histological analysis. All immunostaining was conducted by the author unless otherwise stated.

3.7. Masson Trichrome Staining

As the name implies, three dyes are employed selectively staining muscle, collagen fibres, fibrin, and erythrocytes. The general rule in trichrome staining is that the less porous tissues are colored by the smallest dye molecule; whenever a dye of large molecular size can penetrate, it will always do so at the expense of the smaller molecule. Others suggest that the tissue is stained first with the acid dye, Biebrich Scarlet, which binds with the acidophilic tissue components. Then when treated with the phosphor-acids, the less permeable components retain the red, while the red is pulled out of the collagen.

3.7.1. Interstitial fibrosis determination

A myocardial tissue from the left ventricle was fixed in phosphate-buffered 10% (vol/vol) formaldehyde (pH 7.2) and embedded in paraffin. Three-micron sections were stained with Masson's trichrome to evaluate fibrosis as mentioned in the previous study [203]. Measurements (×20 magnification) were analyzed multiple microscopic fields per section and expressed as a collagen volume fraction. Randomly left ventricle sections were stained from each experimental.

3.8. TUNEL assay

(Terminal deoxynucleotidyl transferase mediated X-dUTP nick end labeling). TUNEL assay has been done on paraffin fixed tissue using the protocol given by kit manufacturers(Sigma Aldrich).

3.9.1. Rehydration

Rehydration was done using xylene for 20 mins, following series of ethanol 100%, 100%, 90%, 80% and 70% 5 mins each. Excess water was removed using Kim-wipe, circles made around sections using PAP pen.

3.9.2. Proteinase digestion

Permeabilization of the tissue section with 15 µg/ml Proteinase K (Gibco BRL) in 5 mM EDTA and 5 mM Tris 7.5. Incubation was done for 20 minutes at room temperature. Proteinase K incubation time was adjusted for 15 minutes.

3.9.3. TUNEL labeling

TUNEL cocktail

The cocktail was prepared each time for new experiment and for control slides only PBS was used. New Eppendorf tubes were used each time for each experiment. 50ul TUNEL solution was used for each section to cover adequately.

Positive control – DNAse1 treatment

Incubation of positive control sections done for 10 min. at room temperature in 1500 U/ml DNAse1 in 20 mMTris pH 7.5, 10 mM MgCl2, 1 mg/ml BSA.

3.9.4. Counterstaining

Counter staining was done with Gel Mount-DAPI (Sigma-Aldrich) as a final step. TUNEL signal observed with blue light excitation (450 – 500 nm), fluorescein emission was green.

3.9.5. Image acquisition

Using the digital camera, because of significantly shorter exposure times as FITC fluorescence is quickly bleached by light. Focus area of interest using UV illumination, acquired DAPI image, then switched to blue illumination without moving the specimen position, captured the TUNEL image.

3.10. Blood samples analysis

At the end of experiments, according to study design blood samples were collected from the abdominal aorta and sent to Hematological and Biochemical Laboratory,

Azienda Ospedaliera Universita di Verona for blood counts, cardiac biomarkers and inflammatory mediator's measurement.

3.11. High-Energy Phosphates

The high-energy phosphates Adenosine MonoPhosphate (AMP), Adenosine Di-Phosphate (ADP), Adenosine Tri-Phosphate (ATP), and phosphocreatine (PCr) were analyzed by high-performance liquid chromatography as described previously[204].[16] Energy charge (EC) was calculated according to the following formula: EC = (ATP + ½ ADP)/(ATP + ADP + AMP).

3.12. Oxidative Stress Measurement

To investigate, Malondialdehyde (MDA) and Reactive Oxygen Species (ROS) after 24 hours, myocardial tissue were processed at 37 °C, 5% CO_2 95% air atmosphere for 60 min according to the manufacturer's protocol. The myocardial tissue were incubated at 37 °C for 30 min in PBS (in mM: 137 NaCl, 2.7 KCl, 9.8 Na2HPO4, 1.5 KH2PO4, pH 7.3) containing 10 μM. Absorbance at 490 nm was measured with a microplate reader (ELx800; Bio-Tek, Winooski, VT, USA).

3.13. Western Blotting

3.13.1. Antibodies

Western blot was used to investigate the levels of proteins in heart tissue homogenate. Details of primary and secondary antibodies and their concentration used can be seen in Table 3.3. Negative control was also used, where membrane was exposed and processed in all aspects except an addition of primary antibodies. Although, the Western blot can be considered reliable, relatively low in cost, time-saving method for quantification of protein. There must be some potential deficits considered while using it including nonspecific binding of antibodies/ nonspecific bands. That can lead to results "false positive". The negative controls are important to rule out this issue in addition to choosing an appropriate blocking solution.

3.13.2. Homogenate Preparation

Then samples were sliced in 10 microns by using cryostat sectioning machine (Leica CM1950 Leica Biosystems Nussloch GmbH) at -20°C. Tissue Homogenization was done in 10 times concentrated volume (e.g. 50μl in 5mg) of homogenization buffer (20mM Tris base, 250mM sucrose, 1mM EGTA,1mM EDTA) to which protease inhibitors cocktail and phosphatase inhibitors were added (Sigma-Aldrich). Following homogenization, samples were centrifuged at 1600 rpm for 10 minutes at

4°C. The Supernatant was collected in fresh Eppendorf tubes for storage at -80°C until use.

3.13.3. Assessment of protein concentration of samples

The protein concentration of samples was determined using the bicinchoninic acid (BCA) protein assay method (Thermo Scientific, Loughborough, UK). This assay is based on the reduction of Cu^{2+} ions to Cu^{+} by protein in an alkaline medium (known as the biuret reaction) and the subsequent reaction of the reduced copper cation with BCA to produce a quantifiable colorimetric reaction.

Table 3-2 Antibodies used for Western blot experiments

Primary antibody	Species Type	Clone	Source	Dilution used	Secondary antibody
Akt	Rabbit	Polyclonal	Cell signalling	1:1000	Goat anti- rabbit
P-Akt	Rabbit	Polyclonal	Cell signalling	1:1000	Goat anti- rabbit
ERk44/42	Rabbit	Polyclonal	Sigma- Aldrich	1:1000	Goat anti- rabbit
P-ERK44/42	Rabbit	Polyclonal	Cell signalling	1:1000	Goat anti- rabbit
β-Actin	Rabbit	Polyclonal	Sigma- Aldrich	1:5000	Goat anti- rabbit

The colour intensity may be measured via spectrophotometry (wavelength 595nm); colour intensity is proportional to protein concentration allowing accurate determination of sample protein concentration between ranges of 20 to 2000 µg/ml. The BCA assay was conducted in a 96 well plate. A series of protein (bovine serum albumin (BSA)) standards 25-2000 µg/ml were made from 2000 µg/ml stock diluted in total homogenization buffer. 1:50 dilutions of total homogenate were prepared, 50 parts of BCA reagent A (Sodium carbonate, bicinchoninic acid, sodium bicarbonate and sodium tartrate in 0.1M sodium hydroxide) was added to 1 part BCA reagent B (4% cupric sulphate) to form the BCA working reagent. 10µl of diluted total homogenate sample was added to the microplate in triplicate, 200µl of BCA working reagent was then added to each well and plates were covered and incubated at 37°C. Plates were analysed on a plate reader (Dynex Technologies, Worthing, UK) and results recorded. Triplicates were examined, and any values greater than +/- 10% were excluded, the value for each sample was taken as the average of each triplicate (or duplicate where a value had been excluded). A

standard curve was constructed from the values of the known standards, and then the protein content of samples was determined via linear regression analysis (Fig. 3.10).

3.13.4. SDS-Page electrophoresis

Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) separates polypeptides based on their electrophoretic mobility. SDS binds proteins denaturing their polypeptide backbone and causes the dissociation of hydrogen bonds which results in an unfolding of tertiary and secondary structures, SDS also confers an overall net negative charge on proteins meaning that they are separated solely on size. Samples are prepared in Laemmli buffer which contains 2-mercaptoethanol, dithiothreitol (DTT), bromophenol blue and glycerol. 2-mercaptoethanol and DTT are reducing agents which reduce inter and intramolecular disulphide bonds ensuring proteins remain as linear structures, bromophenol blue is added as an indicator dye allowing observation of protein migration through the gel, it also makes gel loading easier, glycerol is added to increase the density of the sample ensuring it will sink to the bottom of the well when loaded. All SDS-Page electrophoresis and Western blotting in studies reported in this thesis were conducted using the XCell Surelock mini gel system (Invitrogen, Paisley, UK).

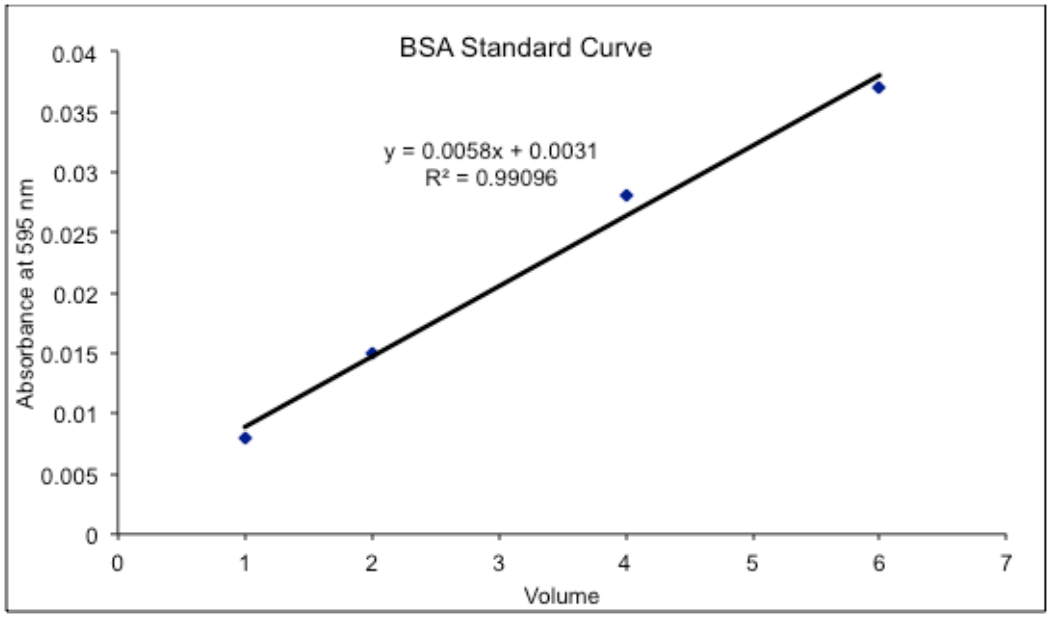


Figure 3.9. *Example of the standard curve for determining proteinconcentration The relative absorbance of BSA standards at 595nm increases with increasing concentration of BSA. Linear regression was used to calculate the protein concentration of samples of total homogenate utilised for Western blotting experiments.*

Following determination of protein concentration of homogenates, samples containing equal amounts of protein were prepared for Western blot analysis. 4x laemmli buffer and RIPA buffer were added to each sample to make up to the required volume. Samples were heated at 70°C in a water bath for ten mins to denature proteins and then vortexed before gel loading protein was separate by SDS-polyacrylamide gel electrophoresis on 4-12% Bis/ Tris gels using 2-(N-morpholino) ethane sulfonic acid (MES) running buffer (Invitrogen, Paisley, UK), gels were run for 1.5 – 2 hours at 115 V unless otherwise stated.

3.13.5. Protein transfer

After running SDS page, when proteins were separated in the gels, gels were removed from the apparatus. By gentle handling, upper gel consist of stacking gel was trimmed off and transferred to filter paper soaked with transfer buffer (6cm x8 cm). Polyvinylidene fluoride (PVDF) membrane (GE Healthcare, Amerbaseline, UK) was dipped into 100% Methanol and distal water for three and one minute respectively, from where membrance and then another layer of soaked filter paper was placed over gel. Blotting paper, membrane, gel and blotting paper 'sandwiches' (Fig 3.11) were adjusted in blot modules (Bio-Rad Laboratories, Inc.). After proper securing the blot transfer apparatus with gel sandwich were filled with transfer buffer solution with 20% methanol. Protein transfer was done using constant 300 m A for the period 2.5 hours.

Following protein transfer, membranes were incubated in Ponceau S solution (Sigma-Aldrich Company Ltd., Dorset, UK) to ensure equal loading of protein. Membranes were then blocked in 10ml Odyssey blocking buffer (Li-Cor Biotechnology UK Ltd., Cambridge, UK) / PBS (1:1) in a light proof box at room temp for one hour. Membranes were incubated with primary antibody in blocking buffer/PBS (1:1), 0.1% Tween20 (Sigma-Aldrich Company Ltd., Dorset, UK) solution (10ml) overnight at 4°C.

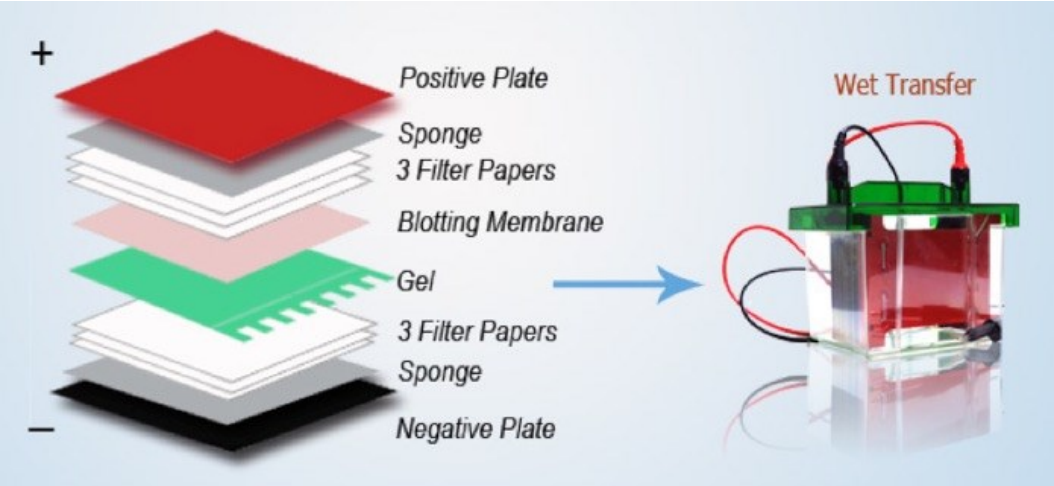


***Figure 3.10**. Schematic diagram of a filter paper, gel, membrane and filter paper "sandwich". This was placed between wet sponges soaked in 20% transfer buffer solution and into a blotting module, a current was passing through negative toward positive plate for protein transfer to PVDF membrane.(Image adapted from a web of Darrel J Killian).*

In Western blot experiments, β-actin (1:5000, Sigma-Aldrich Company Limited, Dorset, UK) used as a loading control. On next day, membranes were washed three times 5 mins each time in TBS-T (0.1% Tween) solution, then incubation with an appropriate secondary antibody (1:10,000 LiCor Biotechnology UK Limited, Cambridge, UK) using blocking solution in TBS-T solution for 50 minutes at room temperature. Following secondary antibody incubation, membranes were washed three times in TBS with 0.1% Tween20 solution for 10 minutes each time. Membranes were developed using Synegene Box. Images were saved in Tif. Version for quantification.

3.13.6. Quantification of protein levels

Actin was used as a loading control in all blotting membranes. To confirm the reliability of actin as a loading control, actin expression was confirmed in myocardial ischemic and reperfusion tissue and results were not influenced by ischemia nor reperfusion. Actin protein was also verified in all groups with the baseline.

3.14. Statistical Analysis

All statistical analysis was performed using SPSS statistical package (version 10.0.7). All values were expressed as means ± SD of the mean. Hemodynamics, apoptosis, and inflammation were compared by using a one-way (ANOVA).A Mann-Whitney *U* test and Student t-test were used for comparison of data between baseline, control and treated groups. Correlations between groups were assess using the Pearson's correlation coefficient(r). The difference was statistically significant considered as a P value ≤ 0.05.

SECTION III-A

4. Cardioprotective effect of fingolimod in Cardiac Arrest (CA) and ExtraCorporeal Life Support (ECLS) resuscitation in experimental model

4.1. Introduction

Sudden cardiac arrest is a major cause of mortality in the developed world[205] and post resuscitation mortality remains more than 50%[206]. CA and cardiopulmonary resuscitation both are associated with global myocardial ischemia reperfusion injury that induce myocardial dysfunction, leading to poor prognosis and adverse outcome[207]. Currently, ECLS is an effective way to treat cardiogenic shock or CA, because of its great potential to provide a quick circulatory support via peripheral vascular access[208]. The sphingosine 1-phosphate (S1P) is known lysolipid mediator for more than 2 decades to play a role in cellular changes, including differentiation, proliferation, migration, contraction and survival[209-214]. Sphingosine 1-phosphate is able to increase the survival of cardiomyocytes during episodes of hypoxia; evidence emerged from in vitro studies [135, 136], it can also reduce the size of the infarcted area in productions of isolated hearts ex vivo and in vivo model [137, 138, 185]. The role of sphingosine 1-phosphate in cytoprotection is under debate by its survival pathways activation. However, pharmacological post-conditioning with sphingosine 1-phosphate activation has been shown for potential application after resucitation in *in-vitro and in vivo* study[215]. Primarily, end points for our study were included functional aspects and level of myocardial protection. Sphingosine 1-phosphate receptors agonist fingolimod act as a pharmacological cardioprotective agent for post CA resuscitation care have not been investigated yet. Our hypothesis is that fingolimod play cardioprotective role in global ischemia reperfusion related to sudden cardiac arrest. Therefore the aim of this study was to assess the cardioprotective effects of S1P receptors activation by fingolimod after resuscitation from CA during ECLS in rat model as compared to control group. To understand cardioprotective role of fingolimod, we investigated functional hemodynamics parameters, apoptosis, inflammation and Akt and Erk11/2 pathways.

4.2. Methods

4.2.1. Animals

Sprague-Dawley Rats (300-350grams) Obtained from Harlan Laboratories (Udine, Italy). They were fed standard rat chow, which they had access to ad libitum. Rats were housed at a density of 3-4 per cage and maintained on a 12-hour light/dark cycle at 21°C. The study was approved by the National Animal Welfare Committee.

4.2.2. Experimental design

At the initiation of ECLS after 10 min of untreated VF, rats were randomly categorized into two groups, Group A was treated with normal saline, and in Group B, fingolimod administration was done at dosage 1 mg/kg through femoral vein cannula. Group C and D were similarly treated with saline and fingolimod respectively. In Group A and B, after 1 hour of reperfusion hemodynamic measurements were done and myocardial tissue was collected to calculate high energy phosphates. During ECLS internal temperature was kept at 36-37°C in both groups. While in Group C and D reperfusion were allowed upto 24 hours. In later groups, ECLS weaning was done with hemodynamic stabilization and rewarming followed by heating lamp. After 24 hours of reperfusion, hemodynamics were measured similarly as at 1 hour and blood and myocardial tissue were collected for further analysis of inflammation, apoptosis and oxidative stress. Schematic review of the experiment is illustrated in Fig. 4.1.

Experimental techniques, tissue and blood analysis protocol is mentioned in section II(Materials and Methods).

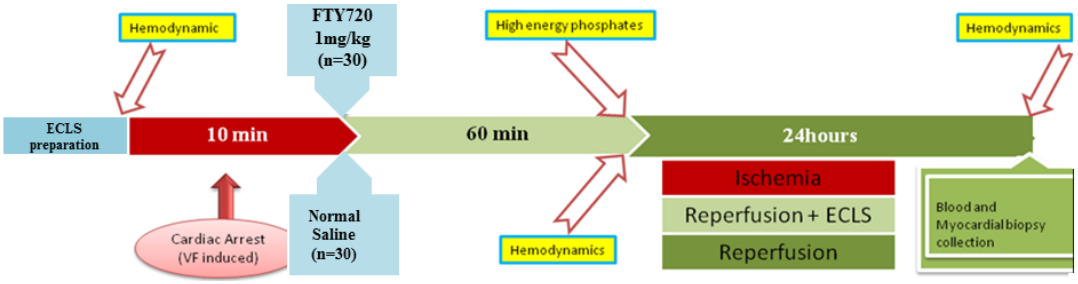


Figure. 4.1. ***Schematic view of experimental study.***

4.3. Results

4.3.1. Left Ventricular Function

Baselines hemodynamic were similar in both groups (Table 4.1). When global ischemia through CA was subjected in all rats following fingolimod treatment in Group B and D (n=15 each group) and saline treatment in Group A and C (n=15 in each group). Hemodynamic measurements were done to evaluate LV performance using the Millar catheter system (Fig. 4.2).

