EFFECTS OF HEME OXYGENASE – 1 MODULATION IN MYOCARDIAL INFARCTION IN RATS

A Thesis submitted to Gujarat Technological University

for the Award of

Doctor of Philosophy

in

Pharmacy

by

Koria Hardik Mukeshkumar

179999901007

under supervision of

Dr. Anita A. Mehta

M. Pharm, Ph. D, MAMS

GUJARAT TECHNOLOGICAL UNIVERSITY

AHMEDABAD

[August – 2026]

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ABSTRACT

Background: Myocardial infarction (MI) has been a major health concern mainly caused by reduced blood flow to the myocardium due to occlusion in the coronary circulation. Although the early reperfusion is vital to salvage the ischemic myocardium, the very reperfusion itself can cause injury to the myocardium. Currently available treatment approaches mostly aim to restore blood flow but they do not adequately address the molecular mechanisms that cause immediate and long term changes in MI. Heme oxygenase-1 (HO-1), a stress-response enzyme, plays an important role to produce cytoprotective effects by catalytic degradation of Heme to biliverdin, carbon monoxide (CO), and free iron.

Objectives: Current study aims to evaluate the cardioprotective effects of Heme oxygenase-1 modulation in MI in rats and its role in activation of downstream components of important pro-survival signaling pathways.

Methods: The current study was conducted in using three methodological approaches: 1) Ex-vivo: IRI was induced in isolated rat heart using Langendorff perfused heart model 2) In vivo: Isoproterenol (ISO)-induced MI in rats; and 3) In vitro: ISO-induced injury in H9c2 cardiomyoblasts. HO-1 was modulated using HO-1 inducer, Hemin and HO-1 inhibitor, Zinc Protoporphyrin IX. For mechanistic evaluation, specific pharmacological inhibitors of PI3K (Wortmannin), Protein Kinase C (Chelerythrine), Nitric Oxide Synthase (L-NAME), and ATP-sensitive potassium (K_{ATP}) channels (Glibenclamide) were used. Various parameters such as cardiac functional parameters, myocardial injury biomarkers, oxidative stress markers, HO-1, PI3K, and histopathology were assessed.

Results: HO-1 induction by Hemin exerted dose-dependent cardioprotection as evident through improvement in cardiac function and electrophysiological stability; reduction in the level of myocardial injury markers; attenuation of oxidative stress and preservation of myocardial architecture. HO-1 inhibition resulted in the abolished aforementioned protective changes. Mechanistic evaluation through the inhibition of PI3K, PKC, NOS, and K_{ATP} pathways revealed involvement of multiple pro-survival mechanisms, with significant involvement of PI3K signaling.

Conclusion: HO-1 induction by Hemin exerts cardioprotection against myocardial injury. The protective effect is mediated through the activation of multiple pro-survival signaling mechanisms with dominant involvement of PI3K signaling.

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- KORIA HARDIK MUKESHKUMAR

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LIST OF ABBREVIATIONS

Abbreviation	Full Form
AMI	Acute myocardial infarction
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
AP-1	Activator protein-1
ARE	Antioxidant response element
ARRIVE	Animal Research: Reporting of In Vivo Experiments
ATP	Adenosine triphosphate
BPM	Beats per minute
BR	Bilirubin
BV	Biliverdin
BVR	Biliverdin reductase
CABG	Coronary artery bypass grafting
CAD	Coronary artery disease
CAT	Catalase
CCSEA	Committee for Control and Supervision of Experiments on Animals
CK-MB	Creatine kinase-myocardial band
CO	Carbon monoxide
CVD	Cardiovascular disease
DALYs	Disability-adjusted life years
DAMPs	Damage-associated molecular patterns
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide

ECG	Electrocardiogram
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ERS	Endoplasmic reticulum stress
ETC	Electron transport chain
FBS	Fetal bovine serum
GPX4	Glutathione peroxidase 4
H&E	Hematoxylin and eosin
HO	Heme oxygenase
HO-1	Heme oxygenase-1
HO-2	Heme oxygenase-2
HO-3	Heme oxygenase-3
I/R	Ischemia/reperfusion
IAEC	Institutional Animal Ethics Committee
IHD	Ischemic heart disease
IP	Intraperitoneal
IPC	Ischemic preconditioning
IPost	Ischemic post-conditioning
IRI	Ischemia-reperfusion injury
ISO	Isoproterenol
K-H	Krebs-Henseleit
KATP	ATP-sensitive potassium channel
L-NAME	N(ω)-nitro-L-arginine methyl ester
LDH	Lactate dehydrogenase

LVDP	Left ventricular developed pressure
LVEDP	Left ventricular end-diastolic pressure
MARE	Maf response element
MDA	Malondialdehyde
MI	Myocardial infarction
mKATP	Mitochondrial ATP-sensitive potassium channel
mPTP	Mitochondrial permeability transition pore
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NBT	Nitro blue tetrazolium
NCX	Sodium-calcium exchanger
NF-κB	Nuclear factor kappa B
NHE	Sodium-hydrogen exchanger
NO	Nitric oxide
NOS	Nitric oxide synthase
Nrf2	Nuclear factor erythroid 2-related factor 2
PAD	Peripheral artery disease
PBS	Phosphate-buffered saline
PCI	Percutaneous coronary intervention
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PKG	Protein kinase G
PMS	Phenazine methosulfate
RIC	Remote ischemic conditioning

RIPK	Receptor-interacting protein kinase
RISK	Reperfusion Injury Salvage Kinase
ROS	Reactive oxygen species
SAFE	Survivor Activating Factor Enhancement
SC	Subcutaneous
SEM	Standard error of the mean
SOD	Superoxide dismutase
SR	Sarcoplasmic reticulum
STEMI	ST-elevation myocardial infarction
StRE	Stress-responsive element
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-alpha
WHO	World Health Organization
ZnPP IX	Zinc protoporphyrin IX

LIST OF SYMBOLS

Symbol	Description / Definition
dP/dt_{max}	Maximum rate of rise of left ventricular pressure / peak rate of contraction
dP/dt_{min}	Maximum rate of fall of left ventricular pressure / peak rate of relaxation
%	Percent
±	Plus-minus; used with mean ± SEM
<	Less than; used in statistical probability values
α	Alpha; used in chemical names and cytokine notation
β	Beta; used in adrenergic receptor and protein notation
κ	Kappa; used in NF-κB
µL	Microlitre
µM	Micromolar
mM	Millimolar
mg/kg	Milligram per kilogram body weight
mL/min	Millilitre per minute
ng/mL	Nanogram per millilitre
U/L	Units per litre
U/mg protein	Units per milligram protein
rpm	Revolutions per minute
p	Probability value used for statistical significance
n	Number of samples/animals/independent observations
pH	Measure of acidity/alkalinity
°C	Degree Celsius

O_2	Molecular oxygen
CO_2	Carbon dioxide
Ca^{2+}	Calcium ion
Na^{+}	Sodium ion
K^{+}	Potassium ion
H^{+}	Hydrogen ion
HCO_3^{-}	Bicarbonate ion
Fe^{2+}	Ferrous ion
400×	Microscopic magnification used for histopathological images

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1. INTRODUCTION

1.1 Background

Cardiovascular disease (CVD) contributes significantly to global mortality and increased healthcare costs (Chong et al., 2025). Cardiovascular disease, also known as heart disease, includes four distinct conditions: coronary artery disease (CAD), peripheral artery disease (PAD), cerebrovascular disease, and aortic atherosclerosis. CAD results in reduced perfusion to the heart, the symptoms of which may range from angina due to ischemia to a myocardial infarction (MI) or heart failure. It makes up one-third to one-half of all cases of cardiovascular disease (Mbewu & Mbanya, 2006). Myocardial infarction represents the most severe coronary event and is a critical clinical manifestation of coronary artery disease (CAD) (Salari et al., 2023). Myocardial infarction is caused by a decrease or cessation in blood supply to a portion of the heart, which results in necrosis of the heart muscle (Saleh & Ambrose, 2018).

In 2022, an estimated 19.8 million people died due to CVDs, accounting for roughly thirty-two percent of all deaths worldwide. Eighty-five percent of these deaths were caused by myocardial infarction or stroke (World Health Organization, 2025). In 2021, ischemic heart disease (IHD) was the leading cause of noncommunicable disease-related health loss, resulting in 8.99 million mortalities along with 188.3 million disability-adjusted life years (DALYs) (Y. Wang et al., 2025). IHD continues to be the main reason for death and disease burden in adults in India. In 2019, IHD was responsible for approximately 1.5 million deaths in India. The IHD-related mortality rate was 109.23 per 100,000 population. Within the span of 2009 and 2019, IHD-related deaths in India rose by 29.8 percent, contributing to 7.79 percent of total disability-adjusted life years (DALYs) (Bhattacharjee et al., 2021). Myocardial infarction, the most prevalent form of IHD, accounts for over fifteen percent of annual deaths. Each year, about 805,000 people in the United States experience a heart attack, with 605,000 being first-time occurrences and 200,000 happening in those with a prior history of heart attacks (Tsao et al., 2023). While the prevalence of MI has decreased in industrialized nations owing to improved health systems and efficient public health measures, rates are rising in developing areas such as South Asia, some regions of Latin America, and Eastern Europe (CHICHE, 1963).

Myocardial infarction is a multifactorial disorder resulting from coronary artery disease. These risk factors contribute to the various changes in the coronary artery, such as atherosclerosis, plaque instability, and coronary thrombosis (Oliveira et al., 2015). These risk factors are classified as modifiable or non-modifiable factors. The primary non-modifiable risk factors include genetic predisposition, male sex, advanced age, and a family history of premature coronary artery disease. These factors increase an individual's baseline susceptibility to atherosclerosis, plaque instability, and thrombotic coronary events (Rathore et al., 2018). In contrast, modifiable risk factors play a dominant role in the development of MI and include cigarette smoking, hypertension, diabetes mellitus, dyslipidemia, obesity, central adiposity, physical inactivity, unhealthy diet, psychosocial stress, and adverse alcohol-use patterns. Smoking promotes endothelial dysfunction, thrombosis, and oxidative stress; hypertension accelerates vascular injury and plaque formation; diabetes enhances inflammation, endothelial damage, and metabolic dysregulation; and abnormal lipids contribute directly to atherogenesis. Similarly, a sedentary lifestyle, obesity, and poor dietary habits worsen the cardiometabolic profile and further elevate MI risk (Yusuf et al., 2004). Therefore, while non-modifiable factors help identify high-risk individuals, modification of lifestyle and metabolic risk factors remains the cornerstone for reducing the global burden of myocardial infarction.

MI results in immediate and long-term changes in the myocardium at the cellular and molecular levels irreversibly, leading to cardiac myocyte death (Lodrini & Goumans, 2021). Although the early reperfusion is vital to salvage the ischemic myocardium, the very reperfusion itself can cause injury to the myocardium, referred to as ischemia-reperfusion injury (IRI). IRI results from a rapid increase in reactive oxygen species (ROS) and pro-inflammatory cytokines as well as calcium overload, which triggers various cell death pathways. Over the time, these pathological changes culminate in adverse cardiac complications and death (Hausenloy & Yellon, 2013). Conventional treatment and management strategies primarily focus on restoring blood flow and maintaining cardiac function (Gunata, 2020). However, these strategies do not adequately address underlying cellular and molecular mechanisms that cause irreversible changes and complications in the myocardium. Therefore, cardioprotection requires multi-target approaches to withstand stressful events and enhance survival (Vilahur et al., 2024).

Heme oxygenase-1 (HO-1) catalyzes the breakdown of heme into carbon monoxide (CO), ferrous iron (Fe^{2+}), and biliverdin (BV). Biliverdin is then converted to bilirubin (BR) by the

action of an enzyme called biliverdin reductase (BVR) (Gozzelino et al., 2010). HO-1 is physiologically induced by cells during stressful events. There are various beneficial roles of HO-1 and catalytic breakdown products of heme, such as immunomodulation, anti-inflammatory, and cytoprotective effects. These cytoprotective effects make HO-1 a suitable candidate to exert protection during pathological changes during cardiovascular diseases, especially MI and its complications (Ryter, 2022).

There are various inducers of HO-1 such as ultraviolet radiation, hypoxia, heavy metals, hydrogen peroxide, and nitric oxide (NO). They induce HO-1 by activating its regulatory mechanisms such as transcription factors nuclear factor erythroid 2-related factor 2 (Nrf2), Activator protein-1 (AP-1), and nuclear factor kappa B (NF-κB) (Liebert, 2005). Hemin also induces HO-1 by activating Nrf2 signaling and thereby promoting cytoprotection (Reichard et al., 2007). Zinc Protoporphyrin IX (ZnPP IX) is a known inhibitor of HO-1. Hemin and ZnPP IX are frequently used for the evaluation of the functional role of HO-1 and its mechanistic role (Nowis et al., 2008).

Various signaling pathways are involved in the pathogenesis and survival of cardiac myocytes. They regulate various processes at cellular and molecular levels, such as inflammation, oxidative stress, apoptosis, myocardial fibrosis, and angiogenesis. All these changes are important targets in the pathogenesis of MI and associated complications (Q. Zhang et al., 2022). HO-1, having a potential to activate prosurvival mechanisms and signaling pathways, could be a promising approach to exert cardioprotection during the pathogenesis of MI. The current study was designed to evaluate the cardioprotective effects of HO-1 induction by hemin and to find its role in the activation of pro-survival signaling pathways, thereby offering mechanistic insights.

2. REVIEW OF LITERATURE

2.1 Myocardial Infarction

Cardiovascular diseases, especially ischemic heart disease (IHD), are the chief contributors of mortality and morbidity worldwide (Jaiswal et al., 2025). Myocardial infarction, being the most severe consequence of coronary artery diseases, results from the death of the cardiac myocytes due to ischemia caused by occlusion in the coronary artery, mainly due to the formation of atheromatous plaque (Palasubramaniam et al., 2019). MI results in immediate and long-term changes in the myocardium at the cellular and molecular levels irreversibly, leading to cardiac myocyte death (Buja, 2024). Although the early reperfusion is vital to salvage the ischemic myocardium, the very reperfusion itself can cause injury to the myocardium, referred to as ischemia-reperfusion injury (IRI). IRI results from a rapid increase in reactive oxygen species (ROS) and pro-inflammatory cytokines as well as calcium overload, which triggers various cell death pathways. Over time, these pathological changes culminate in adverse cardiac complications and death (Fede et al., 2025).

Myocardial infarction is commonly classified according to the Universal Definition of Myocardial Infarction into five major types based on etiology and clinical context.

- Type 1 MI refers to spontaneous MI resulting from a primary coronary atherothrombotic event such as plaque rupture or erosion.
- Type 2 MI occurs as a consequence of an imbalance between myocardial oxygen demand and supply in the absence of acute coronary thrombosis.
- Type 3 MI includes patients who suffer cardiac death with symptoms suggestive of myocardial ischemia before cardiac biomarkers can be obtained or before they become elevated.
- Type 4 MI is procedure-related and includes PCI-related MI (Type 4a), stent thrombosis-associated MI (Type 4b), and restenosis-associated MI (Type 4c).
- Type 5 MI is associated with coronary artery bypass grafting.

In addition to this etiologic classification, myocardial infarction is also clinically categorized into ST-elevation myocardial infarction and non-ST-elevation myocardial infarction based on electrocardiographic findings (Thygesen et al., 2018)

2.2 Pathogenesis of Myocardial Infarction

Myocardial infarction is often triggered by the injury to the coronary artery endothelium (type 1) or the rupture of a susceptible atherosclerotic plaque. Angina necessitates a significant stenosis (>70 percent diameter). However, this kind of stenosis is less likely to cause a type 1 myocardial infarction owing to thick fibrotic caps that are less prone to rupture and the establishment of collateral circulation. In contrast, susceptible plaques have thin fibrous caps, 30-50 percent stenosis, and more inflammatory cells, including lipid-laden macrophages. When the plaque ruptures, thrombogenic chemicals are released, activating platelets, triggering the coagulation cascade, forming mural thrombi, and embolizing atherosclerotic debris downstream (Badimon et al., 2012). This condition of hypercoagulability causes the rupture of additional sensitive fibroatheromas, suggesting that multiple lesions are responsible. Myocyte necrosis is the final outcome, as seen by increased cardiac biomarkers in the peripheral blood. The degree of ischemia is dictated by if the coronary artery was partly or totally blocked, the volume of myocardium fed, the length of the occlusion, the existence of collaterals, and the efficacy of reperfusion after therapy (Reed et al., 2017).

Cardiomyocytes have high energy phosphate reserves, which allow them to contract for a few minutes during total ischemia. However, in experimental models, systolic dysfunction develops significantly faster, since contractile power is rapidly inhibited and ends within 60 seconds of loss of blood supply, despite the presence of energy reserves (Yi-Dan et al., 2021). Interplay of multiple mechanisms causes immediate functional depression. At the beginning, the breakdown of creatine phosphate stores produces inorganic phosphate, which limits the action of proteins involved in contraction. Following that, the intracellular acidosis reduces binding of calcium to contractile proteins, resulting in the inhibition of contraction. Because action potentials and calcium fluxes are retained during the early stages of ischemia, it seems that contractile protein inhibition is the cause of ischemic systolic dysfunction (Handling et al., 2018). Rapid termination of function may extend the lifetime of ischemic myocardium because the available stocks of high-energy phosphates are gradually depleted, allowing longer viability of myocardium in the absence of perfusion. Initial functional deterioration in the ischemic myocardium is entirely reversible if blood flow is immediately restored within 4-5 minutes after coronary artery occlusion. Longer spans of myocardial ischemia, however, are associated with long-term dysfunction despite complete blood flow restoration, even if the length of coronary artery occlusion is insufficient to induce

cardiomyocyte mortality. The inability to restore function after full restoration of blood flow after 10-20 minutes of coronary artery blockage is called "myocardial stunning," and normally continues for less than 24 hours after the reinstatement of coronary flow. (Frangogiannis, 2015).

The only approach to save ischemic myocardium from myocardial infarction is with rapid reperfusion. The successful reperfusion of ischemic myocardium following infarction was originally observed in dogs (Gross & Auchampach, 2007). Soon after, reperfusion methods were applied in patients with acute myocardial infarction, first with pharmaceutical thrombolysis and then with interventional, catheter-based reopening of the blocked coronary artery. Reperfusion, on the other hand, not only saves the ischemic myocardium from infarction but also causes an additional harm. (Heusch, 2020).

2.3 Myocardial Ischemia-Reperfusion Injury

Cardiac ischemia-reperfusion injury (IRI) has been a long-established paradoxical phenomenon in which restoration of blood flow to ischemic myocardium potentially aggravates the condition, although it is important for protecting cardiac tissue. Revascularization therapy, including percutaneous coronary intervention (PCI), thrombolysis, and coronary artery bypass grafting (CABG), aims to minimize ischemic damage and improve clinical outcomes (Ibáñez et al., 2015). However, restoration of blood flow can initiate a cascade of cellular and molecular events that worsen the injury to the heart, and cause death of myocardium that was viable prior to reperfusion. This phenomenon reflects the duality of reperfusion therapy and highlights the complexity of ischemia-reperfusion injury (IRI). IRI is a complex process including several phenomena such as reperfusion arrhythmias, cardiac stunning, no-reflow, and lethal reperfusion injury (J. He et al., 2022). Different types of myocardial dysfunction contribute differently with the lethal reperfusion injury playing a major role in the infarct size and clinical outcomes. During ischemia, the lack of oxygen leads cellular metabolism to shift to anaerobic glycolysis, which results in intracellular acidosis, ATP depletion, and ion imbalances. Reperfusion enhances oxygen availability, but it also produces mitochondrial permeability transition, calcium overload, and oxidative stress, which results in cell death via necrosis and apoptosis (Leivaditis et al., 2024).

