A study on the comparative and combinatorial efficacy of resveratrol with angiotensin-converting enzyme inhibitor and angiotensin receptor blocker/neprilysin inhibitor in myocardial infarction

By

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Abstract

Myocardial infarction (MI) and heart failure are the two major health care burdens around the world. Resveratrol, a plant polyphenol, is well-documented for its benefits in the setting of MI. However, it is also important to understand the comparative and combinatorial efficacy of resveratrol alongside the established therapies to translate the pre-clinical findings to the clinical setting. In the first study, left anterior descending coronary artery ligated (MI-induced) and sham-operated male Sprague Dawley rats were treated with vehicle, resveratrol, angiotensin-converting enzyme inhibitor perindopril, or a combination of both for 8 weeks. Echocardiography was performed to assess the cardiac structure and function. MI rats treated with resveratrol, perindopril and a combination of both had significantly reduced left ventricular (LV) dilatation and improved LV ejection fraction (LVEF). Resveratrol, perindopril and a combination of both significantly decreased cardiac oxidative stress, inflammation and fibrosis and improved the activity of antioxidant enzymes in MI rats. In the second study, MI-induced and sham-operated rats received vehicle, angiotensin receptor blocker/neprilysin inhibitor sacubitril/valsartan, angiotensin receptor blocker valsartan, resveratrol or sacubitril/valsartan + resveratrol for 8 weeks. Echocardiography was performed at the endpoint. Treatment with resveratrol, sacubitril/valsartan, valsartan and sacubitril/valsartan + resveratrol significantly prevented LV dilatation and improved LVEF in MI rats. Resveratrol, sacubitril/valsartan, valsartan and sacubitril/valsartan + resveratrol also significantly reduced oxidative stress, inflammation and brain natriuretic peptide in MI rats. In the final study, adult rat cardiomyocytes were pre-treated with resveratrol or left untreated and exposed to norepinephrine (NE). NE-exposure resulted in significant cardiomyocyte contractile dysfunction, reduced cell viability and an increase in oxidative stress. Resveratrol significantly improved contractile function and cell

viability and reduced oxidative stress in NE-exposed adult rat cardiomyocytes. Pharmacological inhibition of superoxide dismutase (SOD) and Forkhead Box 1 (FOXO1) resulted in a loss of resveratrol mediated protection in NE-exposed adult rat cardiomyocytes. Resveratrol also prevented the proliferation of adult rat cardiac fibroblasts. In conclusion, resveratrol treatment improves cardiac structure and function equivalent to perindopril, valsartan, and sacubitril/valsartan in MI rats and the combination treatment is also effective. Resveratrol protects NE-exposed adult rat cardiomyocytes by a mechanism contingent upon SOD/FOXO1 and reduces cardiac fibroblast proliferation.

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4. List of Abbreviations

ACE	Angiotensin-converting enzyme
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate kinase
Ang II	Angiotensin II
ANP	Atrial natriuretic peptide
ARB	Angiotensin receptor-blocker
ARNI	Angiotensin receptor-blocker/neprilysin inhibitor
AT	3-Amino-1,2,4-triazole
ATP	Adenosine triphosphate
BNP	Brain natriuretic peptide
CABG	Coronary artery bypass grafting
CAD	Coronary artery disease
CAT	Catalase
CF	Calcium fructoborate
CF	Coronary flow
CK-MB	Creatine kinase-muscle/brain
CNP	C-type natriuretic peptide
CREB	The cAMP response element binding *protein*
CSC	Cardiac stem cells
cTn	Cardiac troponin
CVD	Cardiovascular disease
DAMPs	Danger-associated molecular patterns
DAPI	4′,6-Diamidino-2-phenylindole
DDC	Diethyldithiocarbamic acid

DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
ECG	Electrocardiogram
ECM	Extracellular matrix
E-CSC	Explanted heart cardiac stem cells
eGFR	Estimated glomerular filtration rate
FOXO1	Forkhead box protein O1
FOXO3a	Forkhead box protein O3a
FS	Fractional shortening
GSK-3β	Glycogen synthase kinase 3β
HDL	High-density lipoprotein
HF	Heart failure
HFpEF	Heart failure with preserved ejection fraction
HFrEF	Heart failure with reduced ejection fraction
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
IVRT	Isovolumic relaxation time
IVST	Interventricular septum thickness
LAD	Left anterior descending artery
LDL	Low-density lipoprotein
LKB1	Liver kinase B1
L-NAME	N(gamma)-nitro-L-arginine methyl ester
LV	Left ventricle
LVEF	Left ventricle ejection fraction

LVID	Left ventricle internal diameter
LVPWD	Left ventricle posterior wall
MDA	Malondialdehyde
MI	Myocardial infarction
MiRNAs	MicroRNA
mPTP	Mitochondrial permeability transition pore
MRA	Mineralocorticoid receptor antagonist
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Norepinephrine
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NOS	Nitric oxide synthase
NP	Natriuretic peptide
NSTEMI	Non-ST segment elevation myocardial infarction
NYHA	New York Heart Association
PARADIGM-HF	Prospective Comparison of ARNI with ACEI to Determine Impact on Global Mortality and Morbidity in Heart Failure
PARADISE-MI	Prospective ARNI vs ACE Inhibitor Trial to DetermIne Superiority in Reducing HF Events After MI
PBS	Phosphate buffered saline
PKA	Protein kinase A
PPCI	Primary percutaneous coronary intervention

RAAS	Renin angiotensin aldosterone system
ROS	Reactive oxygen Species
SERCA2a	Sarcoplasmic reticular Ca^{2+}/ATPase 2a
SIRT1	Sirtuin-1
SMEM	Serum minimum essential medium
SNS	Sympathetic natriuretic system
SOD	Superoxide dismutase
STEMI	ST-elevation segment myocardial infarction
TAS	Total antioxidant status
TDI	Tissue Doppler imaging
TGF-β 1	Transforming growth factor-β 1
TNF-α	Tumor necrosis factor-α
TNFRs	Tumor necrosis factor-α receptors
UA	Unstable angina
V/Q ratio	ventilation/perfusion ratio
VF	Ventricular fibrillation
VSEL	Very small embryonic-like stem cell
VT	Ventricular tachycardia

CHAPTER 1: Review of literature

1. Myocardial infarction

1.1. Definition and classification

Myocardial infarction (MI) is a term generally used to denote the myocardial cell death that occurs due to prolonged blockages in coronary arteries that supply blood to the myocardium[1, 2]. Dating back to the 1950s, efforts have been made to define and classify MI for both epidemiological and clinical purposes[2]. As per the European Society of Cardiology (ESC), American College of Cardiology (ACC) Foundation, American Heart Association (AHA), and the World Heart Federation (WHF) - 2018 consensus statement on the Universal Definition of MI, MI is an acute myocardial injury characterised by elevation of cardiac specific injury biomarkers with evidence of acute myocardial ischemia (Figure 1). MI is generally diagnosed by an electrocardiogram (ECG), cardiac biomarkers and patient history[2]. MI is associated with an elevation of cardiac troponin (cTn) value due to the myocardial tissue injury[2]. Elevated cTn level in MI patients predicts an adverse prognosis and is a preferred cardiac injury marker[2]. Creatine kinase MB isoform (CK-MB) is plagued with low sensitivity and specificity as cardiac biomarkers, and it is not preferred[2].

The classification of an MI is important for optimal risk stratification and optimal post-MI treatment and is done according to diagnostic, clinical, pathophysiological, and later prognostic factors[2, 3]. An acute MI along with unstable angina (UA) comes under the umbrella term of acute coronary syndrome (ACS)[3-6]. Based on ECG findings, MI patients are clinically classified into two different categories namely non-ST segment elevation MI (NSTEMI) and STEMI[2]. The distinction between NSTEMI and STEMI patients is pivotal to devise optimal treatment

modalities as the medical management varies for these 2 different clinical entities. The third category that comes under ACS is UA. UA may not differ from NSTEMI in ECG characteristics. UA is characterised by the absence of significant myocardial necrosis and elevated cardiac injury markers. As per the ESC/ACC/AHA/WHF, MI is further categorised into 5 types depending upon various diagnostic standards and aetiologies[1]. MI Type 1 occurs as a result of cardiac ischemia secondary to plaque rupture, erosion, fissuring, coronary dissection and distal coronary embolization (Figure 2). This group is characterised by major symptoms of acute ischemia, evidence of ECG changes including pathological Q waves. MI Type 2 occurs mainly because of an imbalance in demand and supply of oxygen and subsequent ischemia. MI Type 2 is not associated with athero-thrombotic plaque complications. Patients show similar ECG changes, irreversible myocardial damage and contractile abnormalities. Type 2 MI occurs more frequently in women than in men[1]. In Type 3 MI, an adverse event may occur before the rapid increase in classical biomarkers and confirmation of an acute MI[1]. Patients mostly succumb to sudden cardiac death in Type 3 MI. Type 4a MI includes MI caused by cardiac procedures, which lead to complications and subsequent myocardial injury[1]. Abnormal cardiac troponin values may be observed in patients who undergo a primary percutaneous coronary intervention (PPCI)[1]. A new MI due to dissections of coronary arteries, a major epicardial artery obstruction, a major coronary side branch obstruction, collateral flow disturbance, and embolisation in distal locations is a characteristic feature of a type 4a MI[1]. MI Type 4b is classified as an MI due to a PPCI associated stent thrombosis and is detected by angiography. An MI may also result from angiography, restenosis in stented arteries, and restenosis due to balloon angioplasty procedure and is designated as type 4c MI[1]. Type 5 MI is a known complication of coronary artery bypass grafting (CABG) having signs and symptoms of MI along with cTn values >10 × 99th percentile

upper reference limit[1]. CABG procedures often result in myocardial injury and elevation of cardiac injury markers. In 2 different types of CABG surgery patients such as off-pump and on-pump CABG patients, a cardiac injury may be present in as high as 30-40% of patients as detected via late gadolinium enhancement cardiac magnetic resonance quantification[1].

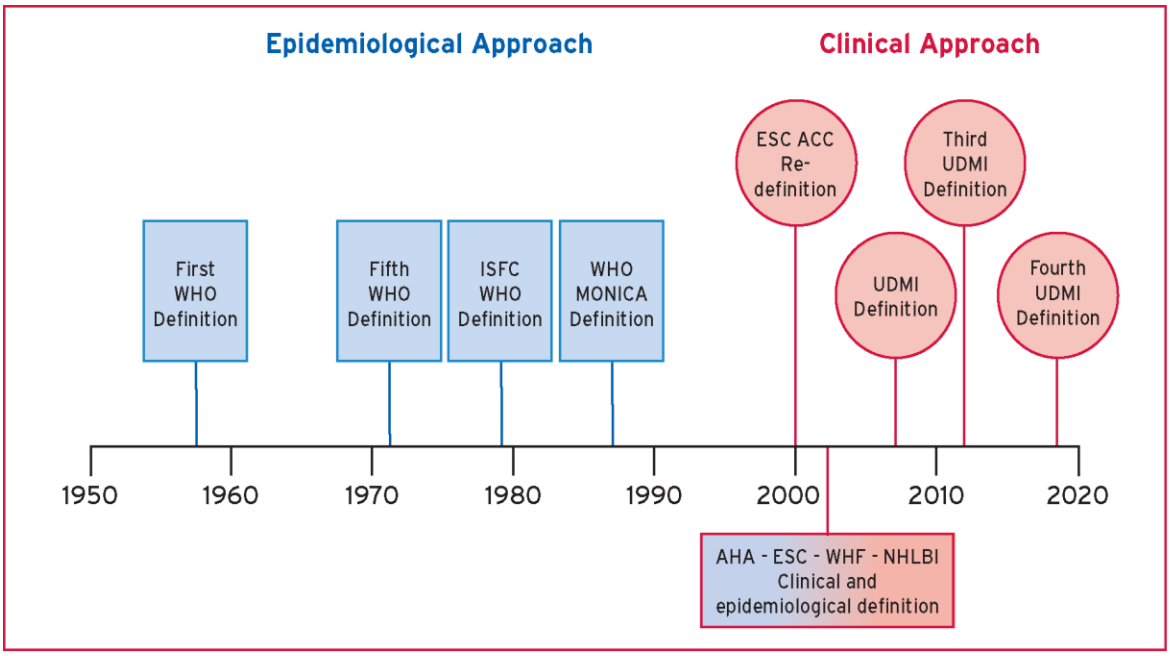


Figure 1. History of documents on the definition of myocardial infarction. ACC = American College of Cardiology; AHA = American Heart Association; ESC = European Society of Cardiology; ISFC = International Society and Federation of Cardiology; MONICA = MONItoring of trends and determinants in CArdiovascular disease; NHLBI = National Heart, Lung, and Blood Institute; UDMI = Universal Definition of Myocardial Infarction; WHF = World Heart Federation; WHO = World Health Organisation[2]. *Thygesen K, Alpert JS, Jaffe AS, Chaitman BR, Bax JJ, Morrow DA, et al. Fourth Universal Definition of Myocardial Infarction (2018). J. Am. Coll. Cardiol. 2018;72(18):2231-64. Reprinted with permission.*

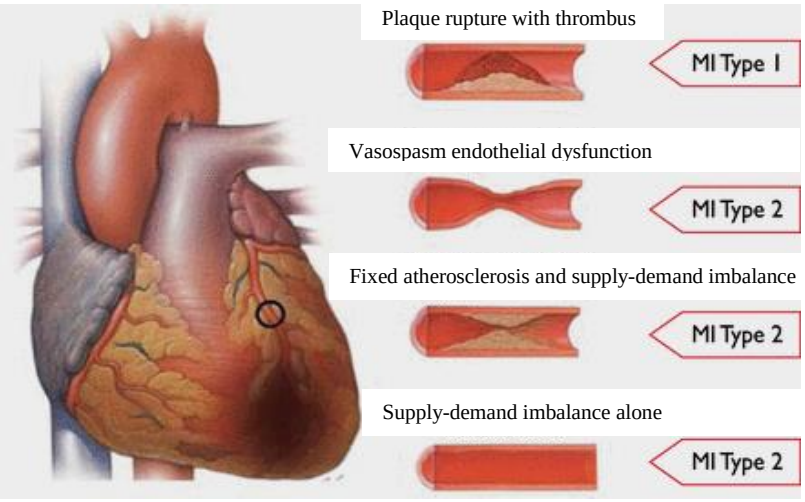


Figure 2. Differentiation between myocardial infarction (MI) types 1 and 2 according to the condition of the coronary arteries[1]. *Thygesen K, Alpert JS, Jaffe AS, Simoons ML, Chaitman BR, White HD, et al. Third universal definition of myocardial infarction. Eur. Heart J. 2012;33(20):2551-67. Reprinted with permission*.

1.2. Prevalence and incidence

According to the Global Burden of Disease report, MI related morbidity and mortality remain a major global health care challenge that needs concerted efforts to meaningfully address the issue[7]. In absolute numbers, ischemic heart disease globally contributes to 50% of 17 million deaths. In addition, it also causes the loss of 329 million disability-adjusted life years[8]. The most recent report estimated that 7.29 million acute MI cases occurred in 2015. In Canada, approximately 2.4 million (or 1 in 12) Canadian adults aged 20 years and older had ischemic heart disease, and 578,000 have had an acute MI in 2012–2013[9]. Notably, age-standardised mortality secondary to ischemic heart disease has shown a decreasing trend during last 2 decades[9]. However, there was an increase in the loss of disability-adjusted life years during the same time period[8]

1.3. Etiology and pathophysiology

An uninterrupted oxygen and nutrient supply is indispensable for the myocardial function, and any prolonged disturbance in this supply of oxygen rich blood through the conduits of the coronary arteries may result in cardiac ischemia[6]. Essentially, the primary etiology of an acute MI is inadequate blood flow via the coronary arteries that provide nourishment to heart muscle cells to keep them in an optimal functional status[10]. As mentioned earlier, coronary blood flow may be interrupted due to multiple factors. Coronary artery disease (CAD) impedes the adequate blood flow in the coronaries due to atherosclerosis. CAD develops and progresses due to both genetic and lifestyle factors. Atherosclerosis is considered as a slowly developing chronic inflammatory disease found in large vessels that results in the build-up of plaques and is considered synonymous with ischemic heart disease[6, 11, 12]. The risk factors for atherosclerosis or ischemic heart disease include non-modifiable and modifiable causes such as age, gender, family history of CAD, genetic predisposition, cigarette smoking, hyperlipidemia, hypertension, and diabetes mellitus[13]. Endothelial dysfunction that alters vascular homeostasis may precede and pave the way for the initiation of atherosclerosis[14]. Branch points and curvatures of the coronary artery bed experience low flow mediated oscillatory endothelial shear stress which is essential for vascular homeostasis. Low flow-mediated oscillatory endothelial shear stress has been implicated in the development of endothelial dysfunction. Atherosclerotic lesion disruption in single or multiple coronary arteries and ensuing thrombosis is the main reason for acute myocardial ischemia (Figure 3)[13]. A growing atherosclerotic plaque in the arteries can also hinder coronary blood flow in a narrowing lumen and this may lead to stable angina pectoris precipitating chest pain. However, stable angina pectoris may not be lethal in most cases unless it perturbs electrical activity due to myocardial injury followed by fibrosis. In adverse scenario,

stable angina pectoris may cause severe arrhythmias and sudden cardiac death. An atheromatous lesion may be present in the arteries in a clinically dormant stage for a long period of time before it becomes unstable and ruptures or erodes[15]. Neoatherosclerosis and accelerated atherosclerosis are phenomena observed in patients undergoing invasive or surgical procedures for the treatment of CAD and are characterised by rapid development[16]. Unstable atherosclerotic plaque, also known as a vulnerable plaque or thin-cap fibroatheroma, rupture causes the activation of platelets due to the exposure of thrombogenic substances and contributes to acute decreased blood flow in the coronary circulation[17, 18]. Plaque erosion is another major mechanism that precipitates platelet aggregation and occluded arteries with the greatest incidence in women aged <50 years[18, 19]. In addition, disruptive calcified nodules may be another natural reason for coronary obstruction in calcified coronary arteries especially in elderly population[16]. Mechanistically, the plaque rupture, erosion and eruptive calcified nodules invariably result in activation of platelets to curtail bleeding. The platelet aggregation depends on its binding to the contents of exposed lesions. The glycoprotein IIb/IIIa and VI receptors on the platelet membrane undergo conformational changes to produce cross-links of platelets via fibrinogen which results in aggregation and superimposed thrombosis[20]. Small atherosclerotic plaques may also suddenly become unstable and lead to thrombosis; in more than half of the patients, significant stenosis may not be present[21]. It is recognised that the severity of stenosis can only predict the symptoms of ischemia but not the chances of having an ischemic event. The thrombus formation in the coronary arteries rapidly obstructs the normal supply of blood through them and leaves the myocardium without nourishment. It is also known that un-stimulated thrombolysis may occur in some patients and nearly 30% of post-MI patients appear to have thrombus occlusion in their arteries after 24 hours[21]. Nonetheless, in most patients, coronary occlusion is found to persist for duration

sufficient to cause significant myocardial injury[21]. Other aetiologies of decreased oxygenation/myocardial ischemia include coronary artery embolism, which accounts for 2.9% of patients, cocaine-induced ischemia, coronary dissection, and coronary vasospasm[21].

The prolonged interruption of the blood supply that precipitates as myocardial ischemia activates a well-described phenomenon of the "wave front" of cardiomyocyte death, which originates from the sub-endocardium and radiates through the affected myocardium to the sub-epicardium[22, 23]. Transmural infarcts encompass the entire myocardium starting from the epicardium through to the endocardium[22]. Transmural infarcts may be identified by the presence of faulty Q waves on the ECGs of the myocardium[22]. In contrast, non-transmural infarcts may not spread through the entire myocardium[22].

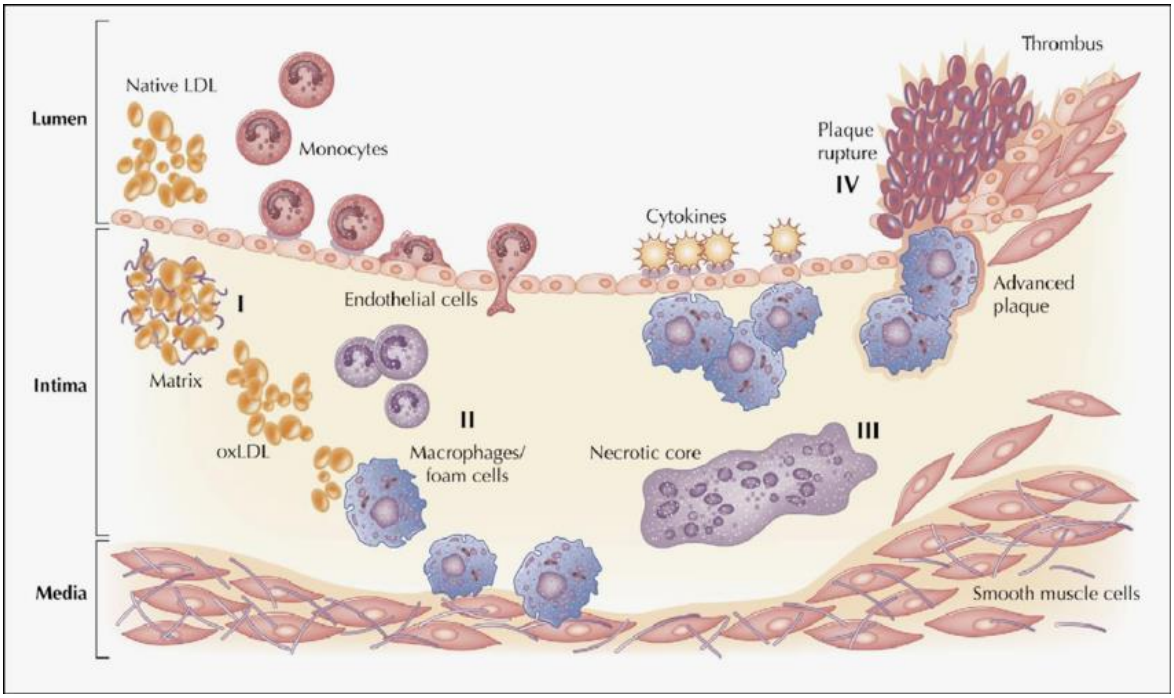


Figure 3. Steps in the progression of atherosclerosis in the artery wall. I, Low-density lipoprotein (LDL) particles accumulate in the sub endothelial cell layer and become trapped within matrix proteins, where they are oxidised (oxLDL) by cell-derived reactive oxygen species. II,

Chemotactic and growth factors stimulate monocytes to transmigrate across the endothelial cell layer and differentiate into macrophages. Macrophages begin to engulf the oxLDL, leading to foam cell formation. III, The foam cells produce a variety of mediators and eventually undergo apoptosis or necrosis, contributing their contents to a growing core of cellular debris and cholesterol. This process is accompanied by the migration of smooth muscle cells into the intima from the media and the formation of a fibrotic cap. As the advanced lesion continues to grow, it becomes increasingly unstable and prone to rupture, which can result in a thrombus or clinical event such as myocardial infarction or stroke[24]. *Tymchuk CN, Hartiala J, Patel PI, Mehrabian M, Allayee H. Nonconventional genetic risk factors for cardiovascular disease. Curr. Atheroscler. Rep. 2006;8(3):184-92. Reprinted with permission.*

At the cellular level, a series of adverse rapid interrelated changes in metabolic and ionic status occur as a result of ischemia which form the targets for many potential interventions. Lack of oxygen affects the energy deriving post-glycolytic pathway that involves the metabolism of pyruvate via citric acid cycle and subsequent oxidative phosphorylation. This leads to a depletion of cellular adenosine triphosphate (ATP) needed for cardiac function[25-28]. The switch from aerobic pyruvate oxidation to anaerobic lactate utilisation by cardiomyocytes under an ischemic condition in order to adapt to the metabolic demand triggers a disruption of overall cardiac energetics[26, 29]. The increased lactate accumulation and uncoupled oxidative phosphorylation mediate a surge of H^+ production that reduces intracellular pH which in turn inhibits glycolysis and perturbs ion channel function in cardiomyocytes[26]. High intracellular H^+ level necessitates sodium/hydrogen exchanger mediated efflux of H^+ and a subsequent increase in demand of ATPs for sodium/potassium ATPase pump function to remove sodium. This leads to a further depletion of the level of ATP and deteriorates the cardiac efficacy. In addition, the depletion of ATP also

adversely affects sarcoendoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a) facilitated intracellular Ca^{2+} homeostasis. Intracellular Ca^{2+} overload induces mitochondrial alterations and leads to the necroptosis of cardiomyocytes in the infarcted heart. Disturbed ion homeostasis during ischemia/MI is believed to contribute to arrhythmogenesis and sudden cardiac death.