Table 4-1 Comparison of baseline characteristics among different groups

Baseline	Group A	Group B	P value	Group C	Group D	P value

HR (beats/min)	281±16	278±21	ns	309±29	295±24	ns
MAP (mmHg)	137±16	132±18	ns	112±14	116±16	ns
CO (ml/min)	42±3.5	45±4.2	ns	44±3	47±5.2	ns

The LV End Systolic Pressure (LVESP) elevated significantly after CA and resuscitation in Group D as compared to Group C ($p \leq 0.05$)(Fig. 4.2A). No difference between Group A and B were observed ($p>0.05$). The LV End Diastolic Pressure (LVEDP) measurements showed a reduction in the Group B and D vs. Group A and C ($p=0.04$) ($p\leq0.05$) respectively(Fig. 4.2B). On the other hand, The minimal pressure relaxation rate (dP/dt min) was also improved in Group B and D as compared to Group A and Group C ($p\leq0.05$) (Fig. 4. 2C). Ventricular systolic performance dp/dt max after CA+reperfusion was increased in Group D vs. Group C ($p \leq 0.05$) (Fig. 4.2D).

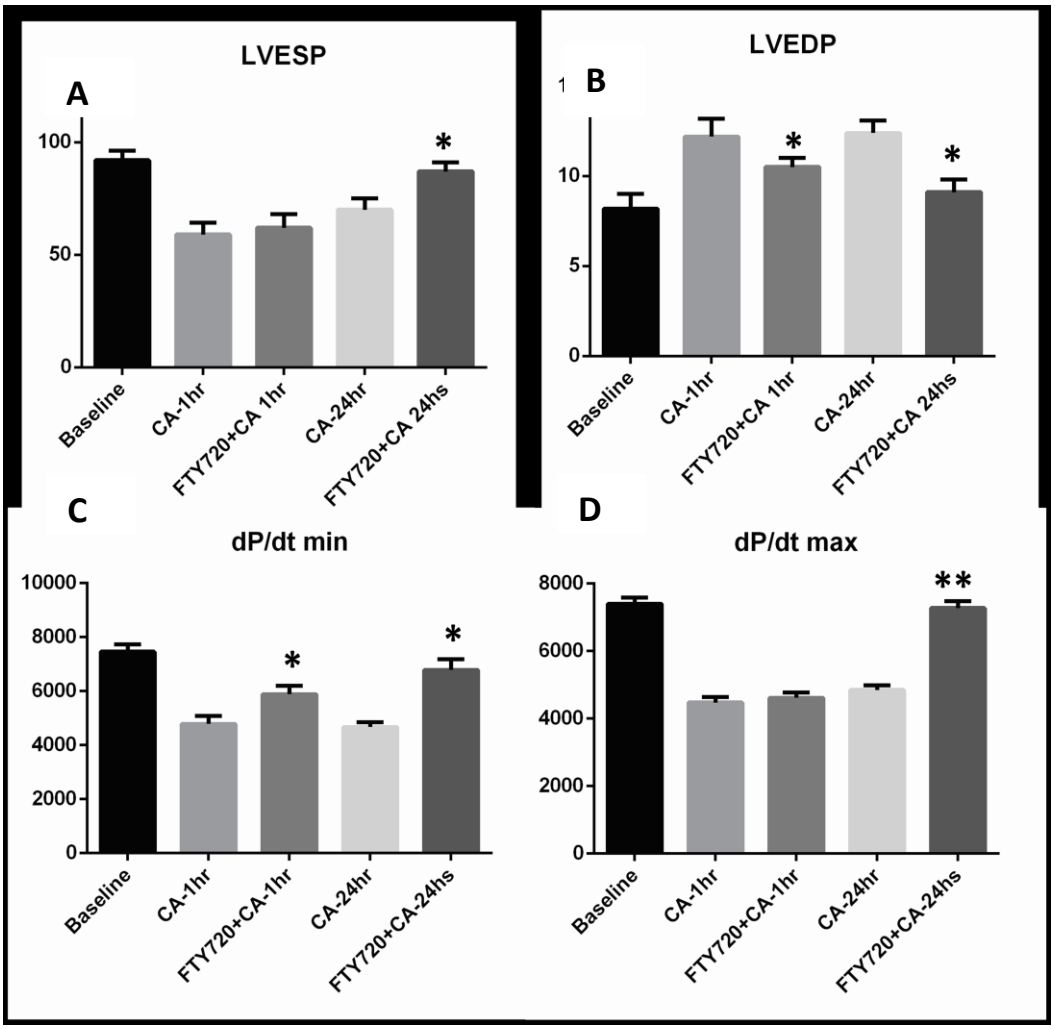


Figure. 4.2. *Hemodynamic parameters measured at baseline, at 1hour and 24 hours of reperfusion. Effects of FTY720 on left ventricular function in the rats with a CA-reperfusion-induced injury. Following 10 mins CA, treatment administered at the start of reperfusion, (A) Effects of FTY720 on LVESP. (B) Effects of FTY720 on LVEDP. (C) Effects of FTY720 on LV dp/dt min. (D) Effects of FTY720 on LV dP/dt max, LVESP and LVEDP were measured using a multichannel physiological recorder. LV dP/dt max and min was expressed as mmHg/sec. LVESP and LVEDP were expressed as mmHg. LV dP/dt max, the rate of maximum positive left ventricular pressure development; LV dP/dt max, the rate of maximum negative left ventricular pressure development; LVESP, left ventricular end-systolic pressure; LVEDP, left ventricular end-diastolic pressure. Values are expressed as the means ± SD.*

4.3.2. Bcl-2 and BAX signaling pathways

Following 10 min of CA and 24 hours of reperfusion, myocardial tissue was excised for immunohistological staining to measure the expression levels of anti-apoptotic (Bcl-2) and pro-apoptotic (Bax) proteins. As presented in (Fig. 4.3), in FTY720

treated(Group D) Bcl-2 expression were significantly increased versus CA-control group (Group C), while expression of BAX was down-regulated in Group D as compared to Group C ($P \leq 0.05$), indicating that fingolimod during CA attenuates apoptosis by up-regulation of Bcl-2 and downregulation of Bax proteins.

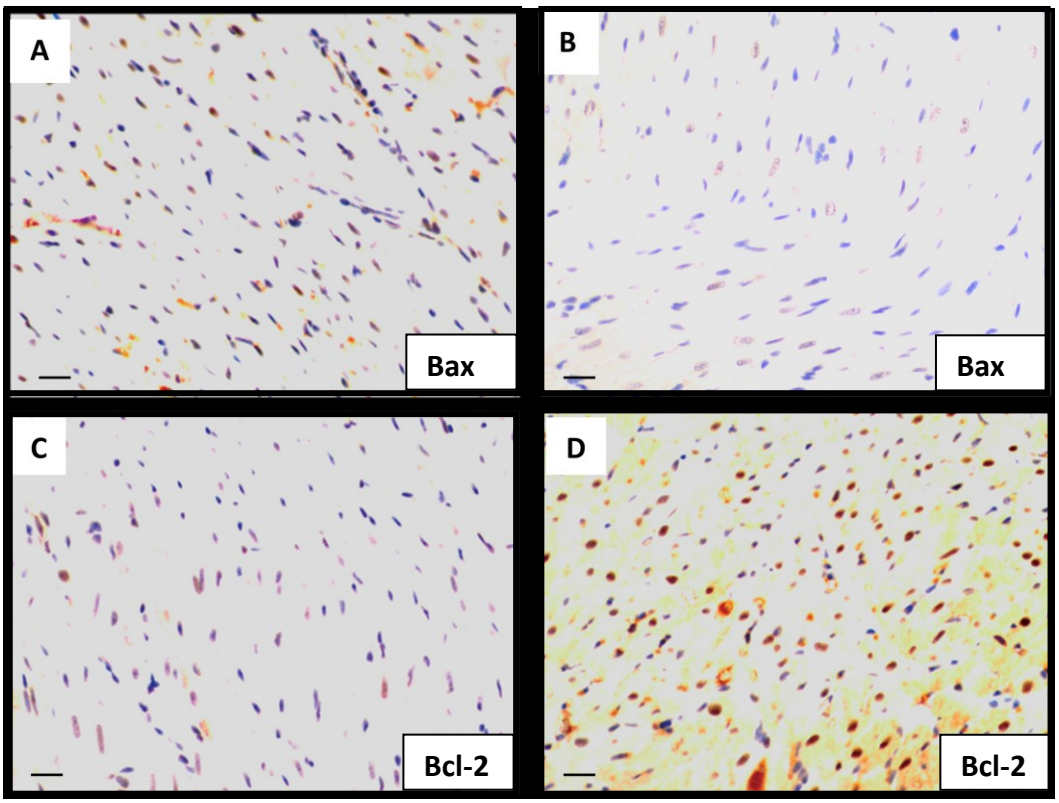


Figure. 4.3. *Myocardial tissue expression levels of Bcl-2 and Bax after10 min of CA and 24 hours reperfusion. The protein expression levels of Bcl-2 and BAX were determined by an immunohistochemistry, (A) BAX expression in CA-control group, (B) Bax expression in CA-FTY720 treated group.(Control vs. Treated $p \leq 0.05$) (C) Bcl-2 expression in Control- CA group and (D) Bcl-2 expression in FTY720 treated group (Control vs. Treated ($p \leq 0.05$)).*

4.3.3. Effect of fingolimod on apoptosis

TUNEL assay is standard way to assess apoptosis, it shows extensive apoptosis in Group C. S1P receptors activation by using fingolimod showed a significant attenuation of TUNEL-positive nuclei in myocardial tissue of cardiac arrest and reperfusion, it indicates that fingolimod exert anti-apoptotic role in I/R related to CA (Fig.4.4).

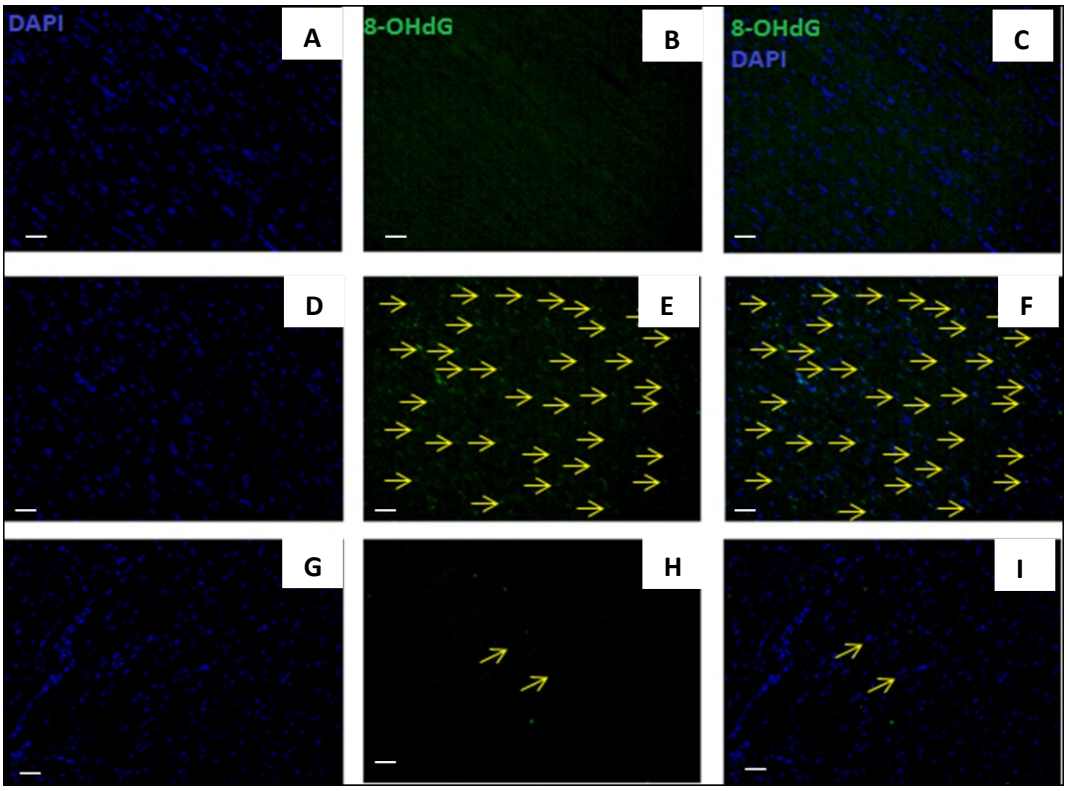


Figure. 4.4. *Representative photomicrographs of immunofluorescent staining for TUNEL-positive nuclei in the baseline, I/R-control, I/R-Fingolimod groups. (A, D, G) shows only DAPI in myocardial tissue, (B, E, H) TUNEL positive signals and (C, F, I) merged images. TUNEL-positive myocytes were much lower in numbers frequently in control CA group than in CA-FTY720 group. Original magnification 20x.*

4.3.4. Interstitial collagen deposition

Interstitial collagen deposition measured by collagen volume fraction (CVF), to evaluate myocardial fibrosis after VF- induced CA and reperfusion for 24 hours. The differences in the interstitial collagen deposition late phase of reperfusion as shown in (Fig.4.5). To calculate CVF, we measured the collagen staining expression (blue) in Masson's trichrome-stained images.(B) Fingolimod (1mg/kg) treated group demonstrated a low level of CVF as(A) compared to control group in CA ($p \leq 0.05$).

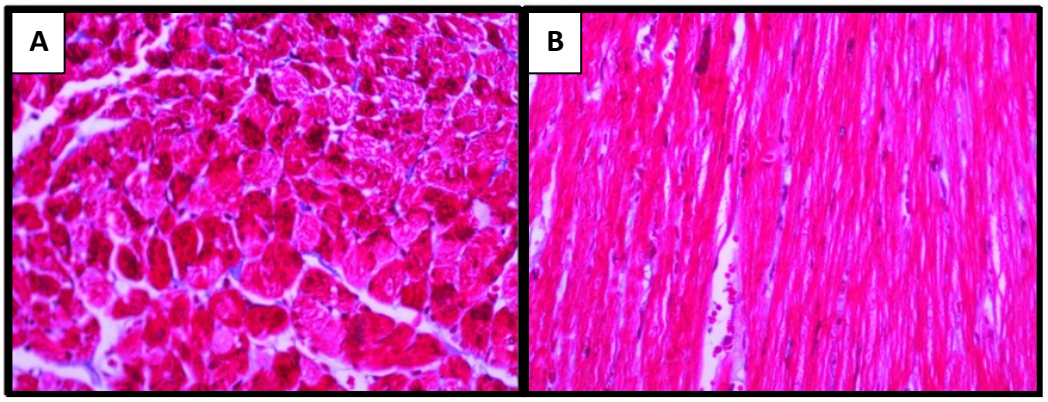


Figure 4.5. *Representative photomicrograph showing the fibrosis in CA induced ischemia-reperfusion (20xMagnification). FTY720 attenuated post- CA myocardial interstitial fibrosis. The viable myocardium is stained bright red. Fibrosis is stained bright blue, (A) CA without fingolimod (n=15) and (B) CA with fingolimod (1mg/kg) (n=15).*

4.3.5. Fingolimod attenuates neutrophil infiltration

In CA -I/R group, there were interstitial edema and structural disarray, and including neutrophils infiltration observed. However, pre ischemia FTY720 treatment, remarkably reduced morphological changes and neutrophil infiltration. Difference between control and treated group were statistically significant ($p \leq 0.05$)(Fig. 4.6).

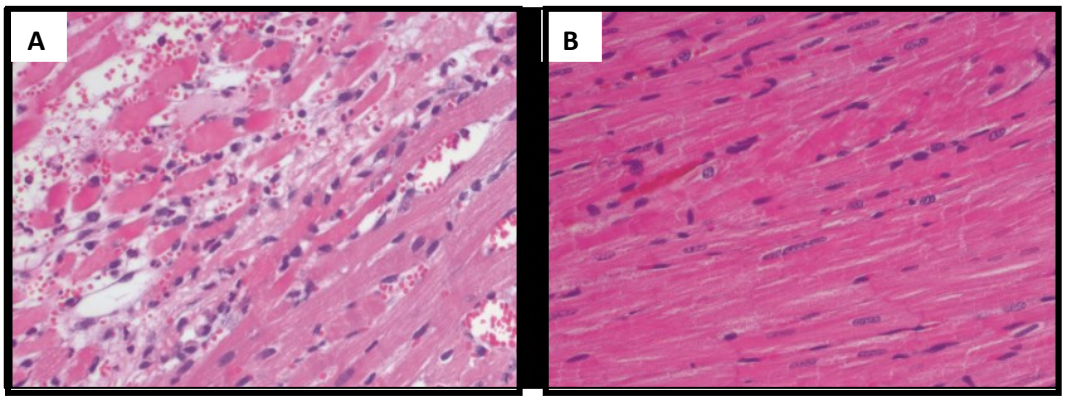


Figure 4.6 *Representative photomicrograph showing the histopathological changes in CA induced ischemia-reperfusion(20X Magnification). A. Cardiac section shows interstitial oedema and neutrophil infiltration in control, B, Heart tissue section showing mild necrosis and band contractions in fingolimod-treated group.*

4.3.6. Fingolimod reduces Nitrative stress

Nitrative stress also plays an important role in myocardial tissue injury under ischemia-reperfusion. Excessive NO production from NOS and the reaction product

between NO and peroxynitrite; superoxide, have been observed to activate apoptotic signalling pathways leading to apoptotic cell death. To determine whether FTY720 may reduce myocardial nitrative stress caused by CA related ischemia-reperfusion and thus attenuation in myocardial apoptosis, we determined peroxynitrite expression. As illustrated in (Fig. 4.7), FTY720 markedly down-regulated peroxynitrite expression, that indicates decrease production of nitrotyrosine.

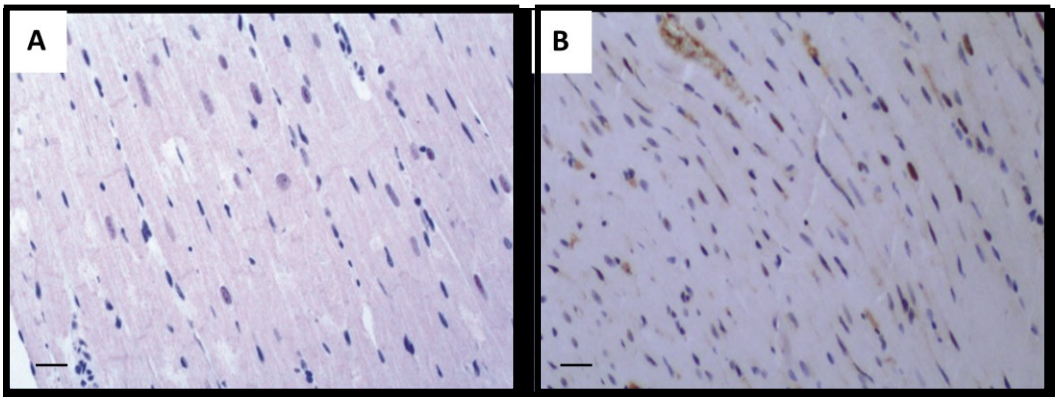


Figure. 4.7. *Myocardial nitrotyrosine staining (A) FTY720-Treated group(n=15), (B) Control group (n=15), Rats were subjected to 10 mins CA followed by 24 hours reperfusion. At the end of the experiment, the heart was removed, and nitrotyrosine localization were determined.*

4.3.7. Serum levels of CK-MB and cTnI

Cardiac markers of cardiomyocyte injury, the serum levels of CK-MB and cTnI were 22±9.3 and 5.35±0.60 U/g protein in the baseline-operated group and were significantly increased in the CA-reperfusion group to 128.8±10.9 and 21.72±0.99 U/g protein, respectively. Following treatment with fingolimod the CK-MB and cTnI levels were significantly decreased as shown in (Fig.4.10).