Acute myocardial ischemia causes the lack of oxygen, which shifts cell metabolism from aerobic to anaerobic respiration. This results in lactate formation and a reduction in

intracellular pH. Ultimately, intracellular Ca^{2+} overload occurs by altering the functions of various channels and transporters. Na^{+}-H^{+} exchanger to extrude H^{+}, which results in intracellular Na^{+} accumulation. This triggers the Na^{+}-Ca^{2+} exchanger to work in the opposite direction, which extrudes Na^{+} and causes intracellular Ca^{2+} overload. (Buja, 2023). MPTP fails to open due to acidic conditions during ischemia, which causes cardiomyocyte hypercontracture. Due to the resumption of ETC (electron transport chain) during reperfusion, ROS are produced. Endothelial cells' xanthine oxidase and NADPH oxidase (neutrophils) also produced ROS. ROS acts as a neutrophil chemoattractant and promotes sarcoplasmic reticulum dysfunction (SR) by causing myocardial reperfusion damage through the opening of the MPTP (Shahbaz et al., 2015). ROS also causes lipid peroxidation, which damages cell membranes, causes enzyme denaturation, and causes direct oxidative damage to DNA. Na^{+}-H^{+} exchanger is reactivated during reperfusion, which causes lactic acid to be washed out. This results in the restoration of physiological pH and release of the inhibition of MPTP opening and cardiomyocyte contraction (Manuscript & Vaccarino, 2008a). Calcium enters mitochondria due to the restoration of the force of mitochondrial membrane potential. Ultimately, MPTP opens. In response to the production of the chemoattractants, neutrophils concentrate in the infarcted myocardial tissue a few hours after reperfusion and produce ROS, cytokines, and activated complement (Hausenloy et al., 2013). The various I/R damage mechanisms are as follows:

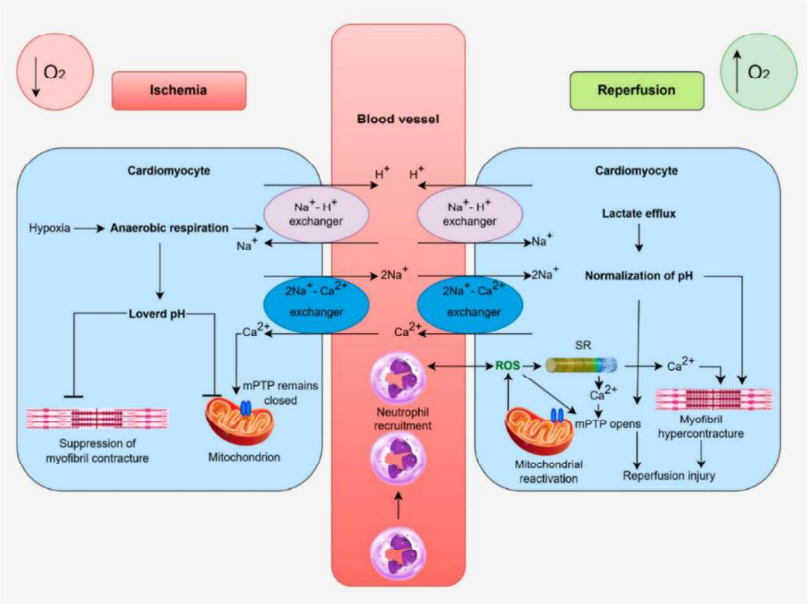


Figure 2.1: Mechanism of Myocardial Ischemia-Reperfusion Injury (Juricic et al., 2026)**:**

2.3.1 Oxidative Stress

Low quantities of oxidants are essential for cell mitosis, homeostasis, and intracellular communication; nevertheless, cell damage occurs when radical production is significantly enhanced. Various endogenous free radical scavenging enzymes, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase, are also present in cardiac myocytes like other cells in the body. However, the oxidant radicals produced after I/R vastly outnumber those that can be eliminated by these enzymes (Kurian et al., 2016). Reperfusion damage is classically associated with the prominent role of oxygen radicals, which is known as the oxygen radical hypothesis. Previous research has demonstrated that oxidative stress lowers the function of Na^+-K^+ ATPase and Ca^{2+} ATPase and impairs cell integrity. Because oxygen radicals induce lipid peroxidation, the cell membrane is damaged, and it may even break down, resulting in cell enlargement (González-Montero et al., 2018). It was discovered that oxygen radicals created during this interval triggered neutrophil chemotaxis and capillary injury. These issues aid in the development of microvascular disorders. Oxidative activities that occur during cardiac tissue reperfusion inhibit or decrease NO production by disrupting intracellular signaling pathways. This diminishes cardioprotective benefits of NO. As a consequence, increased oxidative stress leads to neutrophil buildup, superoxide radical production, and decreased coronary blood flow (Gunata, 2020).

2.3.2 Intracellular Calcium Overload

Following a period of hypoxia in cardiomyocytes, the intracellular anaerobic metabolism may result in the buildup of H^+, lower intracellular pH, and increased levels of intracellular Na^+ via Na^+/H^+ exchange (NHE). The activity of Na^+/Ca^{2+} exchange (NCX) proteins is increased by the accumulation of Na^+ in cardiomyocytes, which reduces internal Na^+ aggregation and transfers external Ca^{2+} into the cells, raising the concentration of Ca^{2+} in the cytoplasm and producing calcium overload (González-Montero et al., 2018). Rapid reoxygenation raises the pH of extracellular fluid and the H^+ gradient across the cell membrane, increasing the activity of NHE and NCX, resulting in intracellular calcium excess. In addition to the NHE and NCX pathways, the H^+/Ca^{2+} exchange mechanism also plays an important role in intracellular calcium overload (H. Liu et al., 2010). Protein channels not only govern calcium processing but also its subsequent influx and efflux. Strict

management of intracellular calcium homeostasis is critical for maintaining the proper function and proliferation of myocardial cells, and disturbance of intracellular calcium homeostasis leads to ERS, exacerbating ischemia damage of myocardial cells (J. He et al., 2022).

2.3.3 The pH Hypothesis

Acute myocardial ischemia decreases intracellular pH to less than 7.0, but later during reperfusion, physiological pH is quickly brought back to normal by the process of lactate removal and by the activation of the Na^+-H^+ exchanger as well as the Na^+-HCO^- symporter. This pH change leads to cardiomyocyte mortality by allowing MPTP to open and cardiomyocyte rigor hypercontracture in the first few minutes after reperfusion. Cohen and Downey referred to this as "the pH theory." (Hausenloy et al., 2013).

2.3.4 Inflammation

Various processes activated during cardiac I/R, such as immunological activity with cytokines, neutrophils, and immune signaling, produce long-term pathophysiological alterations. During reperfusion, damaged cardiomyocytes generate DAMPs. This causes activation of pattern recognition receptors such as Toll-like receptors (TLRs), which are known to trigger inflammatory cascades (Vilahur & Badimon, 2014). TNF-α and IL-6, which are proinflammatory cytokines, attract neutrophils to the myocardium. Neutrophils produce proteolytic enzymes ROS, and pro-inflammatory mediators, which results in endothelial dysfunction, microvascular blockage, and further cardiomyocyte damage, ultimately causing myocardial injury (Alsadder & Hamadah, 2025).

2.3.5 Endothelial Dysfunction

Endothelial nitric oxide synthase (eNOS) produces nitric oxide (NO), which is an important regulator of vascular homeostasis. Nitric oxide orchestrates critical pathophysiological processes behind IRI. Reduction in the NO availability due to oxidative stress results in decreased vasodilation and increased vascular permeability (Manuscript & Vaccarino, 2008b). In addition to that, lower NO levels exacerbate the inflammatory response and cause tissue damage through leukocyte adhesion and infiltration. Endothelial failure and increase in vascular permeability results in extravasation of plasma proteins and fluids into the interstitial space, causing tissue edema. The resultant edema raises interstitial pressure and compresses capillaries. This ultimately reduces microvascular perfusion, aggravates cardiac

ischemia and reduces oxygen supply to cardiomyocytes. This is similar to the reaction seen in microvascular blockage (Yang et al., 2016). The breakdown of the glycocalyx, which is a protective covering on the endothelium surface causes reduced endothelial barrier function leading to increased permeability and promotion of leukocyte adhesion and infiltration. The invading leukocytes emit proteolytic enzymes and ROS, increasing tissue damage and inflammation (Alsadder & Hamadah, 2025).

2.3.6 Mitochondrial Dysfunction

In cardiac IRI, mPTP plays a vital role in mitochondrial dysfunction. The mPTP is present in the inner mitochondrial membrane and remains in a closed condition under the most complicated physiological circumstances. This protects mitochondrial membrane potential and ATP generation. However, during reperfusion, various factors, including calcium overload, oxidative stress, and ATP depletion, cause mPTP activation (Marin et al., 2021). As a result, the mitochondrial membrane potential dissipates, oxidative phosphorylation uncouples, and ATP generation decreases. The opening of the mPTP leads to the release of proapoptotic molecules such as cytochrome c. This activates the cascade of caspases to begin apoptotic pathways. Prolonged opening of the mitochondrial permeability transition pore (mPTP) induces irreversible mitochondrial swelling, membrane rupture, and subsequent cell death via necrosis or apoptosis (Alsadder & Hamadah, 2025).

2.3.7 Apoptosis and Necrosis

Cardiac ischemia-reperfusion injury causes distinct types of cell death (apoptosis and necrosis). These are relevant to clinical outcome. Apoptosis is a type of programmed cell death. Apoptosis is primarily initiated by mitochondrial dysfunction following reperfusion. It is characterized by cytochrome C release and caspase activation (K. Wang et al., 2025). At the same time, extensive ATP depletion and rupture of the plasma membrane result in necrosis. Necrosis is the unregulated mode of cell death. Necrosis results in the release of DAMPs that amplify inflammation. Both processes act synergistically and aggravate myocardial injury. Apoptosis causes delayed loss of cells, and necrosis causes acute structural damage after reperfusion (Alsadder & Hamadah, 2025).

2.3.8 Necroptosis and Ferroptosis

Necroptosis and ferroptosis, which are the other forms of cellular injury, also contribute to cardiac ischemia-reperfusion injury. Ferroptosis is a form of regulated cell death which

causes iron-mediated lipid peroxidation. It has been implicated in cardiomyocyte injury after reperfusion (J. Y. Li et al., 2021). Increased reactive oxygen species (ROS) and low levels of antioxidants such as glutathione peroxidase 4 (GPX4) exacerbate ferroptotic damage. This can be overcome by new therapeutic approaches. Necroptosis is a programmed form of necrosis. Receptor-interacting protein kinases (RIPK1 and RIPK3) regulate the process and cause cardiac damage via membrane rupture and inflammation. Ferroptotic and necroptotic pathways could be targeted to reduce myocardial injury in ischemia/reperfusion. This provides opportunities for the development of novel therapeutics targeting these pathways (Alsadder & Hamadah, 2025).

2.3.9 Autophagy

One of the main mechanisms that contribute to IRI is a process called autophagy. Autophagy is a cellular degradation and recycling process. The extent of modulation and occurrence of autophagy decides whether it would be beneficial or detrimental in ischemia-reperfusion injury. Autophagy in ischemia reduces cellular stress by clearing the damaged organelles and misfolded proteins. This preserves mitochondrial function and energy homeostasis. This adaptive response increases cell survival by preventing accumulation of harmful byproducts (Peng et al., 2024). During reperfusion, the autophagy becomes excessive and dysregulated. This produces deleterious effects on cellular constituents by causing excessive degradation and thus promotes autophagic cell death. Overactivation of autophagy, which is commonly caused by chronic Beclin-1 overexpression and AMP-activated protein kinase (AMPK) activation, might exacerbate myocardial injury. Thus, regulating autophagy to balance its protective and detrimental effects is a possible therapeutic option for decreasing IRI while maintaining heart function (Alsadder & Hamadah, 2025).

2.4 Cardiac Remodeling

Cardiac remodeling is a complicated process in which pathological stressors change cardiac structure, shape, and function. Cardiac remodeling includes both acute events initiated by an ST-elevation myocardial infarction within 90 minutes and long-term effects that occur months or even years after the MI (Berezin & Berezin, 2020). At the initial stages of MI, infarct growth triggers cardiac remodeling. Early alterations are visible within a few hours to days following an acute myocardial injury. Various inflammatory cells, such as macrophages and neutrophils, are infiltrated due to myocardial necrosis. This destroys the collagen framework and alters ventricular shape. These changes cause local thinning and

dilatation of the heart in infarcted regions (Zhao et al., 2020). Over the next several weeks to months, a variety of pathologic processes challenge the surviving myocardium, which

includes protease activation and increased cytokine production. These processes trigger cardiomyocyte death and enhance proinflammatory factor release. The late phase mainly involves reactive myocyte interstitial fibrosis, hypertrophy, and left ventricular dilatation (Schirone et al., 2017). Adverse cardiac remodeling is known to compromise ventricular function and produce heart failure. These are the major cause of death and morbidity in AMI patients. Because of a lack of knowledge of the molecular process of cardiac remodeling current cardiovascular drugs that target it are very limited. The post-MI remodeling process includes inflammatory, proliferative, and maturation stages. Various pathologic adverse changes that results in cardiac remodeling include inflammation (myocarditis), ischemia, biomechanical stress, ischemia-reperfusion damage, excess neurohormonal activation, excess afterload, and cytokine storm (Zhao et al., 2020).

2.5 Current Treatment Approaches and Their Limitations

The current approach to myocardial infarction management is to restore coronary blood flow as quickly as possible while preventing additional thrombotic episodes. Primary percutaneous coronary intervention is the ideal reperfusion method in ST-segment elevation myocardial infarction; however, fibrinolysis may be performed if prompt PCI is not possible (Diletti et al., 2014). Non-ST-elevation acute coronary syndromes are managed with early risk assessment, antiplatelet and anticoagulant treatment, and invasive examination as needed. Standard adjunctive pharmacotherapy frequently includes dual antiplatelet therapy, anticoagulants, statins, beta-blockers, nitrates, and angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, which together have significantly reduced acute mortality and improved long-term outcomes (Gibler et al., 2018; Nadarajah & Gale, 2021).

Despite these advances, current therapies have significant limitations. Reperfusion leads to infarction, adverse ventricular remodeling, arrhythmias, and heart failure through oxidative stress, calcium overload, mitochondrial dysfunction, inflammation, microvascular obstruction, and cardiomyocyte necrosis (Thomas et al., 2025). Conventional treatment is extremely efficient in restoring epicardial blood flow but it fails to fully address ischemia-reperfusion damage and its downstream implications. Moreover, due to the tissue damage that remain at the microvascular and cellular levels, the effective reopening of the occluded coronary artery and complete myocardial salvage is not always achieved. This unmet need

has sparked interest in novel cardioprotective techniques that attempt to reduce myocardial damage in addition to typical reperfusion-based therapy (Bararu Bojan et al., 2026).

2.6 Cardioprotective Strategies

Numerous cardioprotective methods for myocardial ischemia-reperfusion damage (IRI) have been developed. These are roughly classified into four groups depending on the protective mode, time of administration, cellular target, and intracellular target. The use of physical measures such as hypothermia or electrical nerve stimulation, controlled application of brief episodes of ischemia and reperfusion (ischemic conditioning), and the administration of chemical substances (pharmacological) are some of the well-studied cardioprotective approaches (Schirone et al., 2017).

Local post-conditioning (IPost), pre-conditioning (IPC), and remote ischemic conditioning (RIC) are some of the strategies based on ischemia conditioning. Cardioprotective therapies are also time-based, meaning they may be used before, during, or after ischemia. Treatments that protect against reperfusion damage should be administered as soon as feasible during reperfusion, since the majority of cell death occurs within the first minutes of reflow. IPost serves as one illustration of this (Basalay et al., 2012). Drugs should normally be administered as early as feasible during ischemia to achieve appropriate myocardial concentration at the start of reperfusion. There is experimental evidence that certain cardioprotective medicines or treatments may decrease MI size even after the commencement of reperfusion (20 min to 3 h), allowing the cardioprotective agent to be delivered after PPCI. However, this technique is predicated on the problematic assumption that MI size rises with reperfusion time (H. Zhang et al., 2024).

Cardioprotective techniques may also be designed to protect either cardiomyocytes or other noncardiac cells such as platelets or leukocytes. Apart from primary working cardiomyocytes, which are the most vulnerable to IRI, the myocardium also includes a diverse range of supportive cells that play essential roles in myocardial IRI, such as smooth muscle cells, endothelial cells, fibroblasts, and neurons. Endothelial and fibroblast-released substances such as microRNA (miRNA) and exosomes may contribute to cardioprotective signaling (Y. Li et al., 2022).

Cardioprotective techniques may be further classified depending on their intended outcomes. The first category contains molecular targets implicated in mostly necrotic cell death. These

include targets such as components of the mitochondrial permeability transition pore (MPTP), ion exchangers and channels, proteases, reactive oxygen species, and contractile elements. Other types of cell death, such as necroptosis, apoptosis, pyroptosis, and

autophagy, may also give novel targets for cardioprotection, as they may also occur during acute myocardial IRI and ultimately contribute to the infarct development. Endogenous cardioprotective signaling pathways such as the RISK and SAFE pathways, the NO/cGMP/PKG cascade, cardiomyocyte metabolism, and mitochondrial morphology may also be stimulated and include a second set of targets (Pagliaro et al., 2026).

Significant cardioprotection has been reported by many therapies in experimental animal models of IRI. However, translation of these cardioprotective medicines into the clinical situation of acute myocardial infarction (AMI) in terms of patient benefit has been unsuccessful (Hernandez-Resendiz et al., 2025). One key explanation might be because AMI is multifactorial, inducing cardiomyocyte death via several methods while also impacting other cell types such as platelets, fibroblasts, endothelium and smooth muscle cells, and immune cells. Many cardioprotective therapies target common end-effectors and may be ineffective in people with comorbidities. In this context, new results indicate that optimum cardioprotection may need the combination of additive or synergistic multitarget treatments (Davidson et al., 2019).

2.7 Heme Oxygenase-1

2.7.1 Biological Role, Induction and Gene Expression

The enzyme heme oxygenase catalyzes the conversion of heme to three biologically significant end products, namely biliverdin/bilirubin, CO, and ferrous ions. NADPH biliverdin reductase spontaneously reduces the first product, biliverdin, to bilirubin. HO has three isoforms: HO-1, HO-2, and HO-3. The presence of HO-1 is expressed at low basal levels in many tissues under normal settings. HO-2 is abundantly expressed in organs and tissues such as the liver, brain, kidney, spleen, and testes, but nothing is known about HO-3 (Ahmad et al., 2018).

These are various inducers and stimuli such as reactive oxygen species (ROS), nitric oxide (NO), cytokines, heavy metals, electrophilic medicines, endotoxins, hypoxia, and UV irradiation that cause activation of HO-1. There are well-documented important antioxidant and cytoprotective mechanisms that involve elevation of HO-1 expression and function

against inflammation, hypoxia, hyperthermia, radiation, and ischemia/reperfusion. Importantly, HO-1 products are key bioactive molecules for maintaining intracellular redox balance (Ryter, 2022).

Oxidative stress in well known for stimulating expression of HO-1. It stimulates the transcription of the Hmox1 gene in mice and HMOX1 in humans. Previous research has shown that HO-1 expression is activated by a variety of stimuli via distinct methods. DNA analysis identified two significant enhancer regions in the mouse hmox1 promoter sequence at positions -4 and -10 kb. The stress-responsive element (StRE) is the primary enhancer-binding DNA sequence. It overlaps with other elements such as the antioxidant response element (ARE) and the Maf response element (MARE) (Medina et al., 2020). Several transcription factors, including AP-1, Nrf2, CNC-bZIP, NF-κB, and v-Maf oncoprotein, bind to the hmox1/HMOX1 promoter region and control HO-1 production. The Nrf2 transcription factor is a key positive regulator of Heme Oxygenase-1 transcription. When there are no stimuli, in basal condition, the Kelch-like ECH-associated protein (Keap1) interacts with transcription factor Nrf2 through a Cullin 3-based E3 ubiquitin ligase complex. Keap1 essentially sequesters Nrf2 in the cytoplasm. Upon oxidative stimulation, the Keap-Nrf2 complex dissociates, which allows the transcription factor Nrf2 to translocate to the cell nucleus from the cytoplasm and interact with the StRE/ARE sites in the promoters of the target genes (Reichard et al., 2007). Another regulator, Bach1, inhibits the transcription of hmox1/HMOX1 by competing with Nrf2 for DNA binding (Sun et al., 2002). It is interesting that the HMOX1 promoter is largely comparable to the upstream enhancer area of the hmox1 promoter. The only change is in the sequences of heavy metal-responsive elements at -4 kb and metalloporphyrin-responsive element at -9.5 kb for binding of Egr-1 (early growth response-1) (Injury, 2017).

2.7.2 Biological Significance of HO-1 and Products of Heme Catabolism by HO-1

Heme is a critically important molecule that is associated with a variety of cellular heme-containing proteins, such as mitochondrial cytochromes, oxygen transporters (e.g., hemoglobin and myoglobin), and other enzymes as a prosthetic group. In a separate form, heme may accelerate pro-oxidant processes and cause inflammation (Araujo et al., 2012). Heme oxygenase (HO) catalyzes the breakdown of heme into biliverdin-IXα (BV). This process needs NADPH: cytochrome p-450 reductase as an electron source and 3 molecules of O_2. During heme breakdown by HO-1, the α-methene bridge carbon is released in the

form of carbon monoxide (CO), while the core heme iron is released as ferrous iron (Fe-II). An inducible form (HO-1) and a constitutively expressed form (HO-2) represent HO-1 activity. The enzyme NAD(P)H:biliverdin reductase converts BV to bilirubin-IXα (BR). Both BV and BR have high cellular antioxidant activity. BR also has immunomodulatory effects. It has been described as a key modulator of lipid metabolism (Hirvonen et al., 2022). The ferric iron produced during HO activity could be toxic due to its pro-oxidant properties. However, ferritin produced by the cells sequester the heme-derived iron and act as a co-cytoprotective factor. CO generated from HO-1 may influence biological processes such as inflammation, apoptosis, and cellular proliferation. The wide range of biological effects of HO-1 is mainly determined by the integrated functions of the bilirubin, carbon monoxide, and iron handling systems (R. Liu et al., 2023; Ryter, 2022).

2.7.3 Pharmacological Modulation of Heme Oxygenase-1

The pharmacological modulation of heme oxygenase-1 (HO-1) is often used as a tool to investigate the functional role of the enzyme in cardiovascular injury and prevention. HO-1 is an inducible stress-response enzyme. There are various chemical modulators which are known to modulate HO-1 expression or activity. Through HO-1 modulation researchers can determine if a protective effect is HO-1 mediated or not. This approach is particularly useful for the study of myocardial infarction and ischemia-reperfusion injury where HO-1 has been associated with decreased oxidative stress, inflammation, apoptosis and tissue damage. Therefore, the pharmacologic activation and inhibition of HO-1 has emerged as an important strategy to evaluate cardioprotection mechanistically (Stocker & Perrella, 2006).