The human myocardium is not capable of any meaningful large scale regeneration of cardiomyocytes to overcome the ischemic damage and regain normal functional capacity[30]. In contrast, there are a few species which possess the inherent capacity to partially or completely regenerate the heart to a functional status after a major injury to the myocardium including newts, teleostean fish, foetal and neonatal rodents[31]. However, the infarcted heart has the ability to orchestrate a wound healing process that effectively replaces the dead cardiomyocytes with a fibrotic scar[31-33]. Essentially, the post-MI healing encompasses 3 overlapping stages of inflammatory response, proliferation and scar formation, namely[31, 32, 34, 35]. The wound healing process is contingent upon an initial inflammatory cascade initiated by the cells undergoing necroptosis[36]. The inflammatory phase is triggered by endogenous molecules released from necrotic cells via activating pattern recognition receptors such as Toll-like receptors[35]. These molecules are capable of activating the innate immune response and are called danger-associated molecular patterns (DAMPs)[36]. Consequently, DAMPs mediated production and release of chemokines and cytokines will result in the recruitment of leukocytes to the infarcted tissue[36]. The recruitment of white blood cells such as neutrophils and macrophages helps to clear dead cells and extracellular matrix (ECM) debris. A number of macrophage sub-types have been reported to increase in the heart after an MI through the recruitment of bone marrow-derived cells and local self-renewal[36]. Neutrophils and macrophages are recruited to the infarct zone by a variety of myocyte-derived factors, including complement C5a, C-X-C motif chemokine 5,

monocyte chemotactic protein-1, tumor necrosis factor-α (TNF-α), and interleukins (IL)-1β, IL-6, and IL-8[36]. Phagocytes first clear the dead cells and undesired cellular remains and subsequently activate the anti-inflammatory response in an effort to subdue the cytokine and chemokine signalling that may otherwise cause further damage.

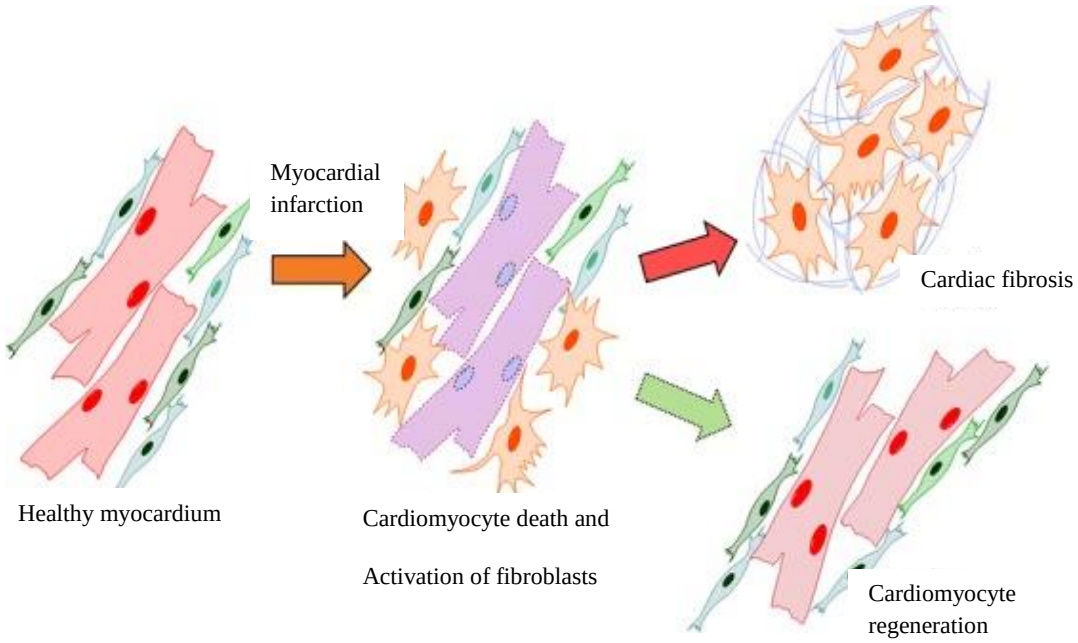


Figure 4. Reparative response following a myocardial infarction. Hypoxia-induced cardiomyocyte death leads to the activation of myofibroblasts and a reparative fibrotic response in the injured area. Right top. In adult mammals, the fibrotic scar formed at the infarcted area is permanent and promotes reactive fibrosis in the uninjured myocardium. Right bottom. In teleost fish and newts and in embryonic and neonatal mammals, the initial formation of a fibrotic scar is followed by regeneration of the cardiac muscle tissue. Induction of post-infarction cardiac regeneration in adult mammals is currently the target of intensive research and drug discovery attempts[31]. *Talman V, Ruskoaho H. Cardiac fibrosis in myocardial infarction-from repair and remodeling to regeneration. Cell and Tissue Res. 2016;365(3):563-81. Reprinted with permission.*

Cardiac fibroblasts are distributed throughout the heart between cardiac muscle fibers[32, 37]. Cardiac fibroblasts are assumed to carry out an ancillary, but indispensable role, in the myocardium by producing ECM components and secreting a host of paracrine mediators. The ECM of the myocardium consists of a variety of constituent proteins which can serve as structural and adhesion agents in dynamic contractile machinery that hold together all other components. Basically, they provide a scaffold that cardiomyocytes can effectively organise themselves into a syncytium that enables their function as a contractile machinery. In addition, the ECM also helps in signalling through the cardiomyocyte syncytium to assist in the excitation and contraction coupling. The paracrine factors released by cardiac fibroblasts help regulate homeostasis in cardiomyocytes, endothelial cells, and immune cells within the heart. Regulation of ECM synthesis is found to play a significant role after myocardial injury. Cardiac fibroblasts are believed to undergo a phenotypic conversion/differentiation as a result of the pathological changes within the myocardium. In order to preserve the myocardial architecture after an injury, ECM synthesis rapidly increases in the post-MI heart.

In this reparation process, different isoforms of collagen are released from cardiac fibroblasts and myofibroblasts and they help in infarct scar formation. Type III collagen has been reported to be abundant initially and replaced with type I collagen in later stages. The scar development progresses by further cross-links formation of collagen fibers to attain sufficient tensile strength to withhold the force generation and to partially maintain the normal geometry of the left ventricle (LV) to ensure that loss of functional tissue does not severely limit cardiac systolic function[32]. Adequate collagen deposition in the scar tissue is pivotal to retain the tensile strength and prevent a rupture of the infarcted LV wall due to the loss of viable tissue[37]. The myocardial scar is also subjected to localised remodelling to ensure its permanent nature mainly by

metalloproteinases and tissue inhibitor proteases which help in maintaining scar homoeostasis. The post-MI scar formation and maturation continues for a few weeks and this well-regulated process culminates with a fully developed scar at around 8 weeks[17]. Simultaneously with the ECM deposition and cross-link formation, the microvasculature also forms in the newly formed scar and surrounding zone to create a network in the infarcted tissue to maintain sufficient blood supply to the proliferating myofibroblasts. The microvasculature is necessary to provide the blood supply needed for myofibroblast survival during the post-MI reparation of myocardial tissue[33]. As mentioned earlier, cardiomyocytes do not increase in number necessitating the permanent presence of scar in the myocardium following an injury[37]. Myofibroblasts depend on the supply of paracrine factors and their interaction with ECM protein to remain active in the scar[37]. Even though they undergo apoptosis as the scar matures, many of them remain in or in the vicinity of the infarct scar and also infiltrate to adjacent areas of the uninjured myocardium. This phenomenon is detrimental to normal cardiac function as non-infarcted cardiac tissue also undergoes remodelling as a result of the unnecessary and excessive ECM synthesis and deposition orchestrated by the proliferating infiltrated myofibroblasts[37]. In addition, the additional workload on non-infarcted myocardial wall and the resultant mechanical stress also contributes to the activation of transforming growth factor-β 1 (TGF-β 1) within the myocardium which has pro-fibrogenic properties[38, 39]. Increased renin-angiotensin-aldosterone system (RAAS) activity and the release of TGF-β 1 are known to cause the pheno-conversion of fibroblasts into myofibroblasts that will result in amplified cardiac fibrosis. Overall, myofibroblasts are characterised by the capacity to release pro-fibrotic factors that ensure their continued survival in the infarcted tissue and scar as well as promote their infiltration to cause interstitial fibrosis and perivascular fibrosis (Figure 4)[33,34]. The healing of the infarcted LV tissue

forms an indispensable process that is coupled with the geometric remodelling of the LV chamber. The necessary evil of scar formation has been shown to predispose the myocardium to a trajectory of progressive LV wall thinning, and dilatation and LV systolic and diastolic dysfunction[33, 34]. Targeting cardiac modelling is generally perceived as a major therapeutic window of opportunity to check the development and progression of HF[40].

1.4. Treatment

Treatment for MI is initiated concurrently with diagnosis at the point of primary medical contact as it is very crucial to rescue the myocardium from extensive damage[4, 41-43]. Out of hospital defibrillator use is also important as post-MI cardiac arrest and sudden cardiac death are frequent in STEMI patients[41]. This routinely involves pre-hospital administration of pain killers, nitrates, aspirin and oxygen[41]. Pain relief is given importance as pain may lead to an aggravation of the condition[41]. In hospital, antiplatelet drugs, anticoagulants, fibrinolytic drugs and nitrates are given to limit the occlusion and restore blood flow[41]. An angiogram needs to be performed in STEMI patients with continuing chest pain even after initiating optimal management options. and also in STEMI patients with complicated issues such as high injury biomarkers, cardiogenic shock, acute mitral regurgitation, reduced septal integrity, and arrhythmogenicity[44]. NSTEMI or UA patients whose conditions have improved may have their angiography done during the initial 24 to 48 hours after diagnosis to identify CAD which can be treated immediately. In reperfusion strategies, a timely PPCI is preferably performed to revascularise the infarcted heart within 12 hours in all STEMI patients[44]. Patients presented with NSTEMI may also undergo PPCI depending on ischemic symptoms and clinical evidence[41]. Fibrinolytics therapy is also a mainstay intervention for post-MI patients with STEMI if PPCI cannot be performed in a proposed time bound manner. Dual antiplatelet therapy with aspirin and a P_2Y12 inhibitor is also

recommended with a high loading dose and low maintenance dose[41, 45]. Glycoprotein IIb/IIIa inhibitors are also recommended for post-MI patients undergoing PPCI or for those who are at high risk. Lifestyle interventions such as quitting smoking, controlling body weight, and increasing physical activity are also recommended[41]. In addition to early therapy, long-term drug therapy strategies are also important in post-MI patients. Angiotensin-converting enzyme (ACE) inhibitors are suggested in MI patients having LV systolic dysfunction as well as for all STEMI patients[41]. Beta-blockers are recommended as early and long-term drug therapy in hemodynamically stable patients undergoing PPCI[41, 46]. Statins, lipid-lowering medication, are effective in secondary prevention of MI and early and intensive statin therapy is suggested to receive maximum benefits[47]. For patients who are known to be intolerant to statin treatment, ezetimibe is used as an excellent lipid-lowering therapy[41].

2. Heart failure

2.1. Definition and classification

Heart failure (HF) is a clinical syndrome characterised by the incapability of the heart to sustain sufficient organ perfusion in the body due to inefficient filling and pumping capacity secondary to the damage and/or weakening of the myocardium[48-50]. HF is a multifactorial life threatening and debilitating syndrome that is identified clinically with a diagnosis process ranging from careful analysis of patient history, physical examination, non-invasive cardiac imaging and biochemical tests and is targeted with a multifaceted treatment regimen[48]. HF is a chronic condition that may sporadically exacerbate to an acute decompensated HF necessitating urgent in hospital treatment[51].

ACC/AHA guidelines follow a novel methodology to understand the pathophysiology of HF that underlines the importance of the development and progression of this clinical entity[48, 51, 52]. This classification system identifies 4 stages of HF enabling the practice of different approaches for its medical management[43, 51]. According to this approach, Stage A patients are considered to be at increased risk for having HF in their lifetime due to the presence of various risk factors; this includes patients who do not have any structural heart disease and symptoms[43]. Patients that come under Stage B may already have structural heart disease but they are devoid of signs or symptoms of HF due to the underlying cardiac abnormalities[43]. Stage C patients are characterised by the presence of already diagnosed structural heart disease with previous or current complicating symptoms of HF[51]. Stage D patients may have refractory HF due to severe cardiac structural and functional defects requiring specialised interventions to maintain the functional status of the heart[51]. As per the most recent ACC/ESC updates, HF is mainly classified into 3 types based on different thresholds of LV functional status represented by LV ejection fraction (LVEF) in patients[51]. HF patients with normal LVEF (which is quantified as ≥50%) comes under the category of HF with preserved LVEF (HFpEF) and patients with reduced LVEF (usually <40%) belong to the category of HF with reduced EF (HFrEF)[51, 53]. Patients that have LVEF of 40–49% are considered as another main category and they come under class of HF with midrange EF (HFmrEF)[53]. The LVEF based classification is deemed important largely because of the diverse demography, primary aetiologies, co-morbidities and HF patients' response to therapies[53]. New York Heart Association (NYHA) functional classification provides further information about exercise capability and the HF related symptomatic grade of the HF patients[48].

2.2. Prevalence and incidence

HF is considered as an emerging debilitating epidemic[54, 55]. HF is one of the major health care issues around the world, with a prevalence of more than 26 million worldwide and 1 million hospitalisations annually in both the United States and Europe. The age-standardised prevalence of HF in Canada, among individuals aged 40 years and older, remains stable at about 3.5%. About 670,000 are Canadians living with HF[14]. The survival rate in HF patients has improved substantially over the last few decades as a result of better medical management. However, the absolute mortality rate in HF patients continues to be approximately as high as 50% within 5 years of diagnosis[9]. HF incidence increases with age and the elderly population is the most affected. For example, HF incidence is 20 per 1,000 persons in the age range of 65 to 69 years and >80 per 1,000 individuals in those people older than 85 years[9].

2.3. Etiology and pathophysiology

According to the Framingham Heart Study, CAD is one of the chief underlying reasons for HF and the risk of a previous MI as a reason for HF increased by 26% in men and 48% in women per decade in comparison with other aetiologies of HF such as hypertension and valvular diseases[50, 56, 57]. Acute MI patients may present with HF or severe cardiac dysfunction and are at a higher risk of subsequent HF[50, 57, 58]. Of note, the presence of HF on admission has also been shown to increase the odds of in-hospital mortality in MI patients[59]. CAD is also independently associated with a negative long-term outcome in HF patients[50]. Post-MI patients may also have multiple coronary arteries with atherosclerosis plaque burden excluding the occluded artery which can further increase the severity of adverse complications[50]. Infarct size is another key factor in the development and prognosis of HF in post-MI patients[3, 60]. Myocardial cell death and

myocardial stunning due to CAD may lead to acute HF[58]. Irrespective of aetiologies, HF is characterised by impaired contractile function[51]. HF develops due to underlying LV systolic or diastolic functional abnormalities or a combination of LV systolic dysfunction and diastolic dysfunction as MI related cardiac remodelling progresses[51]. In addition to the immediate loss of a large number of cardiomyocytes, cardiac ischemia contributes to changes in cardiac metabolism, electrical conduction, and contractility which further exacerbate HF progression.

In ischemic HF, a failing myocardium undergoes a series of complex pathophysiological processes, starting from an initial ischemic insult to the myocardium before going to precipitating acute or chronic HF[50]. Specifically, ischemic HF develops as a continuum of adverse LV structural alterations coupled with a reduction in LV functional capacity as well[61]. These are typically manifested as moderate LV structural changes and preserved systolic function or significant LV structural changes and severely reduced systolic function. These cardiac structural alterations are known as post-MI LV remodelling and encompass changes in chamber size, volume, and LV wall tissue composition due to injury and in response to the stress and strain and systemic neuro-hormonal overactivation[61]. The ensuing LV remodelling is orchestrated by significant modifications of infarcted tissue, surrounding infarct border area, and the distant non-infarcted LV tissue. This remodelling process has been described as a 2-stage pathophysiological event categorised into phases such as an early and late phase. The early phase is believed to involve the gradual expansion of infarct size accompanied by significant thinning and subsequent distention of the infarcted LV wall which may also give rise to mitral regurgitation and arrhythmias. In contrast, the late phase includes hypertrophy of the viable LV and dilatation of LV chamber. Even though LV remodelling serves as a process to cope with the

overload on the viable myocardium, it plays a central role in the initiation and advancement of HF.

Post-MI neuro-humoral response occurs as a compensatory mechanism and is followed by an overactivation of neuro-humoral signalling that contributes to the progressive deterioration of beneficial effects[57]. Evidently, neuro-humoral overactivation eventually becomes a counter-productive mechanism that exacerbates HF[62]. The neuro-humoral response due to increased cardiac demand involves activation of the sympathetic nervous system (SNS), and the RAAS, and the natriuretic peptide (NP) system and an increased release of antidiuretic hormone (vasopressin) which helps to improve stroke volume and reduce fluid retention. Neuro-humoral responses cause an increased constriction of arteries and veins to increase LV filling and cardiac output. In patients with HF, the activation of the SNS plays a critical role[57]. Unfortunately, an uninterrupted chronic overactivation has been shown to adversely affect the prognosis of HF patients. The adrenergic receptor agonist norepinephrine (NE) is released by the sympathetic nerves in order to increase cardiac demand and it helps in increasing cardiac output by exerting direct cardiac or vascular effects[50]. Elevated levels of plasma NE are reported in HF patient[63]. RAAS is another major determinant that influences the pathophysiology of HF. RAAS consists of a cascade of angiotensinogen, renin, and ACE, which generates angiotensin II (Ang II) as the biologically active product[50]. Renin is secreted from kidneys by juxtaglomerular cells, and angiotensinogen is released by the liver[64]. Renin breaks down angiotensinogen to angiotensinogen I and Ang II synthesis occurs in the circulation by the action of ACE which results in conversion of the Ang I to Ang II[65]. Ang II has an affinity to 2 types of receptors named Ang II type 1 and Ang II type 2 (AT_1 and AT_2)[50]. Ang II is a vasoconstrictor and a mitogen which has deleterious direct effects on cardiomyocytes and cardiac fibroblasts. Ang II is

known to produce deleterious effects via its AT_1 receptor based downstream signalling, which is the predominant receptor in the heart, whereas AT_2 is thought to produce beneficial effects[50]. The NP system has also been shown to perform a role in the development and progression of HF. The NP system mainly acts as a counter mechanism that reverses some of the deleterious effects of SNS and RAAS[66]. The NP system includes atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). ANP, BNP and CNP help in diuresis, natriuresis and vasodilation in the setting of HF[66]. Overall, the SNS and RAAS are compensatory machineries that turn deleterious due to their chronic overactivation, whereas the NP system is a counter-regulatory mechanism that invariably produces beneficial effects in HF patients.

Clinically, ischemic HF may patients present with a dilated LV chamber with significantly thinned LV wall due to an increased post-MI hemodynamic overload. In ischemic HF patients, a severe reduction in systolic functional capacity and pump failure are generally seen mainly due to the failure of the compensatory advantage afforded by early the post-MI LV remodelling. In HF due to post-MI LV systolic dysfunction, the LVEF significantly reduces and this affects the perfusion around the body. Normal LVEF ranges from 52% to 72% in men[67]. In women, the normal LVEF is 54% to 74%. A reduction in LVEF value to below 40% is considered as HFrEF as it serves as a diagnostic criterion[67]. Importantly, all large scale landmark drug and device therapy clinical trials have been conducted with HFrEF patients, specifically consisting of a large number of ischemic HF patients, with a cut off value of LVEF $\leq$ 40% or even LVEF <30%[53]. Evidently, these studies have led to a great advancement in drug and device therapy improved survival and decreased HF hospitalisation rates in patients with HFrEF with ischemic aetiology. However, it is also recognised that a large number of patients with HFrEF also develop diastolic dysfunction. In contrast, HFpEF is characterised by a normal LVEF without significant systolic

dysfunction. Demographically, patients with HFpEF tend to be elderly and women with underlying aetiologies such as atrial fibrillation, hypertension, diabetes, and obesity[54]. In HFpEF, ischemic heart disease appears to play a less causative role as well. Post-MI cardiac remodelling may also lead to LV diastolic dysfunction[57]. Diastolic dysfunction mainly arises due to impaired relaxation due to the increased stiffness of the LV wall and reduced compliance which prevents adequate filling of the LV. HFpEF with ischemic aetiology is characterised by an impairment in LV filling, a diastolic functional indicator. Reduced LV filling leads to reduced end-diastolic volume and less cardiac output[68]. Restrictive filling may occur because of a decrease in cardiomyocyte number or a reduction in cardiomyocyte relaxation with cardiac hypertrophy, cardiac fibrosis or impaired calcium handling[57]. As opposed to HFrEF clinical trials, drug therapy has not yet shown any substantial benefits in HFpEF patients.