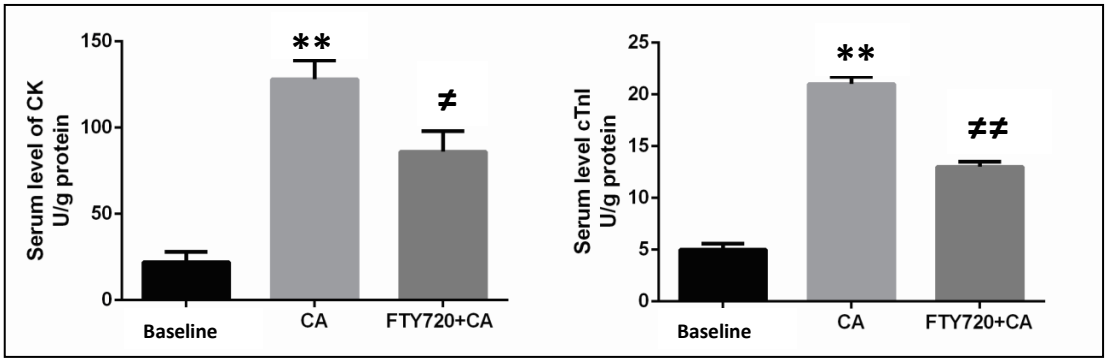


Figure. 4.8. *Serum Levels of creatine kinase-MB (CK-MB) and cardiac troponin I (cTnI) in the serum of rats in the baseline, CA- reperfusion and CA-reperfusion+Fingolimod*

*group(1mg/kg) groups. all Values were expressed as the means ± SD (n=10). (**P≤0.01 vs. baseline; ##P≤0.01 and #P≤0.05 vs. Control group).*

4.3.8. Serum levels of Inflammatory Mediators

In comparison with baseline, CA-related I/R injury caused by marked elevation in the levels of cytokines level mainly TNF-α, IL-1β, IL-6 and ICAM-1. All these cytokines increase in serum in response to ischemia and reperfusion. In present study, in CA-reperfusion related I/R injury these mediators have been measured. Although, ICAM-1 is independently increased in response to I/R, in CA-related ischemia-reperfusion elevation has been observed. Compared to baseline TNF-α, IL-6, IL-1β and ICAM-1 serum levels were significantly increased in CA-reperfusion group (p≤0.001). On administration of fingolimod (1mg/kg) TNF-α, IL-6, 1L-1β and ICAM-1 vs. control group attenuated (p≤0.05, p≤0.05, p≤0.05, and p≤0.001) respectively as shown in (Fig. 4.9).

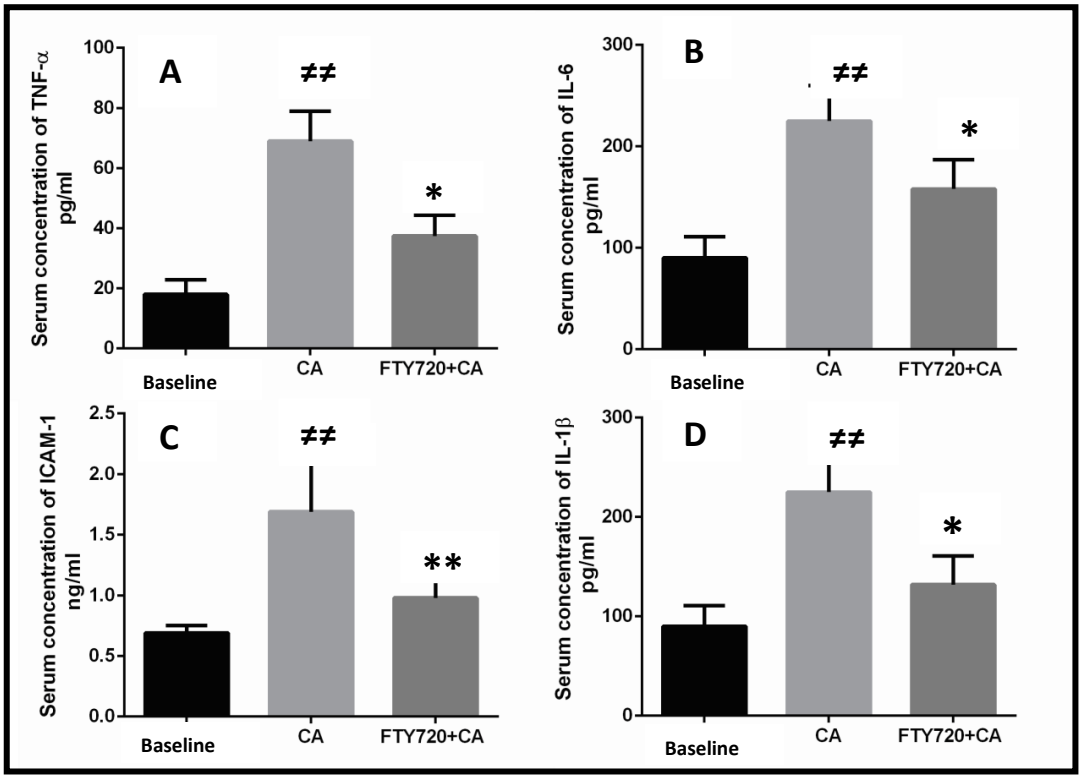


Figure. 4.9. *Myocardial production of TNF-α (A), IL-6 (B) and ICAM-1 (C) and IL-1β (D) after 10 min CA and 24 hours of reperfusion, (A) CA model without fingolimod treatment shows high expression of TNF-α as compared to fingolimod treatment. (B)CA-Reperfusion induced significant high IL-6 after 24 hours of reperfusion compared with the fingolimod treated and baseline group, (C) FTY720 treated*

*group remarkably reduced the production of the ICAM-1 as compared to Control (D) this section of the panel presents, production of 1L-1β higher in control vs FTY720 treated group in CA-reperfusion group. Each bar height represents the mean ± SD (each group n=15). (##P ≤ 0.01 vs. baseline. *P ≤ 0.05 and **P ≤ 0.01 vs. CA-control group).*

4.3.9. Effect of fingolimod on Erk1/2 and Akt1/2 signaling pathways

Pro-survival signalling pathways were measured in the myocardial tissue.Akt/ PI3 kinase pathway is important survival signalling pathway. We measured the phosphorylation level of Akt in Group C and D in the myocardial tissue. As shown in (Fig. 4.10A), phosphorylation levels of Akt1/2 increase over control group ($P \leq 0.05$). ERK1/2 phosphorylation were analysed by Western blot in rat myocardial samples. As shown in (Fig. 4.10B), phosphorylation level of ERK1/2 increased in Group D versus Group C ($P \leq 0.05$).

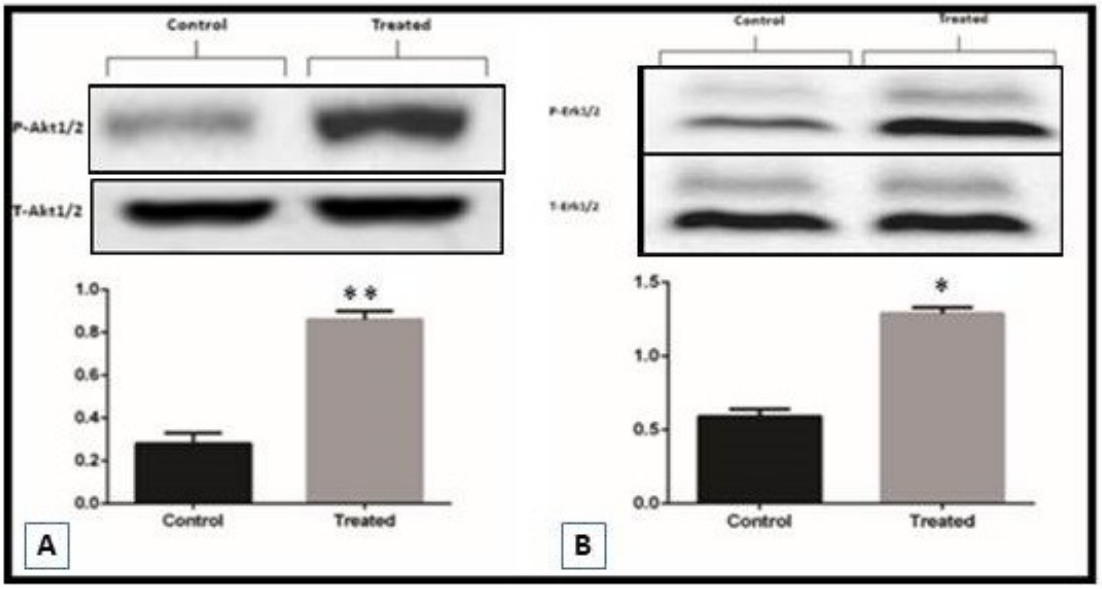
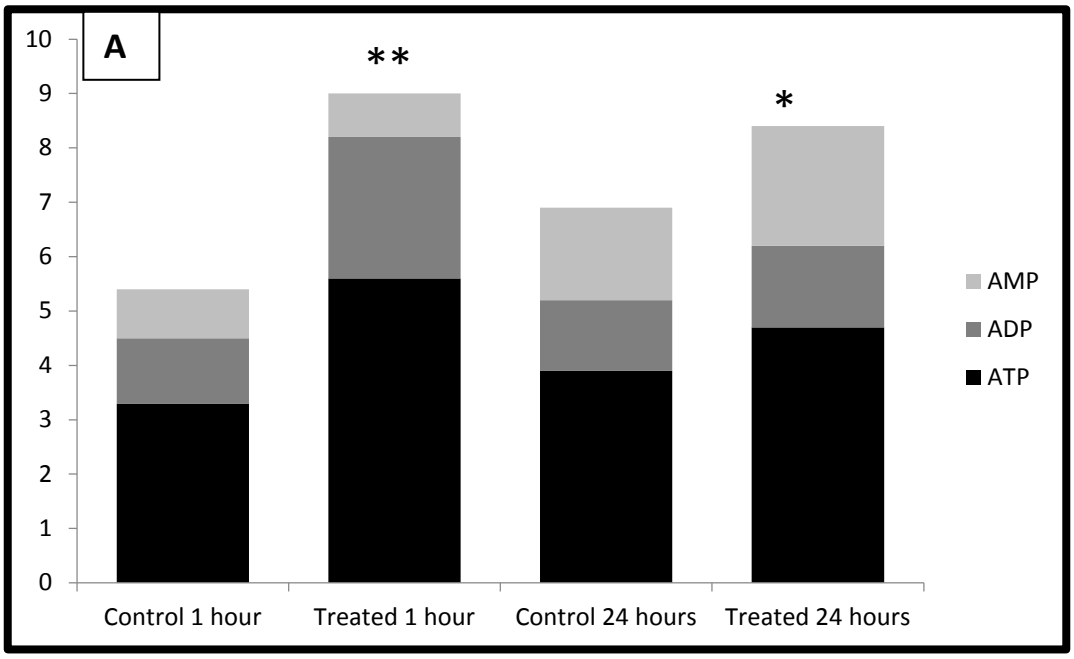


Figure 4.10 *Representative Western blot and relative density ratio of the phosphorylated (p) form of Akt1/2 (A) ERK1/2 (B), samples of left ventricle at the end of 24hours reperfusion. Relative Densities shows Fingolimod activate phosphorylation of these proteins. Values are means ± SD; (n =15 samples/group). (*p≤0.05, **p ≤ 0.001).*

4.3.10. High Energy Phosphates

The measurement of high-energy phosphates in model of CA and resuscitation revealed the superior preservation in myocardium using fingolimod treatment (Fig. 4.12). Phosphocreatine, the buffering energy source for ATP in situations of energy demand, was significantly higher in the fingolimod administered group of animals compared with the control group tissue ($P \leq .01$), whereas ATP was significantly increased after both the 1 hour and 24 hours of reperfusion in treated group as compared with the control group ($P \leq .04$). Energy charge was significantly elevated after 1 hour of reperfusion in fingolimod treatment group as compared with the control group (0.85 ± 0.12 vs 0.63 ± 0.15; $P \leq .05$), whereas the after 24 hour reperfusion, it was not statistically significant (0.83±0.13)(p>0.05).



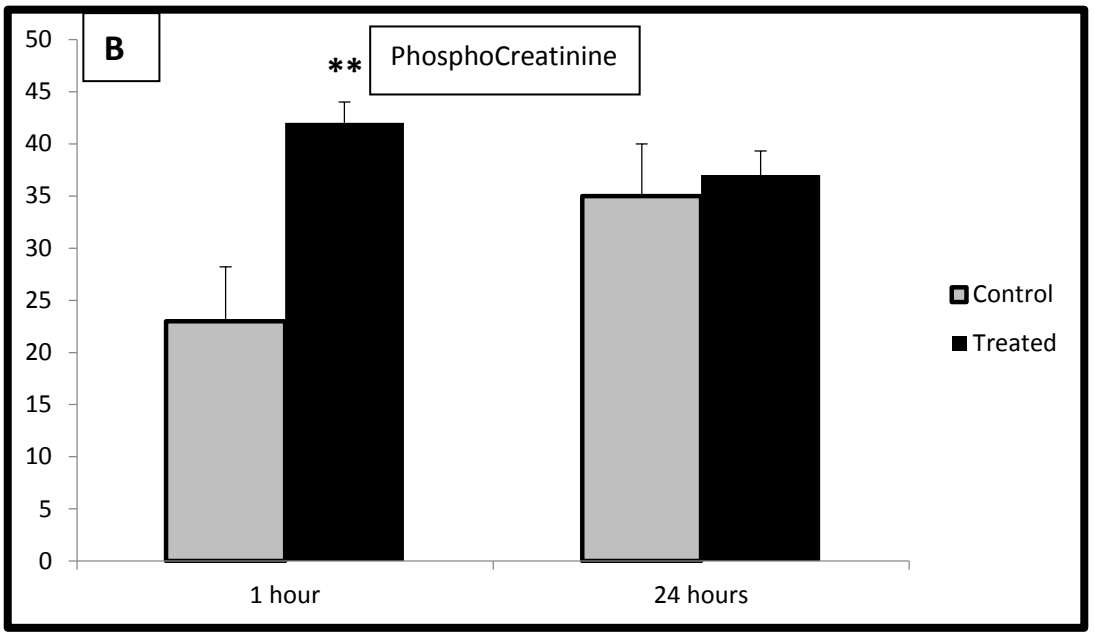


Figure. 4.10. *High-energy phosphates in myocardial tissue of the LV in the fingolimod treated groups compared with the control group. ((A) AMP, ADP and ATP levels at 1 hour and 24 hour of reperfusion B) Changes in phosphocreatine , (*p≤0.05, **p≤0.001) AMP, Adenosine Monophosphate; ADP, Adenosine DiPhosphate; ATP, Adenosine Tri-Phosphate.*

4.3.11. Oxidative Stress

To examine whether fingolimod regulates the free radicals production on ischemia-reperfusion, we examined free oxygen radicals and aldehydes (lipid peroxidation derivatives) in the frozen myocardial sample. In CA-control group, reactive oxygen species level found higher as compared to CA-fingolimod treated group. Particularly, we analysed free reactive oxygen species and malondialdehyde in myocardial tissue by chromatography and mass spectrometry. Collectively, our results suggest fingolimod treatment in CA decrease oxidative stress (Fig. 4.12).

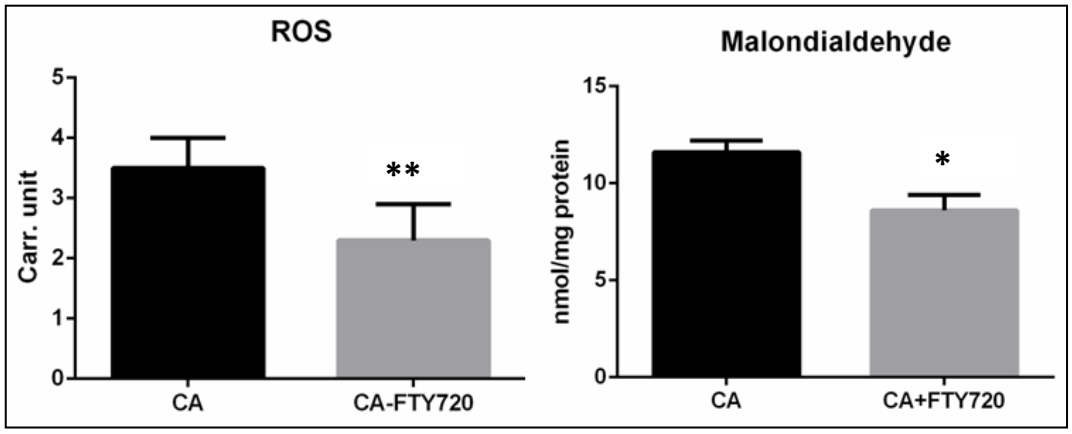


Figure. 4.11. *Oxidative stress Comparison of oxidative stress in fingolimod treated and control group. ROS. Reactive oxygen species, CA. Cardiac Arrest, FTY720. Fingolimod, Carr. Unit. Carratelli Unit. (**p≤0.001,*p≤0.05).*

4.4. Discussion

The cardioprotective strategies to prevent acute global ischemia reperfusion injury have been tested with different approaches. During last two decades, ~1000 experimental studies have been investigated for cardioprotective role including volatile anesthetic agents[216-218], sodium hydrogen exchange inhibitors[219], statins[220], pharmacological preconditioning[221, 222] and anti-inflammatory strategies. Majority of the preclinical strategies could not work in clinical settings.

In this study, administration of fingolimod demonstrated inhibition of apoptosis by inhibiting inflammation, oxidative stress and preservation of high energy phosphates. In our investigations on molecular and protein level both, cardioprotection has been observed. We have also demonstrated inhibition of pro-apototic and activation of anti-apoptotic proteins, reduction in oxidative and nitrative stress, preservation of high energy phosphates. The phosphorylation of Akt1/2 and Erk1/2, are important for cell survival pathways [223-225], were also investigated in this study.

In literature, many studies suggested cardioprotective role of fingolimod by activating survival pathways *ex-vivo* in mouse and rat hearts and *in vivo* models[137, 138]. Recently, a study reported knock-out mice of S1P receptors produced a high level of myocardial damage as compared to wild type[179]. In another study, mice with the lack of Sphingosine kinases demonstrated a big infraction size as compared to control mice. In addition to activation of S1P receptors, metabolism of S1P have also significance in pre and post conditioning cardioprotective mechanisms[138, 177, 178, 226].

Ischemia reperfusion injury can be caused by different mechanisms and pathways. Activation of apoptotic pathways, complement system activation, increased inflammation, and oxidative stress can cause myocardial injury following ischemia reperfusion[227]. Fingolimod shows potential to deal with most of the above mentioned myocardial damaging mechanisms to prevent I/R injury.

Reperfusion after transient ischemia in myocardium leads to apoptosis in cardiomyocytes and cardiac dysfunction[228, 229]. TUNEL positive nuclei staining i.e. standard method to measure level of apoptosis have been used in this study. Consistent with the previous results, the rats treated with fingolimod after 10 min of CA, expressed a lower frequency of TUNEL positive nuclei after 24 hours of reperfusion. It indicates the cardioprotective role of fingolimod by activating anti-apoptotic cascade.

S1P receptor agonists have important role in immune suppression[230]. Porcine model of I/R[185] and spontaneous obstructive coronary atherosclerosis murine model[231], tested for immunosuppression, showed reduction in infarct size and mortality in fingolimod treated group. We measured inflammation in blood and tissue. In blood, we have found significantly low concentration of pro-inflammatory cytokines.

The ICAM-1, IL-6, IL-1β and TNF-α contribute as a pro-inflammatory cytokines to develop inflammatory mechanisms [232-234]. The correlation was found between anti-inflammatory effects of fingolimod on cardioprotection. I/R increased ICAM-1, IL-6, IL-1β and TNF-α level in control group, whereas fingolimod treatment decreased the concentrations of these cytokines. Therefore, the suppression of inflammatory cytokines by fingolimod treatment protects myocardium from I/R injury caused by pro-inflammatory cytokines.

One of the main targets of this drug is to mitigate apoptosis in I/R. During I/R, molecular signaling RISK and SAFE pathways activation have been reported [138, 175]. The RISK (Akt1/2, Erk1/2 and GSK-3β) and SAFE (JAK and STAT3) pathways are main source for mitigation of apoptosis by preventing opening of mitochondrial permeability transition pore [3, 173, 174]. Consistent with the previous findings, activation of RISK and SAFE signaling pathways were observed following decreased level of apoptosis in treated group as compared to the control. The inhibition of pro-apoptotic proteins BAX and enhanced immuno-reaction for anti-apoptotic protein Bcl-2 were found after 10 min of CA and 24 hours of reperfusion.