Hemin is an oxidized heme product and is a potent inducer of HO-1. It has been extensively used experimentally to induce endogenous HO-1-mediated cytoprotection. Hemin induces heme catabolism by upregulating HO-1 expression. Heme catabolism consequently results in production of useful substances like bilirubin and carbon monoxide, which exhibit antioxidant, anti-inflammatory, vasoregulatory and anti-apoptotic activities. In cardiovascular models, hemin-induced HO-1 has been linked to reduced oxidative damage, enhanced tissue resilience to ischemic stress and better post-ischemic recovery. Therefore, hemin is often used as a pharmacological approach to investigate whether enhancement of the HO-1 system would be protective against myocardial injury or not (Funes et al., 2020).

Zinc Protoporphyrin IX (ZnPP IX) is a well-known inhibitor of heme oxygenase activity. ZnPP IX, a metalloporphyrin inhibitor, is commonly used to determine if a beneficial effect

is specific for HO activity or not. In experimental research, the abolition or reversal of protection by ZnPP IX provide evidence that the protective effect is HO-1 mediated. Thus, the hemin/ZnPP IX combination could be useful in mechanistic research to assess the role of HO-1 in cardioprotection (Nowis et al., 2008).

Deaspite having many pharmacological and indirect modulators of HO-1 available, hemin and ZnPP IX remain the most commonly used experimental agents for HO-1 induction and inhibition, respectively because their use allows a direct evaluation of the role of HO-1 activation in the protection of myocardium.

2.7.4 HO-1 in Myocardial Injury and Cardioprotection

Heme oxygenase-1 (HO-1) is now well acknowledged as an essential endogenous protection mechanism in myocardial damage. During myocardial infarction and ischemia-reperfusion, the myocardium experiences severe oxidative stress, inflammatory activation, mitochondrial dysfunction, and progressive cardiomyocyte loss. Because HO-1 is highly inducible under these circumstances, it has received a lot of attention as part of the heart's adaptive defensive response. Current research reveals that HO-1 is more than just a cellular stress marker; it is also an active mediator of myocardial protection and tissue adaptability after ischemia damage (Wu et al., 2011).

The cardioprotective properties of HO-1 are strongly connected to its biological activities. By decomposing free heme, HO-1 alleviates the load of a powerful pro-oxidant and pro-inflammatory molecule. At the same time, its reaction products promote cytoprotection via complimentary pathways. Biliverdin and bilirubin are endogenous antioxidants, while carbon monoxide serves as a signaling mediator with anti-inflammatory, anti-apoptotic, and vasoregulatory characteristics. HO-1's coordinated effects may affect redox balance, inflammatory signaling, vascular function, mitochondrial homeostasis, and cell survival, all of which are important in the pathobiology of myocardial damage (Ryter et al., 2006).

The role of HO-1 in myocardial protection has therefore been studied in terms of reduction in cardiac damage during ischemia and reperfusion as well as during phase of cardiac remodeling. Current evidence suggests that increased HO-1 activity may be associated with an improved response to myocardial injury. It possesses antioxidant, anti-inflammatory, anti-apoptotic and cytoprotective properties. These properties make it an attractive endogenous target in myocardial infarction and ischemia-reperfusion injury. Further studies related to its

protective effects and the downstream signaling pathways could reveal more about the role of HO-1 during myocardial adverse myocardial events (Idriss et al., 2008).

2.8 Signaling Mechanisms of Cardioprotection

It is well established that cardioprotection is mediated by networks of interconnected intrinsic survival mechanisms and not by a single molecular mechanism. In myocardial ischemia and reperfusion a variety of protective signals are activated to withstand the stress. These activated signals preserve cellular integrity and contractile function and prevent irreversible damage (Jovanović, 2025). Oxidative stress, calcium overload, mitochondrial dysfunction, inflammation and cardiomyocyte apoptosis which are the major determinants of the cardiac injury are opposed by the protective signals. Hence, cardioprotection is best understood as an integrated biological process, in which multiple signaling pathways contribute to enhance myocardium resilience to ischemic stress and reperfusion injury. (Transduction & Conditioning, 2015).

Cardioprotective signaling is often defined in terms of triggers, mediators, and end effectors. Protective triggers may include receptor-dependent agonists, autacoids, reactive oxygen species at low signaling concentrations, metabolic cues, and conditioning-related stimuli. These triggers activate intracellular mediators, namely protein kinases and gaseous signaling pathways, which send the protective signal to downstream effectors (Maslov et al., 2015). End effectors are responsible for limiting myocardial damage by maintaining ionic homeostasis, stabilizing mitochondrial activity, and avoiding the transition from reversible to irreversible cell death. This approach is valuable because it illustrates how many preventive therapies might focus on a small number of common survival pathways inside the myocardium (Shahbaz et al., 2015).

Several main cardioprotective mechanisms have been identified in the literature. These include the RISK (Reperfusion Injury Salvage Kinase) pathway, the SAFE (Survivor Activating Factor Enhancement) pathway, NO/cGMP/PKG-related signaling, mitochondrial preservation pathways, and downstream end-effector mechanisms such as modulation of mitochondrial ATP-sensitive potassium channels (mK_{ATP}) and inhibition of mitochondrial permeability transition pore (mPTP) opening (Heusch, 2015). Importantly, these pathways do not work separately. Extensive cross-talk happens between them, and many eventually converge at the mitochondria, which are commonly recognized as critical determinants of cardiomyocyte survival after reperfusion. Thus, successful cardioprotection relies not only

on activating individual pathways but also on their coordinated interaction within a wider signaling network (Rossello & Yellon, 2018).

Although many signaling systems play a role in cardioprotection, this review focuses on PI3K, PKC, NOS/NO, and K_{ATP} signaling because these pathways are among the most well-established components of endogenous cardioprotective signaling and are directly relevant to the current study's mechanistic objectives. PI3K is a vital pro-survival pathway and an essential component of the RISK cascade (Cohen & Downey, 2015). PKC serves as a crucial mediator between upstream protective stimuli and downstream intracellular responses. NOS/NO signaling helps to protect endothelial cells, regulate blood flow, and activate cGMP-dependent survival pathways. K_{ATP} channels, especially mitochondrial K_{ATP} channels, are thought to be essential end effectors that aid in mitochondrial function and minimize reperfusion-induced harm (Hausenloy & Yellon, 2004). Therefore, these routes are described independently in the next subsections.

2.8.1 PI3K Signaling

Phosphoinositide 3-kinase (PI3K) signaling is one of the most well-studied pro-survival pathways in cardioprotection following cardiac ischemia-reperfusion damage. It is a key component of the reperfusion injury salvage kinase (RISK) pathway, which is mostly active during the early stages of reperfusion. Activation of PI3K leads to the activation of downstream Akt signaling (Su et al., 2015). Akt is involved in the various protective cellular responses such as cell survival promotion, apoptotic signaling suppression and the enhancement of myocardial resistance to ischemic injury. The PI3K/Akt signaling pathway regulates also regulate other downstream effectors such as glycogen synthase kinase-3β. GSK-3β is involved in the maintenance of the mitochondrial integrity and it also mitigates irreversible injury to cardiomyocytes. Because of these effects, PI3K is usually considered an important intracellular mediator for the infarct limitation and post-ischemic recovery (Yellon et al., 2023).

PI3K signaling also regulates oxidative stress, calcium homeostasis and inflammatory response. Activation of the PI3K/Akt pathway has been linked to the reduced opening of the mitochondrial permeability transition pore and subsequent reduction in damage caused by the reactive oxygen species. This results in improved endothelial cell and cardiomyocyte survival. This pathway also functions in coordinated manner with other protective signaling mechanism and forms a part of a larger cardioprotective network. PI3K signaling is an

important component in the RISK pathway and is generally associated with myocardial salvage. It is considered an essential mechanism of endogenous cardioprotection in ischemic cardiac injury (Ghafouri-Fard et al., 2022).

2.8.2 PKC Signaling

Protein kinase C (PKC) is a classic regulator of cardioprotective signaling. It has traditionally been linked to the myocardial protection induced by ischemic conditioning. PKCε is regarded as the most important isoform in cardioprotection. Other isoforms may have different or even opposite effects depending on the experimental conditions. The activation and translocation of PKCε has been linked to increased resistance of the heart to ischemia-reperfusion injury. It does so by modulating mitochondrial function and downstream effector pathways (Loubani & Galiñanes, 2002).

PKC mediates cardioprotection by coupling upstream stimuli to downstream mechanisms. It is involved in the opening of mitochondrial KATP channels, reduction of calcium overload, limitation of oxidative damage and inhibition of cell death signaling. It also indicates cross-talk with PI3K/Akt and NOS/NO-dependent pathways. This highlights its role as an integrative mediator rather than an autonomous signal. PKC is a crucial signaling pathway in experimental cardioprotection because it plays an important role in relaying protective signals from membrane receptors to various effectors (Singh et al., 2017).

2.8.3 NOS/NO Signaling

NOS/NO signaling is an important pathway in cardioprotection. Endothelium-derived nitric oxide and different isoforms of nitric oxide synthase (NOS) exert several beneficial effects on the ischemic and reperfused myocardium. It causes vasodilation, improves coronary perfusion, inhibits leukocyte and platelet activation, and regulates of intracellular survival pathways. Besides its vascular effect, NO also acts as a signaling molecule in cardiomyocytes. As a signaling molecule it activates soluble guanylate cyclase, increases cyclic GMP levels and activates protein kinase G (PKG) to induce cytoprotective responses (Weerateerangkul et al., 2011).

The cardioprotective effects of the NOS/NO signaling are closely associated with other intracellular pathways. NO signaling is involved in the maintenance of mitochondrial function, reduction of oxidative damage, and reduction of cardiomyocyte mortality after reperfusion. NOS/NO signaling also serves as a vascular regulatory mechanism and an

essential element of intrinsic pro-survival signaling in cardiac ischemia-reperfusion injury (Schulz et al., 2004).

2.8.4 K_{ATP} Channels

ATP-sensitive potassium (K_{ATP}) channels are known as major end effectors of cardioprotection. These channels link cellular energy status to membrane excitability and mitochondrial function. The sarcolemmal and mitochondrial K_{ATP} channels have been linked to cardiac ischemia-reperfusion injury. However, activation of mitochondrial K_{ATP} results in promotion of mitochondrial homeostasis, reduction of calcium overload, attenuation of oxidative damage, and increased resistance of cardiomyocytes to reperfusion injury. (O'Rourke, 2004).

K_{ATP} channels are involved in several upstream cardioprotective signaling pathways. Often these upstream mediators activate K_{ATP} channels that ultimately preserve mitochondrial integrity and reduce reperfusion injury. Because of their presence at the effector level, K_{ATP} channels are often considered as an important mechanism in reducing infarct size and improving of post-ischemic recovery by several protective stimuli (Grover & Garlid, 2000).

2.9 Experimental Models for the Evaluation of Cardioprotection

Experimental models allow to evaluate protective effects of various therapies and help to understand the mechanisms of myocardial injury in a controlled setting. Single model may not simulate the complexity of an actual myocardial infarction. Due to which complementary ex vivo, in vivo and in vitro systems are often used (Lindsey et al., 2018). This combination approach enables the assessment of cardiac function and mechanisms of protection at the organ level, whole organism level, and at the cellular level. By using Langendorff isolated rat heart, isoproterenol-induced myocardial injury in rats and H9c2 cardiomyoblast model, a complete experimental system can be established to study myocardial injury and cardioprotection from functional, biochemical, histological and cellular perspective. (Lindsey et al., 2018).

2.9.1 Langendorff Isolated Rat Heart Model

The Langendorff isolated heart preparation is a classical ex vivo model to assess cardiac ischemia-reperfusion injury and cardioprotective approaches. The method involves retrograde perfusions of the excised heart through the aorta. This technique is carried out under carefully controlled conditions, which keeps the myocardium in a viable state and

allows to study localized effects in the heart (Lateef et al., 2015). This model has several advantages. It allows exact control of perfusion pressure, perfusate composition, ischemic

time and reperfusion time. It also allows the direct measurement of cardiac functional parameters such as heart rate, coronary flow, left ventricular developed pressure and infarct size. Because this model prevents the systemic effects of the whole organism, such as neurological, hormonal and hemodynamic factors, it is very useful for identifying direct myocardial effects of pharmacological agents and for exploring intrinsic cardioprotective mechanisms. (Aune et al., 2015).

The main drawback is that, isolated study of the heart does not allow complete understanding of complex neurohumoral, inflammatory and systemic metabolic interactions that occur in the entire organism. However, it is widely used in mechanistic studies of myocardial protection as a major link between cellular and whole animal studies (Bell et al., 2011).

2.9.2 Isoproterenol-Induced Myocardial Injury in Rats

The isoproterenol (ISO) induced myocardial injury model is an in vivo experimental model to study of myocardial infarction-like damage and cardioprotection in experimental animals. Isoproterenol is a synthetic β-adrenergic agonist. At high doses it causes significant cardiac stress. Excessive stimulation of β-adrenergic receptors increases myocardial oxygen consumption, disturbs calcium homeostasis, causes oxidative stress and inflammatory activation. This ultimately results in myocardial necrosis and causes functional impairment. The ISO model mimics several important biochemical, electrocardiographic and histological features associated with myocardial injury such as increased cardiac biomarkers, oxidative stress, inflammatory changes and structural damage to the myocardium (Shukla et al., 2015).

This in vivo method is widely used for the evaluation of cardioprotective drugs because of its effectiveness, simplicity, and reproducibility. Moreover, it does not require surgical coronary ligation. This method helps to evaluate the effectiveness of cardioprotective therapies to manage oxidative stress, inflammation, apoptosis, electrocardiogram abnormalities and histological changes in myocardial injury (George et al., 2010).

2.9.3 H9c2 Cardiomyoblast Model

H9c2 cell line is a commonly used in vitro model in cardiovascular research. H9c2 cardiomyoblasts are derived from embryonic rat ventricular tissue. They retain several characteristics of cardiac biology. They are used to study oxidative stress,

hypoxia/reoxygenation injury, mitochondrial dysfunction, apoptosis, drug-induced cardiotoxicity and their signaling pathways. H9c2 cells are not perfectly identical to mature adult cardiomyocytes. However, they are a reliable and reproducible cellular model for mechanistic studies. They are particularly useful for studying intracellular signaling pathways and cytoprotective responses under defined experimental conditions (Dallons et al., 2020).

This model allows to study the direct cellular effects of injurious stimuli on cardiomyoblasts. These results are not fully representative of the whole heart, as they are based on a modified cardiomyoblast line rather than fully developed contractile cardiomyocytes. However, the H9c2 cell line remains one of the most used in vitro models for basic and mechanistic cardioprotective studies. (J. Liu et al., 2008).

Overall, combining these three models results in a reasonable experimental design for evaluating cardioprotection. The Langendorff preparation offers direct assessment of cardiac function under regulated ex vivo ischemia-reperfusion settings; the isoproterenol-induced rat model allows whole-animal evaluation of myocardial damage; and the H9c2 model allows mechanistic research at the cellular level. Together, these systems give more data and improve the experimental assessment of cardioprotective therapies (Son et al., 2020).

2.10 Rationale of the Present Study

Heme Oxygenase-1 (HO-1) is a highly conserved, stress-inducible enzyme that degrades pro-oxidant heme into biliverdin, bilirubin, carbon monoxide (CO), and free iron. These metabolites have been proven to prevent tissue damage in hepatic, renal, pulmonary, and neurological injury models (Wegiel et al., 2014).

HO-1 has been extensively explored for its cytoprotective properties. Although HO-1 has been investigated in cardiovascular injury, limited evidence is available from integrated ex vivo, in vivo and in vitro myocardial injury models combined with pharmacological pathway-inhibitor analysis.

Multiple biological and molecular processes control myocardial survival during IRI. Targeting these prosurvival pathways may minimize IRI-induced damage and provide cardioprotection. Several investigations have identified PI3K, PKC, NOS, and K_{ATP} channels as key prosurvival signaling pathways during myocardial IRI (Comità et al., 2023). PI3K signaling protects the heart from ischemia-reperfusion damage by inhibiting apoptosis,

lowering oxidative stress, regulating inflammation, and boosting angiogenesis and tissue repair. PKC protects cardiac ischemia/reperfusion damage via controlling redox signaling, calcium homeostasis, cell death, oxidative stress, and mitochondrial function. Nitric oxide causes vasodilation, anti-inflammatory effects, and mitochondrial stability, providing a protective impact during IRI. ATP-sensitive potassium channels protect the heart during IRI by avoiding calcium overload, lowering oxidative stress, saving energy, preserving mitochondrial function, increasing blood flow, and controlling inflammation (Q. Zhang et al., 2022). HO-1 may provide cardioprotection by activating one or more of the aforementioned pro-survival signaling pathways during MI and related alterations.

The current research seeks to examine the cardioprotective effects of HO-1 modulation by hemin (inducer) and zinc protoporphyrin IX (inhibitor). The research also looks at the ability of HO-1 to activate prosurvival processes by employing pathway-specific inhibitors of PI3K, PKC, NOS, and K_{ATP} to determine their involvement in myocardial survival in ex vivo, in vivo, and in vitro models. This might disclose new treatment targets outside traditional reperfusion techniques, allowing for the development of innovative supplementary cardioprotective therapies. This would also set the stage for therapeutic translation of HO-1 regulation in ischemic heart disease**.**

2.11 Research Hypothesis

It was hypothesized that pharmacological induction of heme oxygenase-1 (HO-1) by Hemin would attenuate myocardial injury and improve functional, biochemical, histopathological, and cellular outcomes in ex vivo ischemia-reperfusion, in vivo isoproterenol-induced myocardial injury, and in vitro isoproterenol-induced H9c2 cardiomyoblast injury models. It was further hypothesized that, in the ex vivo ischemia-reperfusion model, HO-1-mediated cardioprotection would involve PI3K, PKC, NOS, and KATP-dependent pro-survival signaling mechanisms and pharmacological inhibition of HO-1 or blockade of these pathways would attenuate the protective effects associated with HO-1 induction.

3. AIM AND OBJECTIVES

3.1 Aim of the Study

- To evaluate the effects of Heme oxygenase-1 modulation in Myocardial Infarction in rats.

3.2 Objectives of the Study

3.2.1 Primary objective:

- The primary objective of the current investigation is to evaluate the effects of Heme Oxygenase-1 (HO-1) modulation in myocardial infarction (MI) and to examine its potential interaction with key pro-survival Signaling pathways using ex vivo, in vivo, and in vitro experimental models.

3.2.2 Specific Objectives:

- To evaluate the effects of HO-1 modulation in myocardial ischemia–reperfusion injury using isolated rat heart preparations (ex vivo).
- To evaluate the effects of HO-1 modulation in isoproterenol-induced myocardial infarction in rats (in vivo).
- To evaluate the effects of HO-1 induction against isoproterenol-induced myocardial injury in H9c2 cardiomyoblasts (in vitro).
- To assess the changes in cardiac functional, biochemical, and histopathological parameters following HO-1 modulation across ex vivo, in vivo, and in vitro models.
- To study the effects of HO-1 induction and inhibition using Hemin and Zinc Protoporphyrin IX (ZnPP IX) respectively, in the ex vivo model.
- To investigate the potential role of prosurvival Signaling pathways such as PI3K, PKC, NOS, and K_{ATP}, in HO-1-mediated effects using pathway-specific inhibitors in the ex vivo model.

4. RESEARCH METHODOLOGY

4.1 Drugs, Chemicals, And Reagents

Sigma-Aldrich supplied hemin, zinc protoporphyrin IX, and chelerythrine. Tokyo Chemical Industry India Pvt. Ltd. provided isoproterenol (ISO), L-NAME (N(ω)-nitro-L-arginine methyl ester), and wortmannin for the study. ARKRAY, Inc. supplied biochemical kits for creatine kinase-myocardial band (CK-MB) and lactate dehydrogenase (LDH). The Bioassay Technology Laboratory, Shanghai Korain Biotech Co., Ltd., provided an ELISA (enzyme-linked immunosorbent assay) kit for HO-1, while Allianz Biosciences Pvt. Ltd. provided a PI3K ELISA kit.

4.2 Animals

Male Wistar albino rats weighing between 250-300 g were procured from Jai Research Foundation. Animals were maintained and all experimental procedures were performed following the established guidelines set by CCSEA (Committee for Control and Supervision of Experiments on Animals) and the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. The Institutional Animal Ethics Committee (IAEC) reviewed and approved the experimental protocol under approval number RPCP/IAEC/2020-2021/R16.

4.3 Ex Vivo Study: Ischemia–Reperfusion Injury in Isolated Perfused Rat Heart Preparation

4.3.1 Isolated Perfused Rat Heart Preparation

In the ex vivo experiment we used an isolated rat heart model. We used a Langendorff perfusion technique for the study (Montero-Bullon et al., 2021). The rats were heparinized and anesthetized with Xylazine (10 mg/kg) and Ketamine (75 mg/kg) administered intraperitoneally. After confirming the deep state of anaesthesia in animals by checking necessary stimuli for the same, the hearts of the rats were excised in accordance with compliance with CPCSEA criteria and IAEC-approved procedures. The excised hearts were quickly placed into a chilled Krebs-Henseleit (K-H) buffer containing potassium chloride (4.7 mM), sodium dihydrogen phosphate (1.2 mM), magnesium sulphate (1.2 mM), sodium chloride (118 mM), calcium chloride (2.5 mM), sodium bicarbonate (25 mM), and glucose (11 mM) with pH adjusted to 7.4. Hearts were cannulated via the aorta and mounted on

Langendorff equipment. The hearts were perfused retrogradely at a constant flow rate of 15 ml/min with Krebs-Henseleit buffer kept at 37°C. The Krebs-Henseleit buffer was continuously infused with carbogen gas (95% O_2 and 5% CO_2 mixture). To monitor heart function, a fluid-filled latex balloon linked to a pressure transducer (SS13L) was put into the left ventricle, and preload was adjusted to 5-10 mm Hg via balloon inflation. The cannulated hearts were allowed to stabilize. Ischemia-reperfusion injury (IRI) was then produced by subjecting the hearts to 30 minutes of ischemia and 90 minutes of reperfusion (Watanabe & Okada, 2001).