As HF is a clinical syndrome characterised by a lack of the functional capacity of the heart to sustain sufficient oxygen supply via perfusion, it is important to understand the systemic response elicited by the pathogenesis of HF[51]. In addition to the inherent contractile function of the heart, hemodynamic characteristics such as preload and afterload also act as determinants of perfusion contingent upon the Frank-Starling mechanism[69]. The injured myocardium depends on increased contractility, and preload and afterload to improve SV. However, over a period of time, the initial coping mechanisms largely become maladaptive and insufficient to maintain normal cardiac function. The cardiopulmonary system is also involved in the pathogenesis of HF and manifests as major signs and symptoms of HF such as pulmonary edema and dyspnea, respectively[69]. HF is often referred to as congestive HF due to these salient features. Impaired contraction, restricted LV filling capacity, and increased venous pressure due to vascular resistance leads to increased pulmonary capillary pressure (24 mm Hg) and subsequent fluid

extravasation from the capillaries into the interstitium[69]. As a result, lymphatic drainage also increases to counter the pulmonary edema by draining excess fluid. However, it fails to significantly improve the pulmonary edema due to increased fluid overload in HF patients. Significant fluid retention in alveoli disturbs the ventilation-perfusion (V/Q) relationships (the ratio of air in the alveoli to the amount of blood passing through alveoli per minute) as deoxygenated pulmonary arterial blood passes through the alveoli with low ventilation. This results in low systemic arterial oxygenation (PaO2) and precipitates as dyspnea[50]. However, elevated pulmonary venous pressure may also contribute to the development of dyspnea. Elevated left atrial pressures may also cause pulmonary congestion[57]. In HF, the increased systemic venous pressure also causes fluid extravasation into the surrounding tissue and results in fluid build-up in feet and ankles and abdominal viscera leading to edema. Stomach, intestine and liver are affected by edema in HF patients; fluid accumulation in the peritoneal cavity (ascites) is also observed in HF patients[50].

2.4. Treatment

HF treatment guidelines suggest that HF can be delayed or prevented by controlling risk factors such as CAD, hypertension, diabetes, obesity, and alcohol consumption through lifestyle changes or drug treatment[51, 70]. Medical management of HF is mainly aimed at decreasing mortality and hospital admission as well as improving the clinical status, and quality of life. Neuro-hormonal antagonists form the cornerstone of HF drug therapy. These agents are known to decrease mortality and improve HF related symptoms in patients with HFrEF in clinical trials and hence guideline-directed medical therapy for patients with HFrEF involves their optimal use unless contraindicated or not tolerated[51]. Angiotensin-converting enzyme inhibitors (ACE inhibitors), angiotensin-receptor blockers (ARBs), beta-blockers, aldosterone antagonists, and angiotensin-

receptor blocker/neprilysin inhibitor (ARNI) are the main classes of drugs for HF pharmacological treatment as many large scale clinical trials have reported their efficacy in HF patients[51, 53]. Before the arrival of ARNI, ACE inhibitors, beta-blockers, and aldosterone antagonists were considered as the 3 essential drugs for patients with HFrEF and was the basis of a therapeutic strategy called "triple therapy" in HF[51]. ACE inhibitors are recommended for all post-MI patients with systolic dysfunction to reduce hospitalisation and mortality. ARBs are also beneficial in HF patients and are used when ACE inhibitors are not well-tolerated due to the side effects of cough or hyperkalemia. In addition, in asymptomatic post-MI patients with low LVEF, ACE inhibitors are effective in reducing the risk of HF related hospitalisation. Beta-blockers are also known to produce a highly significant reduction in morbidity and mortality in symptomatic patients with HFrEF when combined with other front-line medications. The beta-blockers and ACE inhibitors are used together in HF patients, and are often started after a diagnosis of HFrEF is made[65]. However, evidence is lacking in using a beta-blocker before treatment with ACE inhibitors is initiated. Mineralocorticoid receptor antagonists (MRAs) such as spironolactone or eplerenone which block aldosterone signalling are also recommended in all symptomatic patients with HFrEF (LVEF $\leq$35%) as part of the triple therapy as they have been reported to decrease mortality and HF hospitalisation. First-in-class ARNI, sacubitril/valsartan, is superior to the ACE inhibitor enalapril in chronic HFrEF patients reducing mortality and HF related hospitalisation and are currently recommended as a better replacement for ACE inhibitors or ARBs if tolerated. The recommendation for the use of sacubitril/valsartan in HFrEF patients was first put forward by the Canadian Cardiovascular Society in 2015, followed by ESC and AHA[70].

Cardiac device therapy is recommended for HF patients as well due to its proven efficacy in clinical trials[51, 71]. Implantable cardioverter-defibrillator (ICD) and cardiac resynchronisation

therapy (CRT) have been used in HF patients when appropriate. ICD may be recommended for HF patients with symptomatic ventricular tachycardia (VT) or ventricular fibrillation (VF) and LVEF <30%[51, 71]. Importantly, ICD use is more beneficial in ischemic HF patients than non-ischemic HF patients. CRT helps to improve major HF related symptoms and reduce HF hospitalisations in patients with HFrEF patients <35%, and abnormal QRS complex and left bundle branch block findings on ECG. CRT devices may also be coupled with an ICD for their use in HF patients[65]. Implantable pumps known as LV assist devices may be used to mechanically augment cardiac function by unloading the blood from the LV in advanced HF patients[72, 73]. LV assist devices are mainly recommended for patients having refractory HF who are heart transplantation candidates[72, 73]. They may also be used as destination therapy in some patients who are not eligible to receive a heart transplant[72, 73]. The heart transplantation procedure is performed for HF patients that have advanced, refractory end-stage HF and no other debilitating conditions if they strictly follow the treatment regimen[51]. Older patients (60 to 70 years) with good health may also receive heart transplantation if they meet all the criteria for successful transplantation[51]. In heart transplant patients, the 1-year survival rate is 85 to 90%, and annual mortality after one year is typically 4%/year. In addition, the mortality rate in transplant candidates who are waiting for a donor heart is 12 to 15%[74].

3. Myocardial oxidative imbalance and pro-inflammatory response in MI

Myocardial ischemia is known to cause an imbalance in the normal redox status which leads to the development of oxidative stress[75, 76]. The abnormal redox status due to the excessive levels of various reactive oxygen species (ROS) adversely affects the cellular organelles and their constituents by oxidising them, which are already compromised by an ischemia[75, 76, 77]. The toxic effect of ROS is considered to be a major contributor to the ischemic myocardial cell injury. In

addition, the abnormal redox status perturbs cardiomyocyte energetics, contractile function and ion homeostasis. Oxidative stress also plays a key role in post-MI cardiac remodelling[78]. The evidence suggests that the various pathophysiological changes associated with cardiac remodelling such as concentric and eccentric hypertrophy, and ECM protein dysregulation are influenced by oxidative stress by interfering with various signalling pathways[79]. Previous studies have shown that enzymatic and non-enzymatic antioxidant mechanism contribute to protection against cardiac ischemia mainly by superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase and thioredoxin peroxidase, as well as vitamin C, vitamin E, glutathione and other dietary antioxidants[80-85].

An acute MI is characterised by an elicitation of a pro-inflammatory response that is evoked by the injured and dying cardiomyocytes[86-88]. This is an indispensable process that initiates a series of tightly regulated complex processes which are involved in the post-MI reparation of the infarct. However, the persistent chronic pro-inflammatory response is known to contribute to the adverse post-MI cardiac remodelling. A number of immune modulatory cell types and cytokines including IL-6, TNF-α, and IL-1β are implicated in the development of post-MI LV remodelling that contributes to a functional decline[87, 89]. TNF-α and IL-6 may also contribute to the contractile dysfunction by altering intracellular Ca^{2+} handling and myofilament sensitivity[90]. Given the perceived role of chronic inflammation in adverse cardiac remodelling and dysfunction, anti-inflammatory therapies have been pursued by targeting the pro-inflammatory cytokines.

4. Nutraceuticals

The link between human health and nourishment has profoundly influenced our understanding of human physiology and pathophysiology. The discovery of vitamins and minerals as agents that

cure diseases including scurvy, beriberi, pellagra, rickets, xerophthalmia, and anaemia is a prime example of how our understanding of the role of nutrients informs evidence-based medical practice. Plant based bio-actives have also been used for the treatment of many diseases[91]. The isolation and characterisation of plant based bio-actives as lead compounds and establishing their synthetic analogues have been instrumental in setting up the modern drug discovery and development platforms. It is not surprising that repertoire of pharmaceutical drugs used today to prevent and treat a large portion of the diseases is originally derived from plants[91]. Notably, approximately half of the globally approved pharmaceutical drugs in the past 3 decades are known to have a plant origin[91]. In recent years, the renaissance of the concept "Let thy food be thy medicine and medicine be thy food" spurred great interest in exploring the potential opportunities of food and food-derived compounds in preventing and treating chronic human diseases[92]. Nutraceuticals are food derived compounds that have beneficial physiological and protective actions against chronic diseases[92]. The utility of nutraceuticals as a preventative or treatment agent presents an opportunity to integrate them into existing therapeutic strategies for the management of cardiovascular diseases (CVDs)[92]. Dietary fibers, polyunsaturated fatty acids, phytosterols, vitamins and polyphenols are some of the groups of compounds that have been reported to have cardiovascular benefits.

5. Polyphenols

Polyphenols are a family of compounds synthesised by plants as secondary metabolites that have received increased attention in medical research[92]. These compounds serve a wide variety of functions in plants ranging from normal growth to reproductive processes including pollination[92]. They are also essential for plant pathogen defense[92]. Such as the term implies, polyphenols are distinguished by the occurrence of multiple phenol rings in their chemical structure. Depending

on the number of phenol rings, and the structural properties of their binding to each other, polyphenols are divided into subclasses including phenolic acids, flavonoids, stilbenes, and lignans[91]. Because of their anti-microbial, antioxidant and anti-inflammatory properties, they became compounds of medicinal importance and they have been widely studied for their potential as pharmacological lead molecules for a variety of diseases[91]. Among the plant polyphenols, one of the most studied is resveratrol[92].

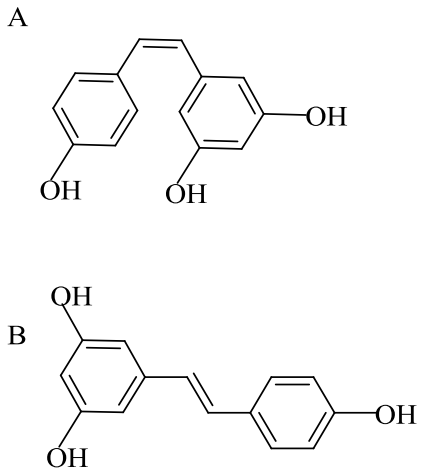
6. Resveratrol

6.1. Classification and structure

Resveratrol (3, 5, 4′-trihydroxy-trans-stilbene) belongs to a class of compounds called plant stilbenes. Resveratrol is present in many different dietary items such as red and green grapes, a variety of berries, plums, and nuts including peanuts in varying amounts[92]. Resveratrol naturally occurs in dual structural isomeric forms, cis-resveratrol and trans-resveratrol (molecular weight: 228.24); both are lipophilic in nature, trans-resveratrol being the more biologically active form (Figure 5A and B)[93].

Plant biologists believe that resveratrol is also a phytoalexin produced by plants during environmental stress and infections to help overcome adverse effects[92]. Phytoalexins form an integral part of the innate immune response by plants against a wide variety of infectious diseases[94-96]. This inherent ability of resveratrol to provide protection against stress conditions in plants has raised a lot of attention in medical research. This was reasoned as the main feature that makes resveratrol an attractive candidate for effectively utilising it for the prevention and treatment of human diseases, considering the evolutionary principle that biosynthetic pathways and hormone signalling may have originated from a common evolutionary tree. For example,

salicylic acid in plants offers protection against plant pathogens and blocks jasmonic acid synthesis in plants and prostaglandin synthesis in animals. It is argued that stress-induced plant compounds may provide protection against stresses in animals as well.



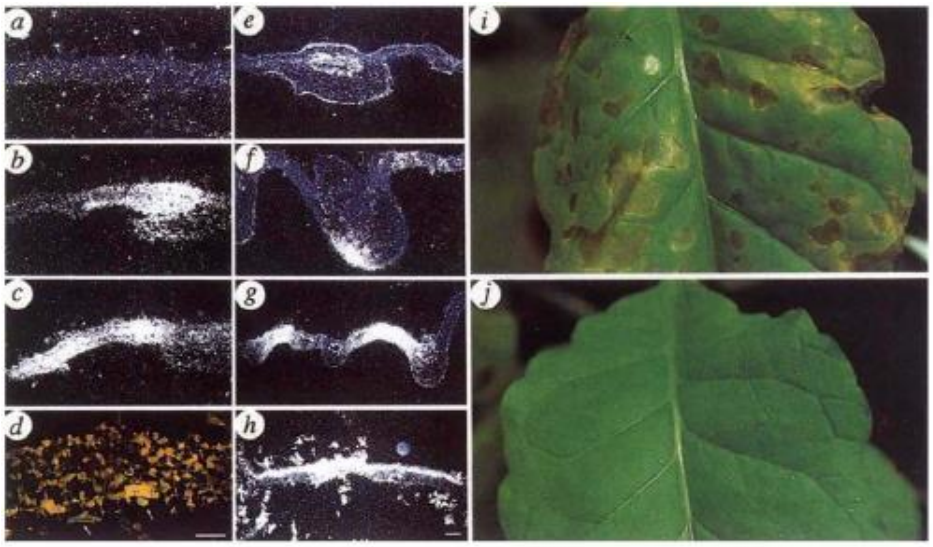


Figure 5. Resveratrol structure and stilbene synthase gene based phytoalexin mediated protection of Tobacco plants. A. Cis-resveratrol. B. trans-resveratrol (molecular weight: 228.24), (C) Tobacco plants containing stilbene biosynthetic genes from grapevine are more resistant to infection by Botrytis cinerea than (right side below) those do not contain stilbene biosynthetic genes (right side above)[97]. *Hain R, Reif HJ, Krause E, Langebartels R, Kindl H, Vornam B, et al.*

Disease resistance results from foreign phytoalexin expression in a novel plant. Nature. 1993;361(6408):153-6. Reprinted with permission.

Resveratrol is produced in the plants by the formation of precursors p-coumaroyl CoA and malonyl CoA in a molar ratio of 1:3. This condensation reaction is catalysed by the enzyme called stilbene synthase, or resveratrol synthase and protects plants when produced enough (Figure 5C)[92]. The highest concentration of this polyphenolic compound has been in the roots of *Polygonum cuspidatum*, a plant which has been used in traditional oriental medicine. The cardioprotective capacity of resveratrol has been extensively studied after it started getting a great amount of attention because of the speculated role of red wine in an observation termed the "French paradox", and also due to the detection of resveratrol's presence in red wine[92]. However, this suggested correlation may not be definitive as there are several factors that can influence the observations that form the basis of the "French paradox". In the past 2 decades, several pre-clinical studies have established the cardioprotective role of resveratrol in various models of heart disease. However, these findings should be further investigated in randomised clinical trials in appropriate human patient populations to understand the true potential of resveratrol as a cardioprotective agent. Recently, a few clinical studies have also described the efficacy of resveratrol as a cardioprotective agent.

6.2. Pharmacokinetics of resveratrol in animals and humans

The absorption, distribution, metabolism and elimination (ADME) of resveratrol have been extensively studied in animals and humans as many studies have reported the role of resveratrol in preventing various diseases including CVDs[98]. The ADME of resveratrol is of great consequence with regards to its action on targeted tissues *in vivo* to replicate the effects

demonstrated in the *in vitro* studies. The minimal bioavailability of the free form of ingested resveratrol is often considered as a main obstacle in achieving the beneficial results demonstrated in *in vitro* studies[98]. Justifiably, it also leads to skepticism regarding the beneficial effects of resveratrol reported from different animal models of various diseases. Adding to this challenge, resveratrol metabolites have also been implicated in the resveratrol mediated beneficial effects. Although many studies have evaluated the metabolism of resveratrol after administration through different routes in animals and oral administration in humans, it is apparent that little information is available about the activities of the metabolic conjugates of resveratrol as well as the therapeutically optimal dosage for its use in clinical conditions. That being said, novel formulations that can circumvent the drawbacks such as limited solubility, and low bioavailability along with a targeted delivery approach may also help to improve the bioavailability and efficacy of resveratrol in the clinical setting.

The recent advances in the efficacy studies using resveratrol in different forms have shed light on the various challenging aspects such as bioavailability, safety, effective dosage for therapeutic use, use of derivatives and inter-individual variations in response[98]. In a seminal work, Bertelli et al. reported the intestinal absorption, the concentration in plasma, urine, heart, liver and kidney as well as the excretion of natural trans-resveratrol and *cis*-resveratrol (6.5 mg/L) after oral administration of red wine to rats[99]. Resveratrol was detected in plasma after 30 minutes, which peaked to the maximum in 1 hour[99]. In another study, it was concluded that half of the orally administrated trans-resveratrol in rat was absorbed from the intestine very rapidly[100]. The intestinal uptake of resveratrol was also demonstrated with the system of isolated rat small intestine perfusion, which reported the presence of resveratrol in enterocytes[101]. The direction-independent trans-epithelial diffusion mediated resveratrol transport in human intestinal Caco-2

cells has also been reported in the *in vitro* setting[102]. In addition, hepatic uptake of resveratrol occurs through carrier mediated transport even though it binds to serum protein albumin which may partly explain the efficiency of the influx[103].

Bioavailability research involving humans used varying doses of resveratrol in different formulations to study the achievable plasma or tissue levels of the molecule using a wide range of analytical techniques. A study involving human subjects by Soleas et al. reported that the level of free resveratrol in plasma is very low[104]. Resveratrol is absorbed into the systemic circulation in the form of glucuronide conjugate as resveratrol is being metabolised rapidly in the intestine in its natural form[105]. Resveratrol also undergoes sulfation in liver and duodenum by sulfotransferases[106]. Interestingly, the oral bioavailability of resveratrol is quite low albeit the total absorption in the intestine is as high as 70%, which is very high compared to other polyphenols[98]. The maximum reported oral bioavailability of resveratrol is only 20% as most of the resveratrol absorbed in the intestine rapidly undergoes conversion to its conjugates in the intestinal epithelial cells and by first-pass metabolism and gets eliminated through kidney. The different metabolites along with trace amounts of unchanged resveratrol namely, 2 resveratrol monoglucuronides, 2 monosulfates, and dihydroresveratrol glucuronide and sulfate were identified after oral and intravenous administration of radio isotope labelled [$_{14}$C] resveratrol. It is also suggested that this bio-transformation phase is a rate limiting factor in terms of resveratrol's bioavailability as it affects the fate of the absorbed resveratrol in the body. Importantly, the detection of dihydroresveratrol also pointed out the role of microbial action on the native resveratrol as it is a by-product of the microbial hydrogenation[98].

Certainly, the plasma concentration of resveratrol depends on a dose and time dependent regimen and maximum total resveratrol concentration was estimated as 2.4 µM (~500 ng/mL) after oral

administration of a high dose of 5 g whereas a low dose of 25 mg resulted in a very negligible concentration (5 ng/mL)[98, 107]. Interestingly, Brown et al. reported an even greater plasma concentration of resveratrol (900ng/mL) using the same high dose of 5 g in a 29 days study[108]. Of note, the small sample size prevented them from drawing valid conclusions on the safety profile of the molecule at this dose. Resveratrol half-life was calculated as 1-3 hours following single doses and 2-5 hours following repeated dosing of 25, 50, 100, and 150 mg and the mean peak plasma resveratrol levels of 3.89, 7.39, 23.1 and 63.8 nm/L, respectively[109]. Although repeated doses did not result in high bioavailability, a linear relationship was seen between the dose and plasma concentration[109].

Apart from plasma concentration, tissue distribution is an important criterion in determining the target specific action of any molecule of therapeutic interest. Resveratrol and its metabolites may also accumulate in different tissues and the latter may bring about the desired effect in target sites due to the enzymatic breakdown of resveratrol metabolites into resveratrol via tissue beta-glucuronidases and sulfatases. Resveratrol was found in varying degrees in various tissues such as kidney, liver, intestine, and heart in rats as well as in swine[99,110]. In addition, in colon cancer patients who have been undergoing surgery with resveratrol treatment prior to the procedure, tissue specific distribution of resveratrol metabolites was identified[111]. Additionally, recent studies reported that resveratrol glucuronides and sulfate inhibit colon cancer cell growth[112,113]. It is likely that accumulation of resveratrol in tissues by virtue of its lipophilic nature contributes to some of the beneficial effects observed in various CVD models. Although major toxic effects were observed at higher doses such as 3000 mg/kg body weight/day, administration of 300 mg/ kg body weight/day produced no negative effects in rats [114] and resveratrol up to 750 mg/kg body weight/day is also well-tolerated without any adverse effect on reproductive system or embryo–

fetal development [115]. Brown et al. also reported the toxicity profile from a short duration clinical trial consisting of 44 healthy subjects who consumed resveratrol at daily doses of 0.5, 1.0, 2.5, or 5.0 g[108]. Boocock et al. also reported that high oral doses of resveratrol such as up to 5 g are well tolerated without any serious consequences[107]. The outcome of these studies supports the notion that resveratrol is safe for use by humans as no adverse effects were reported from the biochemical analysis. However, a few gastrointestinal difficulties including diarrhea, flatulence, and stomach ache were observed at high doses[108]. Another clinical study involving healthy volunteers also reported no adverse toxicity issues when they underwent treatment with 1 g resveratrol for 4 weeks[116]. Nevertheless, these short-term studies are not adequate enough to fully validate the safety profile of resveratrol intake for 2 reasons. First of all, the therapeutic use of resveratrol by patients may be for longer periods compared to the shorter duration of these studies. Secondly, the health status of patients who take resveratrol as a supplement may influence its safety profile. If taking medications, drug interactions may also have a bearing on the final safety and therapeutic outcomes with resveratrol.

To overcome the limitations observed in the pharmacokinetic studies, different approaches have been proposed to fully exploit the potential of resveratrol in clinical conditions[117]. Incorporation of metabolic inhibitors of resveratrol biotransformation has been shown to help achieve higher concentration in the circulation after its administration[117]. It is reported that resveratrol provides a combinational effect with other dietary molecules. Piperine has been shown to inhibit the glucuronidation of resveratrol and improve the pharmacokinetics and slow down its rapid elimination[117]. All of the measures that have been used to enhance the bioavailability, the use of resveratrol derivatives is particularly important as it may contribute to the improvement in

efficacy by overcoming the pitfalls associated with natural resveratrol. These include synthetic derivatives or testing naturally occurring derivatives. Pterostilbene, a dimethyl derivative of resveratrol, was shown to have improved bioavailability[118, 119]. In addition, a natural pharmacologically active analogue of resveratrol, 3,5,4-trimethoxytrans-stilbene was demonstrated to have a longer plasma exposure and half-life, and less clearance than resveratrol[120].

Last but not least, novel formulations and targeted drug delivery system could also improve the bioavailability many fold through improving the solubility and tissue specific delivery. Current formulations used for resveratrol administration lack efficiency in improving pharmacokinetic properties due to poor aqueous solubility and stabilisation. Cyclodextrin carrier systems have been found to improve the solubility of resveratrol but it did not significantly augment its bioavailability. Targeted delivery of resveratrol to the desired tissues or increasing the stabilisation of resveratrol in the body by developing sustained release systems can overcome its early degradation in the intestine and liver and its elimination, and thereby augment the bioavailability. Multiparticulate forms and colloidal carriers have been proven as efficient agents for sustained release of resveratrol in specific areas as described elsewhere[121]. Promising outcomes from these innovative approaches to address pharmacokinetic challenges that are associated with resveratrol may help to improve the efficacy of resveratrol in humans.