4.6. Conclusion

In conclusion, fingolimod play important role in preservation of cardiac mechanical functions, reduction in myocardial apoptosis, inflammation, oxidative and nitrative stress. Furthermore, potential mechanism in cardioprotection is associated with

activation of Reperfusion Injury Salvage Kinase and Survivor Activating Factor Enhancement pathways. As a result, hemodynamic parameters improved in late phase of reperfusion.

SECTION III-B

5. Cardioprotective effect of Sphingosine 1-phosphate in Cardioplegic Arrest model

5.1 Introduction

Cardiopulmonary bypass (CPB) assisted open-heart surgery is important for the surgeon to perform routine cardiac surgeries in a blood free operating field. Cardioplegic arrest (CPA) means heart stops functioning and is separated from systemic circulation while protected from ischemic injury by the cardioplegic solution. After the unavoidable operating ischemia, immediate myocardial blood flow causes reperfusion injury that leads to further myocardial tissue damage[235]. Ischemia-reperfusion injury is a consequence of the release of reactive oxygen species, inflammatory mediators and apoptosis, which may lead to compromised mechanical cardiac function and arrhythmias [236-238]. The risk of cardiac surgery has increased for the last few years especially for the high-risk patients undergoing complicated and prolonged cardiac surgical procedures[239]. Various conditions like aging, high rate of co-morbidities, redo-operations due to complications, multiples procedures, coronary artery bypass grafting (CABG) plus valvular surgeries may be categorised as high-risk conditions[240]. These high-risk cardiac surgery patients are more susceptible to perioperative reperfusion injury and infarction. These patients are usually with multiple co-morbidities and are at a high risk of ischemia-reperfusion (I/R) injury, acute renal failure[241] and stroke[242]. Recent studies have demonstrated that other than inflammatory cascade and oxidative stress, nitrative stress also plays an important role in I/R-induced myocardial tissue injury[243]. Excessive production of nitric oxide (NO) from NOS and its highly reactive products such as peroxynitrite and superoxide have been reported to activate several apoptotic pathways leading to apoptotic cell death[244]. For the last few decades, the role of sphingosine 1-phosphate (S1P)- a derivative of ceramide has been a hot topic of discussion for cytoprotection by causing activation of its survival pathways and inhibition of proinflammatory pathways, nitro-oxidative stress and apoptosis. Previous studies have shown the role of SIP in hibernation where it also act as a key player in mediating effects of hypothermia [245]. However, pharmacological pre-conditioning and activation of sphingosine 1-phosphate have shown potential application in cardioplegic arrest commonly seen in CABG [215]. Primarily, end points for our study were functional aspects and level of myocardial apoptosis. Sphingosine 1-phosphate receptors agonist FTY720 might act as potential pharmacological agent for cardioprotection. Therefore the aim of this study was to assess the cardioprotective effects of S1P receptors activation by FTY720 in the clinical setting of cardioplegic arrest in a rat model. To understand the cardioprotective role of FTY720, we also aim at investigating Reperfusion Injury Salvage Kinase (RISK) and Survivor Activating Factor Enhancement (SAFE) pathways, apoptosis, role of inflammatory mediators and oxidative stress and their relationship with functional hemodynamics parameters.

5.2. Methods

5.2.1. Animals

After approval from the institutional Animal Care Committee, 30 male Sprague-Dawley rats (300-350 grams) were obtained from Harlan Laboratories (Udine, Italy) and fed standard rat chow, which they had access ad libitum. The rats were housed at a density of 3-4 per cage and maintained on a 12-hour light/dark cycle at 21°C.

Rats were anesthetized (sodium pentobarbital, 30 mg/kg intraperitoneally) and intubated using oropharynx with a 14-gauge polyethylene tube. Mechanical ventilation was done with a rodent ventilator (Harvard Apparatus Inc., Holliston, Massachusetts). The tidal volume was 6 mL/kg and the respiratory rate was 50 to 60 breaths/min with an air-oxygen mixture (inspired oxygen fraction = 0.5)[246]. Rats were secured supine on a heating board to maintain rectal temperature at 37°C during the surgical procedure before the initiation of cardioplegic arrest. The left femoral artery was cannulated with a heparinized 24-gauge Teflon catheter to monitor systemic arterial pressure and central cannulation was performed as described previously [247] . In brief, after complete sternotomy, a venous cannula (a modified 4-hole 16-gauge Angiocath catheter) was advanced into the right atrium with good drainage. The left common carotid artery was cannulated using an 18-gauge catheter advanced to the aortic arch.

Full heparinization (500 IU/kg) was assured after surgical preparation and immediately before cardioplegic arrest initiation to reduce overall blood loss. The setup consisted of a venous reservoir, a roller pump, a hollow fiber oxygenator (Sorin, Mirandola, Italy), and a vacuum regulator with an applied pressure of −30 mm H_2O to facilitate venous drainage, all connected by 1.6-mm internal diameter plastic tubing. Total priming volume was 10.5 mL, gas exchange surface was 450 cm^2, and heat exchange surface was 15.8 cm^2.

5.2.2. Experimental design

In this study, 30 rats were randomized into two groups with 15 animals in each group. The first (Group A) was treated with FTY720 1 mg/kg through I/V cannula and the second (Group B) was treated with saline to serve as the control group. After 15 min of both treatments, rats underwent cardioplegic arrest (CPA) using cold St. Thomas solution and aortic clamps for 10 min following initiation of Extra Corporeal Life Support (ECLS) for next 1 hour as described in a previous study[247]. During ECLS, internal temperature was kept at 36-37°C in both the groups. After 1 hour of reperfusion, in all rats, ECLS weaning was done and blood and heart tissue

were collected for analysis. Schematic review of the experiment is illustrated in Figure 1.

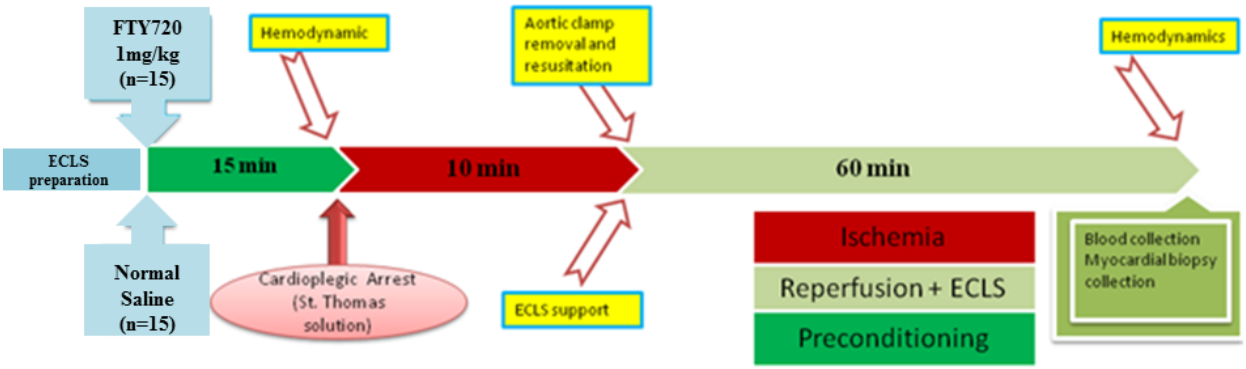


Figure. 5.1 Schematic view of experimental design.

5.4. Results

5.4.1. Left ventricular function

Baseline hemodynamics was similar in both groups (Table 1). No significant difference was observed in Groups A and B. Hemodynamic measurements were done to evaluate LV performance using the Millar catheter system in both groups at baseline and after 1 hour of reperfusion (Figure 2).

Table 1. Comparison of baseline hemodynamics in Group A and Group B.

Variables	Group A (treated) N=15	Group B (control) N=15	p-value
HR /min	370	326	Ns*
MAP mmHg	130	129	Ns
LVESP mmHg	95,2	92,2	NS
LVEDP mmHg	8,2	8,0	Ns
dP/dt max mmHg/s	7305	7427	Ns
dP/dt min mmHg/s	7672	7511	Ns

*Ns denotes not significant

The LV end systolic pressure (LVESP) was improved after 1 hour but statistically did not reach the level of significance (p=0.06). The LV end-diastolic pressure (LVEDP) measurements showed a reduction after 1 hour of reperfusion in the treated vs. control group (p=0.03). Ventricular systolic performance dP/dt max after CPB was improved as in the FTY720-treated vs. control group but didn't reach statistically significance (p=0.2).On the other hand, the minimal pressure relaxation rate (dP/dt min) was significantly lower in the control group compared to the treated group (p=0.02).

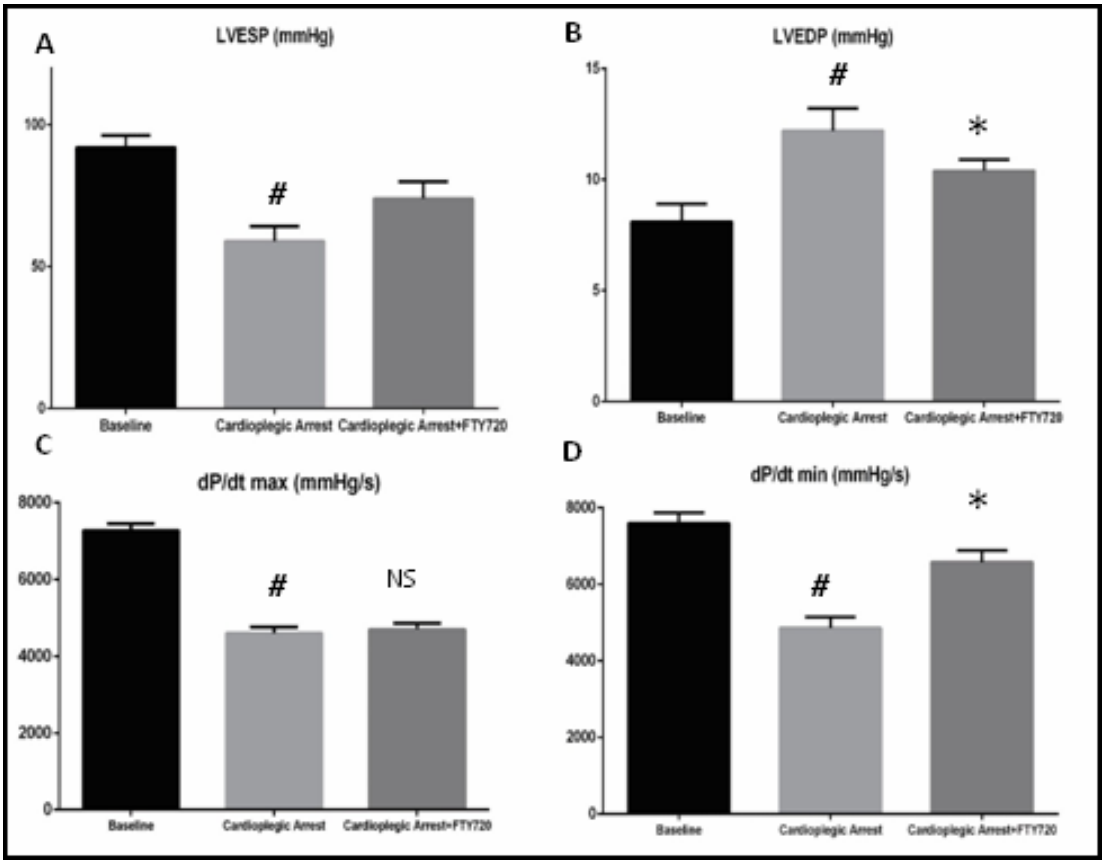


Figure 5.2: Hemodynamic parameters measured at baseline and 1 hour after *reperfusion. Effects of FTY720 on left ventricular function in the rats with CPA-reperfusion-induced injury. Treatment (FTY720 or saline) was done 15 min prior to cardioplegic arrest, (A) Effects of FTY720 on LVESP. (B) Effects of FTY720 on LVEDP. (C) Effects of FTY720 on LV dp/dt max. (D) Effects of FTY720 on LV dP/dt min, LVESP and LVEDP were measured using a multichannel physiological recorder. LV dP/dt max and min was expressed as mmHg/sec. LVESP and LVEDP were expressed as mmHg. LV dP/dt max, the rate of maximum positive left ventricular pressure development; LV dP/dt max, the rate of maximum negative left ventricular pressure development; LVESP, left ventricular end-systolic pressure; LVEDP, left ventricular end-diastolic pressure. Values are expressed as the means* ±

*SD,(n=15 in each group). (#p ≤ 0.01 vs. baseline. *p ≤ 0.05 vs. CPA-control group, NS –not significant).*

5.4.2. Serum levels of inflammatory mediators

Inflammatory mediators have been described to contribute to the CPA-Reperfusion related I/R injury[248]. In the present study, serums levels of some of the inflammatory mediators in CPA-reperfusion related I/R injury were measured. Although, ICAM-1 is independently increased in response to I/R, yet in CPA-related ischemia-reperfusion elevation in its level has been observed. Compared to baseline TNF-α, IL-6, IL-1β, and ICAM-1 serum levels were significantly increased in CPA-reperfusion group (p≤0001). Upon administration of FTY720 (1mg/kg), levels of TNF-α, IL-6, 1L-1β, and ICAM-1 in the treated group were significantly decreased as compared to the control group (p≤0.001, p=0.04, p=0.01, and p=0.001, respectively) as shown in Figure 5.3.

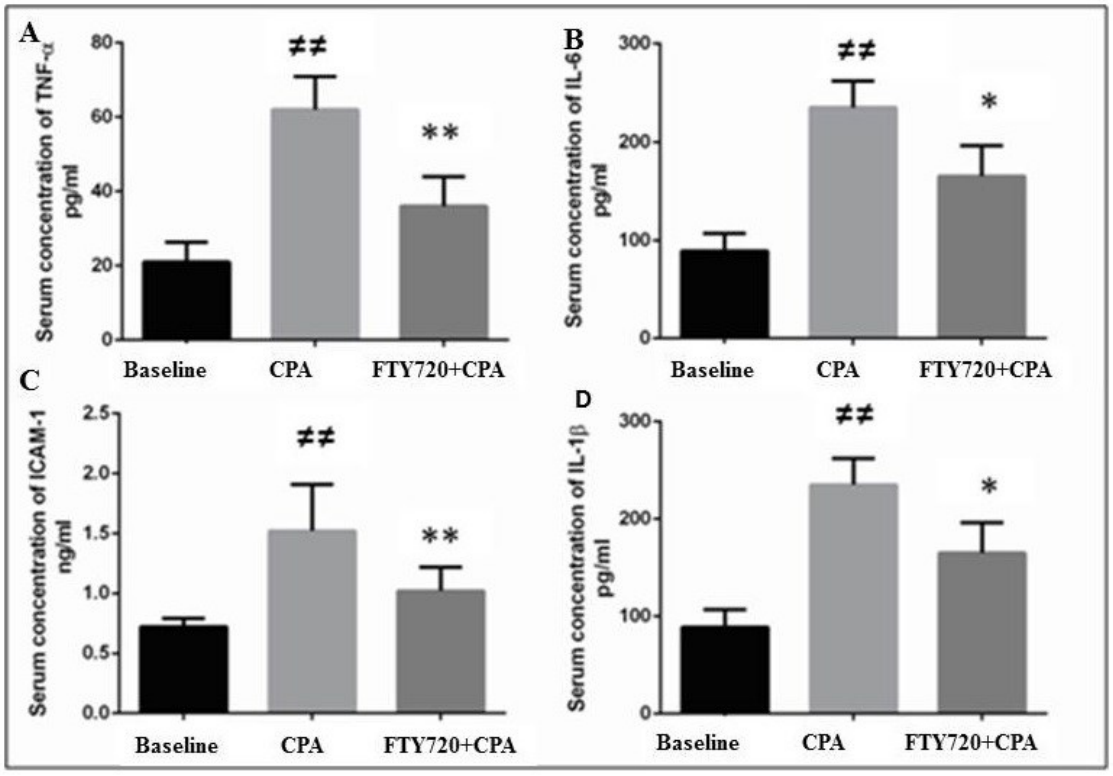


Figure 5.3: *Myocardial production of TNF-α (A) IL-6 (B) and ICAM-1 (C) and IL-1β (D), (A) CPA model without FTY720 treatment shows high expression of TNF-α as compared to FTY720 treatment. (B) CPA-Reperfusion induced significant high ICAM- after 1 hour of reperfusion compared with the FTY720-treated and baseline, (C) FTY720 treated group remarkably reduced the production of the ICAM-1 as compared to Control (D) this section of the panel presents, production of 1L-1β*

*higher in control vs. FTY720 treated group in CPA-reperfusion group. Each bar height represents the mean ± SD (each group n=15). (#p≤ 0.05 and ##p ≤ 0.01 vs. baseline. *p ≤ 0.05 and **p ≤ 0.01 vs. CPA-control group).*

5.4.3. Nitro-oxidative stress

To examine whether FTY720 regulates the free radicals production on ischemia-reperfusion, we quantitated free radicals and aldehydes (lipid peroxidation derivatives) in the frozen perfused myocardial samples. In CPA control group, reactive oxygen species and malondialdehyde levels were found to be higher as compared to CPA-FTY720 treated group. . Collectively, our results suggest FTY720 treatment in CPA decreased oxidative stress (Figure 4 A & B).

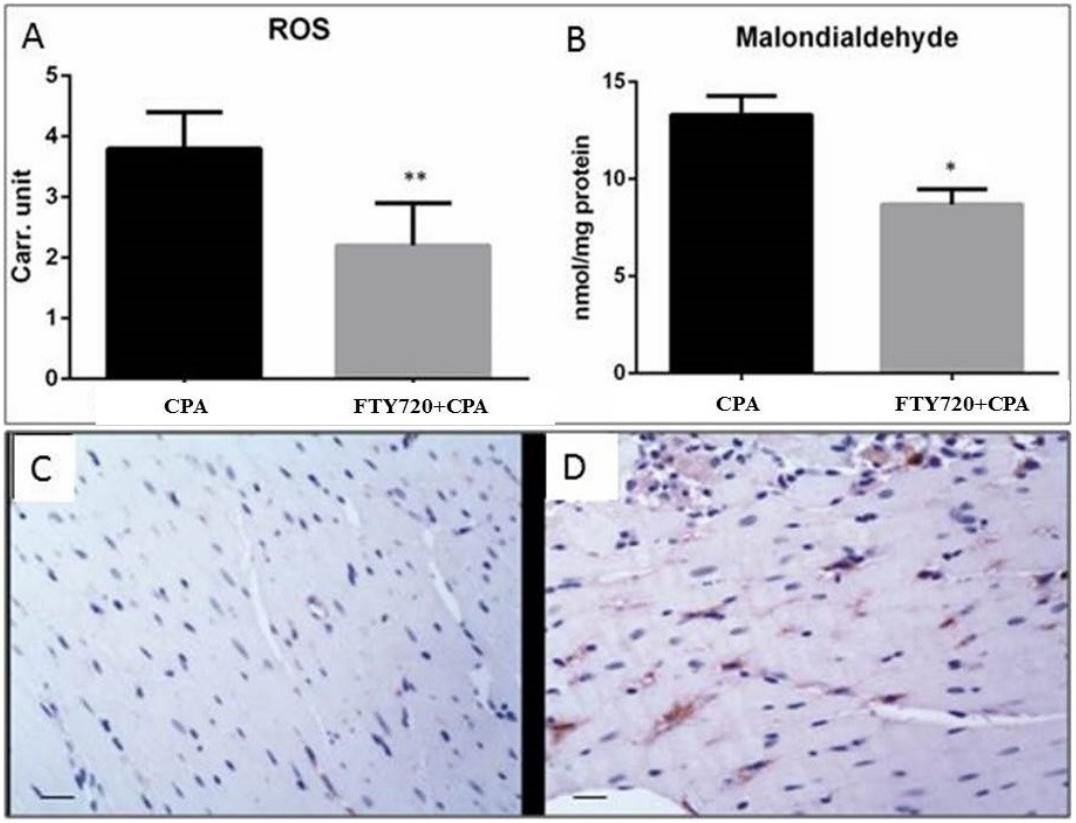


Figure 5.4: *Oxidative stress Comparison of oxidative and nitrosative stress in FTY720-treated and control group in cardiopulmonary bypass model with CPA(A & B). ROS. Reactive oxygen species; FTY720. FTY720; Carr. Unit. Carratelli Unit. (**p≤0.001,*p≤0.05). Myocardial nitrotyrosine staining (C) FTY720-Treated group,*

(D) control group (n=15), Rats were subjected to 10 mins CPA followed by 1-hour reperfusion.