4.3.2 Experimental protocol

The IAEC approved a maximum of 90 animals for the ex vivo study. Animals were allocated to nine experimental groups, with six successfully established Langendorff heart preparations included in each group. A few preparations could not be successfully established or maintained during the isolated-heart procedure and were therefore excluded from the final analysis.

The experiment was divided into two parts.

4.3.2.1 Part 1: Dose Determination of Hemin (Figure 4.1)

The initial part of the study was aimed at determining the optimal dose of Hemin for HO-1 induction and assessing its cardioprotective role.

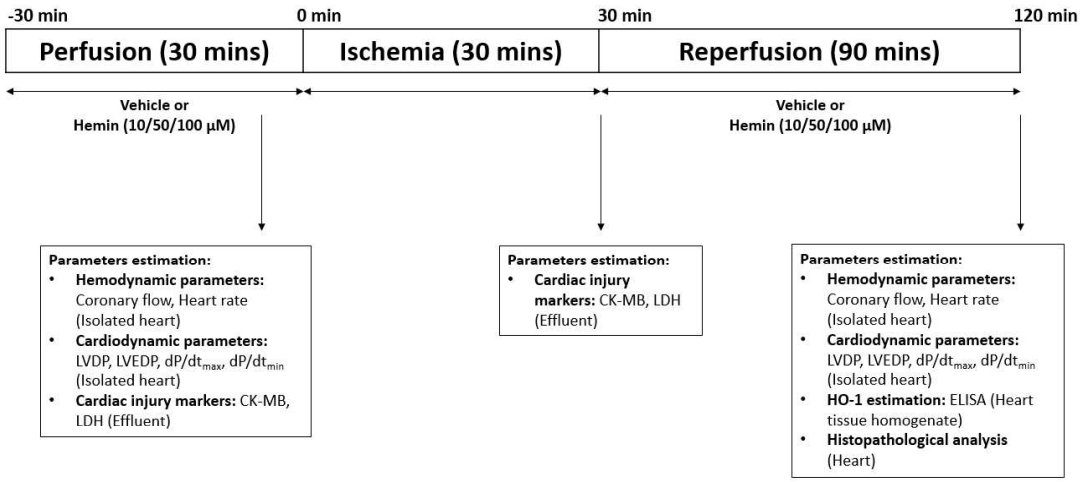


Figure 4.1: Experimental Protocol for Hemin Dose Determination in Ex Vivo I/R Injury (Part 1)

- **Group 1:** Isolated hearts were infused with K-H buffer and subsequently exposed to ischemia-reperfusion damage.
- **Group 2:** Isolated hearts were infused with K-H buffer with a 10 µM Hemin as an HO-1 inducer and subsequently exposed to ischemia-reperfusion damage.
- **Group 3:** Isolated hearts were infused with K-H buffer with 50 µM Hemin as an HO-1 inducer and subsequently exposed to ischemia-reperfusion damage.
- **Group 4:** Isolated hearts were infused with K-H buffer with 100 µM Hemin as an HO-1 inducer and subsequently exposed to ischemia-reperfusion damage.

4.3.2.2 Part 2: Mechanistic Study Using Pathway Inhibitors (Figure 4.2)

- The second part of the study looked into the role of Signaling pathways involved in pro-survival in cardioprotection mediated by HO-1. Pathway-specific pharmacological inhibitors were chosen for their proven roles to inhibit protective mechanism of myocardial ischemia-reperfusion injury. Chelerythrine as an inhibitor of PKC (Protein Kinase C), L-NAME (L-arginine methyl ester) as an inhibitor of NOS (Nitric oxide synthase), wortmannin as an inhibitor of PI3K (Phosphatidylinositol 3-kinase), and glibenclamide as an inhibitor of K_{ATP} (ATP-dependent potassium channel) were used in the mechanistic study (Bril et al., 1992; Kaur et al., 2019; Soni et al., 2012). Their inhibition enabled us to assess the effects of these pathways in mediating the protective effects following HO-1 induction.

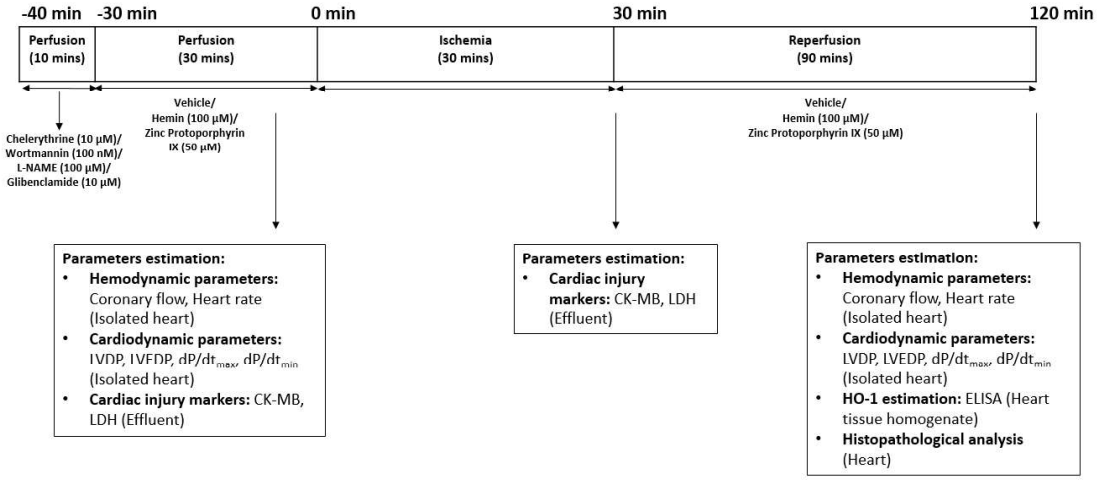


Figure 4.2: Experimental Protocol for Mechanistic Evaluation Using Pathway Inhibitors (Part 2)

- **Group 5:** Isolated hearts were infused with K-H buffer with 50 µM Zinc Protoporphyrin IX as an HO-1 inhibitor and subsequently exposed to ischemia-reperfusion damage.
- **Group 6:** Isolated hearts were infused with K-H buffer with 10 µM Chelerythrine as PKC inhibitor and 100 µM Hemin as an HO-1 inducer and subsequently exposed to ischemia-reperfusion damage.
- **Group 7:** Isolated hearts were infused with K-H buffer with 100 µM L-NAME as an NOS inhibitor and 100 µM Hemin as an HO-1 inducer and subsequently exposed to ischemia-reperfusion damage.
- **Group 8:** Isolated hearts were infused with K-H buffer with 100 nM Wortmannin as PI3K inhibitor and 100 µM Hemin as an HO-1 inducer and subsequently exposed to ischemia-reperfusion damage.
- **Group 9:** Isolated hearts were infused with K-H buffer with 10 µM Glibenclamide as K_{ATP} blocker and 100 µM Hemin as an HO-1 inducer and subsequently exposed to ischemia-reperfusion damage.

4.3.3 Heme Oxygenase-1 assay

HO-1 levels in heart tissue homogenate were quantified using a Rat Heme Oxygenase 1 ELISA Kit (Bioassay Technology Laboratory, Shanghai Korain Biotech Co., Ltd., China; Cat. No. E0676Ra) as per the manufacturer's instructions. The assay is based on the sandwich ELISA principle. Heart tissues were rinsed with PBS (pH 7.4), minced, homogenized in PBS on ice, and centrifuged at 2000–3000 rpm for approximately 20 min. The clear supernatant was used for analysis. Standards were prepared as instructed by serial dilution of the standard stock solution. To perform the assay, 50 µL of standard was taken into the standard wells. In the sample wells, 40 µL of sample was added followed by 10 µL of anti-HO-1 antibody. The 50 µL of streptavidin-HRP was then added to both the standard and sample wells. After which, the plate was incubated for 60 minutes at 37°C. Then all the wells were washed five times. To which 50 µL of substrate solutions A and B were added. The plate was then incubated for 10 minutes at 37°C in the dark. To halt the reaction, 50 µL of stop solution was added. Microplate reader was used to detect absorbance at 450 nm for 10 minutes. A standard curve was prepared, and the HO-1 concentration in the samples was determined and represented in ng/mL.

4.3.4 Assessment of Haemodynamic and Cardiodynamic Parameters

During the investigation, cardiac functional parameters such as left ventricular end-diastolic pressure (LVEDP), left ventricular developed pressure (LVDP), peak rate of relaxation (dp/dt_{min}), peak rate of contraction (dp/dt_{max}), coronary flow, and beats per minute (BPM /heart rate) were recorded using various component of the Biopac data acquisition system. The parameters were analysed before induction of ischemia and after completion of reperfusion phase. For the evaluation of the effect of various treatment, the percentage recovery was computed as the ratio of post-ischemia-reperfusion (post-I/R) values by their corresponding baseline (pre-ischemic) values, and reported as a percentage. The formula for the calculation was: (Post-I/R value ÷ Baseline [pre-ischemic] value) ×100. This estimate reflects the fraction of cardiac function maintained by Hemin therapy after I/R injury (Mahmoudabady et al., 2017; Xu et al., 2020).

4.3.5 Measurement of Myocardial Injury Markers

Cardiac injury biomarkers, namely CK-MB and LDH, were estimated using commercially available diagnostic kits procured from ARKRAY, Inc. and analysed on a biochemical analyzer by the kinetic UV method according to the manufacturer's instructions. For LDH estimation, the working reagent was prepared by mixing Reagent 1 and Reagent 2 in a 4:1 ratio. A volume of 0.03 mL of sample was used, and absorbance was recorded at 340 nm after 60 s and then every 30 s for the next 120 s. LDH activity was calculated from the mean change in absorbance per minute using a kinetic factor of 5520 and expressed in U/L. For CK-MB estimation, the working reagent was similarly prepared in a 4:1 ratio, and 0.05 mL of sample was mixed with 1.0 mL of working reagent. Absorbance was measured at 340 nm at 300 s and then every 60 s for the next 120 s. CK-MB activity was calculated from the mean change in absorbance per minute using a kinetic factor of 6752 and expressed in U/L.

4.3.6 Determination of Oxidative Stress and Antioxidant Enzyme Activity

Following the reperfusion phase, the hearts were cleaned with phosphate buffered saline (PBS). After which homogenising was done in 10 mM Tris-HCl buffer. The heart tissue homogenates were then centrifuged at 5000 rpm at 4 °C for 10 minutes. SOD (Superoxide Dismutase) and MDA (Malondialdehyde) were analysed to determine oxidative stress and antioxidant defence.

4.3.6.1 Malondialdehyde (MDA) Estimation

For the estimation of lipid peroxidation in heart tissue, thiobarbituric acid reactive substances (TBARS) were measured and expressed as malondialdehyde (MDA) equivalents. Briefly, 2.0 mL of TBA-TCA-HCl reagent was mixed with 0.1 mL of tissue homogenate and thoroughly vortexed. The reaction mixture was then heated in a boiling water bath for 15 minutes. The reaction mixture was brought to the room temperature for further processing. Afterwards reaction mixture was centrifuged at 1000 rpm for 10 minutes. The clear supernatant was separated and then measured at 535 nm using a reagent blank. Standard solutions were treated similarly, and the MDA concentration was measured from the standard curve and expressed as nmol/mg (Afzal et al., 2021).

4.3.6.2 Superoxide Dismutase (SOD) Estimation

Superoxide dismutase (SOD) activity was estimated by the PMS-NBT-NADH method. 0.1 mL of tissue homogenate supernatant, 0.1 mL of phenazine methosulfate (PMS, 186 µM), 0.3 mL of nitro blue tetrazolium (NBT, 300 µM) and 1.2 mL of sodium pyrophosphate buffer (0.052 M, pH 8.3) were mixed. To activate the reaction, 0.2 mL NADH (780 µM) was added and then incubated at 30°C for 90 seconds. The reaction was halted by adding 1.0 mL glacial acetic acid followed by 4.0 mL n-butanol. The mixture was shaken vigorously, and allowed to stand for phase separation. After separation it was centrifuged at 3000 rpm for 10 min. The absorbance of the butanol layer was measured at 560 nm using a spectrophotometer. SOD activity was reported as U/mg protein (John & Arockiasamy, 2022).

4.3.7 Histopathological Analysis

Following the experiment, the hearts were washed with PBS and fixed in 10% formalin for cell fixation. The formalin-fixed samples were sent for histopathological analysis to a pathology laboratory. For the staining of tissue slices of histopathological investigation hematoxylin and eosin (H&E) were used. The stained sections were then examined for signs of ischemia-reperfusion injury, such as interstitial oedema (widened gaps between fibres), contraction band development (dense eosinophilic transverse bands), myocardial necrosis (eosinophilic fibres with nuclear loss), and fibre separation.

4.3.8 Statistical Analysis

The findings of the various evaluated parameters of the study are reported as mean ± standard error of the mean (SEM). For further analysis One-way ANOVA was used, followed by

Tukey's multiple comparison test as a post-hoc analysis. We kept the *p*-value of <0.05 as statistically significant results. All the parameters in the study are analysed with the help of GraphPad Prism version 10.1.0 (GraphPad Software, San Diego, CA, USA).

4.4 In Vivo Study: Isoproterenol-Induced Myocardial Infarction in Rats

4.4.1 Experimental Design

The IAEC approved a maximum of 30 animals for the in vivo study. Animals were allocated to three experimental groups, with six animals completing the experimental protocol in each group. A few animals did not complete the experimental protocol because of mortality and were therefore excluded from the final analysis.

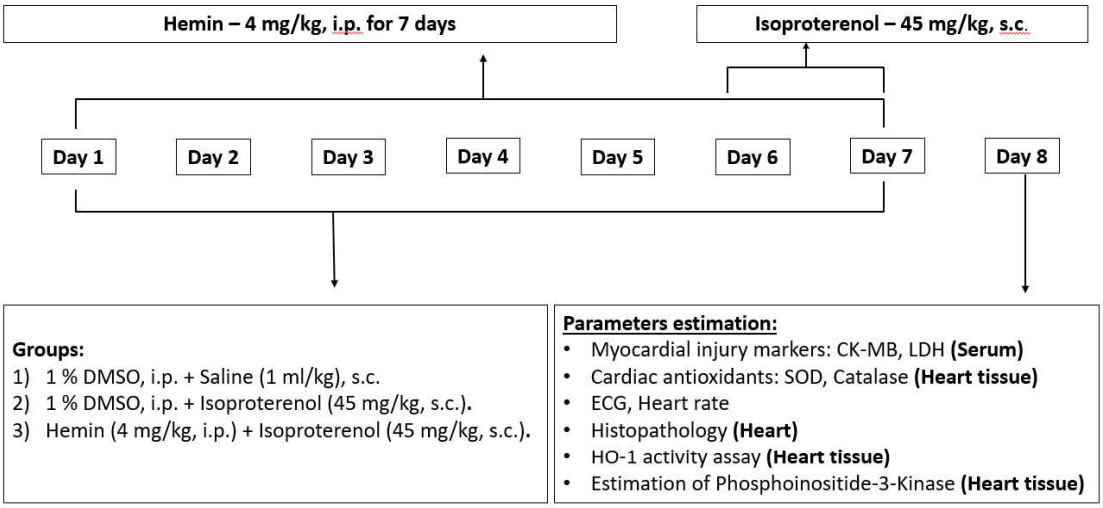


Figure 4.3 Study Design for Isoproterenol-Induced Myocardial Injury in Rats

- Normal control group: Animals were given Dimethyl Sulfoxide (DMSO) through intraperitoneal (IP) route on alternate days for 7 days. Normal saline was administered to the animals via subcutaneous (SC) route for the last two days of the study .
- Disease control group: Animals were given DMSO through intraperitoneal (IP) route on alternate days for 7 days. Isoproterenol (ISO) (45 mg/kg) was administered via SC route for the last 2 days of the study.
- Hemin treated group: Hemin (4mg/kg) was given on alternate days via intraperitoneal (IP) route for 7 days and ISO (45 mg/kg) was administered for the last 2 days of the study via SC route (Collino et al., 2013; Huang et al., 2018; Pham et al., 2023).

4.4.2 ECG Analysis

Electrocardiogram (ECG) analysis was performed to assess cardiac abnormalities induced by isoproterenol (ISO). Anaesthesia was induced in the experimental rats via intraperitoneal administration of ketamine (75 mg/kg) and xylazine (10 mg/kg). The ECG recordings were obtained using the BIOPAC MP36 System. Electrodes were positioned on the right forelimb, left forelimb, and left hind limb to capture the ECG signals. Key parameters including the ST segment, Q wave and heart rate expressed as beats per minute (BPM) were evaluated (Khattak et al., 2025).

4.4.3 Detection of Serum Cardiac Injury Markers

For estimation of serum cardiac injury biomarkers, blood was collected from the retro-orbital plexus under ketamine (75 mg/kg, IP) and xylazine (10 mg/kg, IP) anaesthesia (Sotoudeh & Namavar, 2022). The collected blood samples were subjected to centrifugation at 5000 rpm at 4°C for 10 minutes. The separated serum was stored at -20°C in the deep freezer until further analysis. Cardiac injury biomarkers were quantified using commercially available assay kits, following manufacturer's protocols.

4.4.4 Estimation of Oxidative Stress and Antioxidant Parameters

For tissue biochemical estimation, rats were euthanized with a high dose of barbiturate, after which the hearts were excised and rinsed with phosphate-buffered saline (PBS). The cardiac tissues were then homogenized using 10 mM Tris-HCl Buffer and later centrifuged at 5000 rpm at 4°C for 10 minutes. The resulting supernatant was used to evaluate oxidative stress marker malondialdehyde (MDA) along with anti-oxidant enzyme, including catalase (CAT), and superoxide dismutase (SOD) (Afzal et al., 2021; John & Arockiasamy, 2022).

4.4.5 HO-1 Assay

HO-1 levels in heart tissue homogenate were quantified using a Rat Heme Oxygenase 1 ELISA kit (The Bioassay Technology Laboratory, Shanghai Korain Biotech Co., Ltd., China) as per the manufacturer specified protocol. The assay is based on the sandwich ELISA principle, and absorbance was measured at 450 nm using a microplate reader. Tissue homogenate supernatant was used as the test sample (Kitchin et al., 2001).

4.4.6 Estimation of Phosphoinositide-3-Kinase (PI3K)

The levels of PI3K in heart tissue homogenates from each experimental group were quantified using an enzyme-linked immunosorbent assay (ELISA) kit (Olejnik et al., 2023) following the protocol provided by Allianz Biosciences Pvt Ltd.

4.4.7 Histopathological Analysis

After euthanasia, hearts from each group were excised and rinsed with normal saline to eliminate residual blood. The cardiac tissues were then fixed in 10% formalin for preservation. The fixed samples were sent to a pathology laboratory for histological examination to assess structural modifications in the cardiac tissue (Ghafoor et al., 2020).

4.4.8 Statistical Analysis

The findings of the various evaluated parameters of the study are reported as mean ± standard error of the mean (SEM). For further analysis One-way ANOVA was used, followed by Tukey's multiple comparison test as a post-hoc analysis. We kept the *p*-value of <0.05 as statistically significant results. All the parameters in the study are analysed with the help of GraphPad Prism version 10.1.0 (GraphPad Software, San Diego, CA, USA)..

4.5 In Vitro Study: ISO-Induced Myocardial Injury in H9c2 Cardiomyoblasts

H9c2 rat cardiomyoblast cells were obtained from the National Center for Cell Science (NCCS) in Pune, India. We used Dulbecco's Modified Eagle Medium (DMEM) with 10% foetal bovine serum (FBS) and an antibiotic solution containing penicillin (100 U/mL) and streptomycin (100 U/mL) for the growth of the cells. H9c2 cells were cultured at 37°C in a humidified atmosphere of 5% CO_2. The culture medium was replaced two to three times per week, and subculturing was performed when the cells reached 80–90% confluency. An isoproterenol (ISO)-induced H9c2 cell injury model was used to mimic myocardial injury in vitro. For treatment, cells were pretreated with Hemin at different concentrations for 2 h, followed by exposure to isoproterenol (80 µM) for 24 h (Han et al., 2016).

4.5.1 Experimental Protocol

- **Group 1:** Control
- **Group 2:** Isoproterenol (80 µM)
- **Group 3:** Hemin (10 µM) + Isoproterenol (80 µM)

- **Group 4:** Hemin (30 µM) + Isoproterenol (80 µM)
- **Group 5:** Hemin (60 µM) + Isoproterenol (80 µM)
- **Group 6:** Hemin (90 µM) + Isoproterenol (80 µM)

4.5.2 MTT Assay: Cell Viability

Cell viability was assessed by the MTT assay. At the end of treatment, MTT reagent was added to the cells and by using microplate reader the absorbance was measured at 570 nm. The absorbance values were used as an index of viable metabolically active cells.