7. Resveratrol in ischemic heart disease and HF

7.1. Pre-treatment effects of resveratrol on ischemia/reperfusion *ex vivo*

Approaches targeting the pre-conditioning of the myocardium to combat ischemic injuries are being actively pursued as a preventative strategy. Conceptually, ischemic pre-conditioning involves attaining protection against a lethal ischemic injury by subjecting a tissue or organ to

mild ischemic episodes prior to an actual episode of prolonged ischemia. Many studies have examined the preconditioning effect of acute or chronic administration of resveratrol (pre-treatment) in an *ex vivo* setting of myocardial ischemia/reperfusion, i.e subjecting the isolated perfused hearts to ischemia for the desired duration and then re-establishing normal perfusion (Table. 1).

Acute pre-treatment: Acute pre-treatment with resveratrol has been studied in animals subjected to myocardial ischemia/reperfusion *ex vivo*. Pre-treatment of isolated perfused hearts with resveratrol (10 μM), starting 5 minutes prior to the onset of ischemia (30 minutes) followed by reperfusion (120 minutes), was reported to reduce infarct size when compared with the control ischemia/reperfused hearts[122.] In isolated perfused hearts that were subjected to no-flow global ischemia for 20 minutes and reperfusion for 30 minutes, resveratrol pre-treatment (20 and 100 μM), administered 15 minutes prior to the start of ischemia, improved LV functional recovery and reduced infarct size[123]. Resveratrol (3–100 μM) was also shown to reverse polymorphic ventricular tachyarrhythmia induced by 20 minutes occlusion of LAD followed by reperfusion in isolated rat hearts[124]. In addition, perfusion with resveratrol (2.3 mg/L) reduced infarct size, when rat hearts were subjected to 30 minutes of regional ischemia and subsequent reperfusion[68.]

Chronic pre-treatment: The effects of long-term pre-treatment with resveratrol were also reported in normal animals subjected to myocardial ischemia/reperfusion injury *ex vivo*. For example, intraperitoneal administration of resveratrol (25 mg/kg body weight/day) for 7 days was shown to be effective in preserving contractile function after ischemia/reperfusion injury *ex vivo*[125.] Moreover, administration of resveratrol for 7 days (2.5. 10, 25, and 50 mg/kg body weight/day) was reported to improve the post-ischemic myocardial contractile function *ex vivo* in a dose dependant manner[126.] The hearts from rats treated with 25 mg/L resveratrol for a longer

duration such as 15 days showed better cardiac functional recovery when subjected to 60 minutes low-flow ischemia/reperfusion[127.] Pre-treatment of mice for 6 weeks at a dose of 2 mg/kg body weight/day also improved LV pressure, coronary flow (CF) and reduced infarct size in the hearts that were isolated and subjected to ischemia/reperfusion injury[128]. Furthermore, chronic pre-treatment with resveratrol (25 mg/kg body weight/day in diet) for 16 weeks reduced infarct size in rat hearts subjected to ischemia/reperfusion *ex vivo*[129.]

A few studies have also reported long-term pre-treatment with resveratrol in diseased animals subjected to ischemia/reperfusion injury *ex vivo*. For instance, chronic pre-treatment with resveratrol (5 mg/kg body weight/day) for 2 weeks in Zucker obese rats treated with or without 10% glucose reduced the incidence of VF and infarct size as well as improved LV developed pressure, CF and aortic flow when the hearts were subjected to 30 minutes of global ischemia and 120 min of reperfusion[130]. Resveratrol pre-treatment (2.5 mg/kg body weight/day) in streptozotocin-induced diabetic rats for 15 days improved LV developed pressure, and reduced infarct size when the hearts were subjected to 30 minutes global ischemia followed by 2 hours reperfusion[131]. Resveratrol (20 mg/kg body weight/day) pre-treatment for 2 weeks in rats which received a hypercholesteraemic diet was also found to improve the first derivative of LV developed pressure and decrease infarct size following ischemia/reperfusion *ex vivo*[132]. In another study, when isolated rat hearts were subjected to ischemia/reperfusion after 2 weeks of pre-treatment with resveratrol (20 mg/kg body weight), an improvement in peak systolic pressure,+dP/dt max, and CF and a reduction in end diastolic pressure and infarct size were observed, suggesting a resveratrol mediated cardioprotection[132].

The results from the above mentioned studies indicate that acute or chronic resveratrol treatments prior to the induction of ischemia/reperfusion *ex vivo* exert beneficial effects on the

myocardium by a possible pre-conditioning action. It is, however, not clear whether pre-treatment with resveratrol is specifically more effective during ischemia or at the time of reperfusion, because most studies discussed here reported the endpoint results after ischemia/reperfusion, not after ischemia alone. Interestingly, the ability of resveratrol to prevent arrhythmias is a remarkable finding as MI is associated with increased arrhythmogenesis due to conduction abnormalities that leads to sudden cardiac death, especially, given that current antiarrhythmic drugs are suboptimal in safety. The ability to reduce infarct size with resveratrol treatment during ischemia/reperfusion is also vital as it may help to prevent the development of cardiac dysfunction. This finding also points to the fact that there is an opportunity to use resveratrol in conjunction with a PPCI to reduce reperfusion injury and no-reflow phenomenon. The finding that resveratrol is effective in reducing *ex vivo* ischemia/reperfusion injury in rats with a pre-existing disease conditions may be further explored in *in vivo* studies[131, 132], given that pre-existing disease will predispose the myocardium to more ischemia/reperfusion injury.

7.2. Pre-treatment or combined pre-treatment and post-treatment effects of resveratrol on ischemia and ischemia/reperfusion *in vivo*

***Acute studies*:** Some studies have examined the effects of short-term resveratrol administration on myocardial ischemia and ischemia/reperfusion *in vivo*. Pre-treatment with resveratrol (10 μM) just 15 minutes before induction of ischemia by coronary artery ligation (for a duration of 30 minutes) followed by 120 minutes reperfusion limits the decrease in LV systolic pressure and limit infarct size[133]. In another study, resveratrol (100 μM) reduced infarct size following the induction of ischemia and subsequent reperfusion *in vivo*, when administered 5 minutes prior to reperfusion[134]. Administration of resveratrol (0.05 and 1 mg/kg) in rats 60 minutes prior to induction of ischemia (60 minutes) and subsequent reperfusion for 3 hours also was reported to

reduce infarct size and decrease the incidence of ischemia/reperfusion-induced ventricular VT and VF[135]. In a similarly designed study, resveratrol pre-treatment (1 μM) reduced the incidence and duration of VT and VF when left main coronary artery was occluded for 5 or 30 minutes followed by 30 minutes reperfusion in rats[136]. In left anterior descending (LAD) artery ligated rats, resveratrol shortened the duration of arrhythmia, and decreased incidence of VT and mortality in a dose dependant manner (5, 15, and 45 mg/kg) when administered 10 minutes prior to the surgery[137]. Intraperitoneal (IP) administration of resveratrol (5 mg/kg) 24 hours prior to 30 minutes occlusion of the LAD artery followed by 24 hours reperfusion was also reported to decrease infarct size in mice[138].

***Chronic studies*:** Several studies have also examined the effects of long-term resveratrol administration in animals subjected to myocardial ischemia and/or reperfusion injury *in vivo*. Pre-treatment with resveratrol (1 mg/kg body weight/day) for 2 weeks was reported to reduce infarct size and improve cardiac function (under normal and pharmacologic stress testing with dobutamine), perfused capillary density and myocardial blood flow, in 24 hour coronary artery occluded rats[139]. Resveratrol pre-treatment (5 mg/kg body weight/day) started 1 week before LAD ligation and administered for another 3 weeks also decreased infarct size and cardiac hypertrophy, and suppressed ventricular arrhythmias such as VT and VF[140]. Acute and long-term survival rate was also higher in the resveratrol treated MI-induced rats[140]. Pre-treatment with resveratrol for 1 week (10 mg/kg body weight/day) also reduced infarct size 24 hours after ischemic injury, with a concomitant improvement in capillary density and the first derivative of LV developed pressure at 3 weeks post-coronary artery occlusion[141].

A few studies have also described the effects of long-term pre-treatment with resveratrol in diseased animals subjected to ischemia injury *in vivo*. Resveratrol pre-treatment (100 mg/kg

body weight/day) in Yorkshire swine on a hypercholesterolemia diet for 4 weeks followed by another 3 more weeks after induction of left circumflex artery constriction (to produce ischemia) resulted in conserved local wall motion in the ischemic area, improved flow to the ischemic zone during LV pacing, and increased vasodilation in the ischemic myocardium (at 7 weeks)[142]. Resveratrol also decreased cholesterol levels by about 30% in hypercholesterolemia swine[142]. The observed beneficial effects were attributed to both pre and post-conditioning effects of resveratrol. Resveratrol pre-supplementation improved regional LV function and perfusion to the myocardium which was distant from a zone of ischemia in a similar model of metabolic syndrome and chronic myocardial ischemia mentioned above[143].

7.3. Post-treatment effects of resveratrol on permanent ischemia by coronary artery occlusion

A few studies have investigated the efficacy of resveratrol in improving cardiac structure and function after permanent ischemia. These studies are particularly important to understanding the cardioprotective effect of resveratrol in an injured and healed myocardium that undergoes progressive adverse remodelling and develops LV systolic dysfunction over a period of time after the initial insult. Daily administration of resveratrol (1 mg/kg body weight/day) for 4 weeks by intraperitoneal (IP) injection after induction of an MI was reported to improve LV systolic and diastolic function and reduce LV dilatation and infarct size in rats[144]. Resveratrol, when administered following an MI (for 42 days at a dosage of 20 mg/kg body weight/day), was also able to decrease infarct size, and cardiac remodelling, improve LV function and increase the survival rate in mice[145]. Another long-term study that investigated the efficacy of a post-MI resveratrol treatment at a low dosage of 2.5 mg/kg body weight/day for 16 weeks reported an improvement in cardiac structure and function, and an increase in the survival rate in rats[146].

A large number of acute MI patients present with severe LV dysfunction and HF. Notably, resveratrol has also been found to be effective in reversing established ischemic HF. For instance, resveratrol treatment (50 mg/kg body weight/day by osmotic pump) for 2 weeks in mice, which was started 4 weeks after the induction of MI, drastically improved cardiac structure and function[147]. It should be noted that MI mice had developed HF (LVEF <40%) prior to the commencement of resveratrol treatment and therefore, this study suggests that resveratrol treatment is able to induce reverse cardiac remodelling HF and thereby improve LV dysfunction[147].

Of note, there are studies which reported that resveratrol treatment did not result in cardioprotection in the setting of experimental MI. Firstly, in male New Zealand white rabbits, resveratrol (0.15 mg/kg body weight and 1.5 mg/kg body weight) administration had no effect on the infarct size and regional myocardial blood flow, when given 15 minutes before coronary artery occlusion/reperfusion[148]. In another study, 13 weeks (17 mg/kg body weight/day) combined pre and post-treatment with resveratrol, started 1 week prior to coronary artery ligation in rats, and continued for 12 weeks after the surgery, was not able to improve cardiac function in MI-induced rats[149]. There was, nevertheless, an improvement in cardiac performance with the dobutamine stress test in MI rats[149]. Likewise, a very low dose (0.1 mg/kg body weight/day) and a moderate dose (5 mg/kg body weight/day) treatment with resveratrol in permanent coronary artery ligated rats[24] and mice[27] for 4 weeks and 2 weeks, respectively, did not afford cardioprotection[144, 147]. The different types of delivery methods (gavage, IP, and in diet) and vehicles (ethanol, Dimethyl Sulfoxide (DMSO), and water) may have influenced the outcomes of the studies.

Despite a few studies showing a lack of effect of resveratrol, there is sufficient evidence to suggest that both pre-treatment or combined pre-treatment and post-treatment with resveratrol is beneficial in small and large animal models of ischemia and ischemia/reperfusion *in vivo* and permanent ischemia. Overall, acute and chronic studies show that the beneficial effects of resveratrol have been observed in animal models of ischemic heart disease as well as in overt HF resulting from myocardial ischemia. These encouraging reports have paved the way for clinical trials in patients with ischemic heart disease for a possible translation of these promising pre-clinical findings into clinical practice.

7.4. Efficacy of resveratrol in combination with drugs

The stand-alone cardioprotective effect of resveratrol has been established as discussed in the previous sections. From a clinical point of view, resveratrol should be complementary to existing medications and able to offer additional beneficial patient outcomes for its effective translation and incorporation into the ischemic heart disease management strategies. To date, only 2 studies have investigated the efficacy of resveratrol in combination with standard cardiac medication in the setting of ischemic reperfusion injury[132]. In the first study, isolated hearts from rats fed a hypercholesterolemic diet received a combination of resveratrol and statin before being subjected to ischemia/reperfusion. The hearts exhibited a significant increase in LV functional recovery and a reduction in infarct size in comparison to ischemia-reperfused hearts from rats fed a hypercholesterolemic diet and treated with either of the interventions alone[132]. This study suggested that a combination of resveratrol and statin is superior to treatment with either of the 2 agents alone[132]. However, the efficacy of this combination treatment was not assessed in MI setting *in vivo*, hence the question as to whether a combination of statin and resveratrol could be effective in improving the cardiac structure and function after ischemia or ischemia/reperfusion

injury *in vivo* remains to be investigated. In another study, combination therapy with resveratrol and hydralazine was also shown to attenuate myocardial ischemia/reperfusion injury *ex vivo* in wild type and endothelial nitric oxide synthase (NOS) deficient mice[129].

7.5. Role of resveratrol in regenerative therapy

The injured myocardium is incapable of regenerating and replacing the large number of cardiomyocytes lost after an ischemic insult. Therefore, myocardial regenerative therapy is considered to be an invaluable tool for improving the prognosis of post-MI patients[44, 150]. Regenerative therapy using various types of stem cells has advanced greatly as evident from the large number of pre-clinical studies and emerging clinical evidence[44, 150]. Not surprisingly, stem cell therapy is also faced with major unresolved challenges such as impaired cell engraftment, retention, and survival. Recently, pharmacological approaches are also being explored with a view to improve the efficacy of stem cell therapy[44].

Resveratrol is also being studied as a molecule which can enhance mobilisation of stem cells in the myocardium in the setting of MI[151]. Autologous Sca-1(+)Lin(-)CD45(-)(CXCR(+)) very small embryonic-like stem cell (VSEL) mobilisation has been reported to be beneficial in improving cardiac function after acute MI[151]. Treatment with resveratrol for a period of 1 week resulted in increased recruitment of VSEL to the injured myocardium[151]; it was interesting to note that statin did not improve the mobilisation of VSEL. Cardiac stem cells (CSC) from the explanted decompensated hearts from explanted decompensated hearts (E-CSC) are potential candidates for myocardial regenerative therapy. However, these cells may undergo cellular senescence in thus affecting their regenerative capacity. In this regard, a recent study reported that *ex vivo* pre-treatment with resveratrol and rapamycin reduced senescence of E-CSC[26].

Transplantation of these pre-treated E-CSC to MI-induced mice also mediated cardiac repair with a reduction in cardiomyocyte senescence and apoptosis along with an increase in the endogenous c-Kit(+) CSC in the peri-infarct zone, suggesting the potential of resveratrol in improving the therapeutic efficacy of stem cells[152].

Insufficient perfusion due to impaired vascularisation of a stem cell engrafted area, oxidative stress, increased pro-inflammatory response, and apoptosis are some of the pathological factors that interfere with the capacity of stem cells to differentiate, and thus limiting their beneficial effect[44]. Due to its inherent properties (discussed in section below - Mechanistic insight into the action of resveratrol), resveratrol may be effective in offering a better myocardial environment for cardiac regenerative therapy by controlling some of the pathological factors mentioned above. In light of these promising findings, the efficacy of resveratrol (*ex vivo* stem cell treatment with resveratrol or *in vivo* administration of resveratrol along with stem cells into the ischemic myocardium) in enhancing homing, survival and differentiation of various types of endogenous and transplanted stem cells needs to be further investigated.

7.6. Promise of resveratrol in microRNA therapeutics and gene therapy

MicroRNAs (miRs) are shorter length non-coding RNAs which are capable of regulating various normal and pathophysiological processes in the heart[153]. miR based therapeutic strategies have been shown to be promising in many pre-clinical studies[153]. Interestingly, few studies have suggested that resveratrol may be beneficial in positively modulating miRs under the diseased states[154, 155]. Specifically, pre-treatment with resveratrol resulted in significant alterations in the miR profile in the heart subjected to ischemia/reperfusion *ex vivo*, and these changes were implicated in the improvement in cardiac function and reduction in infarct size[155, 156]. Resveratrol

also positively modified the miR expression profile in ischemic heart disease patients[157]. More studies are needed to understand the potential of resveratrol in regulating miRs in the *in vivo* settings. It is also imperative to investigate how resveratrol regulates the miR expression patterns as well as to compare its efficacy alongside other miR based therapeutics such as anti- miRs to evaluate the potential of resveratrol.

Gene therapy offers great promise for HF patients as a strategy to increase the expression of downregulated defective genes in the dysfunctional myocardium via delivering expression vectors that carry the gene of interest to help recover the normal function of the myocardium[158]. Pilot clinical trials with adenoviral vector 6-SERCA2a have already been deemed successful in HF patients; the follow up trials that were conducted to further evaluate the efficacy this approach failed to show benefits[158]. Interestingly, resveratrol was found to act as a potent inducer of Egr-1 promoter in the adenoviral.Egr.TNF vector (Egr-1 promoter is a construct that helps to express the candidate therapeutic gene of interest) in both in *in vitro* and tumor xenografts *in vivo*[35], suggesting that resveratrol may be effective in improving the efficacy of adenoviral vector mediated gene therapy in CVD[159]. Studies examining the combination of resveratrol and gene therapy will be needed to explore this promising approach.

Ex vivo experimental design	*Resveratrol pre-treatment*	*Effects of treatment*
Ischemia (30 minutes) and reperfusion (120 minutes)	10 μM starting 5 minutes prior to the onset of ischemia	Reduction in infarct size[122]
No-flow global ischemia for (20 minutes) and reperfusion (30 minutes)	20 and 100 μM 15 minutes prior to the start of ischemia	Reduction in infarct size and improvement in LV function[123]
Ischemia (20 minutes) and reperfusion	3–100 μM	Reduction in polymorphic ventricular tachyarrhythmia

Ischemia (30 minutes) and reperfusion (45 minutes)	2.3 mg/L	Reduction in infarct size[124]
Ischemia (45 minutes) and reperfusion (10 minutes)	25 mg/kg body weight/day for 7 days	Improvement in contractile function[125]
Ischemia (15 minutes) and reperfusion (10 minutes)	2.5. 10, 25, and 50 mg/kg body weight/day for 7 days	Improvement in contractile function[126]
Ischemia (15 minutes) and reperfusion (10 minutes)	25 mg/L for 15 days	Better cardiac functional recovery and vasodilation[127]
Ischemia (30 minutes) and reperfusion (30 minutes)	2 mg/kg body weight/day for 6 weeks	LV pressure, CF and reduced infarct size[128]
Ischemia (15 minutes) and reperfusion (30 minutes)	25 mg/kg body weight/day in diet for 16 weeks	Reduction in infarct size[129]
Global ischemia (30 minutes) and reperfusion (120 minutes) in Zucker obese rats	5 mg/kg body weight /day for 2 weeks	Improvement in contractile function[130]
Global ischemia (30 minutes) and reperfusion (120 minutes) in streptozotocin-induced diabetic rats	2.5 mg/kg body weight/day for 15 days	LV developed pressure, and reduced the infarct size[131]
Ischemia (30 minutes) and reperfusion (120 minutes) in rats on hypercholesterolemic diet	20 mg/kg body weight/day pre-treatment for 2 weeks	Improvement in first derivative of LV developed pressure and reduction in infarct size[132]
In vivo experimental design	*Resveratrol pre-treatment or combined pre and post-treatment*	*Effects of treatment*
Ischemia by coronary artery ligation (30 minutes) and reperfusion(120 minutes)	10 μM 15 minutes before induction of ischemia	Decrease in LV systolic pressure and reduction in infarct size[133]

Ischemia by coronary artery ligation (30 minutes) and reperfusion(120 minutes)	100 μM/L administered 5 minutes prior to reperfusion	Reduction in infarct size[134]
Ischemia by coronary artery ligation and subsequent reperfusion	0.05 and 1 mg/kg body weight for 60 minutes prior to induction of ischemia	Reduction in infarct size and decrease in the incidence of ischemia/reperfusion-induced VT and VF[135]
Ischemia by coronary artery ligation (5 or 30 minutes) and reperfusion (30 minutes)	1 μM	Reduced incidence and duration of VT and VF[136]
Left anterior descending artery ligated rats	5, 15, and 45 mg/kg body weight administered 10 minutes prior to the surgery	Shortened duration of arrhythmia, decrease in incidence of VT and mortality in a dose dependent manner[137]
Left anterior descending coronary artery ligated rats	Intraperitoneal administration (5 mg/kg body weight) 24 hour prior to 30 minutes	Reduction in infarct size[138]
Left anterior descending coronary artery ligated rats	1 mg/kg body weight/day for 2 weeks	Reduction in infarct size and improvement in cardiac function[139]
Left anterior descending coronary artery ligated rats	5 mg/kg body weight/day started 1 week prior to ligation and continued for an additional 3 weeks	Reduction in infarct size and cardiac hypertrophy, and suppression of ventricular arrhythmias such as VT and VF[140]
Left anterior descending coronary artery ligated rats	10 mg/kg body weight/day for 1 week	Reduced infarct size (24 hours after ischemic injury). Improvement in capillary density and first derivative of LV developed pressure at 3 weeks post-coronary artery occlusion[141]

Induction of left circumflex artery constriction	100 mg/kg body weight/day for 4 weeks as pre-treatment and post-treatment for another 3 more weeks	Preservation of regional wall motion in the ischemic territory, improvement in flow augmentation to the ischemic territory during ventricular pacing, and increase in vasodilation in the ischemic myocardium[142]
Induction of left circumflex artery constriction	100 mg/kg body weight/day 4 weeks as pre-treatment and post-treatment for another 3 more weeks	Improvement in regional LV function and preservation of perfusion to myocardium remote from an area of ischemia[143]
Left anterior descending coronary artery ligated rats	1 mg/kg body weight for 4 weeks by IP injection	Improvement in LV dilatation, systolic and diastolic function and infarct size[144]
Left anterior descending coronary artery ligated rats	20 mg/kg body weight/day 42 days	Decrease in infarct size, and cardiac remodelling, improvement in LV function and increased survival rate[145]
Left anterior descending coronary artery ligated rats	2.5 mg/kg body weight/day for 16 weeks	Improved cardiac structure and function, and survival[146]
Left anterior descending coronary artery ligated mice	50 mg/kg body weight/day by osmotic pump for 2 weeks (started 4 weeks ligation)	Improved cardiac structure and function[147]
Left anterior descending coronary artery ligated New Zealand rabbits	0.15 mg/kg and 1.5 mg/kg body weight for15 minutes before ligation	Did not affect myocardial blood flow and infarct size[148]
Left anterior descending coronary artery ligated rats	17 mg/kg body weight/day combined pre and post-treatment for 13 weeks	Did not improve cardiac function and infarct size[149]

Table 1. Summary of the effects of resveratrol in the setting of pre-clinical ischemia and ischemia/reperfusion. *Raj P, Zieroth S, Netticadan T. An overview of the efficacy of resveratrol in the management of ischemic heart disease. Annals of the New York Academy of Sciences. 2015. Reprinted with permission*.