In order to investigate whether S1P receptor agonist-mediated attenuation of cardiac cell apoptosis is mediated via reduction in reactive nitrogen species, generation of peroxynitrites was analyzed by measuring the nitrosylation of cardiac proteins using an antibody against nitrotyrosine. As illustrated in Figure 4 C& D, treatment with FTY720 markedly reduced the nitrotyrosine protein content, an indicative of reduced peroxynitrite generation.

5.4.4. Effect of FTY720 on Erk1/2 and Akt1/2 signaling pathways

Activation of pro-survival signaling pathways (MEK/ERK and PI3K/Akt) was analyzed by measuring the phosphorylation state of ERK1/2 and Akt by Western blot analyses. As shown in Figure 5, the phosphorylation levels of ERK1/2 and Akt were increased in treated group (n=15) compared to the control group (n=15) with $p \leq 0.001$ and $p= 0.04$, respectively.

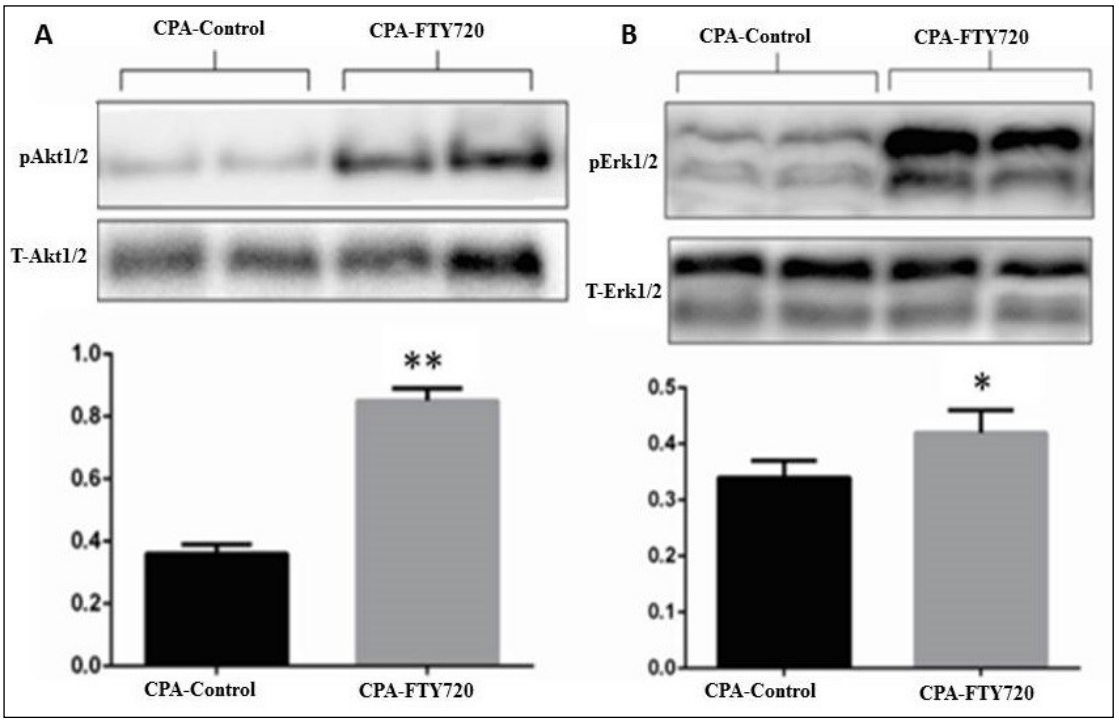


Figure 5 *Representative Western blot and relative density ratio of the phosphorylated (p) form of Akt1/2 (A) ERK1/2 (B), samples of left ventricle at the 1 hour reperfusion. Relative Densities shows FTY720 activate phosphorylation of these proteins. Values are means ± SD; (n =15 samples/group). (*p≤0.05, **p ≤ 0.001).*

5.4.5. Bcl-2 and BAX signaling pathways

Following 10 min of CPA and 1 hour of reperfusion, heart tissue was removed for immunohistochemical staining to measure the expression levels of anti-apoptotic protein, Bcl-2, and its regulator Bax protein. As depicted in Figure 6, in the FTY720 treated group, Bcl-2 expression was significantly increased in CPA related I/R group, while expression of BAX was down regulated (p=0.02), indicating that FTY720 during global ischemia-reperfusion attenuates apoptosis by up-regulation of Bcl-2 and down-regulation of Bax proteins.

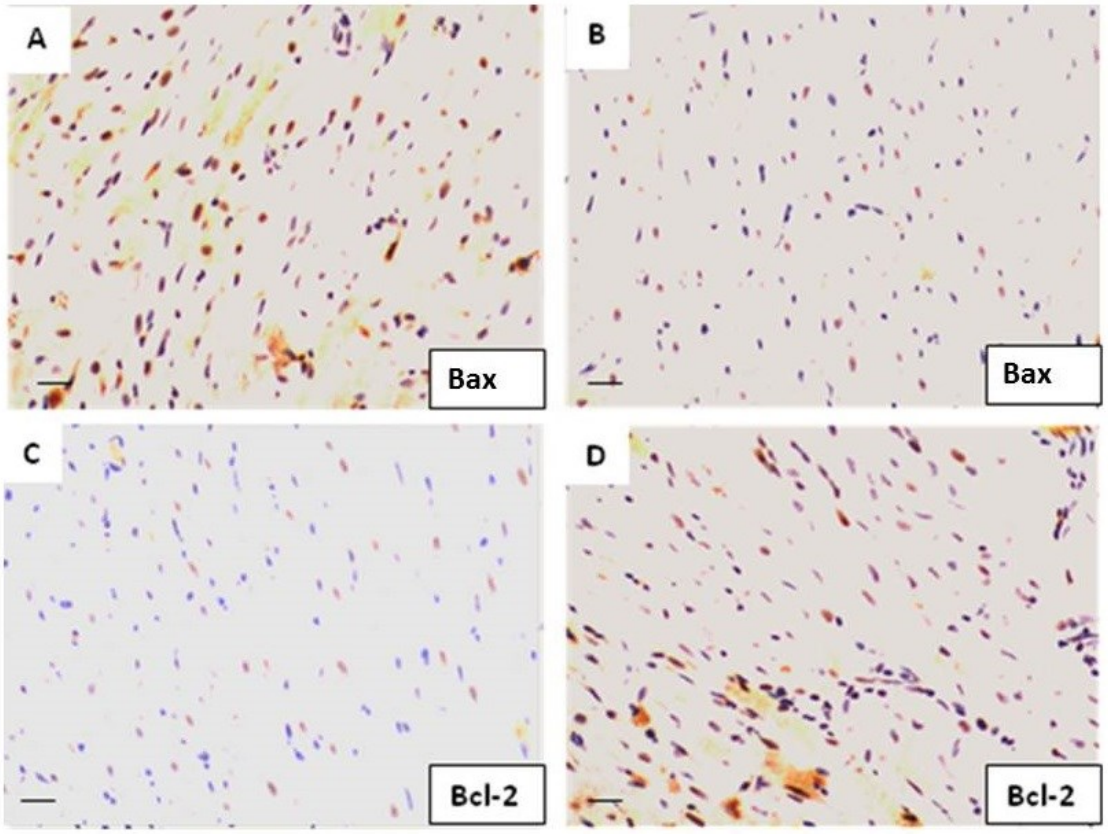


Figure. 6:*The protein expression levels of Bcl-2 and BAX in myocardial tissue were determined by an immunohistochemistry, A. BAX expression in CPA-control group, (B) Bax expression in CPA-FTY720 treated group.(Control vs. Treated p≤0.05) (C) Bcl-2 expression in Control- CPA group and (D) Bcl-2 expression in FTY720 treated group. (Control vs. Treated p≤0.01)*

5.4.6. High energy phosphates

The measurement of high-energy phosphates in model of CPA revealed superior preservation of FTY720-treated myocardium (Figure 7A & B). AMP decreased in treated group, whereas ADP and ATP were significantly increased after 1 hour of

reperfusion in the treated group as compared with the control group (p= 0.03) (Figure 7A). Phosphocreatine, the buffering energy source for ATP in situations of energy demand, was significantly higher in the FTY720 administered group of animals compared to the control group (p=0.01) (Figure 7B).

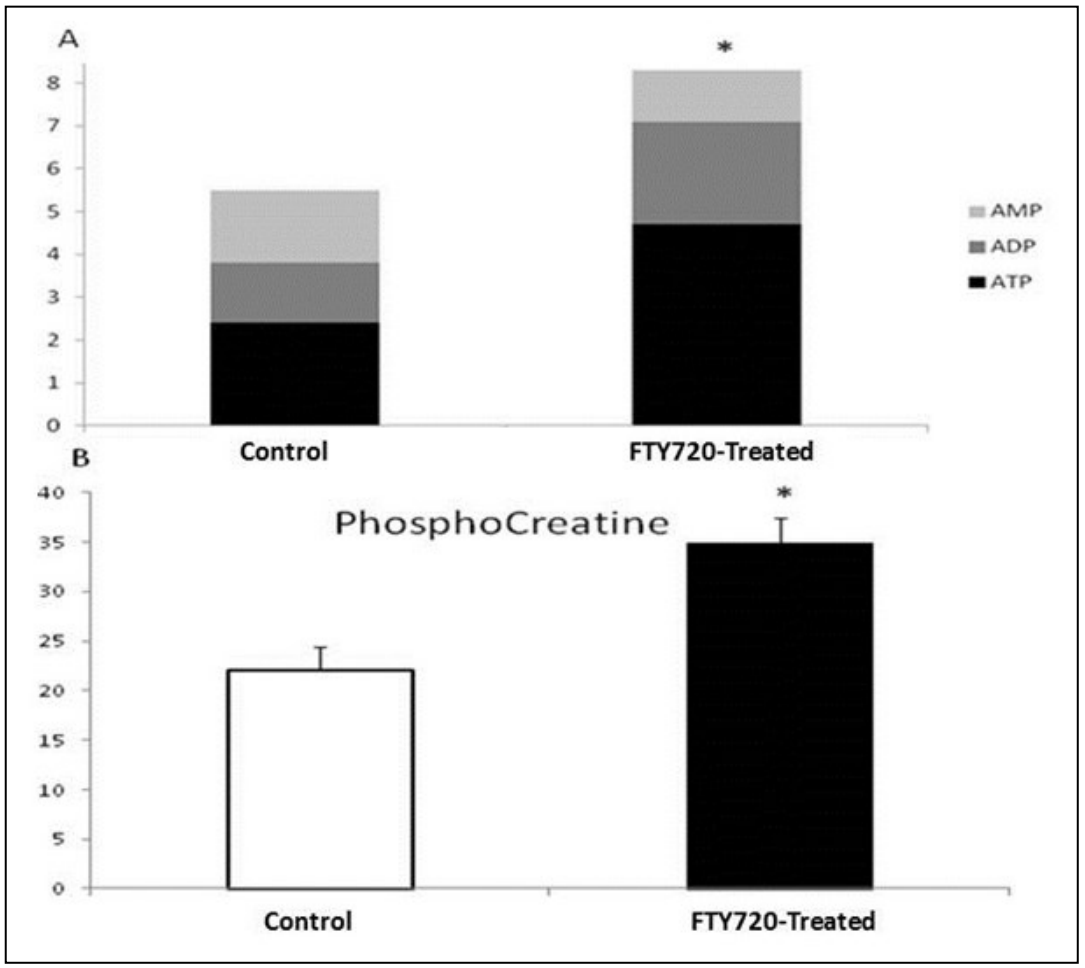


Figure 7: *High-energy phosphates in myocardial tissue of the LV in the FTY720-treated groups compared with the control group. (A) AMP, ADP and ATP levels after 1 hour of reperfusion in CPA, (B) Changes in phosphocreatine. (*p≤0.05, **p≤0.001) AMP, Adenosine Mono Phosphate; ADP, Adenosine Di Phosphate; ATP, adenosine triphosphate.*

5.4.7. Effect of FTY720 on apoptosis

Tissue apoptosis was analyzed by TUNEL assay. As shown in Figure 8, extensive apoptosis was observed after CPA-I/R injury. S1P receptor agonist FTY720 caused a significant attenuation of TUNEL-positive nuclei in the myocardium indicating reduced apoptosis in myocardial cells (Figure 8).

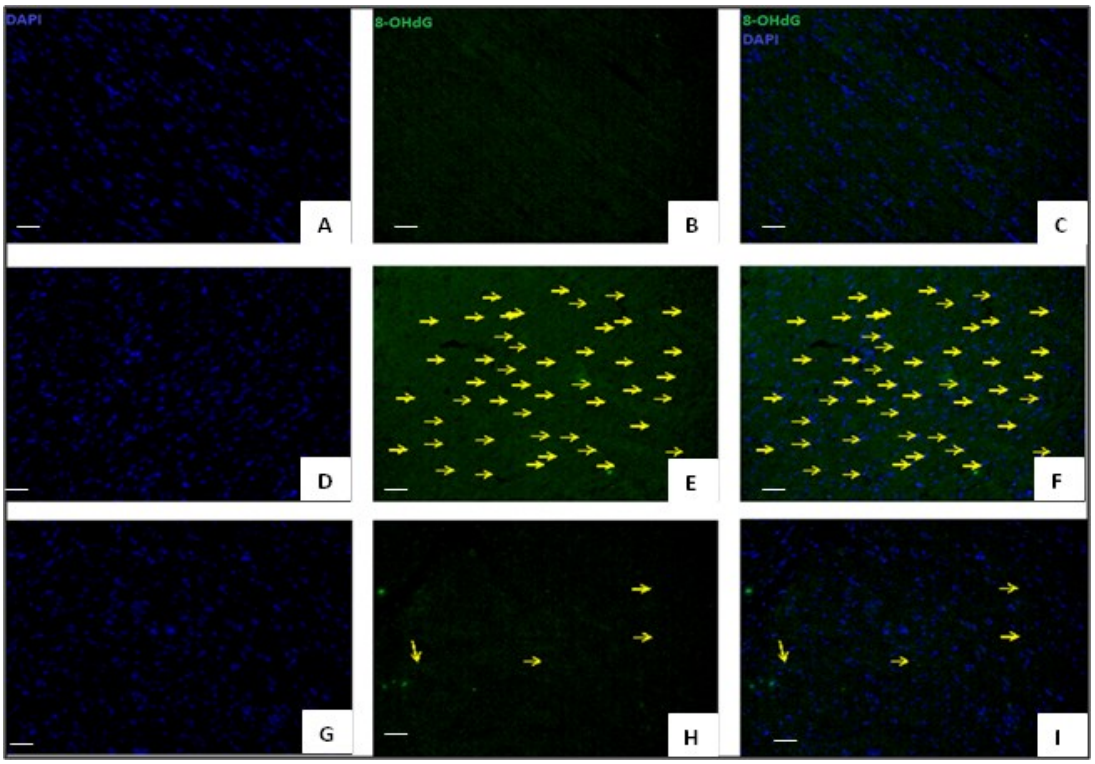


Figure 5.8*: Representative photomicrographs of immunofluorescent staining for TUNEL-positive nuclei in the negative control, I/R-control, and I/R-FTY720 groups. (A, D, G) shows only DAPI in myocardial tissue, (B, E, H) TUNEL positive signals and (C, F, I) merged images. TUNEL-positive myocytes were much lower in numbers frequently in control CPA group than in CPA-FTY720 group.CPA, CardioPlegic Arrest, Original magnification 20x.*

5.4.8. Effect of FTY720 on neutrophil infiltration

In CPA related I/R, development of interstitial edema, structural disarray, and neutrophil infiltration was observed. However, pre-ischemia FTY720 treatment remarkably reduced morphological changes and neutrophil infiltration. In treated group, edema was also attenuated. The difference between control and treated group was statistically significant (p=0.03) indicating that FTY720 attenuates infiltration of neutrophils (Figure 9).

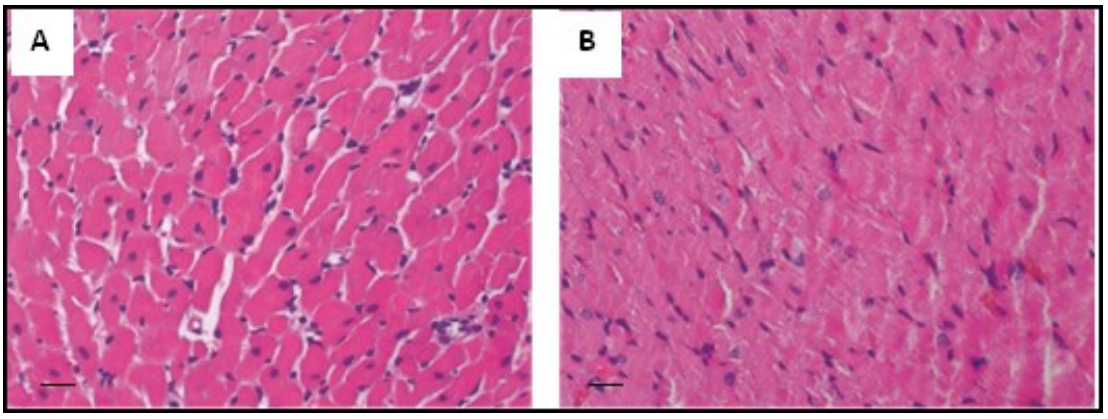


Figure 5.9: *Representative photomicrograph showing the histopathological changes in rats' myocardium related to CPA-induced ischemia-reperfusion (20X Magnification). A. The cardiac section shows interstitial edema and neutrophil infiltration in control group of CPA rat model; B. Heart tissue section showing mild necrosis and band contractions in the FTY720-treated group.*

5.6. Discussion

The pharmacological cardioprotective strategy to prevent acute global I/R injury has been tested using different approaches. Over the last few years, multiple pharmacological agents including volatile anesthetic agents [216-218], sodium hydrogen exchange inhibitors[219],and statins[220], pharmacological preconditioning [221, 222], and anti-inflammatory strategies have been explored as potential cardioprotective therapies. However, majority of preclinical strategies showing cardioprotective effects did not work in clinical settings[249].

In the present study, administration of FTY720 demonstrated inhibition of apoptosis by inhibiting inflammation and oxidative stress. In order to unravel the possible molecular mechanism involved in cardioprotectiondue to FTY720, we have been able to show activation of RISK and SAFE pathways, in addition to inhibition of pro-apoptotic and activation of anti-apoptotic proteins. Akt1/2 and Erk1/2 phosphorylation investigated in this study is an important component of cell survival pathways [223-225].

Previous studies suggested a cardioprotective role of FTY720 by activating survival pathways in *in vitro*, *ex vivo* in mouse and rat hearts and *in vivo* animal models [137, 138]. In a study, Means et al. reported that knock-out mice of S1P receptors produced a high level of myocardial damage as compared to wild type[179]. Moreover, mice with a lack of sphingosine kinases were found to have large infraction size compared to control mice. In addition to activation of S1P receptors, metabolism of S1P has also significance in pre and post conditioning cardioprotective mechanisms [138, 177, 178, 226].

I/R injury can be caused by various mechanisms and pathways. Activation of apoptotic pathways, complement system activation, increased inflammation, and oxidative stress can cause myocardial injury following ischemia-reperfusion[227]. FTY720 shows a potential to deal with most of the myocardial damaging mechanisms to prevent I/R injury.

According to literature, S1P receptor agonists have an important role in immune suppression [230]. Different models have been tested for immunosuppression including a porcine model of I/R [185], and spontaneous obstructive coronary atherosclerosis murine model [231]. Through these models, a reduction in infarct size and mortality has been shown in the FTY720-treated group. We measured inflammatory markers in blood and in heart tissue. In blood, we found a significantly low concentration of neutrophils, lymphocytes, and pro-inflammatory cytokines.

The ICAM-1, IL-6, IL-1β and TNF-α as pro-inflammatory cytokines contribute to develop inflammatory mechanisms [232-234]. . I/R increased ICAM-1, IL-6, IL-1β and TNF-α levels in control group, whereas FTY720 treatment decreased the concentrations of these cytokines. Therefore, the suppression of inflammatory cytokines by FTY720 treatment protects myocardium from I/R injury normally caused by these proinflammatory cytokines.