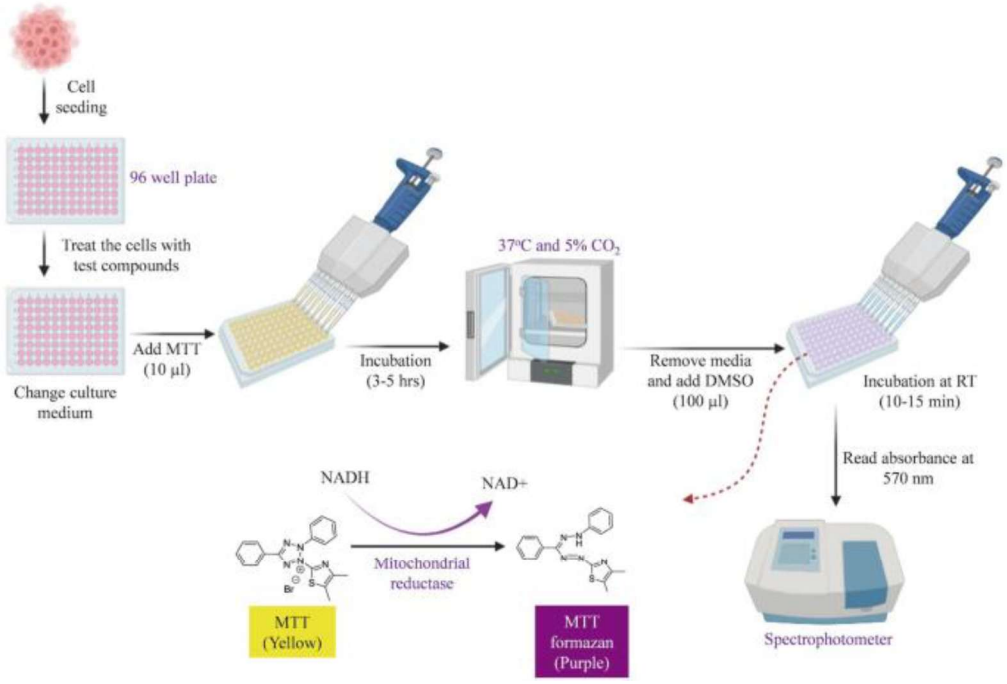


Figure 4.4: Cell culture study: MTT assay

4.5.3 Estimation of Cardiomyocyte Injury Marker: CK-MB

Cell injury was evaluated by measuring creatine kinase-myocardial band (CK-MB) levels in the culture supernatant. CK-MB was estimated using a commercially available ARKRAY diagnostic kit on a biochemical analyser according to the manufacturer's instructions.

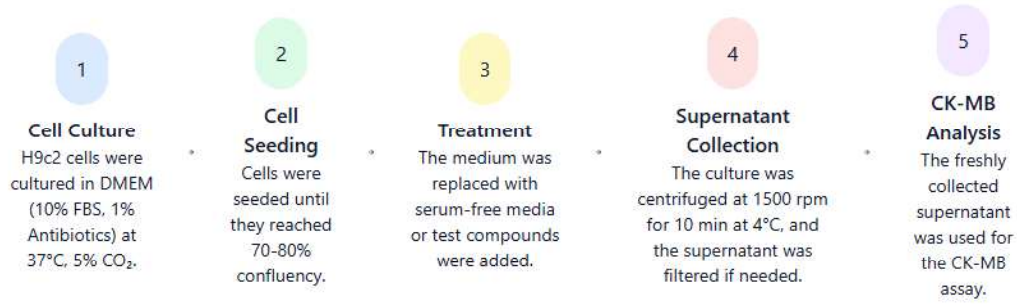


Figure 4.5 : Cell culture study: CK-MB estimation

4.5.4 Estimation of PI3K Levels

PI3K levels in cell lysate were quantified using an enzyme-linked immunosorbent assay (ELISA) kit procured from Allianz Biosciences Pvt Ltd., according to the manufacturer's instructions.

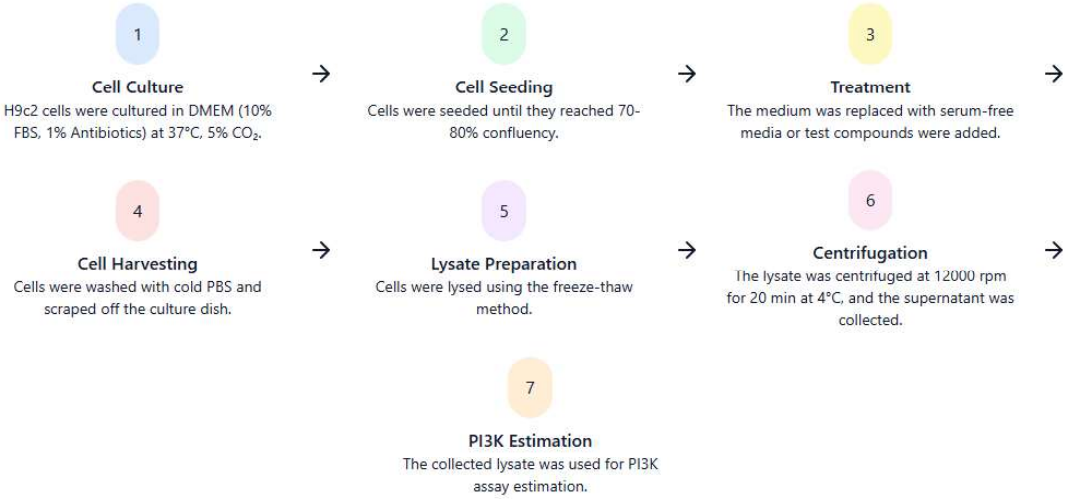


Figure 4.6 : Cell culture study: Cell lysate preparation

4.5.5 Statistical Analysis:

The findings of the various evaluated parameters of the study are reported as mean ± standard error of the mean (SEM). For further analysis One-way ANOVA was used, followed by Tukey's multiple comparison test as a post-hoc analysis. We kept the *p*-value of <0.05 as statistical significant results. All the parameters in the study are analysed with the help of GraphPad Prism version 10.1.0 (GraphPad Software, San Diego, CA, USA).

5. RESULTS

5.1 Ex vivo Study: Ischemia–Reperfusion Injury in Isolated Perfused Rat Heart Preparation

5.1.1 Part 1: Determination of the Optimal Hemin Dose for HO-1 Induction and Cardioprotection.

5.1.1.1 Effect of Hemin on HO-1 Levels:

Following Hemin administration, HO-1 levels rose in a dose-dependent manner. Hearts subjected to the treatment with 10 µM, 50 µM, and 100 µM hemin had considerably higher HO-1 levels than the control group (p<0.05), suggesting effective enzyme induction (Figure 5.1).

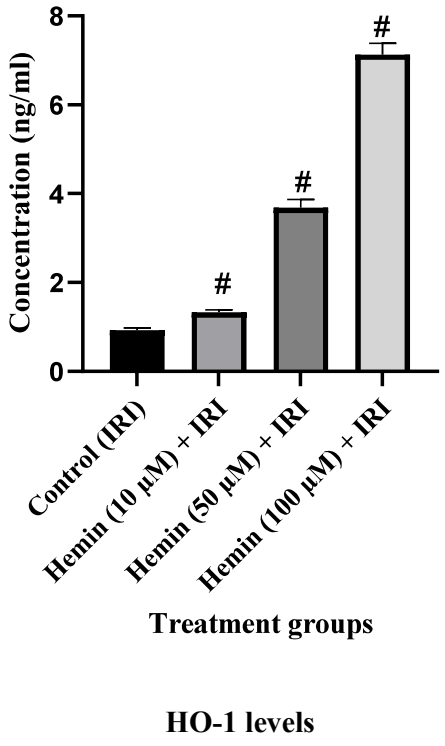
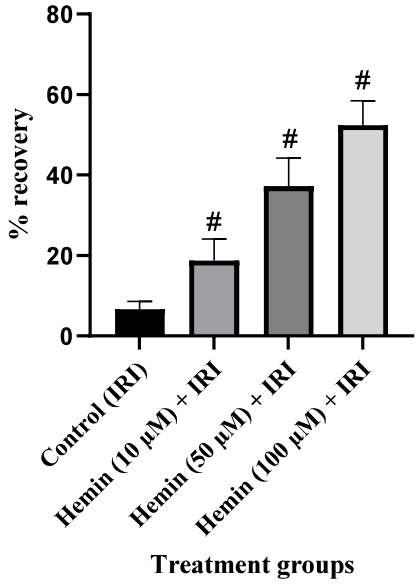


Figure 5.1: Effect of Hemin on HO-1 Levels. HO-1 levels were measured after Ischemia Reperfusion Injury (IRI). The data is reported as mean ± SEM (n = 6). '#' indicated a *p*-value < 0.05 compared to the control group (IRI).

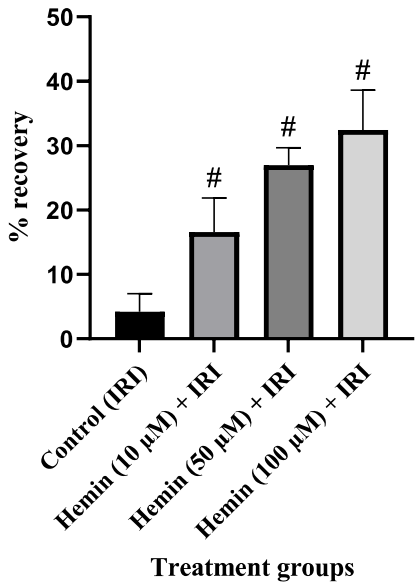
5.1.1.2 Effect of Hemin-Induced HO-1 on Cardiodynamic and Hemodynamic Parameters

Hemin therapy improved heart function significantly after ischemia-reperfusion. Significant recovery (p<0.05) was found in cardiac functional parameters, LVDP and LVEDP in hearts treated with various dosages of hemin in comparison to the group with untreated hearts. **Figure 5.2 and 5.3**. Similarly, there is considerable improvement in dP/dt_{max} in the hearts treated with 100 µM hemin as HO-1 inducer, whereas there was significant improvement in dP/dt_{min} in the hearts treated with 50 µM and 100 µM hemin as HO-1 inducer (p<0.05). **Figure 5.4 and 5.5.** Heart rate and coronary flow increased in a dose-dependent manner and there was substantial recovery in the coronary flow (CF) of hearts subjected to 100 µM hemin as HO-1 inducer (p<0.05). **Figure 5.6 and 5.7**. These data related to cardiodynamic and hemodynamic parameters indicate the functional improvement in cardiodynamic and hemodynamic parameters and could be attributed to HO-1 induction after hemin therapy**.**



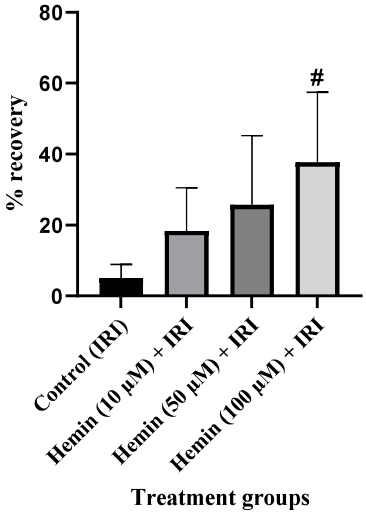
LVDP

Figure 5.2: Effects of Hemin as HO-1 inducer on the cardiodynamic parameter LVDP in terms of % recovery. LVDP was measured before the induction of ischemia and after completion of reperfusion phase. The data is reported as mean ± SEM (n = 6). '#' indicated a *p*-value < 0.05 compared to the control group (IRI).



LVEDP

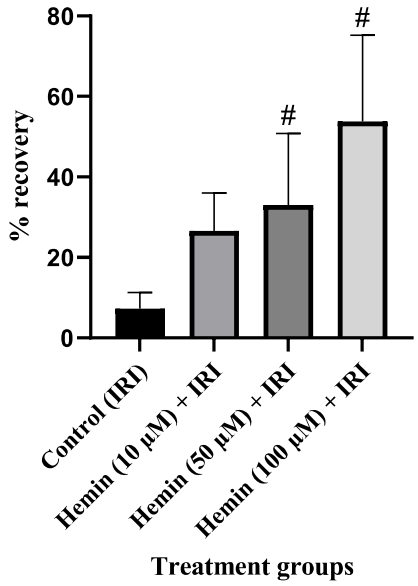
Figure 5.3: Effects of Hemin as HO-1 inducer on the cardiodynamic parameter LVEDP in terms of % recovery. LVEDP was measured before the induction of ischemia and after completion of reperfusion phase. The data is reported as mean ± SEM (n = 6). '#' indicated a *p*-value < 0.05 compared to the control group (IRI).



dP/dt_{max}

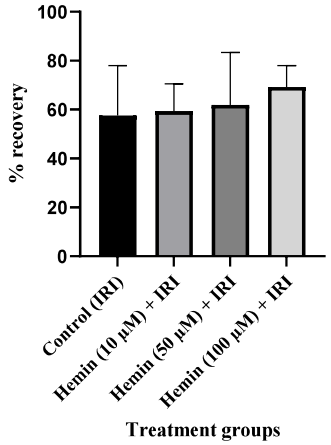
Figure 5.4: Effects of Hemin as HO-1 inducer on the cardiodynamic parameter dP/dt_{max} in terms of % recovery. dP/dt_{max} was measured before the induction of

ischemia and after completion of reperfusion phase. The data is reported as mean ± SEM (n = 6). '#' indicated a *p*-value < 0.05 compared to the control group (IRI).



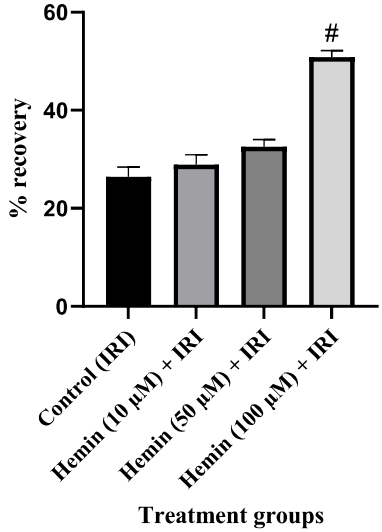
dP/dt_{min}

Figure 5.5: Effects of Hemin as HO-1 inducer on the cardiodynamic parameter dP/dt_{min} in terms of % recovery. dP/dt_{min} was measured before the induction of ischemia and after completion of reperfusion phase. The data is reported as mean ± SEM (n = 6). '#' indicated a *p*-value < 0.05 compared to the control group (IRI).



Heart rate

Figure 5.6: Effects of Hemin as HO-1 inducer on the cardiodynamic parameter Heart rate in terms of % recovery. Heart rate was measured before the induction of ischemia and after completion of reperfusion phase. The data is reported as mean ± SEM (n = 6). '#' indicated a *p*-value < 0.05 compared to the control group (IRI).

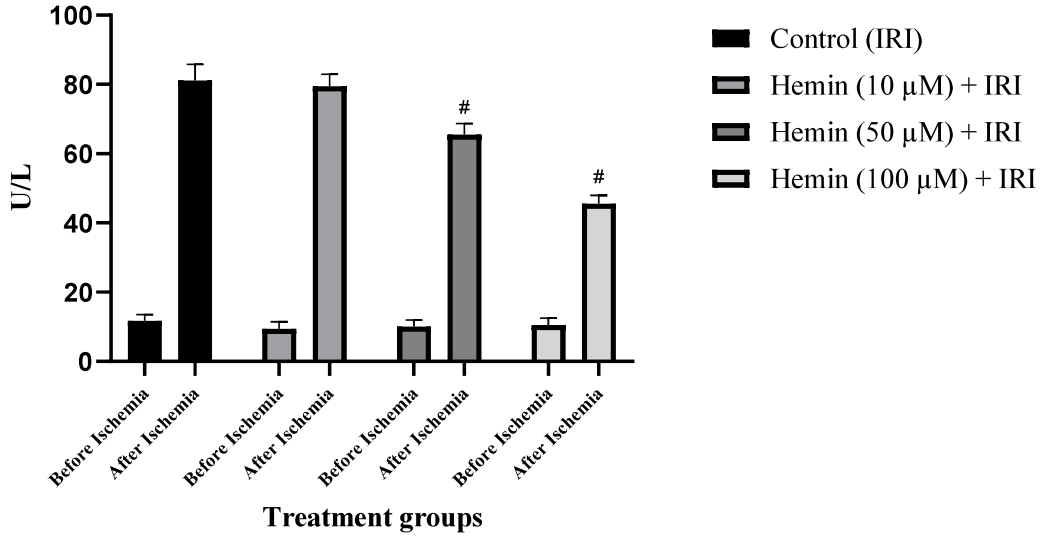


Coronary flow

Figure 5.7: Effects of Hemin as HO-1 inducer on the haemodynamic parameter Coronary flow in terms of % recovery. Coronary flow was measured before the induction of ischemia and after completion of reperfusion phase. The data is reported as mean ± SEM (n = 6). '#' indicated a *p*-value < 0.05 compared to the control group (IRI).

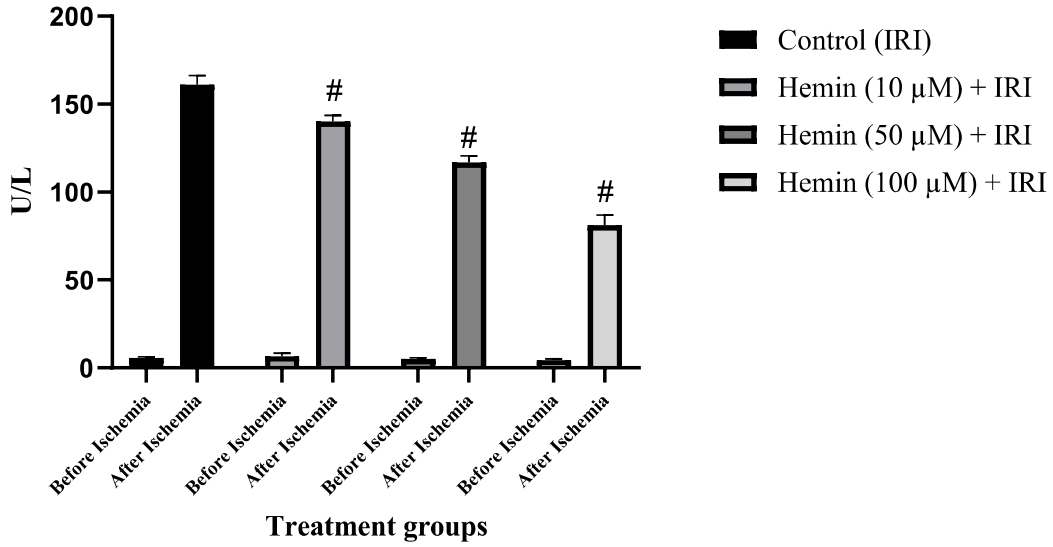
5.1.1.3 Effect of Hemin-Induced HO-1 on Myocardial Injury Markers

Hemin therapy resulted in significant dose-dependent reductions in CK-MB and LDH levels. CK-MB levels were substantially lower in coronary effluent collected from the hearts treated with 50 µM and 100 µM hemin groups (p<0.05), whereas LDH levels in the coronary effluent were significantly lower in all the groups of hearts treated with hemin when compared to the control group (p<0.05). **Figure 5.8 and 5.9**.



CK-MB

Figure 5.8: Effects of Hemin as HO-1 inducer on the cardiac injury marker CK-MB. CK-MB was measured before induction of ischemia and after reperfusion. The data is reported as mean ± SEM (n = 6). '#' indicated a p-value < 0.05 compared to the control group (IRI).

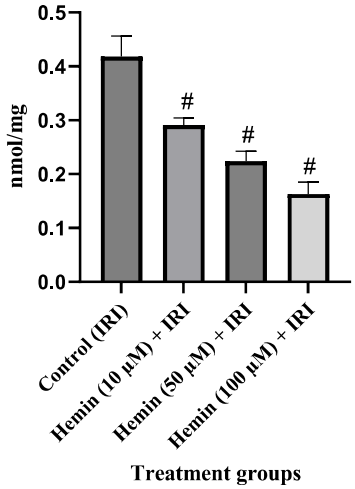


LDH

Figure 5.9: Effects of Hemin as HO-1 inducer on the cardiac injury marker LDH. LDH was measured before induction of ischemia and after reperfusion. The data is reported as mean ± SEM (n = 6). '#' indicated a *p*-value < 0.05 compared to the control group (IRI).

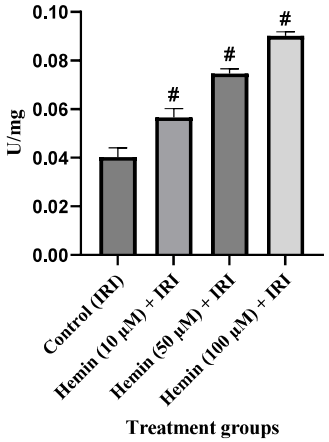
5.1.1.4 Effect of Hemin-Induced HO-1 on Oxidative Stress and Antioxidant Enzyme

Hemin therapy decreased the extent of oxidative stress, as shown by lower level of its marker, MDA levels and at the same time antioxidant enzyme, SOD increased in heart tissue homogenates. MDA levels decreased considerably across all hemin treated groups (p<0.05), whereas SOD levels elevated significantly in the hearts treated with 10 µM, 50 µM, and 100 µM hemin in a dose-dependent manner (p<0.05). **Figure 5.10 and 5.11**.