7.7. Mechanistic insight into the action of resveratrol in ischemic heart disease

Myocardial ischemia and ischemia/reperfusion injury includes a myriad of cellular events which result in cardiac functional deterioration[160]. Previous studies have identified a variety of molecules that contribute to the ischemic preconditioning of the heart. Therefore, agents that can pharmacologically interact with these target molecules and bring about beneficial effects may be explored for their therapeutic application[161]. In this regard, resveratrol has been demonstrated to normalise some of the malfunctioning molecular pathways during ischemia and/or ischemia/reperfusion[62, 92]. Specifically, resveratrol inhibits the detrimental action of pathological effector molecules or enhances the action of some of the beneficial modulators to afford cardioprotection to ischemic and/or ischemia–reperfused myocardium[62, 92]. MI is characterised by dynamic and varied pathophysiological changes; the complexity and heterogeneity are clearly evident from the current therapeutic strategies itself as a number of drugs are used for the management of post-MI patients. Therefore, the pleiotropic nature of resveratrol's action could be beneficial in effectively targeting pathophysiological changes in post-MI patients. Herein, we discuss the major mechanistic targets of resveratrol mediated cardioprotection.

Oxidative stress as a result of the increased level of ROS is a major player in ischemia and ischemia/reperfusion injury as well as the ensuing chronic remodelling that occurs after the initial injury[162]. ROS cause deleterious and irreversible damage to cellular organelles by initiating lipid peroxidation, protein modification and DNA damage[162]. Being a polyphenol, resveratrol possesses an endogenous ability to directly scavenge ROS[62, 92]. Importantly,

resveratrol also mediates antioxidative stress effects by modulating the expression and activity of endogenous enzymes such as SOD and CAT that play a major role in maintaining the normal redox status[92]. For example, resveratrol has been shown to reduce lipid-peroxidation and increase the activity of SOD following myocardial ischemia/reperfusion-induced injury[133, 163]. Resveratrol has also been reported to increase the level of thioredoxin-1 and hemeoxygenase-1 in MI-induced rats, which are involved in the prevention of oxidative stress[131].

Ischemia/reperfusion is also associated with microvascular injury causing vascular permeability and endothelial dysfunction[160]. In addition, impaired vascular function leads to 'no reflow' in the microvasculature during reperfusion and thus increasing the risk of injury[161]. Endothelial derived nitric oxide (NO) contributes to reducing cardiac abnormalities by promoting perfusion of the damaged tissue due to its vasodilator effect as well as its direct effects on myocardial tissue[92, 164]. Treatment with resveratrol also increases the level of NO[62, 92]. NOS are the enzymes that produce NO within the cells[165]. Consistent with an increase in NO, NOS enzymes that produce NO were also reported to be upregulated upon resveratrol treatment[92, 133, 135]. Furthermore, resveratrol mediated cardioprotection was lost in MI-induced rats treated with L-NAME, a chemical inhibitor of NOS, which suggests that NO is a major factor involved in the resveratrol mediated improvement of cardiac structure and function[92, 131].

An MI is associated with major metabolic changes in the myocardium. Specifically, glucose transport in the ischemic myocardium may be altered via alterations of glucose transporters. In this regard, resveratrol has been shown to increase the level of glucose transporter-4 expression in ischemic-reperfused hearts[130]. Mitochondrial permeability transition pore (mPTP) opening is considered to have an essential part in MI related cardiac injury by triggering cell death pathways such as necrosis and apoptosis, whereas glycogen synthase kinase 3β (GSK-3β)

contributes to the modulation of the mPTP opening[122]. Interestingly, resveratrol is believed to prevent myocardial reperfusion injury partly through inhibiting the mPTP opening via cyclophilin D by translocating GSK-3β to the inner mitochondria from the cytoplasm[122]. The AMP-activated protein kinase (AMPK) is a key regulator of glucose and lipid metabolism, protein synthesis and cell survival. AMPK has also been reported to play a major role in resveratrol mediated cardioprotection in the setting of an MI[8]. AMPK inhibits the mammalian target of rapamycin complex 1 activity via tuberous sclerosis complex 1 and 2 and this in turn leads to the activation of autophagy. Resveratrol mediated activation of AMPK has been shown to activate autophagy as evidenced by the significant increase of myocardial microtubule-associated protein-1 light chain 3-II, the level of ATP, and autophagosomes with a concomitant improvement in cardiac structure and function in MI-induced mice. This suggests a role for AMPK mediated autophagy in resveratrol mediated cardioprotection[147]. Treatment with chloroquine (an inhibitor of autophagy) inhibited the improvement in cardiac structure function, suggesting an autophagy mediated cardioprotection by resveratrol[166]. Sirtuin 1 (SIRT1) is a deacetylase. SIRT1 deacetylates many effector molecules involved in the regulation of the cell cycle, cell death pathways, metabolism, oxidative stress, inflammation, and longevity[167]. Resveratrol has also been shown to be a direct and indirect activator of SIRT1 both *in vitro* and *in vivo*[168]. This observation was suggested as a major mechanism of action behind the beneficial effects of resveratrol[168]. In this regard, resveratrol mediated reduction in infarct size was also associated with an increase in the activity of SIRT1 following ischemia/reperfusion[146]. In addition, pre-treatment with sirtinol (a SIRT1 inhibitor) blocked the beneficial effects of resveratrol following ischemia/reperfusion[146].

Inhibition of abnormal ion transfer via cardiac voltage-gated sodium channels and voltage-gated potassium channels may be beneficial as an antiarrhythmic treatment. Interestingly, an electrophysiological study showed that resveratrol blocked late voltage-gated sodium channel currents (I_{Na}) via recombinant human heart $Na_V1.5$ channels expressed in tsA201 cells[169]. In addition, resveratrol has been shown to possess the ability to inhibit late I_{Na} induced by the toxin, ATX II[169]. In field-stimulated myocytes, resveratrol also prevents ATXII-induced increases in diastolic Ca^{2+}[169]. Resveratrol has also been reported to reduce ATXII-induced cardiomyocyte contractile dysfunction[169]. Resveratrol is recognised as an inhibitor of the late I_{Na} in cardiomyocytes and known to reduce the calcium inward current[170]. Furthermore, a previous study showed that the transient and sustained outward potassium currents also decrease after the exposure to resveratrol[170]. The inward rectifier potassium current has been shown to reduce in the presence of resveratrol. Resveratrol can also act as an inhibitor of recombinant $K_v1.5$ currents in single tsA201 cells[171]. Resveratrol similarly prevents H_2O_2-induced increase in late I_{Na} and augments the reverse Na^+-Ca^{2+} exchanger current and diastolic intracellular Ca^{2+} concentration in ventricular myocytes[172]. These studies suggest that resveratrol may be able to act as a potent inhibitor of abnormal ion handling via cardiac voltage-gated sodium channels and voltage-gated potassium channels which could help in preventing lethal arrhythmias.

Overall, these studies suggest that resveratrol acts as a pleiotropic molecule that interacts with a variety of target molecules to afford cardioprotection. However, it should also be noted that these studies differ in the concentration used (very low to very high) and duration (short-term to long-term) of resveratrol treatment, thus precluding the ability to decide on a universal dosage and duration for modulating all of the proposed targets.

7.8. Clinical studies with resveratrol and resveratrol enriched grape formulation in ischemic heart disease patients

A large number of pre-clinical studies provided ample evidence to explore the translational utility of resveratrol for the management of CVDs. In light of the pre-clinical efficacy, a few clinical studies have also been conducted to examine the cardioprotective role of resveratrol in patients with ischemic heart disease. Herein, we will discuss 3 clinical trials that examined the efficacy of resveratrol in patients with stable angina or ACS who were under standard treatment. A double-blinded, placebo-controlled, randomised, 3-month study evaluated the efficacy of resveratrol treatment in 40 Caucasian post-MI patients with CAD (26 men and 14 women)[173]. The 2 intervention arms of the study consisted of placebo or 10 mg/day resveratrol, respectively; both arms also received standard post-MI medication along with placebo or resveratrol. At the end of the study, it was found that resveratrol treatment was able to improve diastolic function (E/A ratio) significantly and slightly improve systolic function (by echocardiography) above baseline values when compared to the placebo arm. Moreover, endothelial function, red blood cell deformability, low density lipoprotein (LDL) cholesterol and platelet aggregation were also positively modulated by 3 months of resveratrol treatment[173]. However, total cholesterol, high density lipoprotein (HDL) cholesterol, triglyceride and C-reactive protein level were not improved with 3 month resveratrol treatment compared to placebo[173].

Calcium fructoborate (CF) is a patented over-the-counter dietary supplement that has calcium, fructose, and boron in a sugar-borate ester as the 3 main ingredients[65]. Current evidence suggests that CF has anti-inflammatory properties[65]. The efficacy of resveratrol and CF treatment has been also reported by a clinical trial involving CAD patients. This clinical trial examined the efficacy of 2-month treatment with resveratrol and CF in 116 patients with stable angina

pectoris. In this trial, 29 patients received placebo treatment, whereas 29 patients each received 20 mg/day resveratrol alone, or a combination of 20 mg/day resveratrol and 112 mg/day CF, or 112 mg/day CF. This study reported that resveratrol, CF and a combination of both agents improved CVD biomarkers[174]. Specifically, a 2-month treatment resulted in significant reductions in high-sensitivity C-reactive protein and the N-terminal pro-hormone of BNP in all 3 groups in comparison to the placebo group. The CF group, the CF and resveratrol group and the resveratrol group had 39.7%, 30.3%, and 24.6 % reductions in C-reactivity protein, respectively. LDL cholesterol and HDL cholesterol and triglycerides were also lower in all the treatment groups[174]. In addition, reduction in the incidence of angina episodes/week, and the number of weekly nitroglycerin consumption, and an improvement in angina class were also observed in all the treatment groups, with the highest improvement in the combination arm (CF + resveratrol group).

A randomised, triple-blind, placebo-controlled trial for a duration of 1-year in 75 patients with stable angina who received either placebo, a regular grape extract, or a grape extract containing resveratrol also reported the beneficial effects of resveratrol[175]. This trial was designed to examine the efficacy of resveratrol in reducing the pro-inflammatory biomarkers and improving anti-inflammatory and fibrinolytic markers, which are considered to be major predictors of CVD risk. All 3 groups consisted of 25 patients each. Specifically, the investigational interventions included grape extract and 350 mg/day grape extract containing resveratrol (8 mg) for the initial 6 months and followed by 700 mg/day (16 mg resveratrol) for the last 6 months of the study along with standard medication. Treatment with the resveratrol enriched grape extract increased the level of the anti-inflammatory adipokine, adiponectin, by 23%, and decreased the level of thrombogenic plasminogen activator inhibitor type 1 by 57% in comparison to the placebo

group[175]. In addition, resveratrol treatment resulted in a down-regulation of pro-inflammatory genes in peripheral blood mononuclear cells along with changes in transcription factors (Kruppel-like factor 2, NF-κB, activator protein-1, c-Jun, activating transcription factor 2, and CREB-binding protein) and miRNAs (miR-21, miR-181b, miR-663, miR-30c2, miR-155, and miR-34a) levels in 2 subpopulations of patients in the same clinical trial[64]. The lipid profile was also improved in both treatment groups compared to the placebo group[59]. Overall, the current clinical evidence suggests that resveratrol may be beneficial for the management of ischemic heart disease. Further clinical studies in ischemic heart disease patients are needed to unequivocally establish the clinical utility of resveratrol.

CHAPTER II: Resveratrol is equipotent to perindopril in attenuating post-infarct cardiac remodelling and contractile dysfunction in rats

1. Rationale

While there are a plethora of reports showing the stand-alone cardioprotective effect of resveratrol, it is clinically imperative to evaluate the efficacy of resveratrol in comparison with current cardiovascular drug therapies in order to better evaluate its true therapeutic value. Importantly, it is also worthwhile to explore the beneficial effects of resveratrol in combination with clinically proven cardiovascular drug therapies to shed light on the efficacy of resveratrol in combinatorial therapeutic strategies. In addition, the possible interactions of resveratrol with other cardiovascular drugs also need careful investigation to ascertain its clinical utility and circumvent unnecessary harmful effects that may occur when used in conjunction with currently prescribed drug therapy. Potential new therapeutic agents are rarely tested against existing drugs for their comparative efficacy in pre-clinical studies. As the current literature suggests, comparative studies on resveratrol alongside cardiovascular drugs are limited and its comparative efficacy remains to be further explored in future studies[176, 177]. In this regard, no study has compared or examined the effects of a stand-alone or combination of resveratrol with a first-line post-MI and HF medication on cardiac structure and function in the setting of acute MI. ACE inhibitor treatment improves morbidity and mortality in post-MI patients[178]. Future prospective clinical trials that aim to test the efficacy of resveratrol in post-MI patients will inevitably involve a drug therapy regimen with both agents at the same time as ACE inhibitors have Class - 1, Level - A indication for post-MI patients. In that context, whether resveratrol could be synergistic or antagonistic with a front-line medication such as an ACE inhibitor is currently not known. The comparative efficacy of resveratrol is also yet to be investigated

alongside a main guideline recommended post-MI drug such as an ACE inhibitor. The unexplored possibility of a combinatorial therapeutic strategy involving resveratrol and an ACE inhibitor also warrants further study as it may provide incremental benefits. In this study, we have addressed these interesting questions of clinical value by examining the equal dose cardiac benefits of resveratrol alongside perindopril (an ACE inhibitor) and an equal dose combination of perindopril and resveratrol on cardiac structure and function in LAD ligated rats, a well-characterised animal model of MI.

2. Hypotheses

1. Resveratrol as a stand-alone low dose treatment will improve cardiac structure and function in LAD ligated rats.
2. Resveratrol in combination with the ACE inhibitor perindopril will improve cardiac structure and function in LAD ligated rats.
3. Resveratrol will attenuate cardiac oxidative stress, chronic inflammation and fibrosis in LAD ligated rats.

3. Materials & methods

3.1.Animal care and experimental design

This study protocol was approved by the University of Manitoba Office of Research Ethics & Compliance and Animal Care Committee and was done in accordance with the guidelines of the Canadian Council for Animal Care. 3 weeks old male Sprague Dawley rats (175-215 g) were housed in a temperature and humidity controlled room with a 12-hour light/dark cycle (Charles River Laboratories, Quebec). Rats were anesthetised with 1-5 % isoflurane with oxygen at a flow rate of 2 l/min and kept in the surgical plane on anesthesia with 2% isoflurane during surgery and

subjected to permanent ligation of LAD to induce MI or sham surgery after baseline echocardiographic examination. A left thoracotomy was done, and the heart was gently exposed from the pericardial sac through the incision. The LAD was located and occluded with 6-0 polypropylene silk suture at about 2 mm from aortic root. The suture was tied and the ligation was estimated to be successful when the anterior wall of the LV turned pale. The heart was repositioned, then the chest compressed to remove any air from the cavity and the incision was closed using a purse string suture. Sham-operated animals that served as the normal controls were subjected to similar surgical procedures except that the LAD was not ligated. Buprenorphine 0.05 mg/kg was administered pre- and post-surgery (2 times a day for 2 days) subcutaneously as an analgesic agent to all rats.

All surviving sham and MI rats were assigned to the following 4 treatment groups. 1. Vehicle (50 % ethanol, 2.5 mL/kg body weight/day); 2. Resveratrol (2.5 mg/kg body weight/day, trans-resveratrol, ≥99%, Sigma-Aldrich, Ltd, Ontario); 3. Perindopril (2.5 mg/kg body weight/day, Servier Inc., Quebec); 4. Perindopril + Resveratrol (both 2.5 mg/kg body weight/day). All 4 groups consisted of sham (*n*= 6-9) and MI (*n*= 10-13) rats and received the treatments by oral gavage for 8 weeks. The dose for the present study was chosen based on previous studies[179-181]. Animals were regularly weighed, and evaluated for well-being.

3.2.Transthoracic echocardiography (TTE)

TTE was done under anesthesia. All experimental rats were weighed and anaesthetised with 3 % isoflurane in a chamber, and then kept under 1.5 – 2 % isoflurane throughout the procedure. TTE was obtained at baseline and at 8 weeks of treatment by 2D guided M-mode and Tissue Doppler Imaging modalities with a 13-MHz probe (Vivid 7; GE Medical Systems, Milwaukee, WI) by a

procedure described elsewhere[2]. Two-D M-mode parasternal long-axis view images were obtained to determine systolic functional parameter such as percentage of LVEF from end-systolic and end-diastolic volumes. The cardiac structural parameters such as interventricular septal wall thickness (IVSTd), and LV posterior wall thickness (LVPWTd) at diastole and LV internal diameter (LVIDd and LVIDs) at diastole and systole were determined from parasternal short-axis view images. All echocardiographic images were analysed to calculate the listed parameters using EchoPAC software (GE Medical Systems, Milwaukee, WI). The values obtained for the mentioned parameters in 3 consecutive cardiac cycles were averaged to obtain final data[182].

3.3.Blood and tissue collection

At 8 weeks, all animals were anesthetised with ketamine/xylazine 9.0 mg/100g and 0.9 mg/100g IM. Depth of anesthesia was assessed by pedal withdrawal reflex. The blood sample was collected from the inferior vena cava by opening the thoracic cavity and the heart was immediately excised. The whole heart was rinsed in PBS and atria, right and LVs, septum, and fibrotic scar tissue were separated, weighed and flash frozen in liquid nitrogen.

3.4.LV scar size (%) and lung wet-to-dry weight ratio determination

Percentage of scarred (infarcted) LV tissue was calculated by dividing the weight of scarred LV tissue by the whole weight of LV tissue[183]. First, LV tissue was removed from septal and left atrial tissue. Then scar tissue was carefully removed from the LV by cutting through the edges of the scar tissue and both portions were weighed. Evidence of HF was assessed by checking the presence of ascites, and by calculating the lung wet-to-dry weight ratio in MI rats that received both vehicle and investigational compounds.

3.5. Oxidative stress marker and antioxidant enzyme activity assays

In order to determine MI associated oxidative stress, the level of the lipid peroxidation product, malondialdehyde (MDA), was assessed using the Oxiselect MDA quantification kit (Cell Biolabs, San Diego) following the manufacturer's instructions[184]. Antioxidant enzyme status was also determined by measuring the activity of major endogenous antioxidant enzymes such as SOD and CAT by following the manufacturer's instructions (Cayman Chemicals, Michigan)[185].

3.6. Pro-inflammatory and cardiac fibrosis marker assays

The level of TNF-α protein in heart tissue was measured by ELISA following the manufacturer's instructions (Thermo Scientific, Illinois). Hydroxyproline in the heart was also assayed following the manufacturer's instructions (Sigma-Aldrich, Ltd, Ontario). Collagen concentration was calculated by multiplying hydroxyproline level by a factor 7.46 because the interstitial collagen contains approximately 13.4 % hydroxyproline as described elsewhere[183].

3.7. Statistical analysis

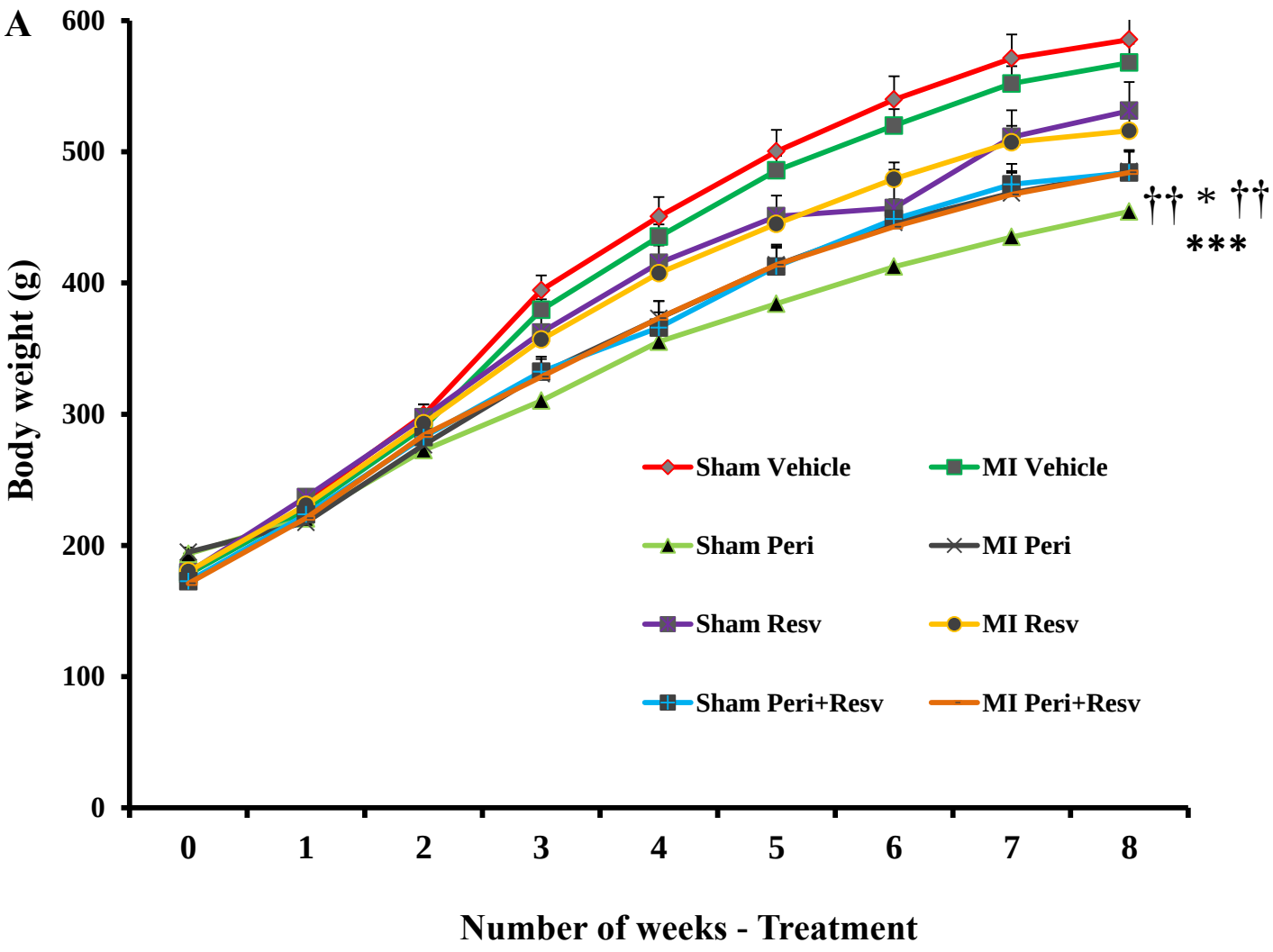
All values are expressed as means ±SEM. One-way analysis of variance was used to analyze variations between the means of the groups. Significant values are defined as $p < 0.05$. When significance was obtained, one-way analysis of variance was followed by Newman-Kuels post hoc test.