One of the main strategies for cardioprotection is to mitigate apoptosis in I/R. Molecular signaling RISK and SAFE pathways activation have been reported during I/R [138, 175]. The RISK (Akt1/2, Erk1/2, and GSK 3β) and SAFE pathways (JAK and STAT3) are the main sources for mitigation of apoptosis by preventing opening of mitochondrial permeability transition pores[3, 173, 174]. Consistent with previous findings, activation of RISK and SAFE signaling pathways was observed along with decreased level of apoptosis in the treated group as compared to the control group. This was further supported by decreased activation of pro-apoptotic proteins BAX and enhanced expression of anti-apoptotic proteins Bcl-2 after 10 min of CPA and 1 hour of reperfusion.

Reperfusion after transient ischemia in myocardium leads to cardiomyocytes apoptosis and cardiac dysfunction[228, 229]. Consistent with previous reports,our TUNEL assay results showed that those rats treated with FTY720 15 min before CPA, expressed a lower frequency of TUNEL positive nuclei after 1 hour of reperfusion. This observation along with above mentioned findings is indicative of cardioprotective role of FTY720 by activating anti-apoptotic cascade.

This study was not without limitations. Coronary artery disease in patients undergoing cardiac surgery is mostly associated with multiple co-morbidities including diabetes, hypertension and hyperlipidemia. Our experimental model did not truly reflect those conditions. Perhaps, this could be the reason that many studies

on experimental models failed to show their beneficial effects inactual clinical practice. However, in a recent study we have shown that the rat model due to similarities in the SIP receptors between rat and human heart would be more appropriate to study the beneficial effects of FTY720 in clinical trials.

In our study, reperfusion time was one hour and we could not study the effect of FTY720 in late phase. Prolonged reperfusion could have provided more strong evidence of reduction in I/R injury. The effect of FTY720 in structural remodeling can be studied only if there is prolonged reperfusion time. However,due to complex surgical procedure, it was hard to achieve in our model.

Furthermore, we administered only one dose before initiating ischemia in our model. There is a possibility that multiple doses of FTY720 could offer better cardioprotection. This would need further investigation.

In spite of these limitations, this study did provide evidence that FTY720 has a great potential to be used for cardioprotection in cardioplegic arrest. On the basis of these results, it can be suggested that sphingosine 1-phosphate receptor activation in patients undergoing cardioplegic arrest during cardiac surgery could be most important for assessment of the potential clinical efficacy of this cardioprotective agent.

5.7. Conclusion

In conclusion, FTY720 preserves, high energy phosphates and cardiac mechanical functions, and attenuates myocardial apoptosis, inflammation and oxidative stress. Furthermore, a cardioprotective mechanism appears to be associated with phosphorylation of Akt and Erk1/2 pro-survival cell signaling pathways (RISK and SAFE pathways) to prevent myocardial damage. In our experimental setting, at early phase no improvement in hemodynamic parameters was observed. Moreover, the question of whether a lower dose of FTY720 in human will bring protective effects on myocardium needs to be addressed in future studies.

SECTION III-C

6. Cardioprotective role of fingolimod (FTY720) in heterotopic heart transplantation

6.1. Introduction

Heart transplantation is the ultimate treatment option for heart failure[238]. Heart Failure (HF) is a major health problem worldwide, with a prevalence of 23 million worldwide and 5.8 million alone in the USA[239]. Since 1997, HF has been singled out as an emerging epidemic in the world with higher morbidity and mortality in 65 and elders[240]. Most common cause of cardiac dysfunction and mortality in cardiac transplant patients is due to ischemia-reperfusion injury[241, 242]. *Ex-vivo* organ transportation is the major factor involved in prolonged ischemia and exacerbation reperfusion injury that cause major post-operative morbidity and mortality. Factors like advanced donor age, smoking, diabetes, prolonged ischemia time also influence ischemia reperfusion injury that is the main cause of early graft dysfunction. Ischemia/reperfusion injury is correlated with advanced donor age, prolonged preservation time, impaired myocardial reserves, is a major cause of primary graft dysfunction[243]. Currently, low potassium solutions give some positive evidence for better cardiac graft preservation [244]. Although low potassium solutions demonstrated attenuation of grafted heart damage related to cold storage and ischemia-reperfusion injury. But it has been observed that this solution is not sufficient to protect myocardial damage due to decreased cellular energy reserves, release of oxidants, myocardial stunning and myocardial dysfunction. Till now, there is no efficient strategy for cardioprotection during heart procurement and transplantation. To achieve optimal cardio protection, other strategies needed to investigate. Sphingosine 1-phosphate agonist fingolimod is phosphorylated form of sphingosine that is derivative of ceramide may also take part in cardioprotection in heart transplantation related I/R injury [245]. Fingolimod has potent anti-inflammatory and anti-oxidant properties that inhibit oxygen free radical[246]. Fingolimod activates RISK and SAFE pathways leading to inhibition of pro-apoptotic proteins and activation of anti-apoptotic proteins[247]. We hypothesized that fingolimod pre-conditioning might achieve optimal cardioprotection with its known anti-apoptotic, anti-inflammatory and pro-survival pathways activation. In Rat heterotopic heart transplantation model, our aim is to investigate the cardioprotective role of fingolimod on apoptosis, inflammation, oxidative stress, nitrative stress and activation of Akt and ERK1/2 signalling pathways.

6.2. Materials and Methods

6.2.1. Animals

Sprague-Dawley Rats (300-350grams) Obtained from Harlan Laboratories (Udine, Italy). They were fed standard rat chow, which they had access to ad libitum. Rats were housed at a density of 4-6 per cage and maintained on a 12-hour light/dark cycle at 21°C. These rats were used as donors and recipients. The study was approved by the National Animal Welfare Committee.

6.2.2. Experimental design

In this study, we used four transplant groups, two for control and two for fingolimod treatment 1-hour reperfusion and 24-hour reperfusion respectively, (n=15 for each group). Treatment was administered 15 mins before explantation. Intravenous injection was given, either fingolimod 1mg/kg (treated) or saline (control group). This dose was given on the basis of a previously reported *in-vivo* study reported by Santos-Gallego et al[185]. In Group A (control) and Group B (treated), hemodynamic measurement for systolic and diastolic function was performed after 1 hour of reperfusion. In next two groups, Group C (control) and Group D (treated), after performing transplantation, abdominal cavity was closed and animals were allowed to recover for 24 hours. Following 24 hours of reperfusion with similar environment and diet for all animals, again hemodynamic measurements were performed, blood and tissue were collected for cardiac, inflammatory, apoptotic, oxidative markers and high energy phosphates.

Experimental techniques, data acquisition and analysis methods are mentioned in Section II(methods and materials).

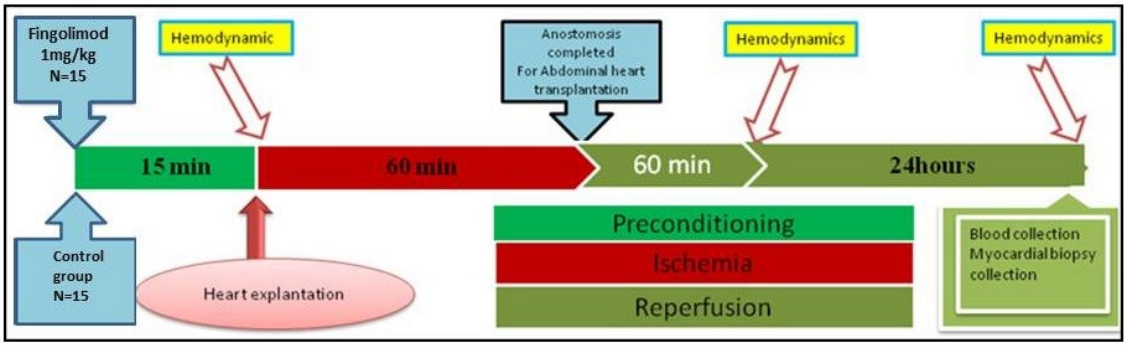


Figure 6.1. Schematic view of experimental design.

6.3. Results

6.3.1. Left Ventricular Function

Baseline hemodynamics were similar in both groups (Table 6.1). In all groups, rats underwent cardioplegic arrest, explantation, and implantation of the graft. The hemodynamics were measured after 1 hour of reperfusion in Group A and B (n=15 in each) and after 24 hours of reperfusion in Group C and D(n=15 in each group). Hemodynamic measurements were done to evaluate LV performance using the Millar catheter system (Fig. 5.B).

Table 0-1 Comparison of baseline hemodynamics in Group A vs. Group B and Group C vs. Group D.

Baseline	Group A	Group B	P value	Group C	Group D	P value
HR (beats/min)	289±17	288±22	ns	309±29	297±17	ns
MAP (mmHg)	127±15	130±17	ns	112±14	115±11	ns
CO (ml/min)	43±3	47±5	ns	44±3	50±4	ns

The LV End Systolic Pressure (LVESP) elevated significantly after HT and resuscitation in Group D as compared to Group C ($P \leq 0.05$)(Fig. 6.2A). No difference between Group A and B were statistically not significant. The LV End Diastolic Pressure (LVEDP) measurements showed a reduction in the Group B and D vs. Group A and C ($p>0.05$)($p\leq0.05$) respectively(Fig. 6.2B). While, Ventricular systolic performance dp/dt max after HT+reperfusion was increased in Group A vs. Group B, Group D vs. Group C ($P\leq0.05$)($P \leq 0.001$) respectively(Fig. 6.2C).The minimal pressure relaxation rate (dP/dt min) was also improved in Group B and D as compared to Group A and Group C ($P\leq0.05$)(Fig. 6. 2D).

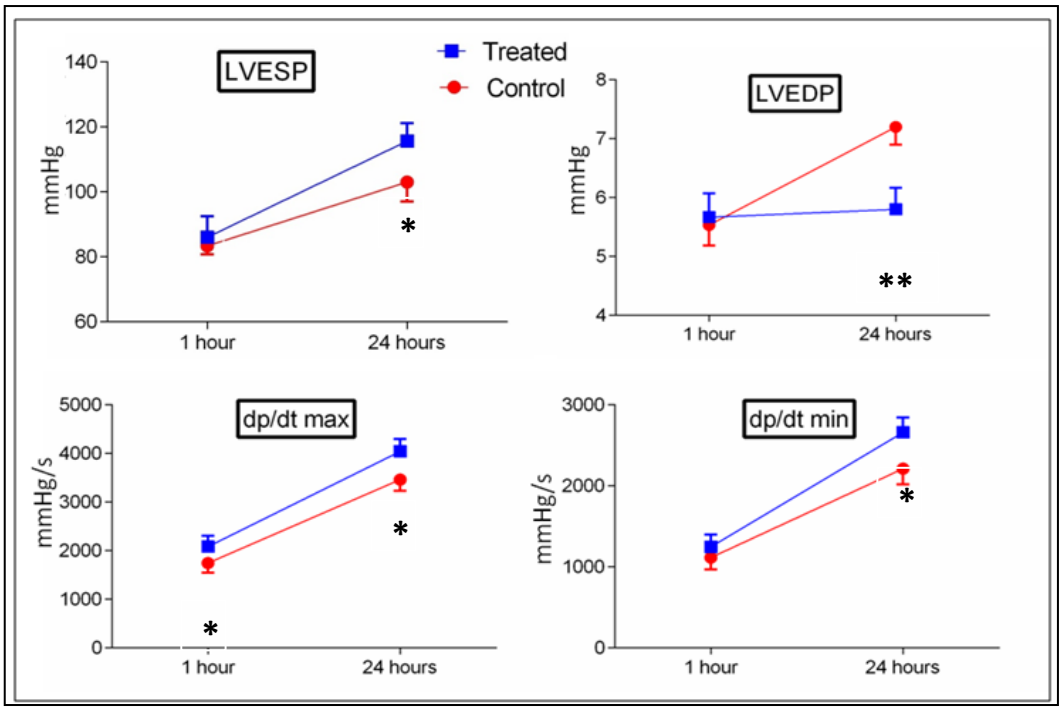


Figure 6.2. *Hemodynamic parameters demonstration, at 1hour and 24 hours of reperfusion. Effects of FTY720 on left ventricular function in the rats with heterotopic transplantation. Treatment was given 15 mins prior to explantation of heart, (A) Effects of FTY720 on LVESP. (B) Effects of FTY720 on LVEDP. (C) Effects of FTY720 on LV dp/dt max. (D) Effects of FTY720 on LV dP/dt min, LVESP and LVEDP were measured using a*

multichannel physiological recorder. LV dP/dt max and min was expressed as mmHg/sec. LVESP and LVEDP were expressed as mmHg. LV dP/dt max, the rate of maximum positive left ventricular pressure development; LV dP/dt max, the rate of maximum negative left ventricular pressure development; LVESP, left ventricular end-systolic pressure; LVEDP, left ventricular end-diastolic pressure. Values are expressed as the means ± SD.

6.3.2. Bcl-2 and BAX signaling pathways

After 24 hours of reperfusion in HT models, heart tissue was excised for immunohistological staining to measure the expression levels of anti-apoptotic (Bcl-2) and pro-apoptotic (Bax) proteins. As presented in (Fig. 6.3), in FTY720, treated (Group D) Bcl-2 expression were significantly increased versus HT control group(Group C), while expression of BAX was downregulated in Group D as compared to Group C ($P \leq 0.05$), indicating that Fingolimod during HT attenuates apoptosis by upregulation of Bcl-2 and downregulation of Bax proteins.

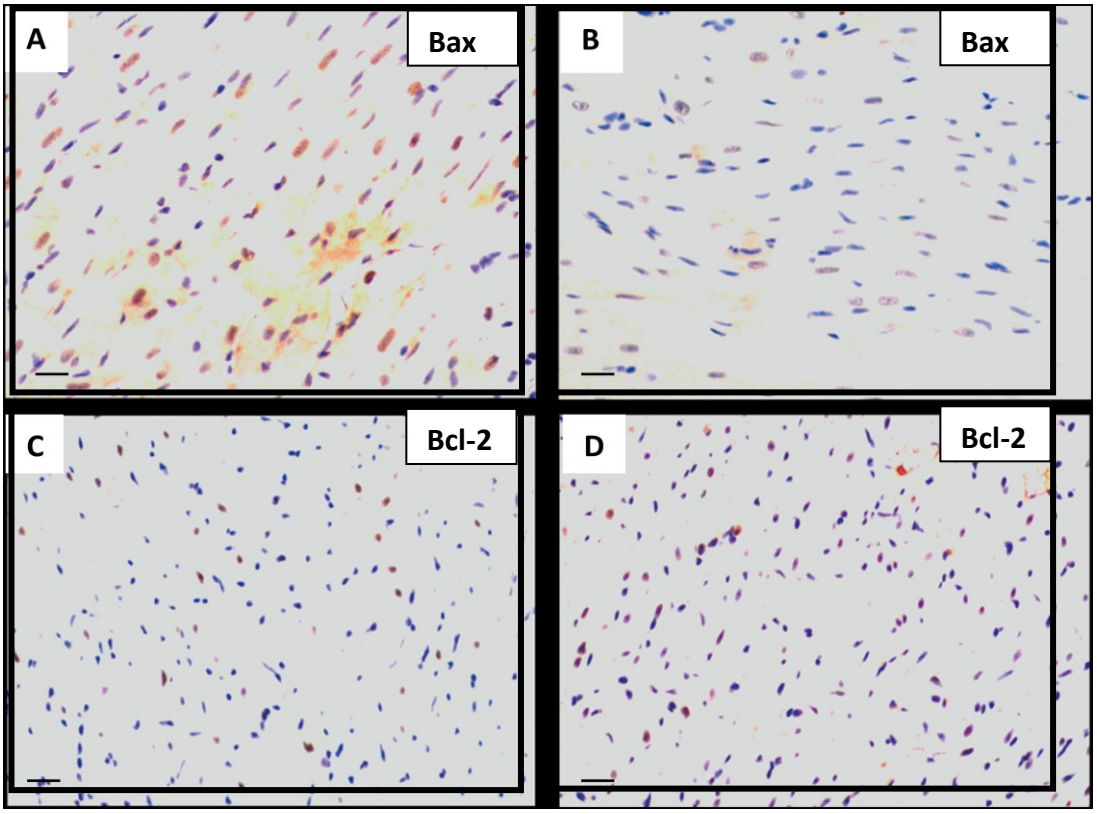


Figure 6.3. *Myocardial tissue expression levels of Bcl-2 and Bax after 30-40mins ischemia and 24 reperfusion. The protein expression levels of Bcl-2 and BAX were determined by an immunohistochemistry, (A) BAX expression in HT-control group(Group C), (B) Bax expression in HT+FTY720 treated group(Group D).(Control vs. Treated p≤0.05) (C) Bcl-2 expression in Group C and (D) Bcl-2 expression in Group D. (Control vs. Treated p≤0.05).*

6.3.3. Effect of fingolimod on apoptosis

TUNEL assay is apoptosis detection marker, its shows extensive apoptosis in HT-related I/R injury. S1P receptors activation by using fingolimod showed a significant attenuation of TUNEL-positive nuclei in myocardium, that is indicates anti-apoptotic effect of fingolimod on myocardial cells apoptosis (Fig.6.4).

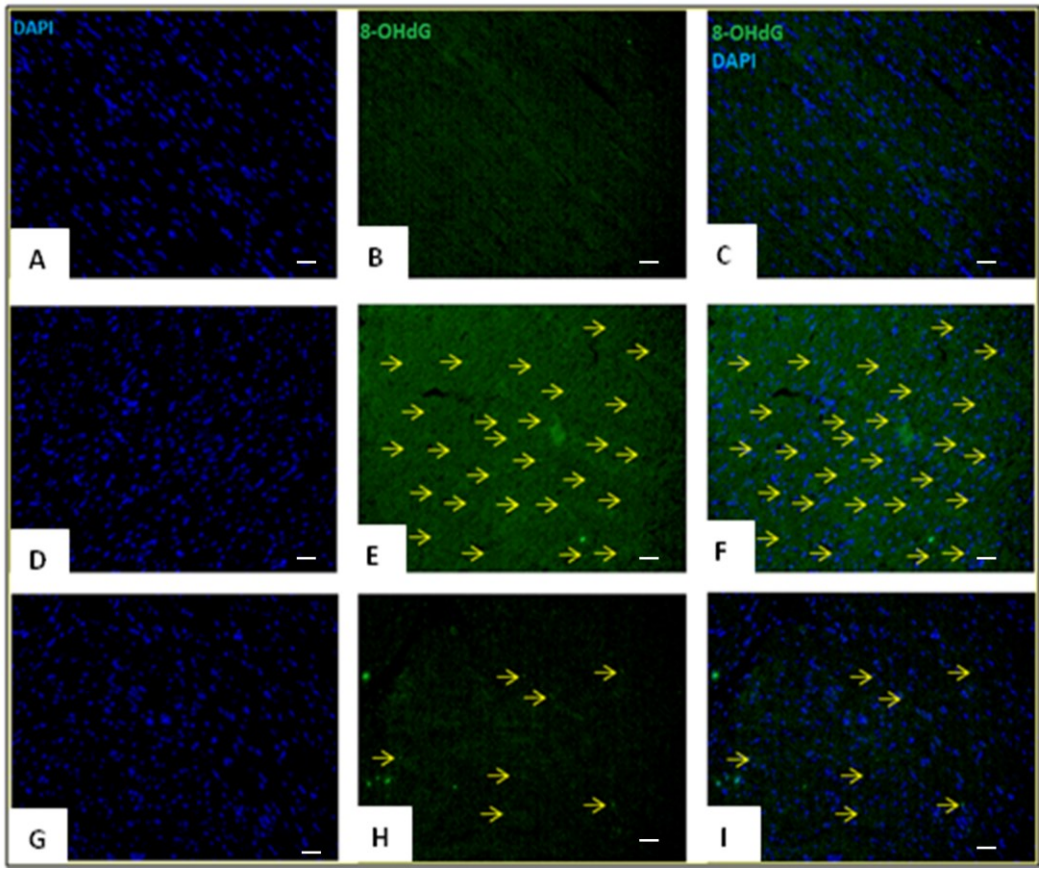


Figure 0.4. *Representative photomicrographs of immunofluorescent staining for TUNEL-positive nuclei in baseline, HT-control, HT-Fingolimod groups(Group C and D). (A, D, G) Shows only DAPI in myocardial tissue, (B, E, H) TUNEL signals and (C, F, I) merged images. TUNEL-positive myocytes were much lower in numbers frequently in control HT group than in HT-FTY720 group. Original magnification 20x.*

6.3.4. Interstitial collagen deposition

Interstitial collagen deposition measured by collagen volume fraction (CVF), to evaluate myocardial fibrosis after HT(60 min ischemia) and reperfusion for 24 hours. The differences in the interstitial collagen deposition in HT group and

HT+FTY720 group as showm in (Fig.6.5). To calculate CVF, we measured the collagen staining expression (blue) in Masson's trichrome-stained images. Fingolimod (1mg/kg) treated group demonstrated low level of CVF as compared to control group ($p \leq 0.05$).