MDA

Figure 5.10: Effects of Hemin as HO-1 inducer on oxidative stress marker MDA. MDA was measured after induction of IRI. The data is reported as mean ± SEM (n = 6). '#' indicated a *p*-value < 0.05 compared to the control group (IRI).



SOD

Figure 5.11: Effects of Hemin as HO-1 inducer on antioxidant enzyme SOD. SOD was measured after induction of IRI. The data is reported as mean ± SEM (n = 6). '#' indicated a *p*-value < 0.05 compared to the control group (IRI).

5.1.1.5 Effect of Hemin-Induced HO-1 on Cardiac Histopathology

Histopathological study demonstrated that IRI induced significant structural changes, such as myocardial necrosis, interstitial oedema as well as contraction bands, and fibre separation. These alterations were significantly reduced in the hearts treated with HO-1 inducer hemin, suggesting structural preservation and less tissue damage. **(Figure 5.12)**.

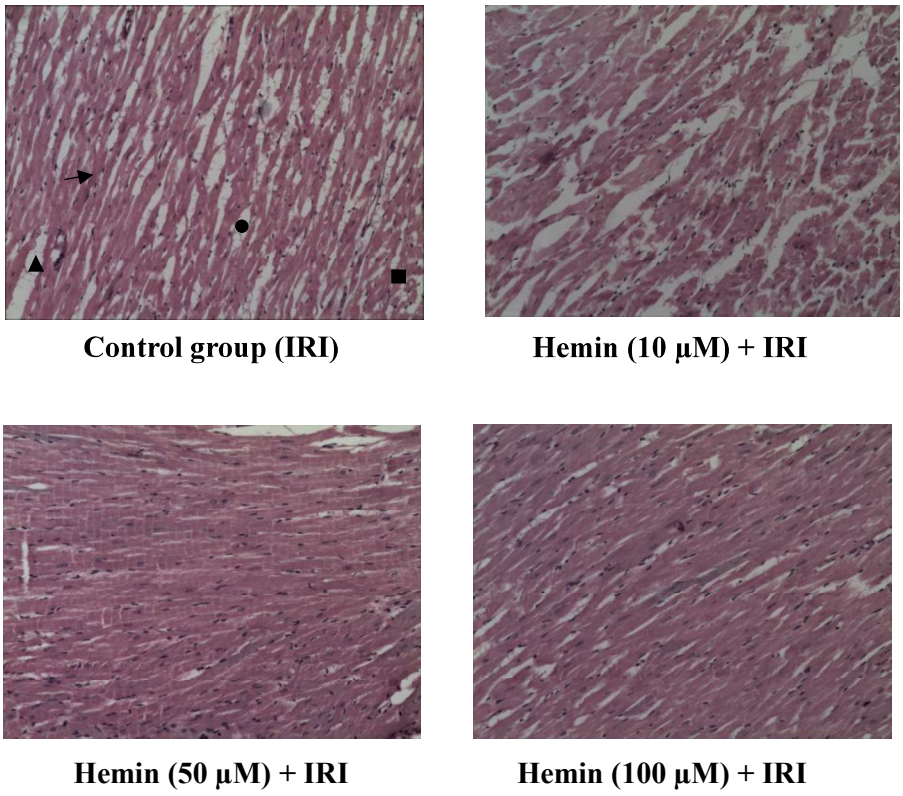


Figure 5.12: Effect of Hemin-Induced HO-1 on Cardiac Histopathology. (H&E Staining, 400x). (Structural changes: Square - myocardial necrosis, Triangle - interstitial oedema, Arrow - contraction bands, Round - fibre separation)

5.1.2 Part 2: Evaluation of Pro-Survival Signaling Pathways Involved in HO-1-Mediated Cardioprotection

5.1.2.1 Effect of Pharmacological Inhibitors of Pro-Survival Signaling Pathways on HO-1 Levels

Hearts treated with hemin had considerably higher HO-1 levels when compared to the control group (p<0.05). ZnPP IX as HO-1 inhibitor dramatically decreased HO-1 levels (p<0.05 vs. Hemin). Hearts treated with HO-1 inducer hemin and different pathway inhibitors retained high HO-1 level, proving that the inhibitors did not influence HO-1 induction. **Figure 5.13**.

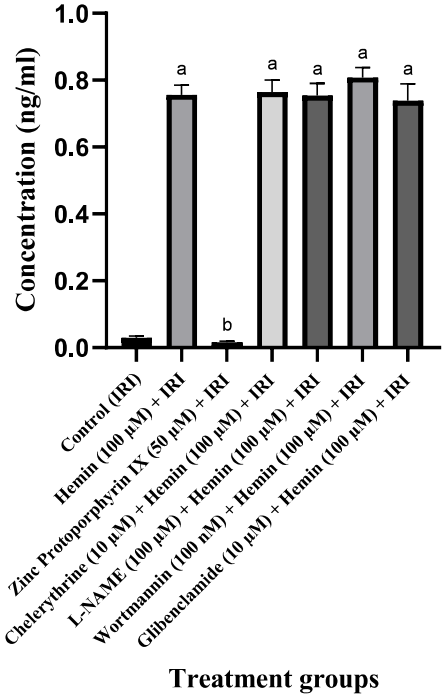
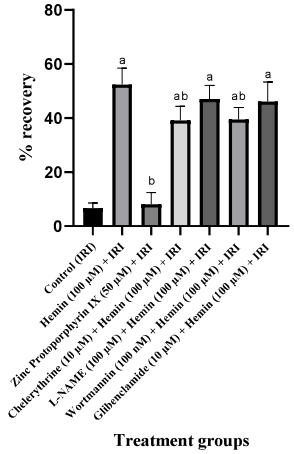


Figure 5.13: Effect of pharmacological inhibitors of various cardioprotective mechanisms on HO-1 levels. HO-1 levels was assessed following the induction of IRI. The data is reported as mean ± SEM (n = 6). 'a' indicated a p-value < 0.05 when compared to the control group (IRI) and 'b' indicated a p-value < 0.05 when compared to Hemin treated group.

5.1.2.2 Effect of Pharmacological Inhibitors of Prosurvival Signaling Pathways on Cardiodynamic and Hemodynamic Parameters

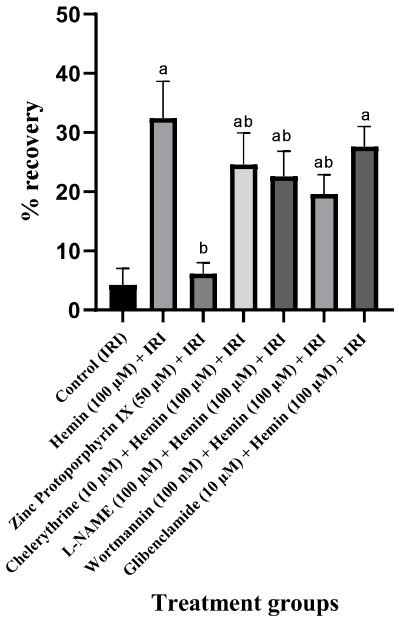
Hemin treatment substantially recovered cardiodynamic parameters dP/dt_{max}, dP/dt_{min}, LVDP, LVEDP and haemodynamic parameter coronary flow when compared to control (p<0.05). Although Heart rate was improved in hearts treated with HO-1 inducer hemin, the change was not statistically significant. HO-1 inhibitor ZnPP IX abolished these effects, showing that these observed effects were mediated by HO-1 induction. Furthermore, co-administration of HO-1 inducer hemin with particular Signaling pathway inhibitors such as Wortmannin (PI3K inhibitor), Chelerythrine (PKC inhibitor), L-NAME (NOS inhibitor), and Glibenclamide (K_{ATP} inhibitor) resulted in partial or complete loss of these functional gains.

For example, the recovery of cardiodynamic parameter LVDP was severely reduced in the group of hearts treated with PKC and PI3K inhibitors. Similar reduction in recovery was also observed in the cardiodynamic parameter LVEDP in the hearts treated with NOS, PI3K, and PKC inhibitors. (p<0.05 vs. Hemin) **Figure 5.14 and 5.15**. Similarly, recovery in dP/dt_{max} was considerably reduced in the NOS inhibitor group, whereas recovery in dP/dt_{min} decreased in both PI3K and NOS inhibitor-treated hearts. (p<0.05 vs. Hemin) **Figure 5.16 and 5.17.** BPM did not exhibit a significant change in any of the inhibitor treated groups, but recovery in coronary flow was considerably decreased with inhibition of NOS, PKC, K_{ATP} channels, and PI3K further validating the role of these prosurvival pathways in HO-1-mediated cardioprotection. (p<0.05 vs. Hemin) **Figure 5.18 and 5.19**.



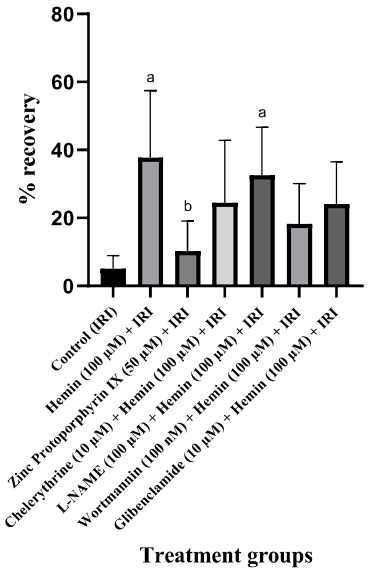
LVDP

Figure 5.14: Effect of pharmacological inhibitors of various cardioprotective mechanisms on LVDP in terms of % recovery. LVDP was before ischemia and after reperfusion. The data is reported as mean ± SEM (n = 6). 'a' indicated a *p*-value < 0.05 when compared to the control group (IRI) and 'b' indicated a *p*-value < 0.05 when compared to Hemin treated group.



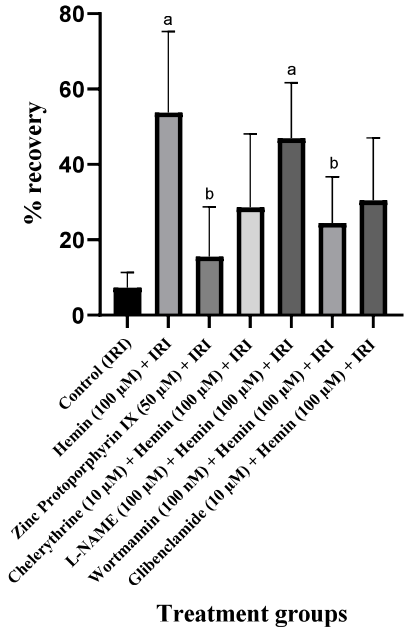
LVEDP

Figure 5.15: Effect of pharmacological inhibitors of various cardioprotective mechanisms on LVEDP in terms of % recovery. LVEDP was measured before ischemia and after reperfusion. The data is reported as mean ± SEM (n = 6). 'a' indicated a *p*-value < 0.05 when compared to the control group (IRI) and 'b' indicated a *p*-value < 0.05 when compared to Hemin treated group.



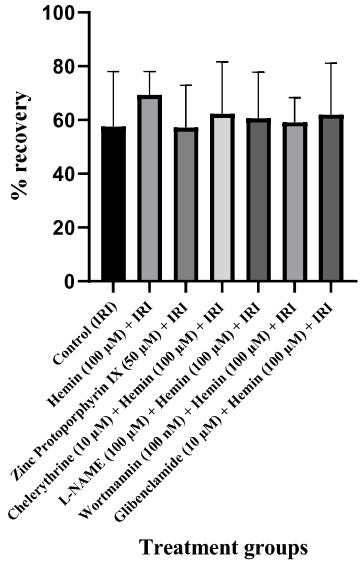
dP/dt$_{max}$

Figure 5.16: Effect of pharmacological inhibitors of various cardioprotective mechanisms on dP/dt$_{max}$ in terms of % recovery. dP/dt$_{max}$ was measured before ischemia and after reperfusion. The data is reported as mean ± SEM (n = 6). 'a' indicated a *p*-value < 0.05 when compared to the control group (IRI) and 'b' indicated a *p*-value < 0.05 when compared to Hemin treated group.



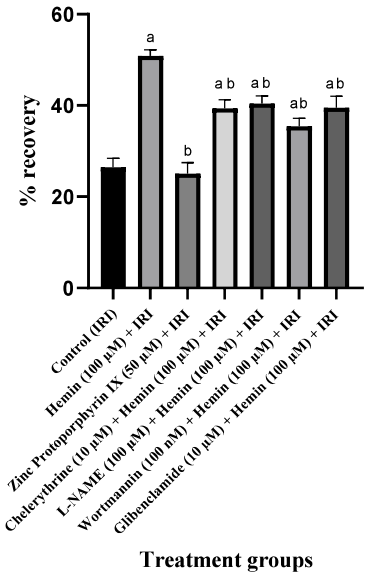
dP/dt_{min}

Figure 5.17: Effect of pharmacological inhibitors of various cardioprotective mechanisms on dP/dt_{min} in terms of % recovery. dP/dt_{min} was measured before ischemia and after reperfusion. The data is reported as mean ± SEM (n = 6). 'a' indicated a *p*-value < 0.05 when compared to the control group (IRI) and 'b' indicated a *p*-value < 0.05 when compared to Hemin treated group.



Heart rate

Figure 5.18: Effect of pharmacological inhibitors of various cardioprotective mechanisms on Heart rate in terms of % recovery. Heart rate was measured before ischemia and after reperfusion. The data is reported as mean ± SEM (n = 6). 'a' indicated a *p*-value < 0.05 when compared to the control group (IRI) and 'b' indicated a *p*-value < 0.05 when compared to Hemin treated group.

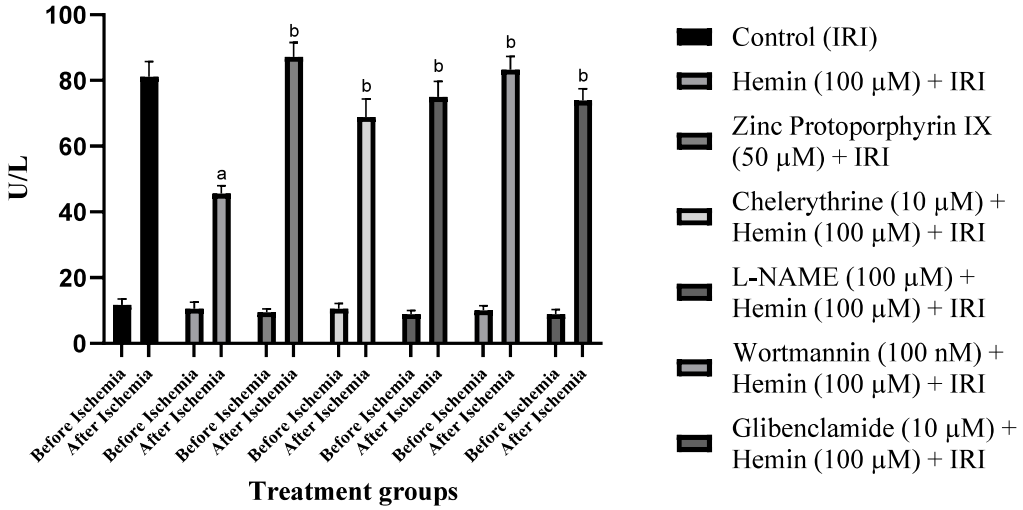


Coronary flow

Figure 5.19: Effect of pharmacological inhibitors of various cardioprotective mechanisms on Coronary flow in terms of % recovery. Coronary flow was measured before ischemia and after reperfusion. The data is reported as mean ± SEM (n = 6). 'a' indicated a *p*-value < 0.05 when compared to the control group (IRI) and 'b' indicated a *p*-value < 0.05 when compared to Hemin treated group.

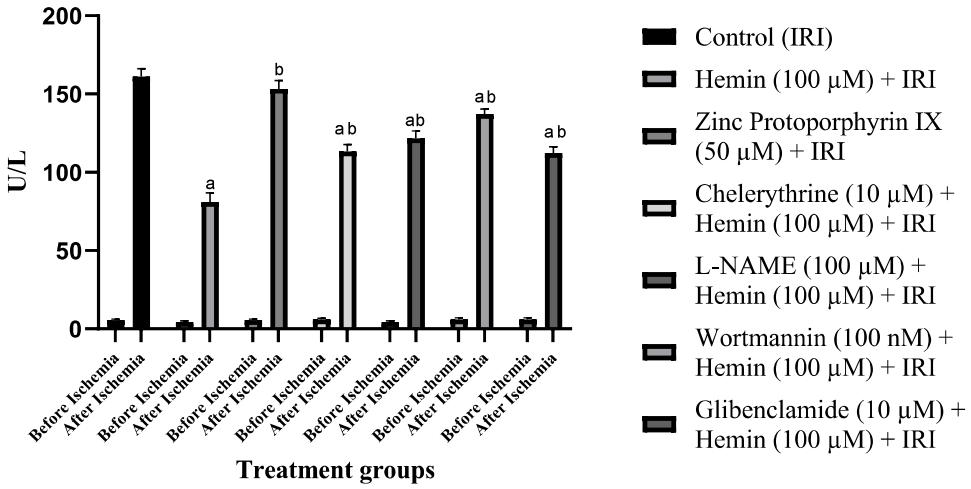
5.1.2.3 Effect of Pharmacological Inhibitors of Prosurvival Signaling Pathways on Myocardial Injury Markers

Co-treatment with ZnPP IX or pathway inhibitors dramatically elevated levels of LDH and CK-MB, which were considerably decreased by hemin (p<0.05). This suggests that HO-1's cardioprotective actions involve these signaling pathways. This pattern was found in all groups treated with pathway inhibitors. **Figure 5.20 and 5.21**.



CK-MB

Figure 5.20: Effect of pharmacological inhibitors of various cardioprotective mechanisms on the cardiac injury marker CK-MB. CK-MB was measured before induction of ischemia and after reperfusion. The data is reported as mean ± SEM (n = 6). 'a' indicated a *p*-value < 0.05 when compared to the control group (IRI) and 'b' indicated a *p*-value < 0.05 when compared to Hemin treated group.

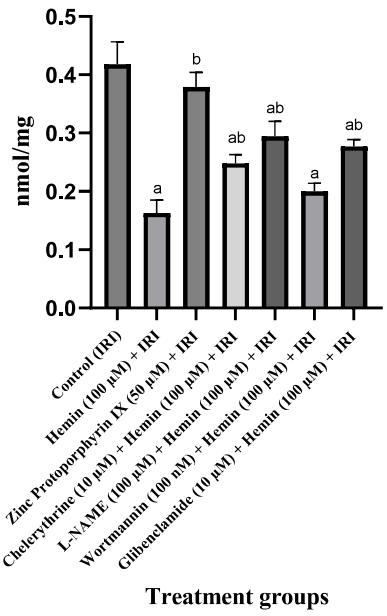


LDH

Figure 5.21: Effect of pharmacological inhibitors of various cardioprotective mechanisms on the cardiac injury marker LDH. LDH was measured before induction of ischemia and after reperfusion. The data is reported as mean ± SEM (n = 6). 'a' indicated a *p*-value < 0.05 when compared to the control group (IRI) and 'b' indicated a *p*-value < 0.05 when compared to Hemin treated group.

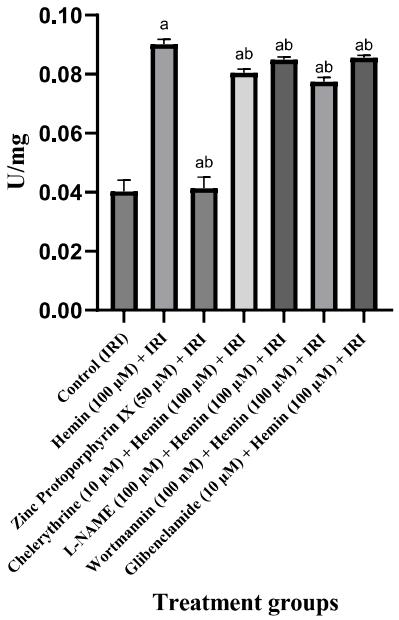
5.1.2.4 Effect of Pharmacological Inhibitors of Prosurvival Signaling Pathways on Oxidative Stress and Antioxidant

Hemin decreased MDA levels, whereas ZnPP IX as HO-1 inhibitor and other pathway inhibitors dramatically raised them (p<0.05 vs. Hemin), suggesting increased lipid peroxidation owing to the effect of oxidative stress **Figure 5.22**. SOD levels, which were increased with hemin treatment, reduced considerably in ZnPP IX treated group and other pathway inhibitor treated groups (p<0.05 vs. Hemin), indicating lower antioxidant defence **Figure 5.23**.



MDA

Figure 5.22: Effect of pharmacological inhibitors of various cardioprotective mechanisms on MDA. MDA was measured after IRI. The data is reported as mean ± SEM (n = 6). 'a' indicated a p-value < 0.05 when compared to the control group (IRI) and 'b' indicated a p-value < 0.05 when compared to Hemin treated group.