4. Results

4.1. General characteristics

Eight weeks post-surgery, body weight was comparable between sham and MI rats in all groups (Figure 1A). However, perindopril and perindopril + resveratrol treated sham and MI rats had

significantly lower body weight compared to vehicle treated sham and MI rats, respectively (Figure 1A). Resveratrol treatment alone did not alter the body weight of sham and MI rats in comparison to vehicle treated sham rats and vehicle treated MI rats (Figure 1A). The heart-to-body weight ratio was also comparable between sham and MI rats in all groups (Figure 1B). Perindopril and perindopril + resveratrol treated sham rats had significantly lower heart-to-body weight ratio compared to vehicle treated sham rats as well as resveratrol treated sham rats. Perindopril and perindopril + resveratrol treated MI rats also had lower heart-to-body weight ratio compared to vehicle treated MI rats as well as resveratrol treated MI rats (Figure 1B). In sham and MI rats, pleural and abdominal cavities were devoid of any effusions or ascites at 8 weeks. There was no significant difference in lung wet-to-dry weight ratio between the groups (Figure 1C). All MI rats included in the study had well-defined scarred LV tissue at the anterior region due to the large anterior infarct that resulted from the LAD ligation surgery. Scar size (infarct size in %) calculated as the percentage of fibrotic LV tissue weight versus total LV weight was comparable between MI groups and was not statistically significant (Figure 1D).


A
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Body weight (g)
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2
3
4
5
6
7
8
Number of weeks - Treatment
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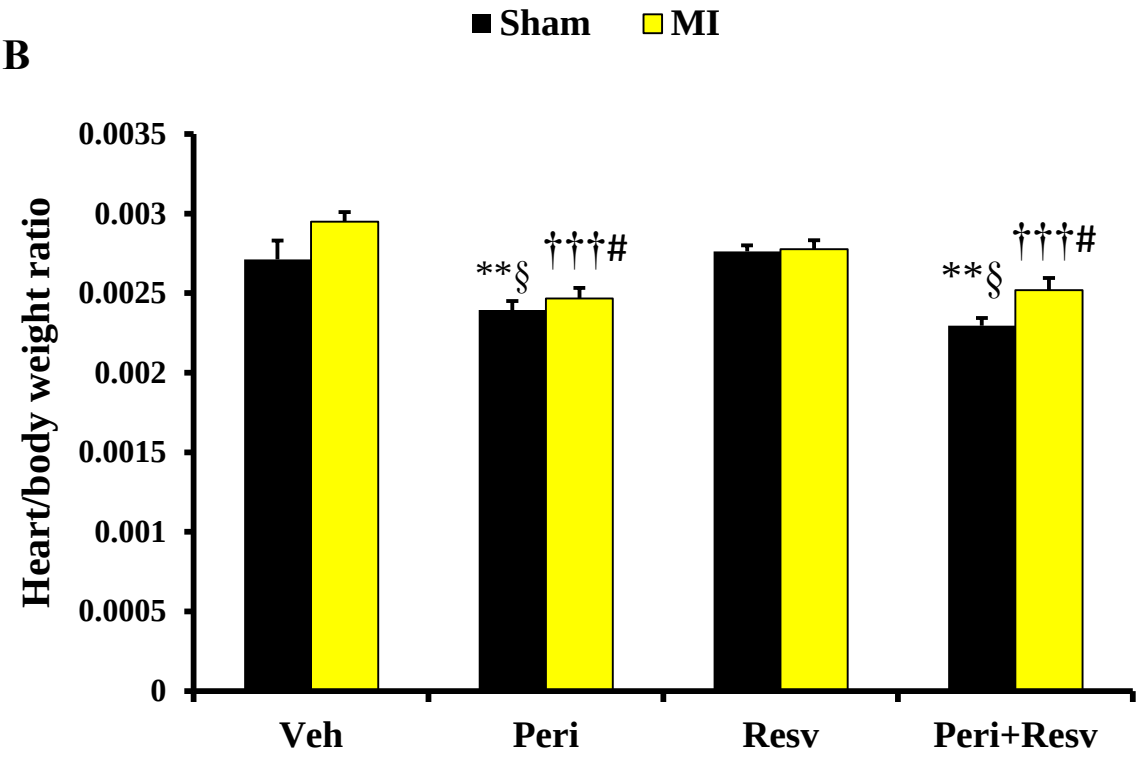
MI Vehicle
Sham Peri
MI Peri
Sham Resv
MI Resv
Sham Peri+Resv
MI Peri+Resv
†† * ††
***

B


Sham
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Peri
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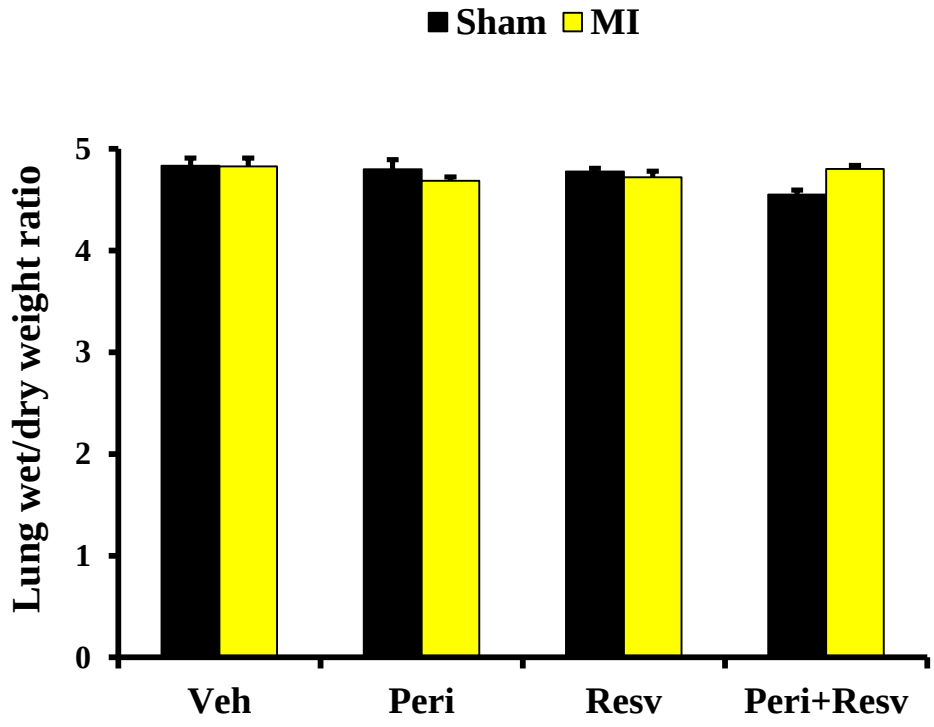
C


Sham
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Lung wet/dry weight ratio
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Peri+Resv

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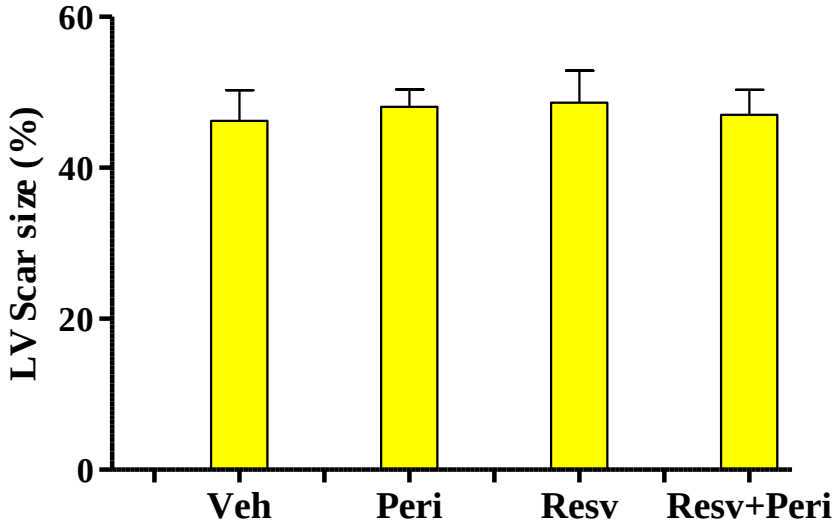
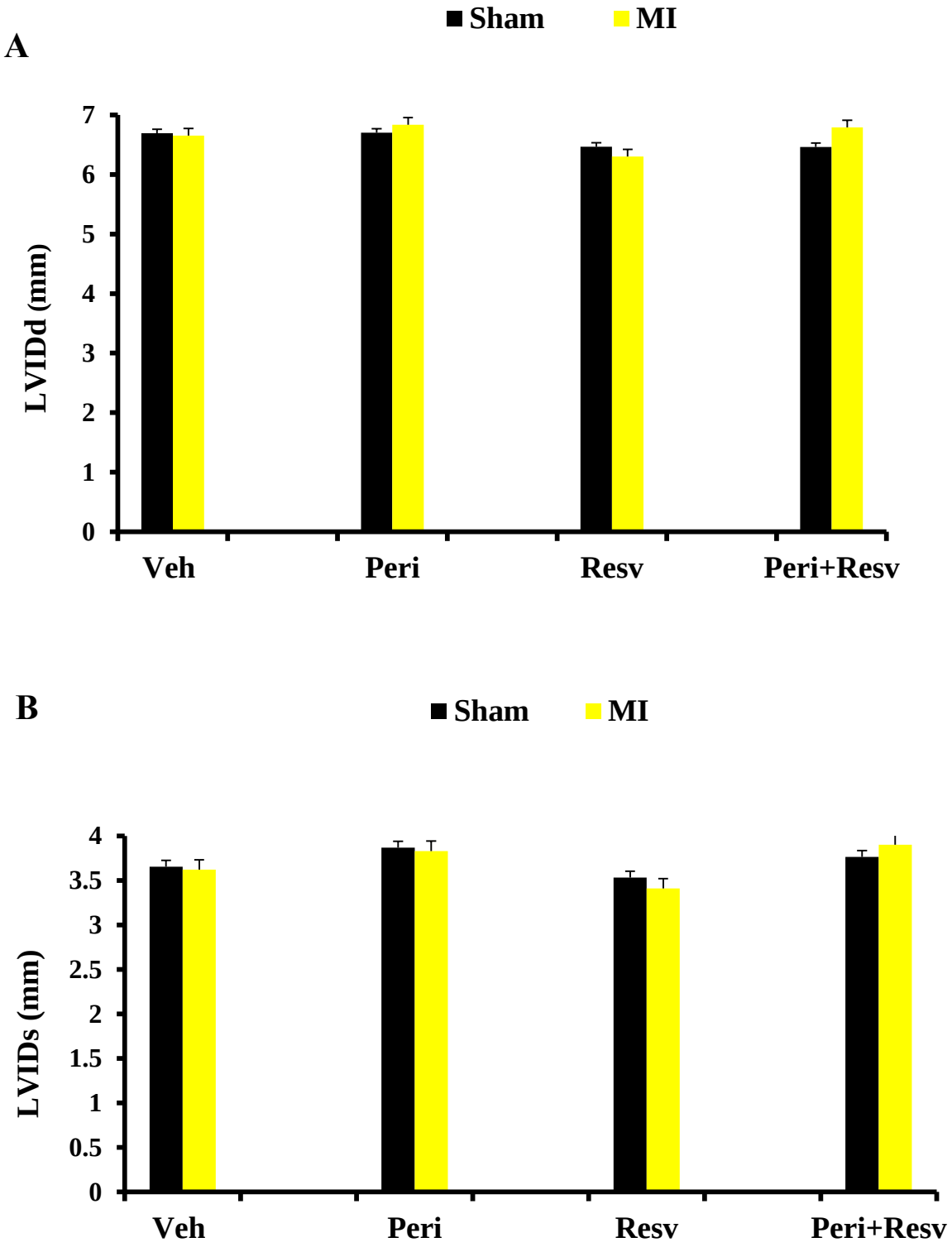


Figure 1. A. Body weight of sham and MI rats from week 1 to week 8 (sham *n*=6-9; MI *n*= 10-13). ***p <0.001 *vs* Sham Veh; * p <0.05 *vs* Sham Veh; †† p <0.01 *vs* MI Veh. B. Heart-to-body weight ratio of sham and MI rats at 8 weeks (sham *n*=6-9; MI *n*= 10-13). ** p <0.01 *vs* Sham Veh; § p <0.05 *vs* Sham Resv; ††† p <0.001 *vs* MI Veh; #p <0.05 *vs* MI Resv. C. Lung wet-to-dry weight of sham and MI rats at 8 weeks (sham *n*=6-9; MI *n*= 10-13). D. LV scar size (%) in MI rats. (*n*=10-13). Vehicle – Veh, Peri – Perindopril, Resv –Resveratrol

4.2. Treatment with perindopril, resveratrol and perindopril + resveratrol prevents post-MI LV dilatation

Baseline M-Mode echocardiography assessment showed that cardiac structural parameters were comparable between all groups (Figure 2). Conversely, vehicle treated MI rats had significantly increased post-infarction LV dilatation as evidenced by the increased LVID when compared with

vehicle treated sham rats at 8 weeks (Table 1. LVID at diastole: 10.60±0.31 *vs* 9.01±0.16, *p <0.001*; LVID at systole: 7.94±0.33 *vs* 4.82±0.14, *p <0.001*). Eight week treatment with perindopril or resveratrol significantly prevented the post-infarction LV dilatation in MI rats compared to vehicle treated MI rats (Table 1. LVID at diastole: 9.54±0.18 *vs* 10.60±0.31; 9.47±0.30 *vs* 10.60±0.31, *p <0.01*; LVID at systole: 6.90±0.23 *vs* 7.94±0.33; 6.60±0.37 *vs* 7.94±0.33, *p <0.01*). Perindopril + resveratrol treated MI rats also had significantly less post-infarction LV dilatation compared to vehicle treated MI rats (Table 1. LVID at diastole: 9.45±0.17 *vs* 10.60±0.31, *p <0.01*; LVID at systole: 7.13±0.22 *vs* 7.94±0.33, *p <0.05*). Furthermore, LVPWTd and IVSTd at diastole were also measured to detect post-MI cardiac concentric hypertrophy as well as the effect of treatment on both parameters at 8 weeks in all groups. There was no significant difference in LVPWTd between sham and MI animals from any of the groups at 8 weeks (Table 1). However, there was a significant reduction in the IVSTd in vehicle treated MI rats in comparison with vehicle treated sham rats at 8 weeks (Table 1). Treatment with perindopril or resveratrol and perindopril + resveratrol did not affect the reduction in IVSTd in MI-induced rats.


A
Sham
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LVIDd (mm)
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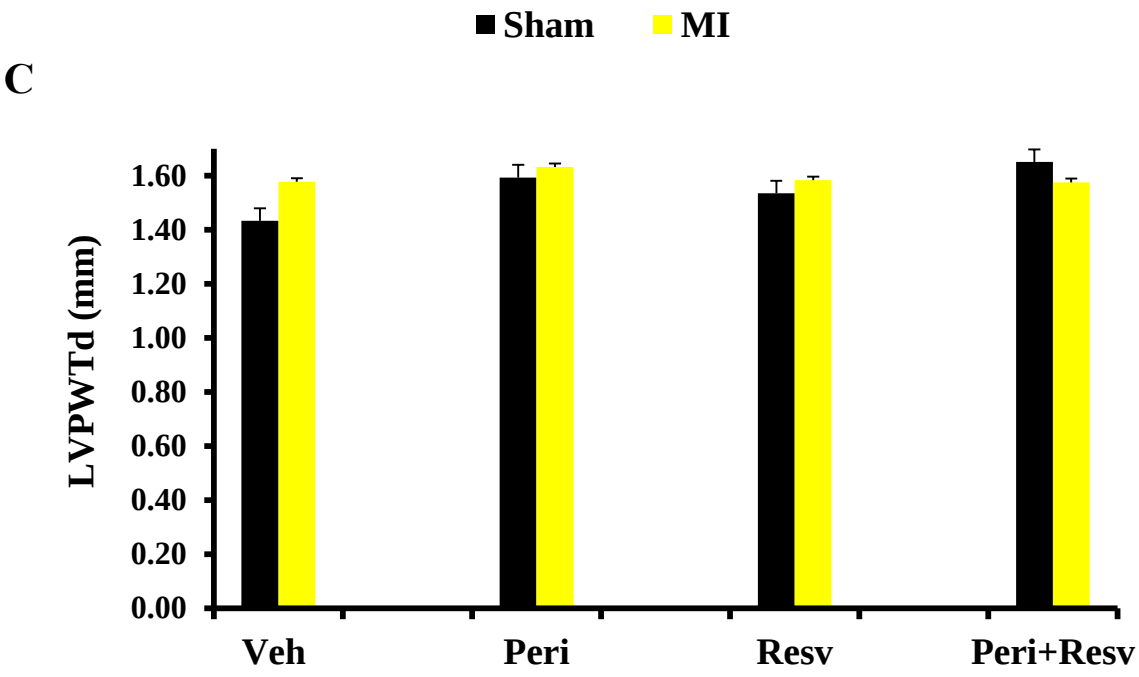


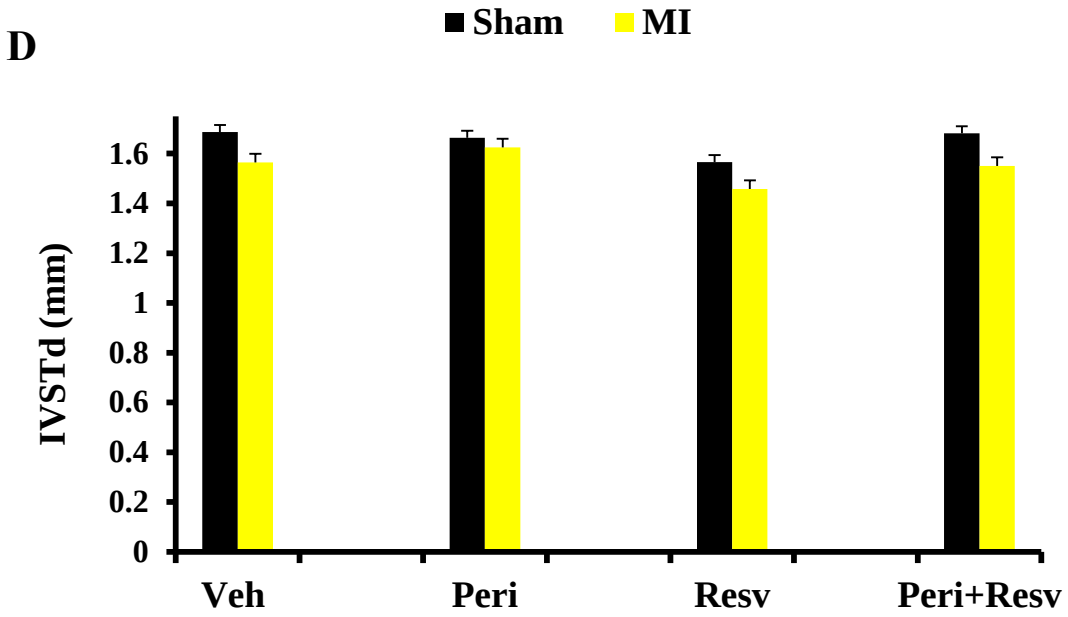


Figure 2. Echocardiographic structural parameters at baseline (n=9-13) A & B. Left ventricular internal diameter at diastole and systole (LVIDd and LVIDs). C & D. Left ventricular posterior wall thickness (LVPWTd) and Interventricular septal wall thickness (IVSTd) at diastole.

	Sham Veh	MI Veh	Sham Peri	MI Peri	Sham Resv	MI Resv	Sham Peri+Resv	MI Peri+Resv
LVIDd (mm)	9.01±0.16	10.60±0.31***	8.55±0.25	9.54±0.16††	8.99±0.23	9.47±0.30††	8.20±0.21	9.45±0.17††
LVIDs (mm)	4.82±0.14	7.94±0.33***	4.96±0.15	6.90±0.23††	4.94±0.25	6.60±0.37††	4.74±0.17	7.13±0.22†
IVSTd (mm)	2.01±0.08	1.44±0.09***	1.85±0.03	1.40±0.08	1.99±0.05	1.52±0.13	1.91±0.04	1.33±0.08
LVPWTd (mm)	2.10±0.08	2.28±0.09	1.99±0.08	2.40±0.12	1.96±0.08	2.44±0.19	1.95±0.04	2.20±0.09
HR	363.38±10.07	355.06± 6.81	347.85±8.39	350.87±8.15	338.24±14.39	373.37±10.63	379.22±11.78	364.44±8.73

Table 1. Echocardiographic parameters of sham and MI rats at 8 weeks. (sham *n*=6-9; MI *n*= 10-13). Left ventricular internal diameter (LVIDd and LVIDs) at diastole and systole, interventricular septal wall thickness (IVSTd) and left ventricular posterior wall thickness (LVPWTd) at diastole, and heart rate (HR). ***p <0.001 *vs* Sham Veh; ††p <0.01 and †p <0.05 *vs* MI Veh.

4.3. Treatment with perindopril, resveratrol and perindopril + resveratrol prevents post-MI cardiac dysfunction

Cardiac function was also assessed by performing echocardiography at baseline and 8 weeks. At baseline, LV systolic function evaluated as LVEF was normal in all rats. LVEF was comparable between all groups as well (Figure 3). However, LVEF was significantly lower (by ~23 %) in vehicle treated MI rats compared to vehicle treated sham rats at 8 weeks post-surgery (Figure 4A. 59.83±1.73 *vs* 77.38±1.42%, $p < 0.001$). Resveratrol or perindopril treated MI rats had significantly improved LVEF (increased by 15% and 14 %) in comparison to vehicle treated MI rats (Figure 4A. 59.83±1.73 *vs* 68.97±2.58 and 68.36± 1.93%, $p < 0.01$). Eight week treatment with perindopril + resveratrol also significantly improved the reduction in LVEF (by 14%) in MI rats when compared with vehicle treated MI rats (Figure 4A. 59.83±1.73 *vs* 66.33±1.62%, $p < 0.05$). TDI was also done to determine the LV posterior endocardial velocity (Vendo) to identify regional LV contraction abnormalities. Our analysis showed that LV posterior Vendo was unaltered in all groups at 8 weeks (Figure 4B and Table 1). Heart rate was also comparable between the groups (Table 1).