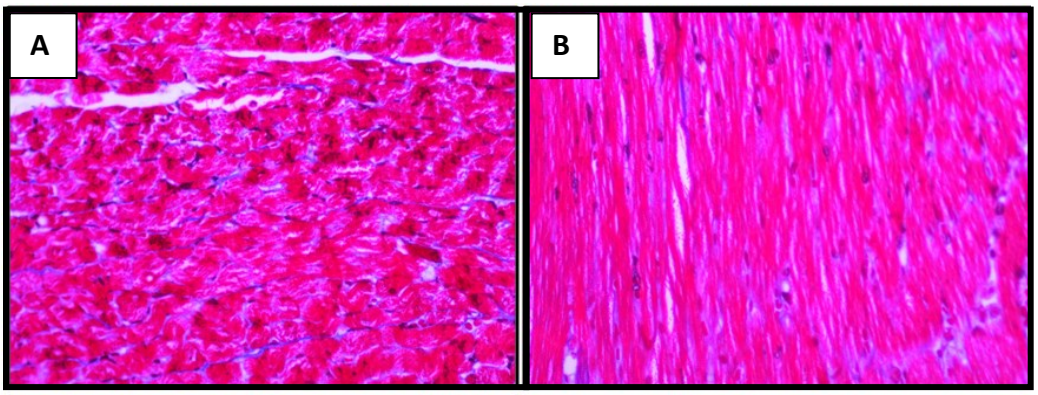


Figure 6.5. *Representative photomicrograph showing the collagen deposition in rats related to HT ischemia-reperfusion injury (20X Magnification). FTY720 decreased post-HT myocardial interstitial fibrosis. The viable myocardium is stained bright red. Fibrosis is stained bright blue. A; HT without fingolimod(n=15) and B; HT with fingolimod (1mg/kg)(n=15).*

6.3.5. Fingolimod attenuates neutrophil infiltration

In HT animals group, there were interstitial edema and structural disarray, and including neutrophils infiltration observed. However, pre-ischemia FTY720 treatment, remarkably reduced morphological changes and neutrophil infiltration. Difference between control and treated group were statistically significant ($p \leq 0.05$).

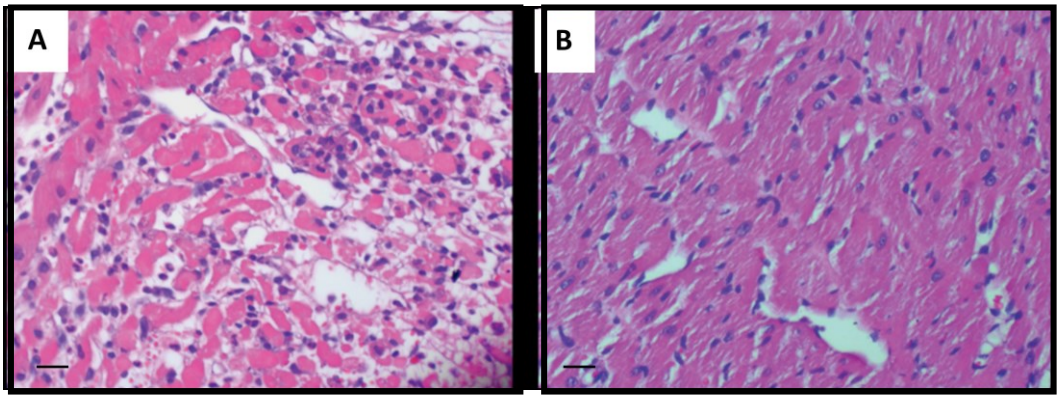


Figure 6.6. *Representative photomicrograph showing the histopathological changes in rats related to HT related ischemia-reperfusion (20X Magnification); A. Cardiac section shows interstitial oedema and neutrophil infiltration in control group(Group C) in HT model; B. Heart tissue section showing mild necrosis and band contractions in fingolimod-treated group(Group D).*

6.3.6. Fingolimod reduces Nitrative stress

Recent studies have demonstrated that other then oxidative stress, nitrative stress also plays a important role in myocardial tissue injury under a ischemia-reperfusion. Excessive NO production from NOS and the reaction product between NO and peroxynitrite, superoxide have been observed to activate apoptotic signallling pathways leading to apoptotic cell death. To determine whether FTY720may reduce myocardial nitrative stress caused by HT related ischemia reperfusion and thus attenuation in myocardial apoptosis, we determined peroxynitrite expression. As illustrated in (Fig. 6.7), FTY720 markedly down-regulated peroxynitrite expression, that indicates decrease production of nitro-tyrosine.

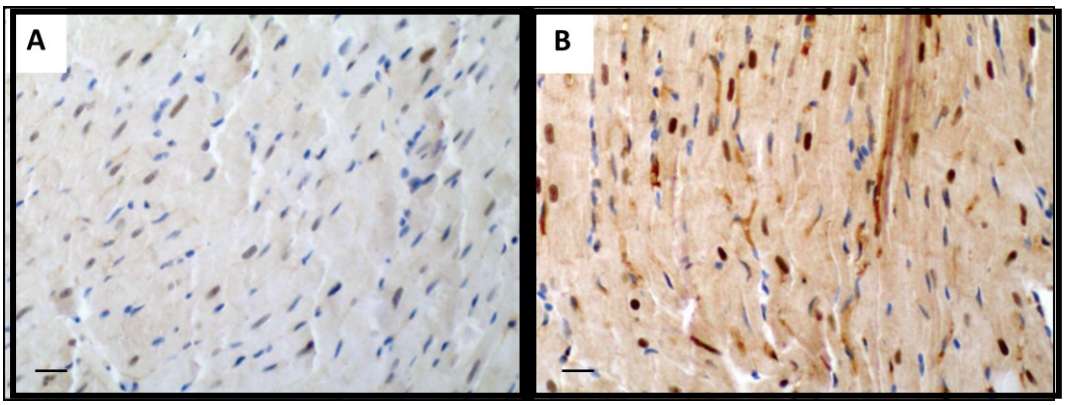


Figure 6.7. *Myocardial nitrotyrosine staining (A) FTY720-Treated group(n=15), (B) Control group (n=15), Rats were subjected to 60 mins ischemia followed by 24 hour reperfusion. At the end of the experiment, the heart was excised, and nitrotyrosine*

localisation was determined.

6.3.7. Serum levels of CK-MB and cTnI

Cardiac markers of cardiomyocyte injury, the serum levels of CK-MB and cTnI were 14±8.2 and 3.22±0.52 U/g protein in the baseline-operated group and were significantly increased in the HT group to 148.8±8.9 and 26.5±0.69 U/g protein, respectively. Following treatment with fingolimod the CK-MB and cTnI levels were significantly decreased (P≤0.01 and P≤0.05 vs. control HT-group) as shown in (Fig. 6.8).

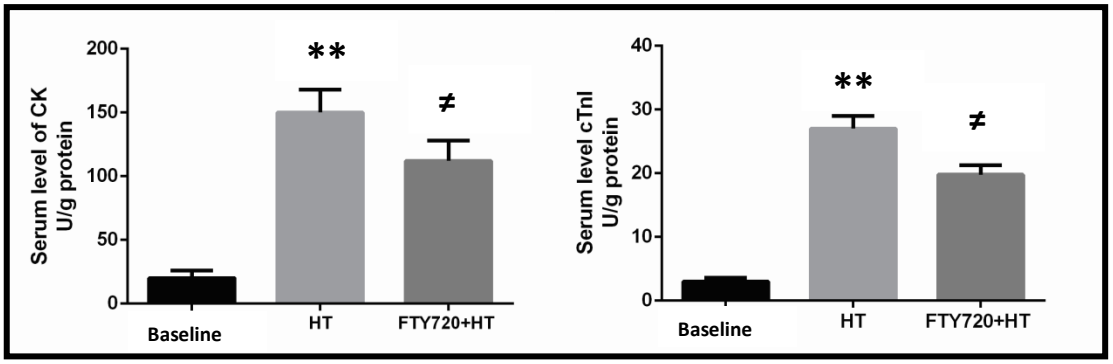


Figure 6.8. *Serum Levels of creatine kinase-MB (CK-MB) and cardiac troponin I (cTnI) in the serum at the baseline, HT- reperfusion and HT-reperfusion+Fingolimod group(1mg/kg). All Values are expressed as the means ± SD (15 rats in each group). (**P≤0.01 vs. baseline; #P≤0.05 vs. Control group).*

6.3.8. Serum levels of Inflammatory Mediators

The inflammatory mediators contribution were identified in the HT related I/R injury. HT-related I/R injury caused by marked elevation in the levels of cytokines mainly TNF-α, IL-1β, IL-6 and ICAM-1. All these cytokines increase in serum in response to ischemia and reperfusion. In the present study, serum levels of these mediators have been measured in HT animals after 24 hours of reperfusion. Compared to the baseline TNF-α, IL-6, IL-1β and ICAM-1 serum levels were significantly increased in HT-reperfusion group. On administration of fingolimod (1mg/kg) TNF-α, IL-6, 1L-1β and ICAM-1 attenuated vs. control group (p≤0.05, p≤0.05, p≤0.05, and p≤0.001) respectively as shown in (Fig. 6.9).

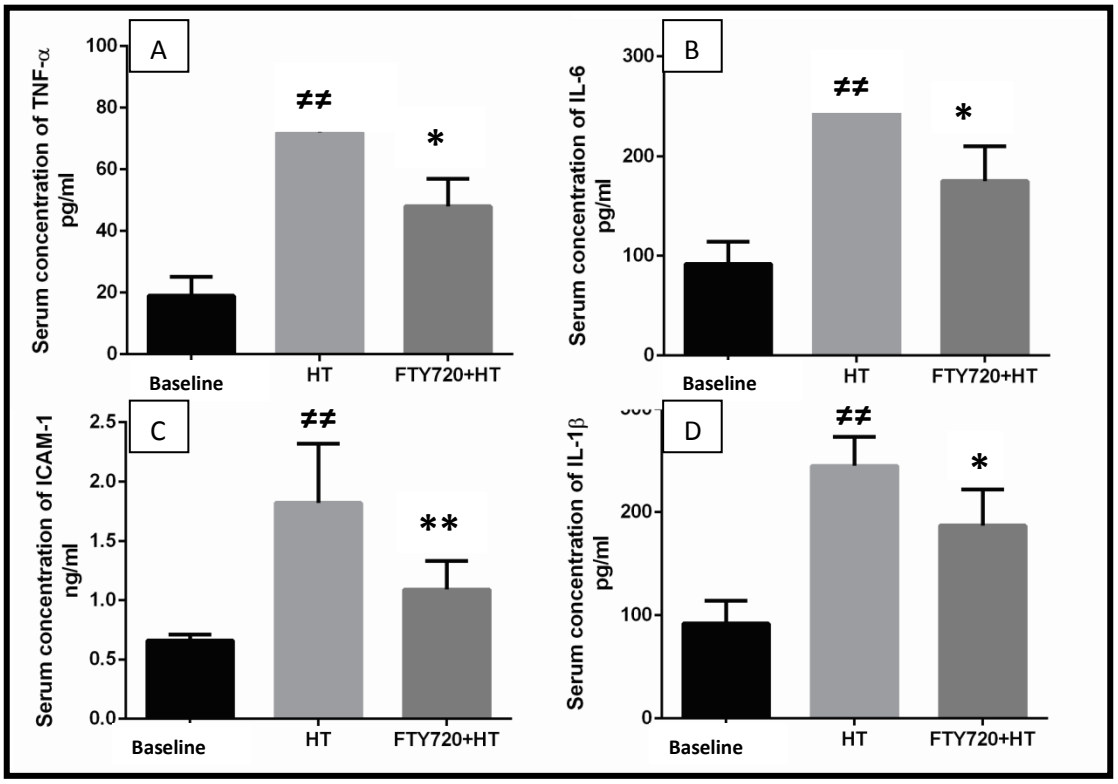


Figure 6.9. *Myocardial production of TNF-α (A), IL-6 (B) and ICAM-1 (C) and IL-1β (D) after 24 hours of reperfusion, (A) HT model without fingolimod treatment shows high expression of TNF-α as compared to fingolimod treatment. (B)HT-Reperfusion induced significant high IL-6 after 24 hours of reperfusion compared with the fingolimod treated and baseline, (C) FTY720 treated group remarkably reduced the production of the ICAM-1 release as compared to Control (D) this section of the panel presents, production of 1L-1β higher in control vs. FTY720 treated group in HT-reperfusion group. Each bar height represents the mean ± SD (each group n=6). ($^{\#\#}P \leq 0.01$ vs. baseline. $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$ vs. HT-control group).*

6.3.9. Effect of fingolimod on Erk1/2 and Akt1/2 signalling pathways

Pro-survival signalling pathways were measured in the myocardial tissue.Akt/ PI3 kinase pathway is important survival signalling pathway. We measured the phosphorylation level of Akt in Group C and D in the myocardial tissue. As shown in (Fig. 6.10A), phosphorylation levels of Akt1/2 increase over control group ($P \leq$ 0.05). ERK1/2 phosphorylation were analysed by Western blot in rat myocardial samples. As shown in (Fig. 6.10B), phosphorylation level of ERK1/2 increased in Group D versus Group C ($P \leq 0.05$).

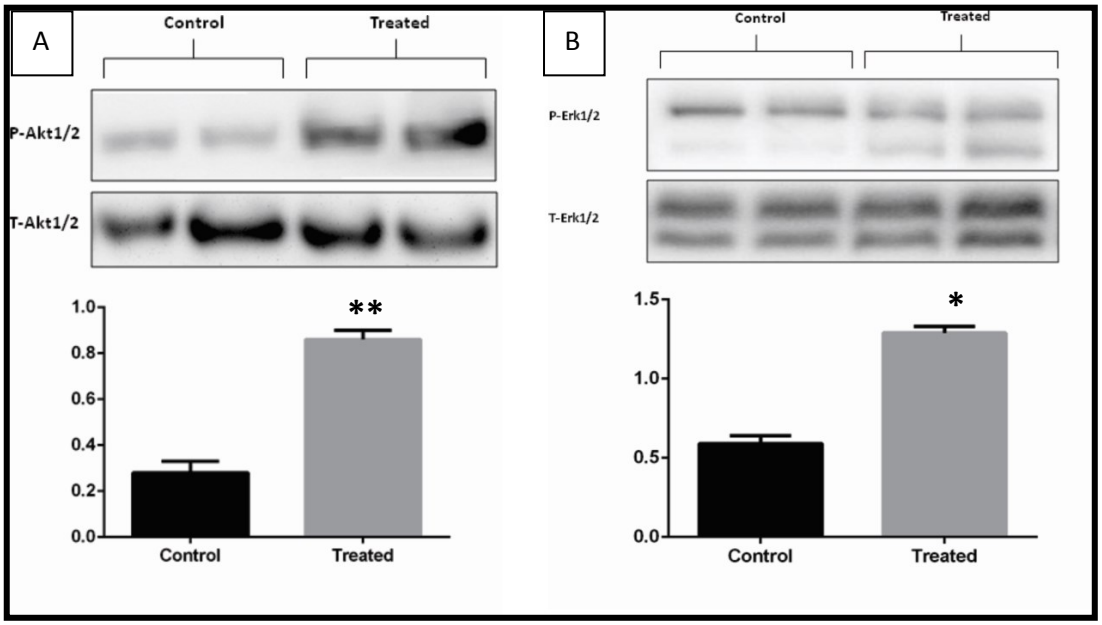


Figure 6.10 *Representative Western blot and relative density of the phosphorylated (p) form of Akt1/2 (A) ERK1/2 (B), samples of the left ventricle at the end of reperfusion. Relative Densities shows Fingolimod activate phosphorylation of these proteins. Values are means ± SD. (*p≤0.05, **P ≤ 0.05).*

6.3.10. High Energy Phosphates

The measurement of high-energy phosphates in model of heterotopic transplantation revealed the superior preservation of fingolimod treated myocardium (Fig. 6.11). Phosphocreatine, the buffering energy source for ATP in situations of energy demand, was significantly higher in the fingolimod administered group of animals compared with the control group tissue ($P \leq 0.05$). While high energy phosphates (AMP, ADP, and ATP) were significantly increased after both the 1 hour and 24 hours of reperfusion in treated group as compared to the control group ($P \leq 0.05$). Energy charge was significantly elevated after 1 hour of reperfusion in Group B versus Group A (0.79 ± 0.02 vs. 0.71 ± 0.01) ;($P \leq 0.001$), whereas the after 24 hour reperfusion, it was not statistically significant (0.78 ± 0.01) ($p>0.05$).

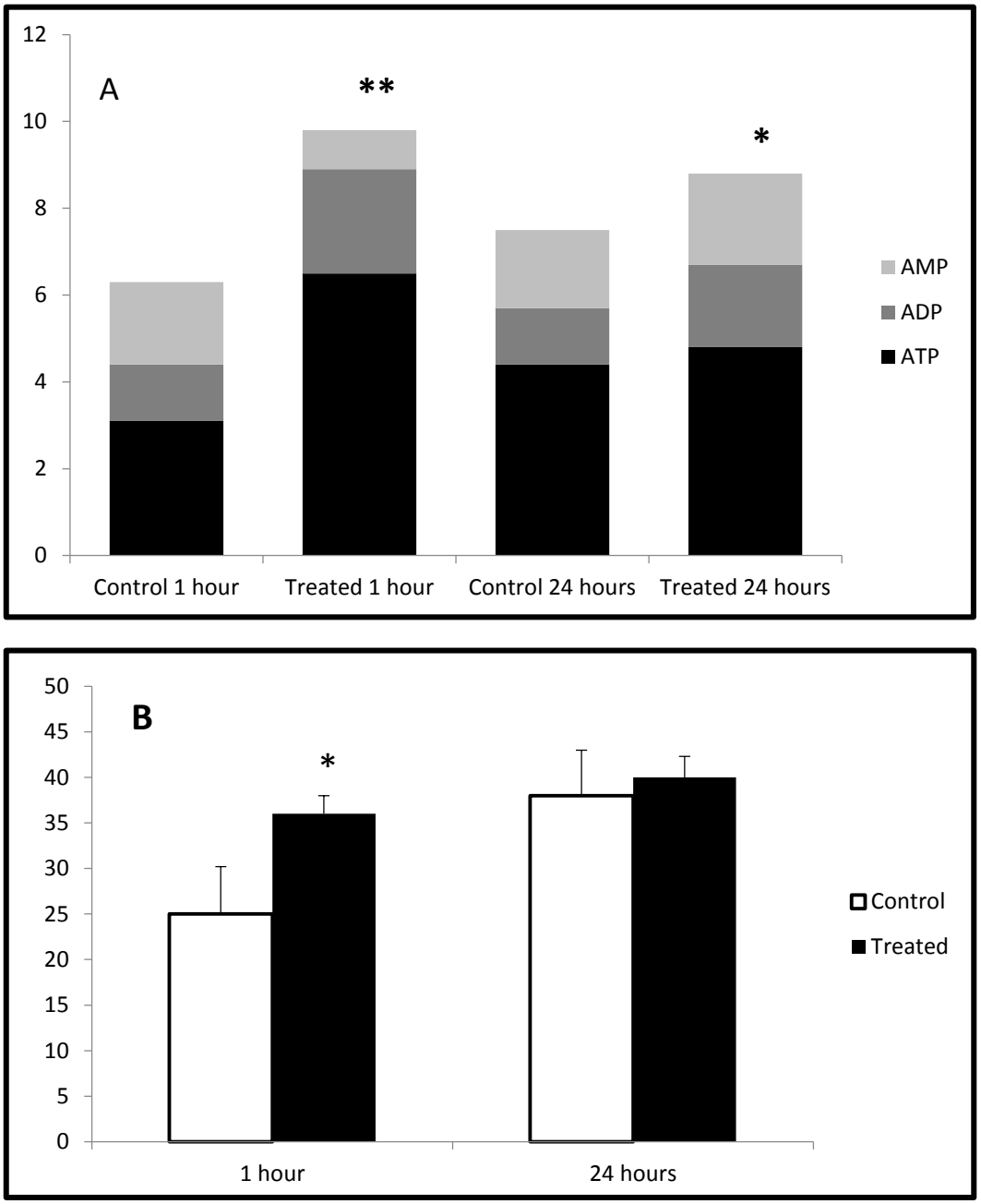


Figure 6.11. *High-energy phosphates in myocardial tissue of the LV in the fingolimod treated groups compared with the control group. (A) Changes in phosphocreatine , (B) ATP levels at 1 hour and 24 hour of reperfusion energy charge. (*p≤0.05, **p≤0.005) AMP, Adenosine MonoPhosphate; ADP, Adenosine DiPhospahte; ATP, adenosine triphosphate.*

6.3.11. Oxidative Stress

To examine whether fingolimod regulates the free radicals production in ischemia-reperfusion, we examined free radicals and aldehydes (lipid peroxidation derivatives) in the frozen perfused myocardial sample. In Group C, reactive oxygen

species level found higher as compared to Group D. Particularly, we analyzed free reactive oxygen species and malondialdehyde in HT animals myocardial tissue by chromatography and mass spectrometry. Collectively, our results suggest fingolimod treatment in HT decrease oxidative stress (Fig.6.12).