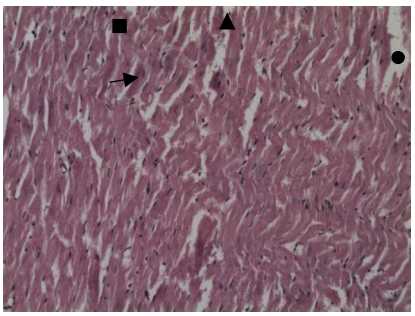


SOD

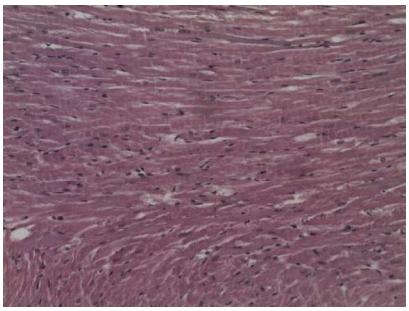
Figure 5.23: Effects of pharmacological inhibitors of various cardioprotective mechanisms on SOD. SOD was measured after IRI. The data is reported as mean ± SEM (n = 6). 'a' indicated *p*-value < 0.05 when compared to the control group (IRI) and 'b' indicated a *p*-value < 0.05 when compared to Hemin treated group.

5.1.2.5 Effect of Pharmacological Inhibitors of Prosurvival Signaling Pathways on Histopathology

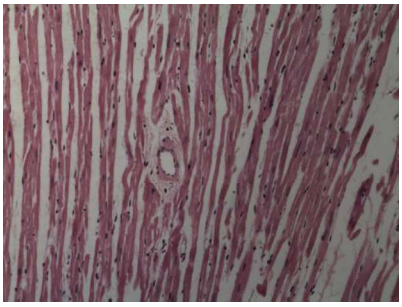
Histological examination demonstrated little tissue changes in the Hemin-treated group, with maintained cardiac architecture and fewer evidence of necrosis and oedema. In contrast, hearts treated with HO-1 inhibitor ZnPP IX or specific pathway inhibitors showed visible IRI features such as myocardial necrosis, contraction band, interstitial oedema, and fibre separation. The observed histological changes were in line with the biochemical and functional results, further validating the role of prosurvival pathways in producing HO-1-induced protection **(Figure 5.24)**.



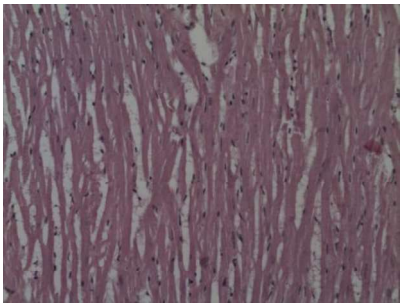
Control group (IRI)



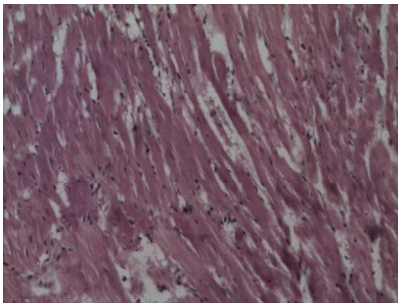
Hemin (100 µM) + IRI



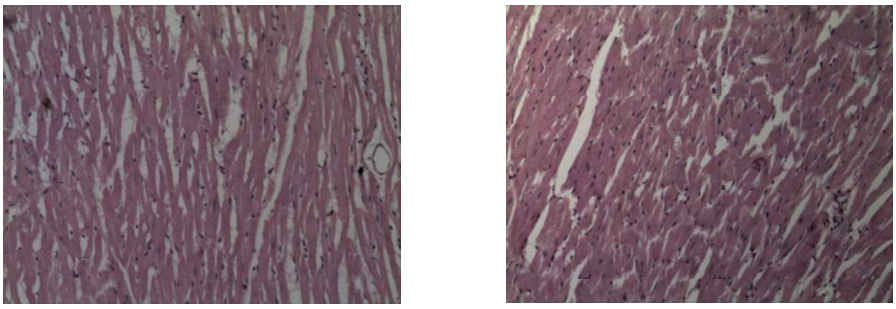
Zinc Protoporphyrin IX (50 µM) + IRI



Chelerythrine (10 µM) + Hemin (100 µM) + IRI



L-NAME (100 µM) + Hemin (100 µM) + IRI



Wortmannin (100 nM) + Hemin (100 µM) + IRI

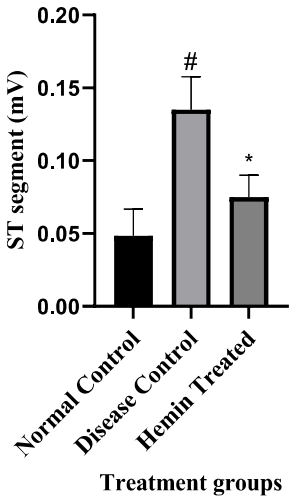
Glibenclamide (10 µM) + Hemin (100 µM) + IRI

Figure 5.24: Effect of pharmacological inhibitors of various cardioprotective mechanisms on Histopathology. (H&E Staining, 400x). (Structural changes: Square - myocardial necrosis, Triangle - interstitial oedema, Arrow - contraction bands, Round - fibre separation)

5.2 In Vivo Study: Isoproterenol-Induced Myocardial Infarction in rats

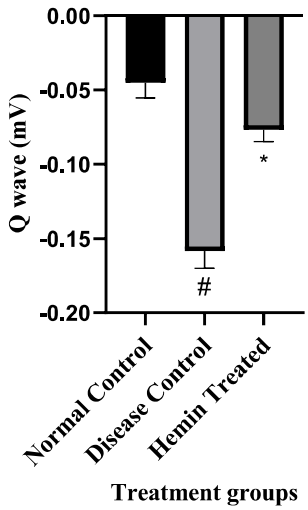
5.2.1 Effects of HO-1 Induction by Hemin on ECG Parameters

Isoproterenol treatment in the disease control group resulted in considerable ST-segment elevation, the emergence of pathological Q waves, and an increase in heart rate (BPM) compared to the normal control group. HO-1 induction by Hemin significantly decreased ST-segment elevation and normalised Q-wave shape, combined with a considerable reduction in heart rate compared to the disease control, indicating an improvement in myocardial electrical stability. **(Figure 5.25, 5.26 and 5.27)**.



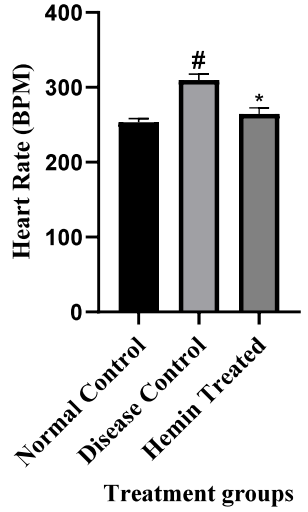
ST-segment

Figure 5.25: Effects of HO-1 induction by Hemin on ST-segment. The data is reported as mean ± SEM (n = 6). '#' indicated a *p*-value < 0.05 compared to the normal control group and '*' indicated a *p*-value < 0.05 compared to the disease control group.



Q-wave

Figure 5.26: Effects of HO-1 induction by Hemin on Q-wave. The data is reported as mean ± SEM (n = 6). '#' indicated a *p*-value < 0.05 compared to the normal control group and '*' indicated a *p*-value < 0.05 compared to the disease control group.

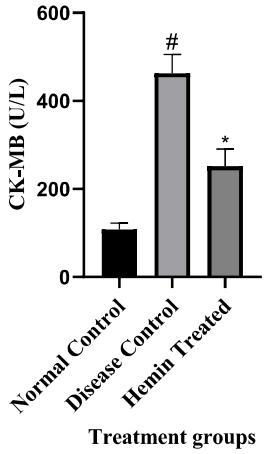


Heart rate (BPM)

Figure 5.27: Effects of HO-1 induction by Hemin on Heart rate (BPM). The data is reported as mean ± SEM (n = 6). '#' indicated a *p*-value < 0.05 compared to the normal control group and '*' indicated a *p*-value < 0.05 compared to the disease control group.

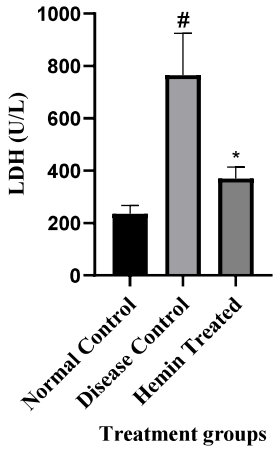
5.2.2 Effects of HO-1 Induction by Hemin on Myocardial Injury Markers

LDH and CK-MB, two indicators of cardiac damage, were measured in the blood serum. LDH and CK-MB levels were significantly raised in the disease control group, demonstrating the severity of isoproterenol-induced heart damage. HO-1 induction by Hemin considerably lowered both CK-MB and LDH levels when compared to the disease control group, demonstrating the protective effect against myocardial damage. **(Figure 5.28 and 5.29)**.



CK-MB

Figure 5.28: Effects of HO-1 induction by Hemin on CK-MB. The data is reported as mean ± SEM (n = 6). '#' indicated a *p*-value < 0.05 compared to the normal control group and '*' indicated a *p*-value < 0.05 compared to the disease control group.

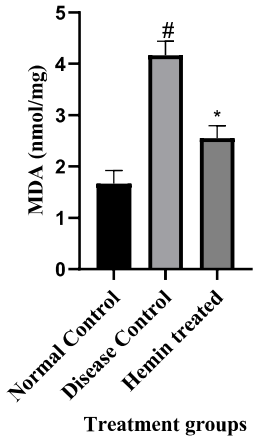


LDH

Figure 5.29: Effects of HO-1 induction by Hemin on LDH. The data is reported as mean ± SEM (n = 6). '#' indicated a *p*-value < 0.05 compared to the normal control group and '*' indicated a *p*-value < 0.05 compared to the disease control group.

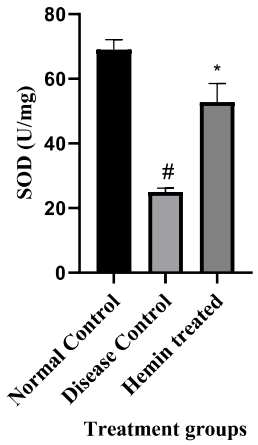
5.2.3 Effects of HO-1 Induction by Hemin on Oxidative Stress Marker and Antioxidant Enzyme

Isoproterenol exposure increased malondialdehyde (MDA) levels in the heart tissue, suggesting lipid peroxidation and oxidative stress. Hemin administration and subsequent HO-1 induction resulted in a considerable reduction in MDA levels, suggesting its involvement in reducing oxidative damage via HO-1 induction. The disease control group had significantly lower superoxide dismutase (SOD) and catalase activity, indicating inadequate antioxidant defence. Hemin therapy restored both SOD and catalase activity around normal ranges, confirming its involvement in improving endogenous antioxidant systems. **(Figure 5.30, 5.31 and 5.32)**.



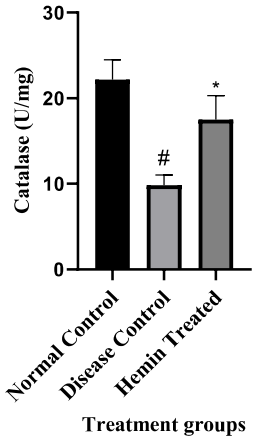
MDA

Figure 5.30: Effects of HO-1 induction by Hemin on oxidative stress marker MDA. The data is reported as mean ± SEM (n = 6). '#' indicated a *p*-value < 0.05 compared to the normal control group and '*' indicated a *p*-value < 0.05 compared to the disease control group.



SOD

Figure 5.31: Effects of HO-1 induction by Hemin on SOD. The data is reported as mean ± SEM (n = 6). '#' indicated a *p*-value < 0.05 compared to the normal control group and '*' indicated a *p*-value < 0.05 compared to the disease control group.



Catalase

Figure 5.32: Effects of HO-1 induction by Hemin on catalase. The data is reported as mean ± SEM (n = 6). '#' indicated a *p*-value < 0.05 compared to the normal control group and '*' indicated a *p*-value < 0.05 compared to the disease control group.

5.2.4 Effects of Hemin on HO-1

HO-1 levels, as measured by ELISA, were considerably higher in the Hemin-treated group compared to both normal and disease control groups, indicating the induction of HO-1 enzyme by Hemin. **(Figure 5.33).**

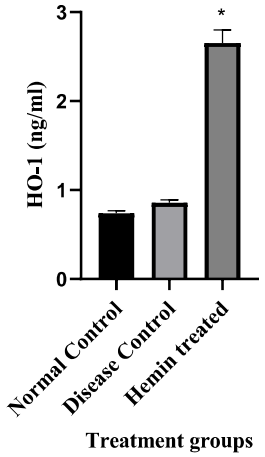


Figure 5.33: Effects of Hemin on HO-1. The data is reported as mean ± SEM (n = 6). '#' indicated a *p*-value < 0.05 compared to the normal control group and '*' indicated a *p*-value < 0.05 compared to the disease control group.

5.2.5 Effects of HO-1 Induction by Hemin on PI3K

PI3K levels were considerably lowered in the disease control group after isoproterenol treatment. Hemin pretreatment substantially increased PI3K levels compared to the disease control group, indicating a possible involvement of PI3K signaling in HO-1-mediated cardioprotection **(Figure 4.34)**.

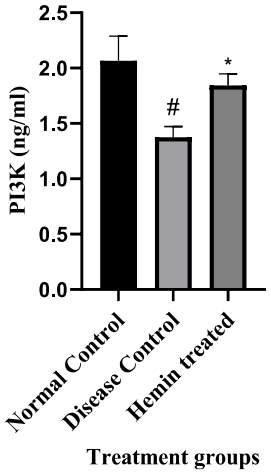
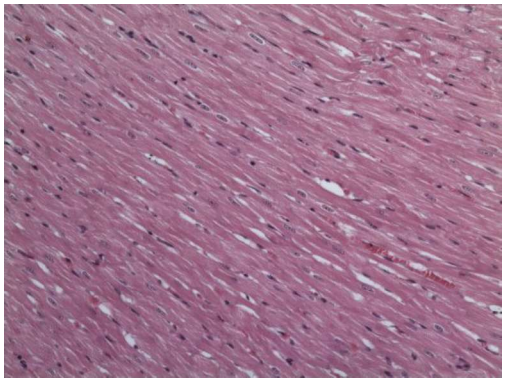


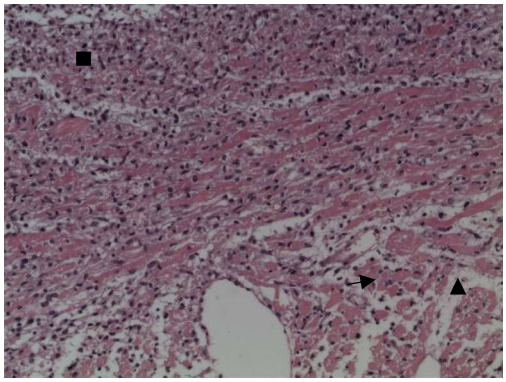
Figure 5.34: Effects of HO-1 induction by Hemin on PI3K. The data is reported as mean ± SEM (n = 6). '#' indicated a *p*-value < 0.05 compared to the normal control group and '*' indicated a *p*-value < 0.05 compared to the disease control group.

5.2.6 Effect of HO-1 Induction by Hemin on Cardiac Histopathology

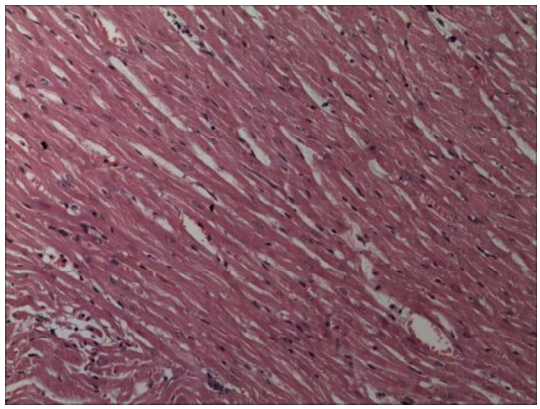
The histopathological analysis of the disease control group's cardiac tissue showed typical myocardial infarction signs such as myocardial fibre rupture, necrosis, inflammatory cell infiltration, and oedema. In contrast, heart tissues from the Hemin treated group showed notably maintained myocardial architecture, decreased necrosis, and low inflammatory infiltration, corroborating prior results of HO-1-mediated cardioprotection. **(Figure 5.35)**.



(A) Normal Control



(B) Disease Control



(C) Hemin Treated

Figure 5.35: Effects of HO-1 induction by Hemin on histopathology (H&E Staining, 400x) (Structural changes: Square – neutrophil infiltration, Triangle - interstitial oedema, Arrow - necrosis)

5.3 In Vitro Study (ISO-Induced Myocardial Injury in H9c2 Cardiomyoblasts)

Cell viability was significantly reduced after ISO exposure, as determined by the MTT test**.** Cellular damage was also found after ISO exposure, as shown by an increase in CK-MB levels in the cell culture supernatant. The cell viability increased significantly in a dose-dependent manner with Hemin pretreatment. These alterations demonstrate that HO-1 has substantial cytoprotective effects against ISO-induced damage **(Figure 5.36)**. There was also a substantial reduction in CK-MB release in Hemin-treated cell culture supernatants **(Figure 5.37)**. Based on these findings, it may be concluded that HO-1 induction protects cardiomyocytes from harm while also increasing survival. There was a substantial reduction in Phosphoinositide 3-kinase (PI3K) levels in the H9c2 cell lysate of the ISO-treated group, showing that ISO inhibited the PI3K signaling. HO-1 induction by Hemin resulted in considerably higher PI3K levels than the ISO-treated group **(Figure 5.38)**.

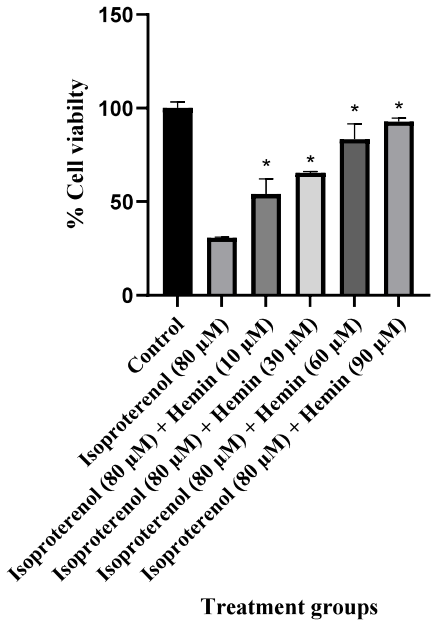


Figure 5.36: Effects of HO-1 induction by Hemin on Cell viability. The data is reported as mean ± SEM (n = 3). '*' indicated a *p*-value < 0.05 compared to the normal control group.

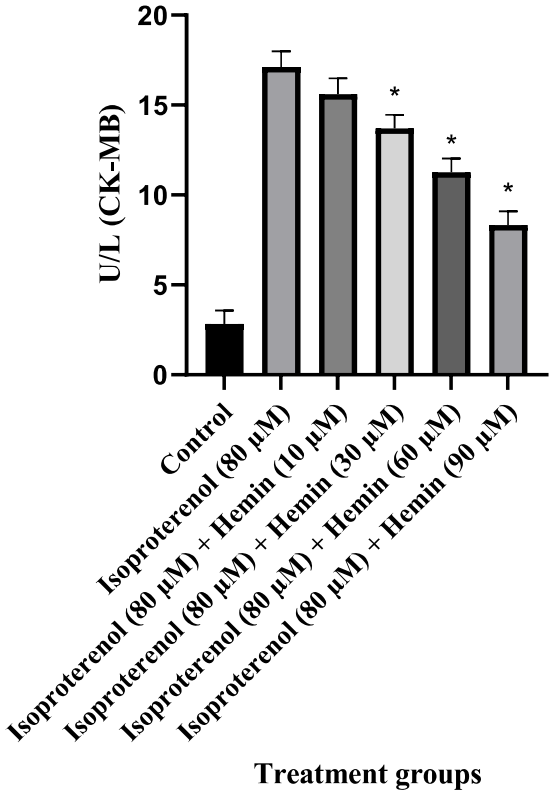


Figure 5.37: Effects of HO-1 induction by Hemin on CK-MB. The data is reported as mean ± SEM (n = 3). '*' indicated a *p*-value < 0.05 compared to the normal control group.

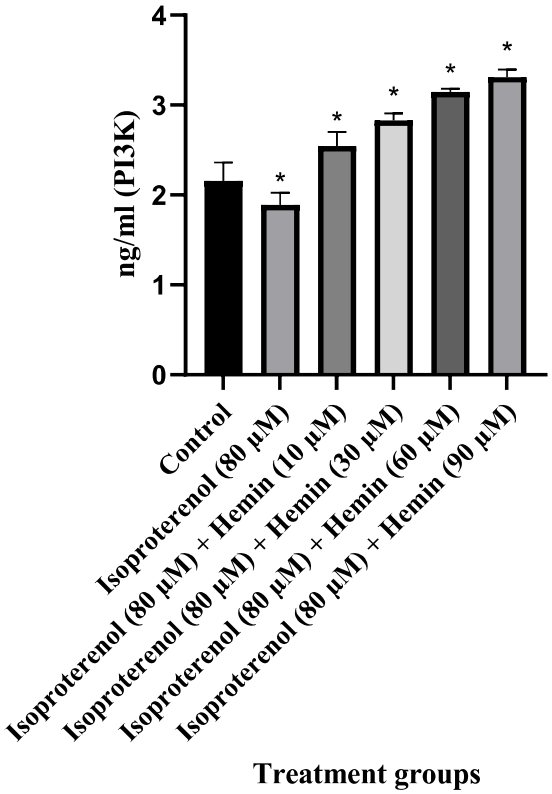


Figure 5.38: Effects of HO-1 induction by Hemin on PI3K. The data is reported as mean ± SEM (n = 3). '*' indicated a *p*-value < 0.05 compared to the normal control group.