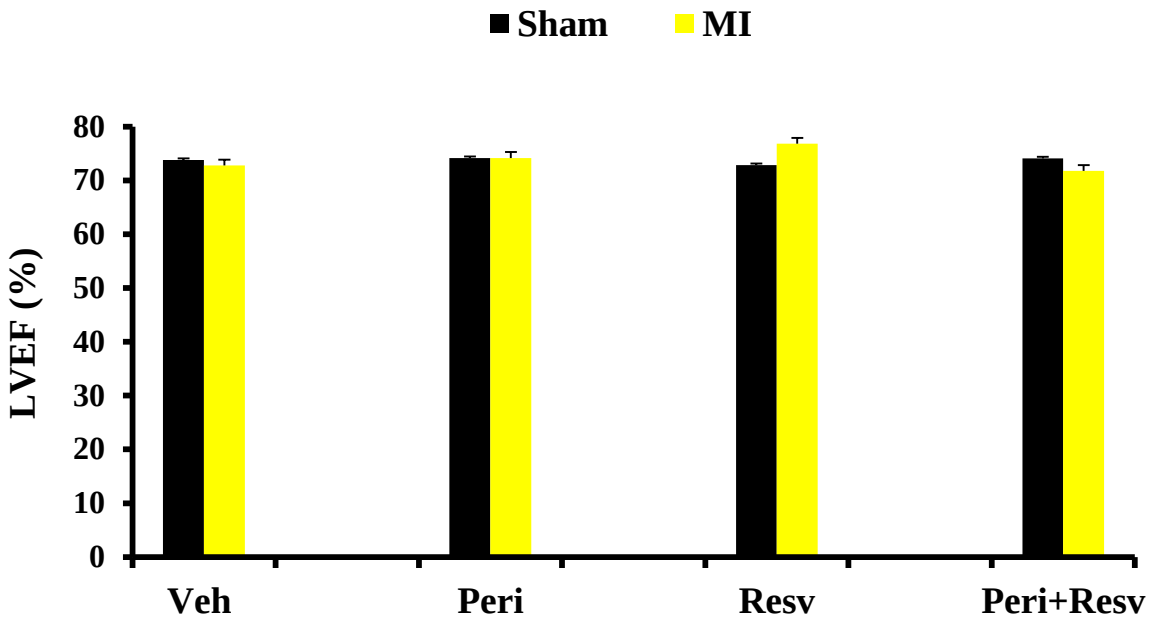
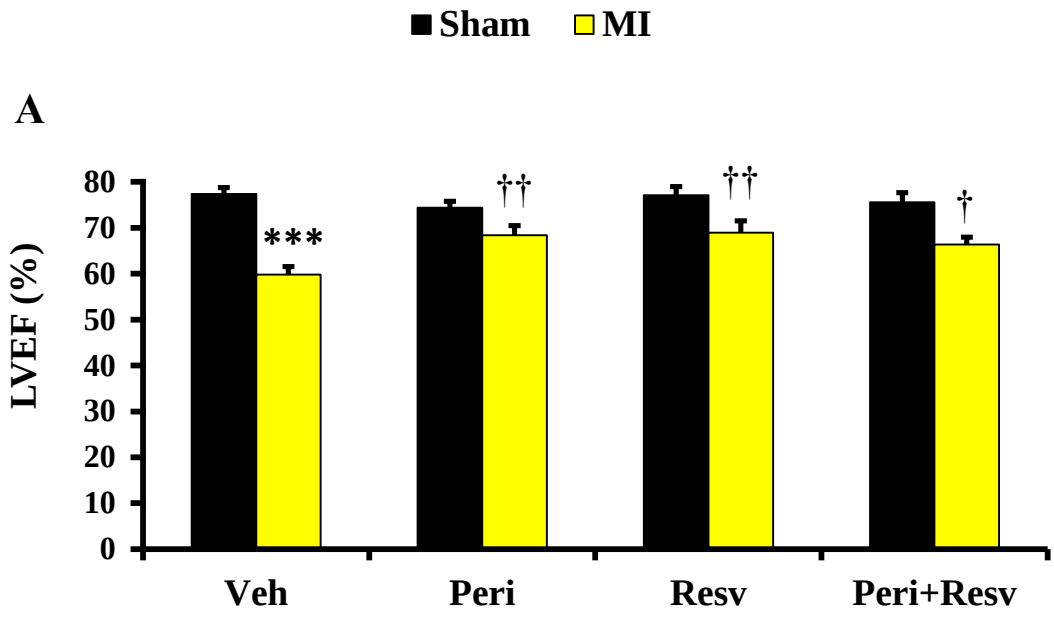


Figure 3. Echocardiographic systolic functional parameter at baseline (n=9-13). Left Ventricular Ejection Fraction % (LVEF).



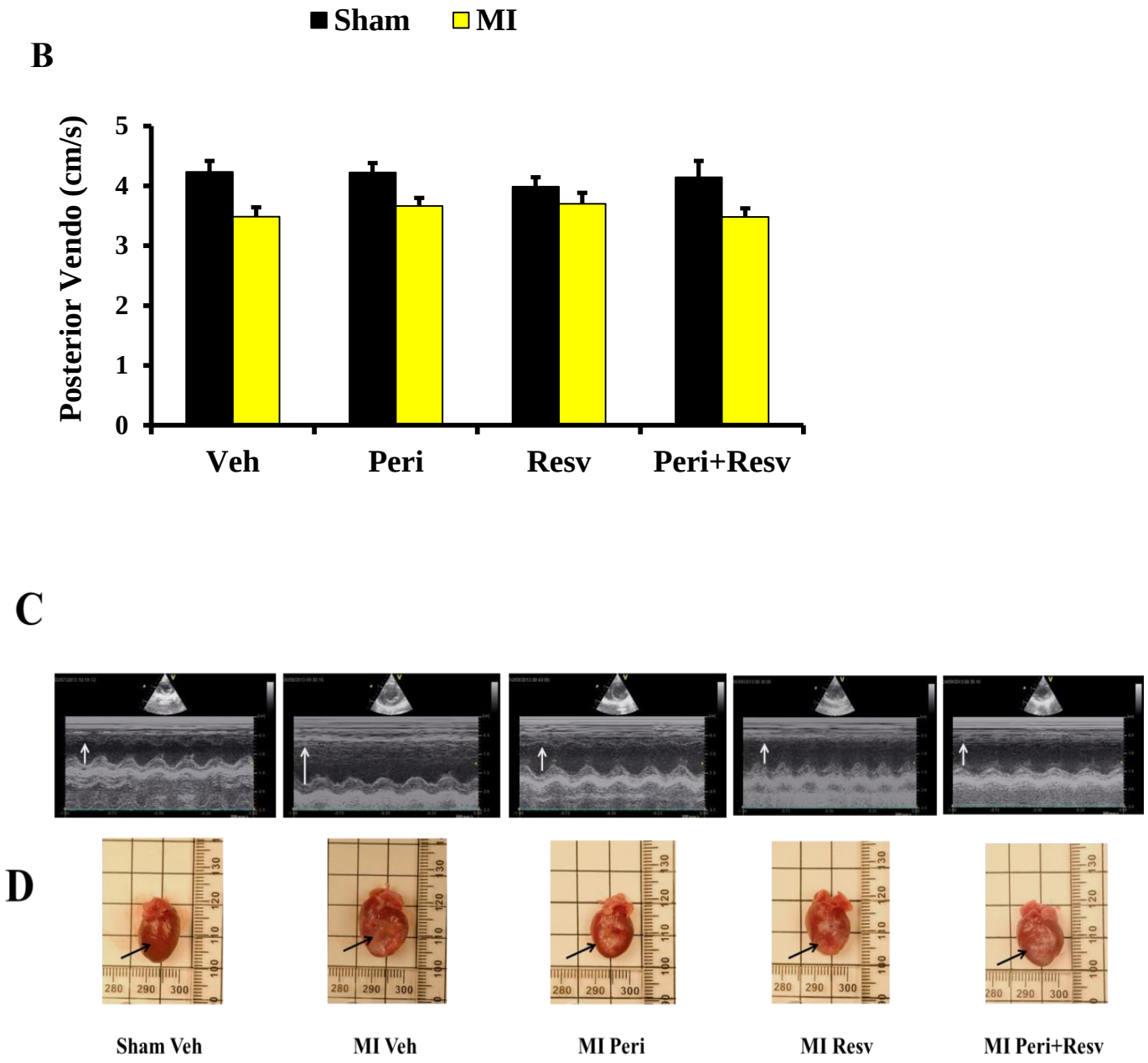


Figure 4. Echocardiographic systolic functional parameters of sham and MI rats at 8 weeks. A. Left ventricular ejection fraction % (LVEF) in sham and MI rats at 8 weeks. B. Posterior wall endocardial velocity in sham and MI rats at 8 weeks. C & D. Representative echocardiographic

and whole heart images showing improvement in LV dilatation. Sham *n*=6-9; MI *n*= 10-13. ***p <0.001 *vs* Sham Veh; ††p <0.01 *vs* MI Veh; †p <0.05 *vs* MI Veh.

4.4. Treatment with perindopril, resveratrol and perindopril + resveratrol prevents post-MI oxidative stress

To explore the mechanisms responsible for cardiac structural and functional abnormalities in MI rats as well as the cardioprotective effects of the treatments, we measured oxidative stress which is known to be associated with post-MI cardiac abnormalities[163, 186]. MDA is a reactive lipid peroxidation product and a widely used biomarker of oxidative stress that has been reported to be elevated in MI animals and MI patients[163, 187, 188]. At 8 weeks, vehicle treated MI rats had significantly increased levels of MDA in both plasma and heart tissue compared with vehicle treated sham rats (Figure 5A. 10.11±1.89 *vs* 1.52±0.35, *p <0.001*; Figure 5B. 11.53±0.84 *vs* 4.48±0.53, *p <0.001*). Treatment with perindopril or resveratrol significantly prevented the elevation of MDA levels in MI rats as compared with vehicle treated MI rats (Figure 5A. 4.49±1.11 *vs* 10.11±1.89, *p <0.05*; 3.13±0.33 *vs* 10.11±1.89, *p <0.001*; Figure 5B. 8.09±0.54 *vs* 11.53±0.84, *p* <0.05; 6.38±1.01 *vs* 11.53±0.84, *p <0.001*). The combination treatment with perindopril + resveratrol also significantly prevented the increase in MDA in MI rats (Figure 5A. 2.41±0.57 *vs* 10.11±1.89, *p <0.001*; Figure 5B. 5.03±1.11 *vs* 11.53±0.84, *p <0.001*).

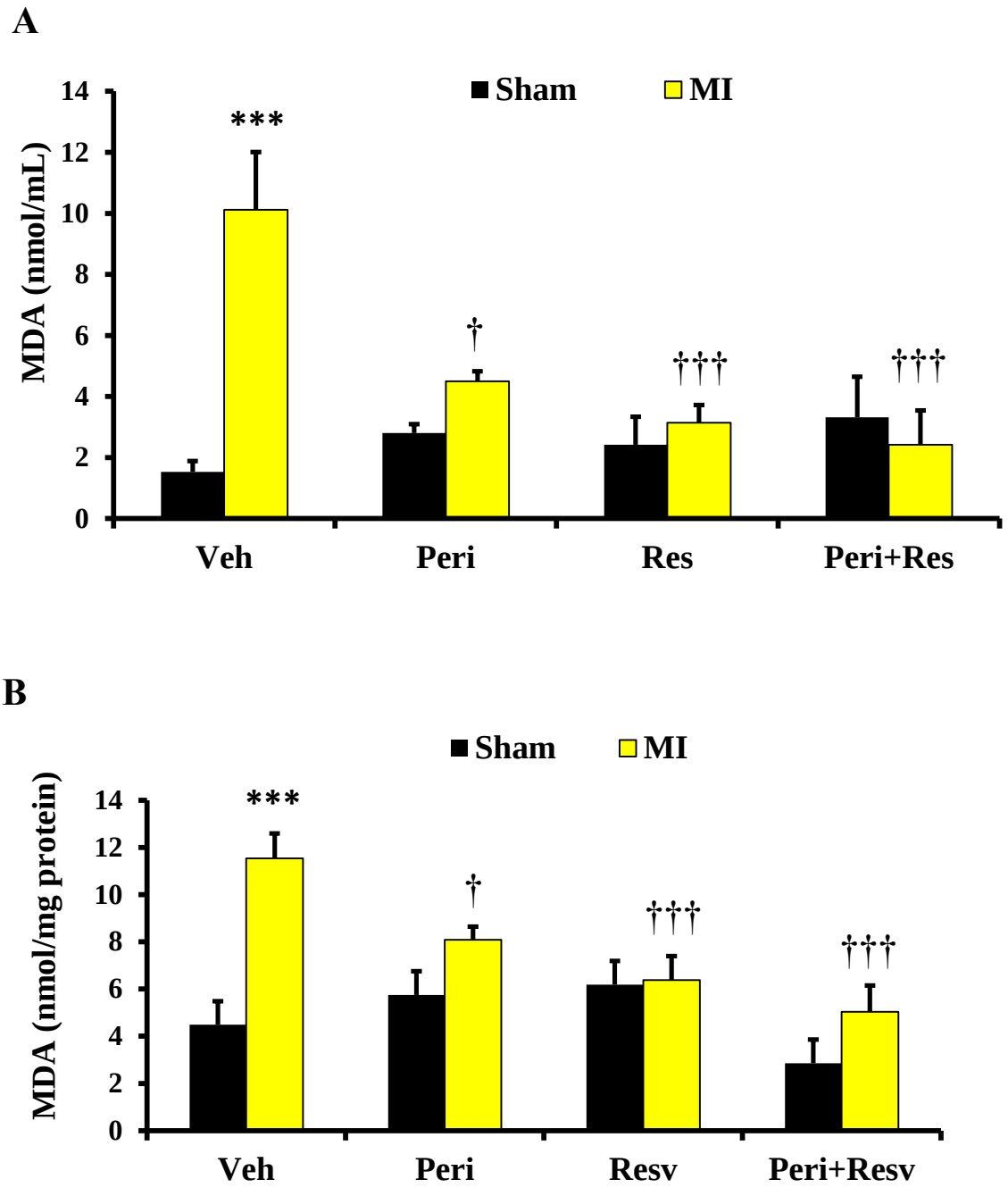
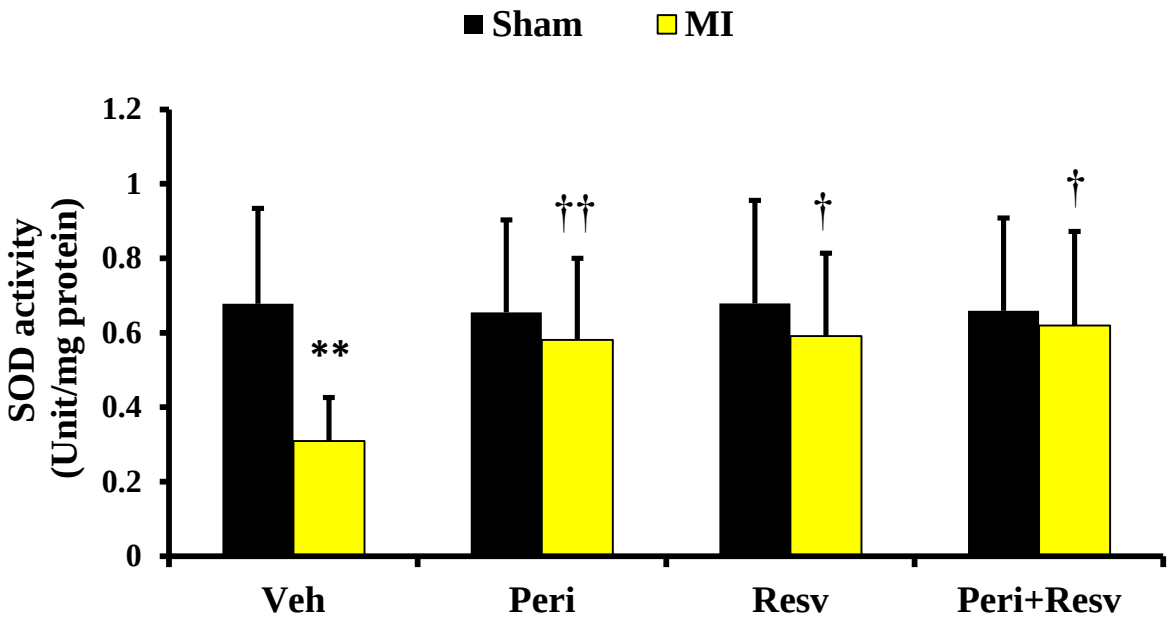


Figure 5. Malondialdehyde (MDA) in the plasma (A) and heart tissue (B) of sham and MI rats at 8 weeks (n = 4-7). ***p <0.001 *vs* Sham Veh; †††p <0.001 *vs* MI Veh; †p <0.05 *vs* MI Veh.

A


Sham
MI
SOD activity (Unit/mg protein)
1.2
1
0.8
0.6
0.4
0.2
0
**
††
†
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Veh
Peri
Resv
Peri+Resv

B

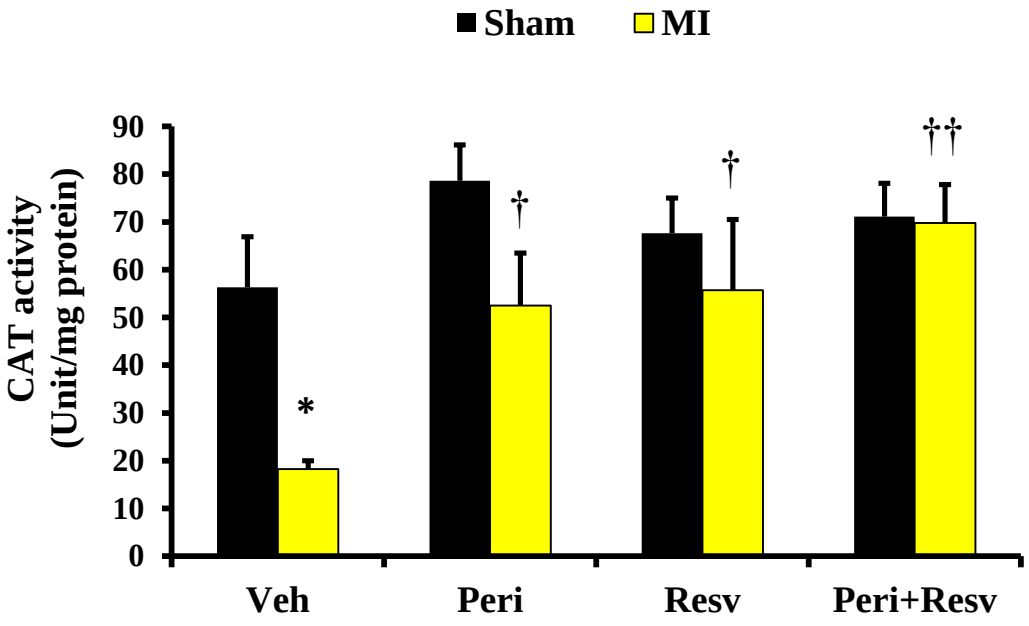

Sham
MI
CAT activity (Unit/mg protein)
90
80
70
60
50
40
30
20
10
0
*
†
†
††
Veh
Peri
Resv
Peri+Resv

Figure 6. A. Activity of superoxide dismutase (SOD) and B. catalase (CAT) in the heart tissue of sham and MI rats at 8 weeks (n = 5-7) and (n = 3-5). **p <0.01 *vs* Sham Veh; * p <0.05 *vs* Sham Veh; †† p <0.01 *vs* MI Veh; †p <0.05 *vs* MI Veh.

4.5. Treatment with perindopril, resveratrol and perindopril + resveratrol improves post-MI antioxidant enzyme status and reduces pro-inflammatory cytokine and cardiac fibrosis

In order to further examine whether the observed increase in oxidative stress marker is associated with a decrease in endogenous oxidative stress defence mechanism, activities of the major enzymes involved in the maintenance of normal redox state were measured. Activity of SOD was significantly decreased in vehicle treated MI rats when compared to vehicle treated sham rats at 8 weeks (Figure 6A. 0.30±0.11 *vs* 0.67±0.25, *p <0.01*). Stand-alone treatments with perindopril and resveratrol significantly prevented the reduction in activity of SOD in MI rats compared with vehicle treated MI rats at 8 weeks (Figure 6A. 0.58±0.21 *vs* 0.30±0.11, *p <0.01*; 0.59±0.22 *vs* 0.30±0.11, *p <0.05*). MI rats treated with a combination of equal dose perindopril + resveratrol also had significantly improved activity of SOD relative to vehicle treated MI rats (Figure 6A 0.61±0.25 *vs* 0.30±0.11, *p <0.05*). Our result indicated that the activity of CAT was also significantly decreased in vehicle treated MI rats in comparison to vehicle treated sham rats (Figure 6B. 18.22±1.73 *vs* 56.29±10.57, *p <0.05*) at 8 weeks. The activity of CAT was significantly higher in MI rats treated with perindopril alone, resveratrol alone or a combination of equal dose perindopril + resveratrol when compared to vehicle treated MI rats at 8 weeks (Figure 6B. 52.51±10.98 *vs* 18.22±1.73, *p <0.05*; 55.67±14.80 *vs* 18.22±1.73, *p <0.05*; 69.78 *vs* 18.22±1.73, *p <0.01*).

MI is also characterised with an acute inflammatory response that is mediated through various immune cells to facilitate wound healing[189]. However, the chronic post-MI inflammatory

response may be disadvantageous and contribute to cardiac remodelling and dysfunction[189]. To further investigate the effect of treatments on the pro-inflammatory cytokine status in the post-MI heart, we measured the protein level of TNF-α in the heart. At 8 weeks, vehicle treated MI rats had a significantly elevated level of TNF-α compared with vehicle treated sham rats (Figure 7. 33.22±2.20 *vs* 13.34±1.09, $p < 0.001$). Treatment with perindopril alone or resveratrol alone significantly prevented the elevation in the protein level of TNF-α in MI rats compared with vehicle treated MI rats (Figure 7. 14.95±1.59 *vs* 33.22±2.20, $p < 0.001$; 20.63±1.82 *vs* 33.22±2.20, $p < 0.001$). Combination treatment with perindopril + resveratrol also significantly prevented the increase in TNF-α in MI rats (Figure 7. 17.12±1.86 *vs* 33.22±2.20, $p < 0.001$). Post-MI cardiac remodelling is also associated with interstitial fibrosis due to an imbalance in the ECM turnover that leads to increased collagen deposition. To determine the effect of treatments on the post-MI cardiac fibrosis, we quantified the level of hydroxyproline, a modified amino acid present in triple-helical mature collagen, as a measure to detect the amount of collagen in cardiac tissue at 8 weeks. Our results showed that there was a significant increase in the level of collagen (based on the level of hydroxyproline) in vehicle treated MI rats when compared to vehicle treated sham rats (Figure 8. 20.11±1.53 *vs* 11.01±0.90, $p < 0.001$). Individual treatment with perindopril and resveratrol significantly prevented the elevation of interstitial collagen in MI rats compared with vehicle treated MI rats (Figure 8. 12.13±1.23 *vs* 20.11±1.53, $p < 0.001$; 13.56±2.25 *vs* 20.11±1.53, $p < 0.001$). Combination treatment with perindopril + resveratrol also significantly prevented the increase in interstitial collagen in MI rats (Figure 8. 11.84±0.93 *vs* 20.11±1.53, $p < 0.001$).