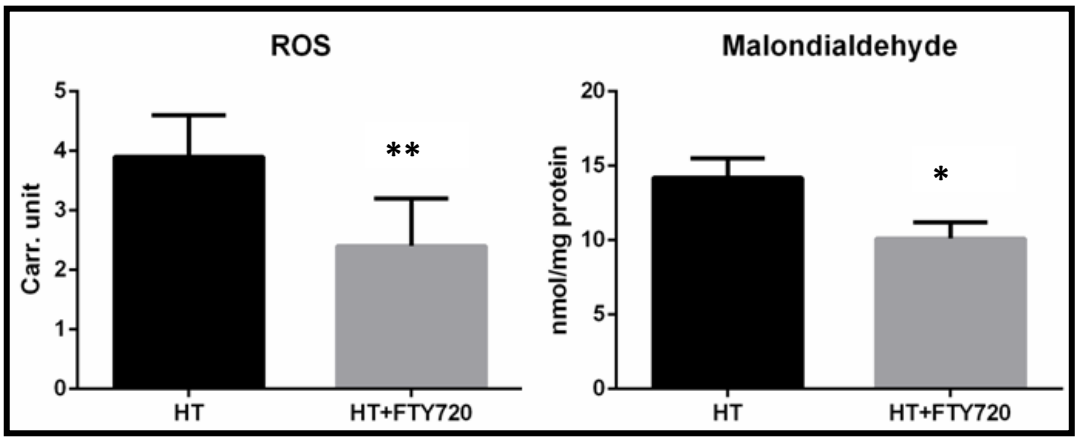


Figure 6.12 *Oxidative Stress Comparison of oxidative stress in heterotopic transplantation model with and without fingolimod treatment. ROS. Reactive oxygen species; HT. Heterotopic Transplantation; FTY720. Fingolimod; Carr. Unit. Carratelli Unit. (**p≤0.001,*p≤0.05).*

6.4. Discussion

In this study, we investigated cardioprotective effect of prolonged organ preservation during *in vivo* heart transplantation model. We found that fingolimod, efficiently works as ischemic preconditioning agent. Fingolimod improved myocardial function as compared to control group after 60 min global ischemia in St. Thomas solution and 24 hours of reperfusion. Our findings suggested that fingolimod plays an important role in the reduction of apoptosis, inflammation and improve mechanical function of the heart.To our knowledge, the present study is first to investigate cardioprotective role of S1P receptor agonist in preclinical heart transplantation model.

Fingolimod is one of the most best treatment option in prevention of multiple sclerosis relapse. In addition to its immune-modulating effect, fingolimod has a number of additional useful actions, including anti-inflammatory, anti-apoptotic, anti-oxidative and anti-nitrative stress[248]. These properties are predicted to improve the myocardial insult related to ischemia-reperfusion during heart transplantation. Previous studies have reported that pretreatment with S1P receptor agonist protected myocardium from ischemia/reperfusion injury[249]. In this experiment, we found that fingolimod treatment remarkably elevated LVESP and LVEF at late phase. This indicates, cardiac function improvement at late phase in fingolimod treated groups

as compared to control group is due to anti-apoptotic and anti-inflammtory pathways activation.

Reperfusion after transient ischemia in myocardium leads to cardiomyocytes apoptosis and cardiac dysfunction[224, 225]. TUNEL positives nuclei staining i.e gold standard method[250] to measure extinct of apoptosis have been used in this study. Consistently with previous results, in the transplanted group those treated with fingolimod 15 min before explantation of heart expressed a lower frequency of TUNEL positive nuclei as compared to control. That indicates the cardioprotective role of fingolimod by activating anti-apoptotic cascade.

One of the primary targets with this drug is to mitigate apoptosis in I/R. The molecular signalling RISK and SAFE pathways activation have been reported with fingolimod [138, 175]. The RISK (Akt1/2, Erk1/2, and GSK 3β) and SAFE pathways (JAK and STAT3) are the main sources for regulation of apoptotic pathways because of control on mitochondrial permeability transition pore [3, 173, 174]. The m PTP opening is vital step in apoptosis. Consistent with previous findings, activation of RISK and SAFE signalling pathways observed following decreased level of apoptosis in the treated group as compared to control. The inhibition of pro-apoptotic protein Bax, enhanced immunostaining for anti-apoptotic protein Bcl-2 found after 24 hours of reperfusion in heterotopic transplanted heart tissue.

According to literature, S1P and its agonist has important role in reduction of inflammatory mediators in I/R injury. Different models have been tested for immunosuppression including the porcine model of I/R, and spontaneous obstructive coronary atherosclerosis murine model showed the better myocardial protection and decrease inflammatory markers in the fingolimod-treated group. We measured inflammatory response in blood and tissue. In blood, we found the reduction of neutrophils and lymphocytes. Recent studies have demonstrated the significance of immune system role in I/R injury. Carlos G et al[185] found improved myocardial salvage in animals using fingolimod and suggested an immunomodulatory role of fingolimod by activation of S1P receptors. [185]. The Inflammatory mechanism is one of the key factors in I/R injury. The ICAM-1, IL-6, and TNF-α contribute in as a pro-inflammatory cytokines to develop myocardial damage[228-230]. The correlation between anti-inflammatory effects of fingolimod with cordioprotection is evident in our experiment. While anti-inflammatory effect of fingolimod is well established as its approved drug for multiple sclerosis.

This is well established that ischemia-reperfusion cause myocardial injury due to oxidative stress[251,252]. This drug also decreases oxidative stress in the ischemia-reperfusion[11,27]. The reduction in oxidative stress mediated by both RISK and SAFE pathways[185]. Oxidative stress is another major element involved in apoptosis. In our

transplantation study, we found decreased level of malondialdehyde and reactive oxygen species in the fingolimod-treated group.

Myocardial ischemia-reperfusion also produces nitric oxide synthase, that release nitric oxide which reacts with ROS, as a result, forms toxic substance peroxynitrite that also behaves as ROS leading to necrosis and apoptosis[253]. Fingolimod treatment partially attenuates nitrative stress in transplanted myocardium[254]. Present findings suggest fingolimod can be efficiently used as a preconditioning agent to improve myocardial salvage.

Together, all these results are suggestive of the myocardial protective role of fingolimod in global ischemia-reperfusion. Fingolimod is the only available FDA-approved agent, containing S1P receptors for prevention of multiple sclerosis relapses. This experimental study has the potential for translation into clinical trials.

6.5. Conclusion

In conclusion, our study supports the cardioprotective role of sphingosine 1-phosphate structural analogue fingolimod for reduction in apoptosis and inflammation in an experimental model of heterotopic heart transplantation. We found preservation of high energy phosphates at early phase of reperfusion associated with fingolimod treatment. This study provides insight for activation of cellular signaling pathways including Akt1/2 and ERK1/2 following improved mechanical activity of myocardium by assessment of left ventricular end diastolic, systolic pressures and positive and negative maximal contractility at late phase of reperfusion.

SECTION-IV

7. GENERAL DISCUSSION

7.1. Restatement of the Hypothesis

The main hypothesis was that an intravenous bolus of fingolimod would provide cardioprotection from ischemia-reperfusion injury by preservation of HEP, reducing inflammation, apoptosis and activating survival pathways. In addition, it was hypothesized that fingolimod would preserve hemodynamic function after global ischemia-reperfusion by reducing myocardial damage. While a few studies have recently used fingolimod for cardioprotective role against regional ischemia reperfusion therapy. According to our best knowledge, effect of fingolimod on global ischemia reperfusion injury has never been studied before. In this thesis, we studied the effect of fingolimod on cardiac arrest and resuscitation, cardioplegic arrest (cardiopulmonary bypass) and cardiac transplantation models.

An intravenous infusion of fingolimod administered before ischemia offered better protection from apoptosis in cardiomyocytes and tissue necrosis than without drug (Section III B and C). When the fingolimod infusion was given in early reperfusion(Section III-A), significantly anti-inflammatory and anti-apoptotic effects have been observed but relatively less as compared to pre-ischemic treatment and hemodynamics were also improved in both protocols at late phase of reperfusion, that indicates fingolimod do not have inotropic property and late phase hemodynamic improvement suggests improved myocardial salvage. The difference between pre and post pharmacological conditioning with fingolimod demonstrated no statistically significance. Although, in heterotopic cardiac transplantation, the cardioprotective effect was relatively lesser than other models, where ischemic time were 10 minutes. But still in transplantation group, results were significantly improved as compared to control group.

From this thesis, it can be concluded that fingolimod play cardioprotective role by preservation of high-energy phosphates, reduction in oxidative stress along with inhibition of apoptosis and inflammation in ischemia and reperfusion injury. Fingolimod therapy maintained [ATP] after ischemia and reperfusion by changes relatively near to the baseline. According to Hofmann U et al[283], a post ischemic treatment do not reduce infract size and increased mortality rate due to pro-arrhythmic property, while in pre and post ischemic treatment group, pro-arrhythmic property was canceled because it's connected with only first dose. In our study, results are suggestive about cardioprotection as also reported by Santos Gallego et al. in his study by demonstrating regional cardioprotection [185].

7.2. Possible mechanism of cardioprotection by Fingolimod

The work in thesis indicates possible mechanism of cardioprotection by fingolimod in global ischemia reperfusion injury (Figure.7.1).

7.2.1. Anti-apoptotic role of fingolimod

Fingolimod demonstrated significant beneficial outcomes in ischemia reperfusion injury, more specifically to reduce reperfusion injury. During any myocardial ischemia reperfusion event, finally upto 50% contribution is caused by reperfusion injury, and in some cases like sudden cardiac arrest, where we can not prevent ischemic injury but reperfusion injury can be reduced by using pharmacological cardioprotective agent. Fingolimod shows promising results to deal the above issues,
because in reperfusion, major issue is apoptosis and mPTP is final and and important step in I/R injury to cause extensive apoptosis. Main pathways to inhibit mPTP opening are the RISK and SAFE signaling pathways [3, 173, 174]. It has been proved that fingolimod may activate these pathways [138, 175]. In our all experimental models of global ischemia reperfusion, Akt1/2 and Erk1/2 demonstrated significant phosphorylation as compared to control group after 1 hour and 24 hours of reperfusion.

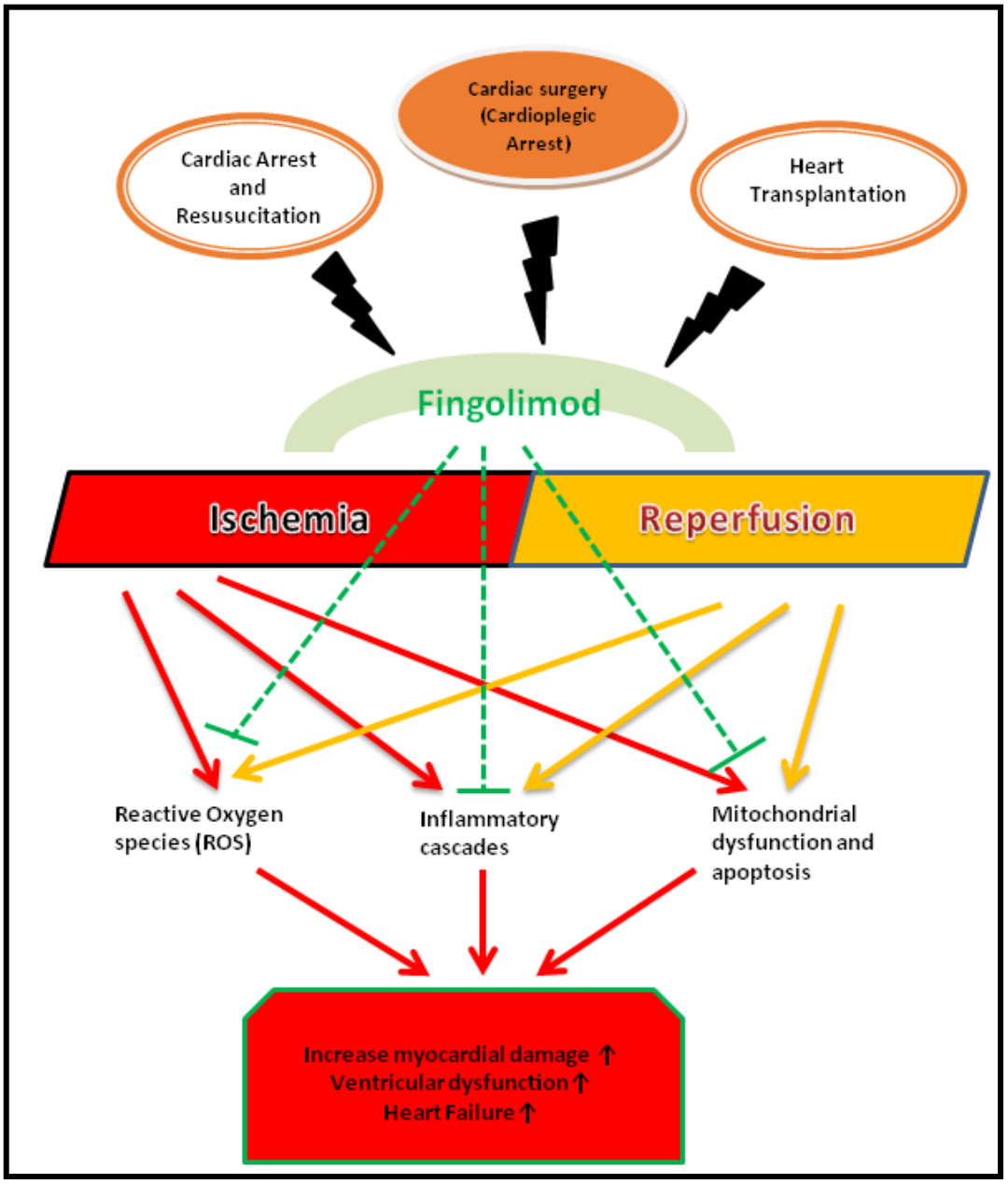


Figure 7.1 Cardioprotective mechanism of fingolimod

Therefore, the current study suggests that fingolimod may serve as a potential reperfusion therapy by preventing apoptosis through RISK and SAFE signalling pathways. In our rat models, consistent with the results of RISK and SAFE pathways activation; reduced apoptosis in TUNEL assay, increased expression of antiapoptotic protein and reduced expression of proapoptotic expression have been found. In previous studies, these pathways have been verified by using inhibitors of these pathways, wortmannin (RISK inhibitor) and AG490 (SAFE inhibitor)[185], suggesting the cardioprotective mechanism mediated through RISK and SAFE pathway.

7.2.2. Anti-Inflammatory role of fingolimod

Inflammatory mediators are one of major factors contributing towards ischemia reperfusion injury. Fingolimod showed adequate anti-inflammatory effect in our all ischemia reperfusion models. Santos *et al.* and Hofmann U *et al.* both achieved anti-inflammatory effects of fingolimod in ischemia reperfusion models. On the basis of known anti-inflammatory actions of fingolimod in multiple sclerosis [284, 285], it may also be useful to reduce the inflammatory response to ischemia-reperfusion injury [185, 283]. Fingolimod is a potent immune modulator by inhibiting the activation of neutrophils [286], monocytes [287] and lymphocytes, which can lead to necrosis of cardiomyocytes [288]. A study by Nakamura *et al.,* [289] confirmed that there is a significant apoptosis in myocardical cell induced by neutrophil infiltration.

In summary, the effects of fingolimod to reduce ischemia-reperfusion injury could also be applied to reducing the adverse effects of the inflammatory process which includes attenuation of the production of free radicals, reduction in capillary plugging and minimizing the direct injury to cardiomyocytes.

7.3. Limitations and clinical significance of fingolimod therapy

Despite of all advances in cardiovascular sciences, ischemic heart disease continues to be the leading cause of mortality and morbidity in developing as well as developed world (World Health Organization 2016). According to a study, acute coronary occlusion will be the most common cause of death in the entire world by 2020 [290]. All these patients may undergo one of the following events 1) PCI, 2) CABG, 3) Sudden cardiac arrest, 4) myocardial infarction leading to heart failure that need heart transplantation in severe cases. Although with advances in percutaneous techniques, majority of the patients undergo PCI but still CABG is main stay of treatment in complex coronary artery disease and/or with valvular diease. That expose to global ischemia and reperfusion as cardioplegic arrest for open surgery.

The work presented in this thesis may offer a new attractive therapeutic strategy that may contribute to myocardial protection in reperfusion injury. Although, usually it's not easy to translate potential therapeutic treatment from animal to clinical settings due to multiple factors mainly including mass specific metabolic rate difference, receptors expression difference in different species [291], and electrophysiological properties [292]. Indeed, rat have higher metabolic and extremely short half- life of fingolimod, while in human half-life is much longer. Consideration of all these factors make translation challenging but as this drug is already in human use with

mimimal serious adverse effect that gives hope to overcome challenges.
In rat models, infection rate and complications due to infection cannot be studied because of high resistance to infections in rats. It needs to be considered prior to human translation of fingolimod because it may cause lyphmphopenia that increases the risk of infection.

Another precaution in comparing data on rats and humans, are differences in collateral circulation of the heart. However, since humans have a greater collateral circulation than the rat [293], in human better cardioprotection can be expected using fingolimod as compared to rat models. Further experiments are already underway to test this hypothesis.

7.4. Concluding remarks

In conclusion, this study has provided evidence that fingolimod infusion is cardioprotective in sudden cardiac arrest, cardiopulmonary bypass and cardiac transplantation in *in vivo* rat model. Moreover, the concept may have therapeutic potential for the treatment of reperfusion injury by acting on apoptotic pathways, inflammatory cascades and activation of survival pathways that consequenctly preserves high energy phosphates and cardiac function. This thesis has identified a new potential pharmacological pre and post conditioning drug for cardioprotection in global ischemia-reperfusion.

In summary, the potential use of fingolimod may therapeutically involve in cardioprotection in global ischemia reperfusion injury in cardioplegic arrest, sudden cardiac arrest with ECLS and during heart procurement and transplantation by,

i. ***Attenuation of apoptosis by inhibition of pro-apoptotic and activation of anti-apoptotic cascades.***
ii. ***Inhibition of pro-inflammatory cytokines and inhibition of complement system activation.***
iii. ***Protecting cardiomyocytes by attenuation of neutrophil infiltration in response to ischemia reperfusion injury.***
iv. ***Activation of Akt1/2 and Erk1/2 signalling survival pathways (RISK and SAFE pathways).***
v. ***Reduction in reactive oxygen species and nitrative stress***
vi. ***In consequences of all above mechanisms, preservation of high energy phosphates and myocardial salvage.***
vii. ***Due to myocardial salvage, ultimate goal can be achieved i.e. preservation of***

mechanical function of heart.

At the end, we speculate that, given mechanisms for cardioprotection by fingolimod may open new windows for prevention of global ischemia-reperfusion injury.

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