6. DISCUSSION

The current study investigated the cardioprotective effects of Heme Oxygenase-1 modulation in myocardial infarction (MI) by its inducer Hemin (Lo et al., 2006), and examined its potential interaction with key pro-survival signaling pathways using ex vivo, in vivo, and in vitro experimental models.

Ex-vivo experimental approach involved the evaluation of the effects of HO-1 modulation in isolated rat hearts subjected to ischemia–reperfusion injury using Langendorff technique. The investigation was carried out in two parts. Investigation in the first part of the study involved assessment of the cardioprotective effect of HO-1 generated by various dosages of Hemin as HO-1 inducer in isolated rat hearts, which were subjected to an ex vivo method of ischemia reperfusion injury. Three doses of HO-1 inducer Hemin were chosen based on prior studies: low (10 μM), medium (50 μM), and high (100 μM). The investigation in the second part of the study involved the exploration of the underlying mechanism of HO-1-mediated cardioprotection.

HO-1 was induced by Hemin in the hearts of the various groups in a dose-dependent manner, as evident by increased HO-1 levels in cardiac tissue homogenate. HO-1 enzyme causes catalytic degradation of heme into biliverdin, carbon monoxide (CO), and iron, which have been shown to have various beneficial effects such as anti-inflammatory, antioxidant, anti-apoptotic, and vasodilatory, effects necessary for protecting the heart during ischemia-reperfusion injury (Drummond et al., 2020). Induction of HO-1 also confers direct cardioprotection independent of its enzymatic products (Consoli et al., 2021). In this study, the recovery of several cardiodynamic and haemodynamic measures (LVEDP, LVDP, dP/dt_{max}, dP/dt_{min}, and coronary flow), a decrease in myocardial injury markers (LDH and CK-MB), and improvement in histopathological findings indicated the HO-1-mediated cardioprotection. The ventricles' ability to generate pressure determines the Cardiac contractile function and performance. Reduced LVDP indicates impaired contractility (Dokainish, 2015). LVEDP indicates ventricular compliance, preload, and cardiac performance. It indicates the pressure and volume in the left ventricle of the heart just before contraction. Abnormal LVEDP indicates diastolic dysfunction (Mielniczuk et al., 2007). Hemin-treated hearts preserved the cardiac function during IRI as indicated by improvement in LVDP and LVEDP. dP/dt_{max} and dP/dt_{min} measure contraction and relaxation of the heart,

respectively (Ogilvie et al., 2020). Hemin treatment improved both dP/dt_{max} and dP/dt_{min}. The heart rate determines the demand and supply balance of myocardial oxygen. Injury to the myocardium makes it difficult to maintain adequate perfusion and causes a mismatch between myocardial perfusion and contraction. The process of IRI causes fluctuations in the heart rate in the form of bradycardia and tachycardia and may also precipitate cardiac arrhythmia (Indolfi & Ross, 1993). Similar changes were also observed in this study, as indicated by the findings of heart rate, across treatment groups. Flow in the coronary circulation was deteriorated during the ischemic phase, resulting in myocardial ischemic injury. Sudden reinstatement of perfusion after ischemic phase, causes many changes such as increased vascular permeability, endothelial dysfunction, and microvascular obstruction (Hausenloy et al., 2019). Hemin treatment improved coronary circulation, as evidenced by increased coronary flow. Myocardial injury can be detected by checking the presence of cardiac biomarkers. Lower CK-MB and LDH levels in hemin-treated groups indicated reduced myocardial injury (Kehl et al., 2012). IRI causes oxidative stress, which overwhelms the antioxidant defence system and damages the structural and functional aspects of the myocardium (Dhalla et al., 2000). Hemin treatment reduced oxidative stress, as measured by MDA and increased antioxidant capacity, and SOD levels. IRI causes a variety of histopathological changes, including myocardial necrosis, interstitial oedema, contraction band, and fibre separation (Duncanson & Mackey-Bojack, 2018). These harmful histopathological changes were also alleviated in hemin-treated hearts.

Interplay between different cellular and molecular pathways controls myocardial survival during IRI. Targeting these prosurvival pathways may reduce IRI-induced damage and exert cardioprotection (M. Zhang et al., 2024). Several studies have identified PI3K, PKC, NOS, and K_{ATP} channels as important prosurvival signaling pathways during myocardial IRI. PI3K signaling protects the myocardium during ischemia-reperfusion injury by inhibiting apoptosis, lowering oxidative stress, suppressing inflammation, and promoting angiogenesis and tissue repair (Deng & Zhou, 2023). PKC regulates redox signaling, calcium homeostasis, cell death, oxidative stress, and mitochondrial function and thus provides cardioprotection in myocardial ischemia/reperfusion injury (Chen et al., 2021). Nitric oxide causes vasodilation, anti-inflammatory actions, and mitochondrial stabilisation, resulting in a protective effect during IRI (Schulz et al., 2004). ATP-sensitive potassium channels prevent calcium overload, reduce oxidative stress, conserve energy, protect mitochondrial function, increase blood flow, and modulate inflammation, thereby protecting the heart during IRI

(Grover & Garlid, 2000). The current study investigates the potential role of the aforementioned prosurvival signaling pathways in mediating cardioprotection conferred by HO-1 induction by hemin. In the present study, co-treatment with inhibitors of pathways (Chelerythrine, Wortmannin, L-NAME, and Glibenclamide) attenuated or abolished the cardioprotective effects of HO-1 induction, which supports the role of these pathways in mediating cardioprotection.

This study makes a significant contribution by demonstrating that HO-1-mediated cardioprotection occurs due to interplay of multiple pro-survival signaling pathways in an ex vivo model of myocardial ischemia-reperfusion injury. Their functional relevance is supported by the attenuation of protective effects upon inhibition of each pathway. Hemin's protective effects were completely abolished by the administration of Zinc Protoporphyrin IX, a selective HO-1 inhibitor, confirming the central role of HO-1 activity in mediating cardiovascular protection. When PI3K was inhibited, the protective effects were mostly lost. This suggests that the PI3K pathway plays an important role in mediating cardioprotective effects. The results strongly support that HO-1 is a key upstream regulator. It helps coordinate several survival mechanisms, such as reducing heart injury, lowering oxidative stress, and preserving heart function and structure after ischemia-reperfusion.

The findings of the ex vivo study were further supported through the evaluation of the cardioprotective effect of Heme oxygenase-1 (HO-1) induced by Hemin using a well-established model of Isoproterenol-induced myocardial infarction (MI) in rats, with particular emphasis on the involvement of the PI3K signaling pathway (Oh & Ishikawa, 2018). Improvements in ECG patterns, cardiac enzyme markers, oxidative stress profiles, antioxidant enzyme activities, and histopathological integrity demonstrated the cardioprotective effect following HO-1 induction. The findings also highlight the possible mechanistic association between HO-1 mediated cardioprotection and the PI3K pathway. This provides further insight into the cellular mechanisms underlying HO-1-mediated cardioprotection.

Isoproterenol-induced myocardial infarction in rats is a well-validated model for mimicking human cardiac injury. The model induces similar electrical conduction abnormalities as well as biochemical and structural derangements as those of the MI in humans (Halim et al., 2018). In the present study, the disease control group exhibited ST-segment elevation, pathological Q-wave formation, and increased heart rate. These changes reflect electrical

abnormality and acute myocardial damage. The ECG findings are consistent with the classic pathophysiological changes of myocardial infarction, where ischemia-induced ion channel dysfunction and conduction block from infarcts lead to ECG abnormalities (Song et al., 2024; White & Chew, 2008). On the other hand, the Hemin-treated group revealed a significant normalization of these abnormal ECG changes indicating a preservation of myocardial electrical stability. The cardioprotective effects related to electrophysiological changes can be mechanistically attributed to the activation of the PI3K pathway. The PI3K pathway controls ion channel activity under various stress conditions to maintain membrane potential stability in cardiomyocytes (Ballou et al., 2015). PI3K signaling is activated and results in phosphorylation of downstream targets including GSK-3beta, eNOS and FOXO. These targets regulate a wide range of cellular functions including calcium homeostasis, myocardial contractility and resistance to apoptotic stimuli (Sugden, 2003). The improved ECG parameters in Hemin-treated groups could be attributed to PI3K-mediated stabilization of ion transport and Signaling mechanisms. This increased electrical conduction and rhythm during ischemic stress.

The elevation of CK-MB and LDH in serum suggests myocardial membrane damage and cell necrosis. Cardiac injury markers were significantly increased in isoproterenol treated group. The anomaly is an indication of irreversible cellular damage and impaired membrane integrity (Nigam, 2007). This pattern is similar to previous studies where CK-MB and LDH were identified as sensitive markers of myocardial injury (Kemp et al., 2004). Hemin pretreatment significantly reduced the levels of cardiac injury markers, suggesting protective effects against membrane rupture and cellular leakage. PI3K signaling activation leads to increased expression of anti-apoptotic proteins, such as Bcl-2, and decreased pro-apoptotic signals, such as Bax and caspase-3, thus preventing the transition from early stress responses to necrotic or apoptotic cell death (X. He et al., 2022). The well documented biochemical protection is likely at least partially mediated by PI3K stimulated enhancement of cellular life preserving pathways that enhance membrane integrity.

Oxidative stress plays a central role in isoproterenol-induced myocardial damage, and this was reflected in our study by the increase in MDA levels and the concurrent suppression of SOD and catalase activity in the disease control group. Elevated MDA level confirm the occurrence of lipid peroxidation, a direct result of free radical build up during the course of MI (Zheng et al., 2024). The ability of Hemin to reduce MDA levels and restore the antioxidant enzyme emphasizes HO-1's regulatory role in redox homeostasis, which has

been reported to occur via both its enzymatic degradation products and its indirect antioxidant signaling (Sorrenti, 2023). PI3K activation exerts a significant antioxidant effect by promoting the nuclear translocation of a key transcription factor Nrf2. The Nrf2 regulates antioxidant gene expression (Lin et al., 2007). The Nrf2/PI3K feedback loop increases enzymatic detoxification and removal of reactive oxygen species (ROS) and thereby increases cellular resilience to oxidative stress. The normalization of MDA, SOD, and CAT levels observed in the Hemin treated group could likely be attributed to this important protective mechanism, in which both HO-1 and PI3K act synergistically to neutralize oxidative damage.

A significant increase in HO-1 levels was observed in the hemin-treated group, as determined by ELISA. HO-1 is an inducible stress-response enzyme that degrades pro-oxidant heme into carbon monoxide, biliverdin, and free iron. Each of them possess distinct cytoprotective functions (Origassa & Câmara, 2013). Bilirubin acts as a potent lipid antioxidant, while carbon monoxide modulates inflammatory pathways and anti-apoptotic signaling. Each of these heme degradation products may contribute to the reduction in myocardial injury.

HO-1 induction by hemin was associated with the restoration of PI3K activity, which was significantly suppressed in the disease control group. The PI3K signaling pathway activation is one of the heart's adaptive responses to the stressful events such as ischemic injury. It is known for its roles in cellular survival, metabolism, angiogenesis and other protective cellular processes (Walkowski et al., 2022). The reduction in PI3K levels in the disease control group likely reflects the inhibitory effects of excessive oxidative stress on survival pathways, as previously reported in models of myocardial infarction and oxidative injury (Ghigo et al., 2017). The restoration of PI3K levels following hemin administration indicates that HO-1-mediated cardioprotection may be mediated through PI3K signaling.

Histopathological analysis also confirmed myocardial protection at the structural level. Isoproterenol exposure induced widespread myocyte necrosis, inflammatory infiltration, and interstitial edema, which are characteristic of infarct pathology (Pasotti et al., 2006). In contrast, Hemin treated hearts showed preserved myocardial architecture with lesser necrosis and inflammation. Cardioprotection through activation of the PI3K pathway has been demonstrated histologically to increase cardiomyocyte survival, reduce oxidative stress and

inflammation, promote angiogenesis and stabilize mitochondrial function. (Z. Zhang & Guo, 2025).

The primary focus of this study was the acute phases of myocardial injury. However, the reduction in cellular necrosis and preservation of cardiac architecture may prevent long-term cardiac remodelling too. Cardiac remodeling is mainly driven by processes such as infarct expansion, inflammation and oxidative stress (Contessotto & Pandit, 2021). These effects were significantly attenuated in current study following HO-1 induction. Moreover, PI3K pathway has also been documented to protect against long-term maladaptive hypertrophy and fibrosis (Aoyagi & Matsui, 2011). This highlights the clinical promise of the HO-1 induction.

Collectively, these findings support the involvement of PI3K Signaling in HO-1 mediated cardioprotection. PI3K activation results in multifaceted cardioprotection through restoration of electrical stability, membrane integrity, oxidative balance, and structural preservation. This establishes PI3K signaling as a critical mediator of HO-1-mediated cardiac resilience.

In vitro experimental approach involved the assessment of the effects of HO-1 induction in H9c2 cardiomyoblasts subjected to isoproterenol-induced injury. There was significant improvement in the cell viability and reduced injury of H9c2 cardiomyoblasts following HO-1 induction. In addition, PI3K levels were increased following HO-1 induction, suggesting that the protective association between HO-1 and PI3K signaling is preserved even at the cellular level. These findings support the view that HO-1 exerts direct cytoprotective effects on cardiomyoblasts and that this response is not solely dependent on systemic or organ-level influences.

The present study has certain limitations. The study is confined to the evaluation of effects of HO-1 modulation in ex vivo, in vivo and in vitro experimental models of myocardial injury and does not extend to clinical evaluation. The pharmacological modulation of HO-1 is restricted to the use of Hemin as HO-1 inducer and Zinc Protoporphyrin IX (ZnPP IX) as an HO-1 inhibitor. The mechanistic investigations are limited to the specific pro-survival signaling pathways such as PI3K, PKC, NOS, and K_{ATP} channels. The interpretation of findings is limited to the assessment of various parameters indicating myocardial injury or survival, including cardiodynamic indices (LVDP, LVEDP, dp/dt_{max}, dp/dt_{min}, BPM, and

coronary flow), biochemical markers of cardiac injury (CK-MB, LDH), oxidative stress markers (MDA, SOD, Catalase), and HO-1 and PI3K levels.

Overall, HO-1 induction led to consistent cardioprotection in the form of improvement in the cardiac functional, biochemical, and histopathological parameters across all experimental models. These findings reinforce the therapeutic potential of HO-1 modulation in myocardial injury. HO-1 modulation was achieved through the use of Hemin and zinc protoporphyrin IX (ZnPP IX) to elucidate the effects of HO-1 induction and inhibition, respectively. Significant cardioprotection was achieved through the HO-1 induction across various models. HO-1 inhibition abolished protective effects in ex vivo model, thereby establishing a relationship between HO-1 activity and cardioprotection. Mechanistic exploration revealed the involvement of multiple pro-survival signaling pathways such as PI3K, PKC, NOS, and K_{ATP} in mediating cardioprotective effects following HO-1 induction. Among these pathways, PI3K signaling was found to exert significant cardioprotection following HO-1 induction. The use of pathway-specific inhibitors demonstrated that inhibition of these pathways significantly reduced the protective responses to varying degree, indicating their contribution to HO-1-mediated cardioprotection. Collectively, the findings of this study provide integrated experimental evidence supporting the cardioprotective role of HO-1 and its interaction with key pro-survival signaling pathways in myocardial injury.

7. CONCLUSION

The current study systematically evaluated the effects of Heme oxygenase-1 modulation across ex-vivo, in-vivo and in-vitro models of myocardial injury and its potential to activate prosurvival mechanisms. HO-1 induction by hemin improved cardiac functional, biochemical and histological parameters, thereby increasing cardiomyocyte survival, and reduced damage. HO-1 inhibition led to reversal of change in the aforementioned parameters, which confirmed the cardioprotective role of HO-1. Moreover, mechanistic evaluation using pathway specific inhibitors of PI3K, PKC, NOS, and K_{ATP} channels confirmed that HO-1 mediated cardioprotection involved activation of multiple prosurvival signaling pathways. Among these pathways, PI3K signaling was found to have pronounced effect on HO-1 mediated cardioprotection, as its inhibition significantly abolished the protective effects of HO-1.

Previous studies on HO-1 have mainly focused on the antioxidant, anti-inflammatory and anti-apoptotic functions of its metabolic byproducts such as bilirubin and carbon monoxide. The current study provide novel insights that extends previous reports of HO-1-mediated cardioprotection. In this study, HO-1 has been revealed as an upstream regulator of important intracellular signaling pathways that are involved in the cardioprotection. This study shows the intricate relationships between the protective effects of HO-1 and the regulation of pro-survival signaling pathways through the integration of in vivo, ex vivo and in vitro experimental models. The finding that PI3K inhibition almost completely abolishes the HO-1-induced protection suggests that PI3K pathway is an important downstream mediator of HO-1 activity.

These findings collectively reveal HO-1 as an important upstream regulator of cardioprotective mechanisms. Cardioprotective effects across various models highlight the translational relevance of HO-1 and makes HO-1 modulation a promising therapeutic strategy for myocardial injury and infarction.

8. CONTRIBUTION OF THE STUDY

The current study contributed to the field of cardiovascular pharmacology by extending current understanding of biological importance of Heme oxygenase-1 (HO-1). The study specifically focuses on elucidating the role of key prosurvival signaling mechanisms activated following HO-1 induction.

The key contributions of this work are outlined below:

- Protective role of HO-1 in the myocardial injury was established trough the improvement in functional, biochemical and histopathological parameters following HO-1 induction across in vivo, ex vivo and in vitro experimental models. HO-1 inhibition by Zinc protoporphyrin IX attenuated these protective effects, confirming the protective role.
- Significant contribution related to the mechanistic insights about HO-1 mediated cardioprotection was also provided through investigation of potential involvement of prosurvival signaling pathways such as PI3K, PKC, NOS, and K_{ATP} following HO-1 induction. A significant role of PI3K signaling in the HO-1 mediated cardioprotection among the investigated pathways was also established through the findings that inhibition of this pathway results in maximum attenuation of the protective effects.
- Overall, this study advances the current understanding of protective effects of HO-1 by providing mechanistic insights through the investigation of prosurvival signaling pathways involved in cardioprotection following HO-1 induction. Based on these observations, future experimental investigations and therapeutic strategies targeting HO-1 could be devised.

9. SCOPE OF FURTHER WORK

- Future studies should investigate the downstream signaling pathways associated with the HO-1–PI3K/Akt axis and examine their interaction with other cardioprotective signaling cascades.
- Apart from the existing pharmacological inhibition approach, other molecular experiments such as targeted gene silencing of HO-1 and PI3K should be performed to further substantiate the evidence of HO-1 mediated cardioprotection via PI3K signaling.
- Future studies should be designed to evaluate the impact of sex, age and comorbid conditions on HO-1 mediated cardioprotection for better translation of these findings to diverse clinical populations.
- Nanoparticle-based delivery systems encapsulating hemin may be explored to achieve targeted delivery to the ischemic myocardium, thereby minimizing systemic exposure and reducing the risk of iron-related toxicity.
- Screening and development of novel non-iron-based synthetic compounds that can activate the HO-1/PI3K/Akt protective pathway may provide safer therapeutic options for long-term cardioprotection.
- Further research is required to evaluate HO-1 gene modulation and its potential role as a clinical biomarker for assessing cardioprotection and therapeutic response.
- The development of adjunctive therapies targeting HO-1-mediated protective mechanisms may offer additional benefit during the management of acute myocardial infarction.

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11. LIST OF PUBLICATIONS

1. Koria H, Mehta A. Hemin-induced HO-1 protects isolated rat hearts from ischemia-reperfusion injury by activation of pro-survival Signaling pathways. Am J Transl Res. 2025;17(10):7626–7639. doi:10.62347/XJXV3554.

2. Koria H, Mehta A. Hemin-Induced Heme Oxygenase-1 Induction Preserves Myocardial Architecture and Functions Via PI3K Signaling in Isoproterenol-Induced Myocardial Infarction in Rats. JBras Patol Med Lab.2025; 61(2): 245-251. doi: 10.1900/JBPML.2025.61.02.0 41.

3. Koria H, Mehta A. Cardioprotection at the Molecular Interface: Extracellular and Intracellular Targets in MI Management. Int. J. Pharm. Sci. Rev. Res. 2025;85(6):8-17. doi: 10.47583/ijpsrr.2025.v85i06.002.

ANNEXURE-I

Certificate

This is to certify that the project proposal no RPCP/IAEC/2020-21/R16 (Extension) entitled Effects of Heme oxygenase - 1 modulation in Myocardial infarction in rats submitted by Mr. Koria Hardik Mukeshkumar has been approved/recommended by the IAEC of Ramanbhai Patel College of Pharmacy (Organization) in its meeting dated 14/09/2021 and has been sanctioned 150 (animals) under this proposal for a duration of next 12 months.

Authorized by	Name	Signature	Date
Chairman:	**Dr. Manan Raval**	Manan	4/9/21
Member Secretary:	**Dr. Nilay Solanki**	NDS	4/9/21
Main Nominee of CPCSEA:	**Dr. Tejal Gandhi**		4/9/21