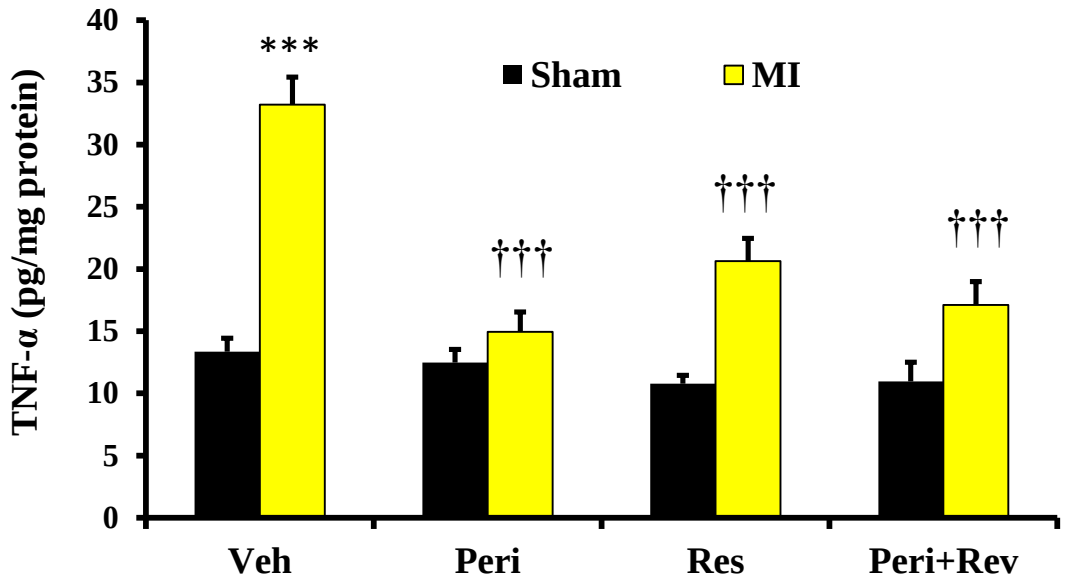


Figure 7. Tumor necrosis factor-α **(**TNF-α) in heart tissue of sham and MI rats at 8 weeks (*n* = 5). ****p* <0.001 *vs* Sham Veh; †††*p* <0.001 *vs* MI Veh.

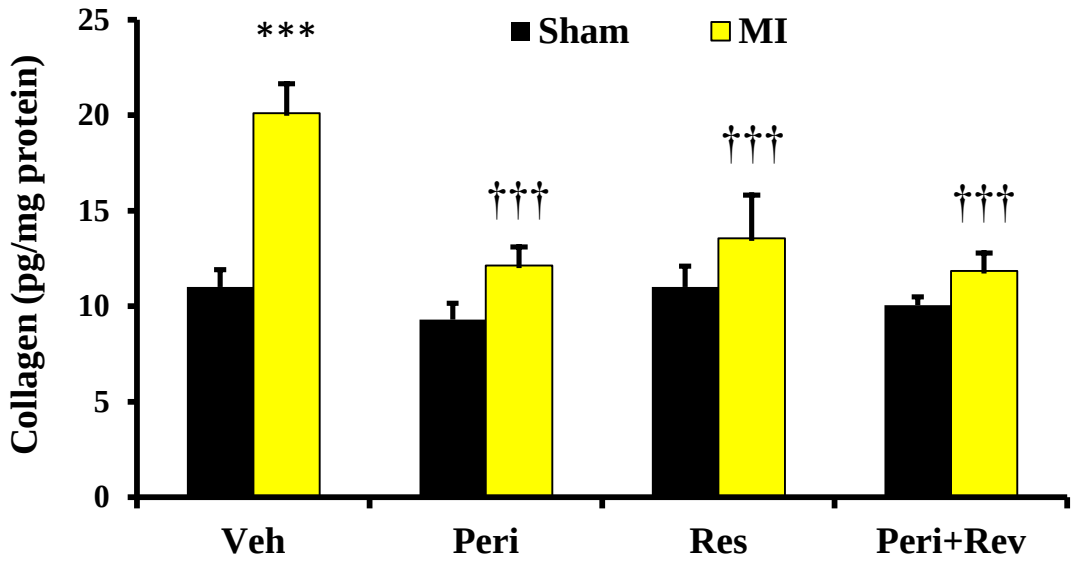


Figure 8. Collagen content in the heart tissue of sham and MI rats at 8 weeks (n = 5). ***p <0.001 *vs* Sham Veh; ††† p <0.001 *vs* MI Veh.

5. Discussion

Earlier studies have reported the varying effects of resveratrol pre-treatment and post-treatment in *ex vivo* and *in vivo* settings of cardiac ischemia and ischemia/reperfusion[92]. Of note, previous post-treatment studies employed resveratrol well after MI, thus precluding the option to assess the beneficial effects of an earlier treatment in a more clinically relevant setting where patients are being treated immediately after the onset and diagnosis of MI. Moreover, it is vital to understand whether resveratrol treatment would have any disadvantageous effect on the wound healing process and reparative fibrosis that are activated immediately after MI, which help to maintain the myocardial architecture after the loss of cardiac cells. Commencing the treatment at a later time point may not provide this valuable information. We investigated, for the first time,

the combinatorial and comparative efficacy of a low dose resveratrol treatment alongside a standard post-MI medication such as ACE inhibitor in improving cardiac structural and functional abnormalities in MI rats. Herein, we demonstrate that resveratrol is as efficacious as a clinically proven long-lasting ACE inhibitor, perindopril, in improving cardiac structural and functional abnormalities associated with acute MI in rats. All the treatments were started shortly after LAD ligation to closely mimic real-life clinical scenarios. We also used an equivalent dose (2.5 mg/kg body weight/day) of perindopril and resveratrol for the comparison of potency their cardioprotective effects.

MI is associated with acute and chronic cardiac remodelling leading to structural and functional abnormalities such as LV dilatation and systolic dysfunction, which are principal indicators of long-term prognosis in MI patients[190]. Post-MI increase in LV dilatation and wall thinning cause an elevation in wall stress which in turn exacerbates dilatation (dilatation begets dilatation) and may lead to mitral insufficiency, altogether effecting a transition from a compensated to a decompensated stage of cardiac function[191]. In fact, LV dilatation and reduced LVEF are the major precursors for the development of HF in MI survivors, and hence approaches to improve these adverse conditions are of great potential therapeutic value and could help to ensure better prognosis in MI patients. Consistent with previous evidence, our study showed that resveratrol significantly prevents the post-MI increase in LV dilatation, and thereby reduces the pathological remodelling similar to the improvement in cardiac remodelling observed in the case of ACE inhibitors in pre-clinical and clinical studies[147, 191]. Furthermore, a combination treatment with resveratrol and perindopril was also effective in reducing the adverse LV remodelling in MI rats. Treatments did not significantly affect the infarct size in MI rats, which suggested that the improvement in LV remodelling was not due to a reduction in infarct size. All MI rats had well-

healed infarct scar (fibrotic tissue) which confirmed that treatments had not interfered with infarct healing and reparative fibrosis. Even though it was beyond the scope of this study, it may be worthwhile to examine whether resveratrol facilitates post-MI wound healing by positively interacting with the various effectors involved in this process. In addition, there was no evidence of significant cardiac hypertrophy in MI rats.

Indeed, loss of cardiomyocytes and increased dilatation significantly affected the systolic function as evidenced by the reduced LVEF in MI rats. LV dysfunction manifested as reduced LVEF not only predisposes the post-MI patients to an increased risk of HF but also constitutes an independent predictor of patient outcome[192]. We observed that there was a 23% reduction in LVEF in vehicle treated MI rats signifying severe LV dysfunction. Consistent with the observed cardiac structural improvement, treatment with resveratrol alone or perindopril alone was able to improve the LVEF to a similar degree in MI rats, which further confirmed the equivalent cardioprotective effect of resveratrol and perindopril in the setting of acute MI. Notably, the combination treatment with equal dose perindopril + resveratrol also improved LVEF in MI rats, suggesting that neither of the treatments offset each other's therapeutic effect through adverse drug interactions. However, the equal dose combination treatment did not provide an incremental benefit in terms of cardiac function as we observed that LVEF was comparable between MI rats that received stand-alone (resveratrol or perindopril) or combination (perindopril + resveratrol) treatment. A previous short-term clinical trial reported that resveratrol provided added beneficial effect over prescribed drug therapy in stable MI patients[173]. Specifically, resveratrol improved diastolic function and flow mediated dilatation and reduced platelet aggregation, and LDL cholesterol in MI patients compared to the placebo group[173]. The improvement in MI-induced abnormalities with combination treatment consisting of resveratrol and perindopril suggests that

there is no negative interaction of the compounds; an antagonistic interaction would have resulted in an offsetting of each other's cardioprotective effect and worsening or no improvement in cardiac abnormalities. Notably, ACE inhibitors are highly effective in blocking the production of Ang II, however, there are other enzymes (chymase and cathepsin-G) that can form Ang II which may reduce the efficacy of chronic ACE inhibitor treatment in post-MI patients (ACE inhibitor escape)[193]. In addition, patient populations resistant or intolerant to ACE inhibitor treatment may also benefit from alternative strategies. For example, cough and angioedema are potential risk factors that often lead to the discontinuation of ACE inhibitors even though the overall benefit-to-risk ratio is favorable[194]. Resveratrol has been shown to possess a broad spectrum of cardiovascular beneficial effects which may be clinically advantageous wherein ACE inhibitors may not be suitable, if it is clinically proven to be equipotent to ACE inhibitors. Resveratrol may also be useful as an adjuvant therapy for a large number of post-MI HF patients presenting with co-morbidities as recent meta-analyses concluded that resveratrol is effective in improving type-2 diabetes and hypertension[195, 196]. The latter view is also consistent with the finding of a recent, 1-year clinical study involving Type-2 diabetes and hypertensive patients with CAD which demonstrated that resveratrol improves pro-inflammatory related miRNAs and cytokine expression in peripheral blood mononuclear cells[64].

It should also be noted that, in our study, MI rats did not develop overt HF, and hence it was not possible to ascertain the effect of combination treatment in the HF setting. Notably, a previous study reported that 2 weeks treatment with resveratrol in mice (started 4 weeks after inducing MI) at a dose of 50 mg/kg body weight/day is effective in reversing HF by improving systolic function[147]. Therefore, it is valuable to investigate the effectiveness of resveratrol treatment alone or in combination with the front-line medications, specifically, at a transition stage that involves

the progression from asymptomatic mild to symptomatic severe HF (further than 8 weeks' time period in the current study, wherein MI rats would begin to exhibit signs of overt HF).

To understand the mechanism underlying the improvement in cardiac structure and function, we examined the status of cardiac oxidative stress, inflammation, and fibrosis, 3 major adverse processes involved in MI-induced HF. Cardiac ischemia is associated with an increase in ROS due to the disruption of the electron transport chain as well as an increased activity of free radical generating enzymes; this leads to oxidative stress when the ROS defence mechanism is overwhelmed and in turn causes irreversible damage to myocardium[186, 197]. Oxidative stress is the major tipping point of ischemic injury cascade and is characterised with lipid peroxidation, protein oxidation, and DNA modifications that lead to cellular damage[198]. Increased ROS level is known to activate cardiac injury, ECM dysregulation, and inflammatory cytokines and modify cardiac contractile and calcium cycling proteins leading to contractile dysfunction[199]. We observed that there was a 2-3 fold elevation in the level of MDA (a widely used marker of oxidative stress) after MI, which corroborates with previous evidence[163, 186]. Given the fact that oxidative stress is capable of causing cardiac abnormalities, our data suggest that the observed impairment in cardiac structure and function is partly due to increased oxidative stress in MI rats. Previous studies have reported that the cardioprotective effect of resveratrol is associated with a decrease in oxidative stress[163, 181]. The current study also showed that perindopril and resveratrol alone or in combination prevented oxidative stress in MI rats.

The 2 major first-line endogenous antioxidant enzymes, SOD and CAT, play a crucial role in scavenging ROS such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), respectively[185 198]. MI is associated with a deleterious increase in O_2^- and H_2O_2 that overwhelms the endogenous scavenging systems, whereas, an increase in the SOD and CAT has been reported to improve

cardiac dysfunction[200-202]. ACE inhibitor treatment has also been reported to decrease oxidative stress and improve the activities of SOD and CAT[203, 204]. We have previously reported that resveratrol preserves the activities of SOD and CAT and prevent injury and cell death in adult rat cardiomyocytes *in vitro* (cardiomyocytes directly exposed to oxidative stress)[185]. Our current study suggests that the mechanism of reduction in post-MI oxidative stress by treatment with resveratrol or perindopril involves an improvement in the activities of SOD and CAT. Our finding is consistent with the earlier studies which reported that resveratrol mediated improvement in cardiac structure and function and mortality involves an increase in the level of SOD[205-207]. Our results also suggest that CAT may also be important in resveratrol mediated cardioprotection. However, further studies are needed to identify how resveratrol mediates the improvement in the activity of SOD and CAT. Based on prior studies, we speculate that resveratrol may have acted via plausible mechanisms such as; first, resveratrol's intrinsic antioxidant action (ability to scavenge ROS due to the presence of polyphenolic structural moieties) may have contributed to the preservation of the activity of SOD and CAT by preventing the oxidative modification of these enzymes[185]. Second, because resveratrol is not a very good ROS scavenger, resveratrol mediated increase in the activity of FOXO3a (Forkhead box 3a) , a transcription factor that upregulates the level of SOD and CAT[208] may have helped to prevent oxidative stress. Third, resveratrol has also been reported to be an activator of SIRT1 (an NAD (+)-dependent histone/protein deacetylase). Resveratrol was reported to reduce cardiac oxidative stress by upregulating manganese SOD via SIRT1[209]. A previous study also showed that moderate overexpression of SIRT1 protects the heart from oxidative stress[210]. Furthermore, SIRT1 positively modulates the transcriptional activity of FOXOs by deacetylation (FOXO activity is inhibited by CBP/p300-mediated acetylation), and in turn induces the expression of

antioxidant enzymes[211]. Even though ACE inhibitors have been shown to decrease oxidative stress via improving SOD and CAT, whether ACE inhibitors mediate their action through FOXOs or SIRT1 is not clearly known, and further studies are needed [203, 204]. Nevertheless, a previous study reported that zofenopril (ACE inhibitor) reverts the Ang II mediated ROS generation and downregulation of SIRT1[212]. Therefore, our results suggest that attenuation of post-MI oxidative stress with perindopril and resveratrol may be effective in improving the cardiac structure and function and preventing the development of HF.

Oxidative stress is also known to contribute to increased pro-inflammatory response and cardiac fibrosis; increased production of pro-inflammatory cytokines may in turn further aggravate both oxidative stress and cardiac fibrosis[186]. Previous studies demonstrated that resveratrol treatment improves the pro-inflammatory status and cardiac fibrosis by reducing TNF-α and collagen deposition, respectively, with a concomitant reduction in oxidative stress[180, 213]. TNF-α has been reported to be associated with an increased risk of HF by directly contributing to cardiac remodelling and dysfunction[214, 215]. Consistent with previous reports (pre-clinical and clinical), this study also showed that treatment with perindopril alone and resveratrol alone as well as their combination prevented the increase of TNF-α in MI rats[175, 216]. Overall, our finding suggests that reduction in the pro-inflammatory cytokine may also be involved in the treatment mediated improvement in cardiac dysfunction in MI rats. Peri-infarct and interstitial fibrosis are mainly attributed to increased deposition of collagen which in turn contributes to the adverse cardiac remodelling (stiffening) and dysfunction[217, 218]. In accordance with previous studies, there was a reduction in collagen content in the MI rats treated with perindopril alone and resveratrol alone or a combination of perindopril + resveratrol[219, 220]. Notably, a recent study also reported that resveratrol improves cardiac structure and function and prevents cardiac fibrosis by inhibiting

TGF-β 1/Smad3 pathway in transverse aortic constricted mice, signifying the role and mechanism of action of resveratrol in interfering with the abnormal activation of fibrotic pathways in the heart[221]. Therefore, our results suggest that a reduction in interstitial fibrosis may also be another mechanism underlying the resveratrol and perindopril mediated attenuation of cardiac remodelling and improvement in function.

The reason for the lack of an incremental effect of combination treatment in improving the post-MI cardiac abnormalities remains unknown at this point. However, the comparable improvement in oxidative stress, inflammatory and cardiac fibrosis indicators in both resveratrol and perindopril treated MI groups suggests a possible overlap in the overall action of these treatments through common signalling pathways in the setting of MI, which could be a possible reason for the absence of an incremental effect with combination treatment. For example, the inhibition of Ang II production (by ACE inhibitor) may lead to the blockade of the initiation of Ang II signalling pathway, whereas resveratrol may prevent the activation of this signalling pathway at downstream sites and prevent the deleterious downstream effects such as oxidative stress, inflammation and cardiac fibrosis[222, 223]. Interestingly, resveratrol has also been reported to downregulate the expression of Ang II type I receptor in the mouse aorta and reduce Ang II–induced hypertension[224], which suggests that ACE inhibitor and resveratrol treatment may invoke a similar end result as observed in our study. Accordingly, the individual action of these compounds may have prevented the MI-induced adverse effects to the maximum possible limit at the dose used in this study, and hence the combination may not have resulted in an incremental effect. In addition, it should be noted that permanent LAD ligation often leads to irreversible damage to a large area of LV, thus the therapeutic effects of the treatments may be limited to a certain extent. Collectively, our study suggests that treatment with resveratrol alone or in

combination with standard therapy begun at the early stage after MI is beneficial and it warrants further investigation in the clinical setting of ischemic HF.

6. Limitations of the study

Resveratrol has been shown to afford cardioprotection by acting through a variety of molecular pathways in the heart. Due to the limited scope of our study, we have examined only the major MI-related cardiac pathological features such as oxidative stress, inflammation and cardiac fibrosis. In this study, we did not check for specific toxicological liver and kidney biomarkers to examine whether combination treatment with resveratrol and perindopril has any adverse effect. We employed only a single dose regimen (2.5 mg/kg body weight of each resveratrol and perindopril/day) for the combination treatment, therefore, it was not possible to examine whether increasing (up-titration) the dose of either of the 2 compounds or both compounds over the treatment period would have resulted in incremental cardioprotective effect. Also, we did not explore the effects of perindopril + resveratrol for a long-term duration after MI, i.e. at the overt HF stage, due to the extensive time frame (16 weeks), and limited resources available.

7. Conclusion

Strategies to prevent the high prevalence of HF in post-MI patients by improving the existing treatment options are extremely important in the current clinical scenario. Our study reports for the first time, the efficacy of a low dose treatment with resveratrol in comparison to a well-established post-MI drug as well as a combination treatment in preventing MI-induced cardiac abnormalities in rats, and suggests its promising translational potential and clinical utility in conjunction with the current standard therapy for post-MI patients.

CHAPTER III: Stand-alone and combinatorial cardioprotective effects of resveratrol and sacubitril/valsartan in MI-induced rats

1. Rationale

Our first study demonstrated that resveratrol is equally beneficial in improving cardiac remodelling and function in MI-induced rats in a direct comparison with the ACE inhibitor perindopril[225]. RAAS blockade is unequivocally established as a first and foremost clinically beneficial strategy in post-MI and HF management[51]. The NP system is also recognised to play an important counter-regulatory role in fluid balance via natriuresis, diuresis and vasodilation in the setting of HF[66, 226]. Intuitively, targeting the main counter-regulatory mechanisms that operate to reduce the adverse effects of RAAS appeared to be a valuable therapeutic strategy for MI and HF patients[227]. Previous studies have shown that resveratrol also enhances nitric oxide bioavailability, and increases endothelium-dependent vasodilation[228, 229]. Evidently, augmenting the circulating NPs that mediate natriuresis, diuresis and vasodilation via pharmacological agents has become an area of avid interest in regards to the development of drug therapy for HF patients[227, 230]. However, the use of biologically active recombinant NP such as nesiritide has met with limited success in a clinical trial after promising initial findings[231, 232]. Hence novel approaches have been pursued to increase the circulating levels of NPs by inhibiting their degradation by peptidases such as neprilysin which breaks down NPs [6]. It has also been recognised that boosting the NP system via a peptidase inhibitor alone without RAAS blockade is not an effective strategy[232]. Unfortunately, an attempt to suppress the RAAS while enhancing the NP system with simultaneous inhibition of both ACE and a peptidase, neprilysin, has been proven fatal in another clinical trial as well[231, 233]. Eventually, Prospective Comparison of ARNI with ACEI to Determine Impact on Global Mortality and Morbidity in HF (PARADIGM-HF)

trial showed that dual blocker/inhibitor of the Ang II receptor and neprilysin, sacubitril/valsartan, is superior to the ACE inhibitor, enalapril, in reducing mortality and morbidity in HFrEF patients[234]. The evidence from PARADIGM-HF trial also suggests that combinational treatment strategies should be pursued with new potential compounds that can act through different therapeutic targets. Resveratrol possesses vasodilatory properties similar to NPs and NP enhancing drugs[229, 235]. As our previous study had investigated resveratrol alongside an ACE inhibitor, it is prudent to compare resveratrol with an ARNI which has been reported to be superior in HFrEF patients versus an ACE inhibitor[225]. Since ARB is also widely used in post-MI patients, it is also important to compare the efficacy of resveratrol alongside an ARB. In light of the PARADIGM-HF finding that a combination drug provides significant benefit, potential combination therapy involving resveratrol and front-line drugs warrants further study. Accordingly, it appears that study on the efficacy of resveratrol alongside sacubitril/valsartan may be a promising approach in developing new strategies to combat MI-induced HF management. In this regard, a pre-clinical study that evaluates the efficacy of sacubitril/valsartan in the setting of acute MI by starting the treatment with sacubitril/valsartan immediately after the ischemic episode may provide valuable information.

2. Hypotheses

1. Resveratrol will be comparable to valsartan and sacubitril/valsartan in improving cardiac structure and function in LAD ligated rats.
2. The combination treatment with resveratrol and sacubitril/valsartan will improve cardiac structure and function in LAD ligated rats.

3. Materials and methods

3.1. Animal care and experimental design

This study protocol was approved by the University of Manitoba Office of Research Ethics & Compliance and Animal Care Committee and was done in accordance with the guidelines by the Canadian Council for Animal Care. Three weeks old male Sprague Dawley rats (175-215 g) were housed in a temperature and humidity controlled room with a 12-hour light/dark cycle (Charles River Laboratories, Quebec). Rats were anesthetised with 1-5 % isoflurane with oxygen at a flow rate of 2 l/min and kept in the surgical plane on anesthesia with 2% isoflurane during surgery and subjected to permanent ligation of LAD to induce MI or sham surgery after baseline echocardiographic examination. A left thoracotomy was done, and the heart was gently exposed from the pericardial sac through the incision. The LAD was located and occluded with 6-0 polypropylene silk suture at about 2 mm from aortic root. The suture was tied and the ligation was estimated to be successful when the anterior wall of the LV turned pale. The heart was repositioned, then chest compressed to remove any air from the cavity and the incision was closed using a purse string suture. Sham-operated animals that served as normal control were subjected to similar surgical procedures except that the LAD was not ligated. Buprenorphine 0.05 mg/kg was administered pre- and post-surgery (2 times a day for 2 days) subcutaneously as an analgesic agent to all rats. All surviving sham and MI rats were assigned to the following 5 treatment groups. 1. Vehicle (50 % ethanol 2.5 mL/kg body weight/day), 2. Sacubitril/Valsartan (68 mg/kg body weight/day, Novartis, Basel), 3. Resveratrol (2.5 mg/kg body weight/day, trans-resveratrol, ≥99%, Sigma-Aldrich, Ltd, Ontario), 4. Valsartan (31 mg/kg body weight/day, Novartis, Basel), 5. Sacubitril/Valsartan Resveratrol (both 2.5 mg/kg body weight/day). Five groups consisted of sham (n= 6-9) and MI (n= 10-13) rats and received the treatments by oral

gavage daily for 8 weeks. The dose for the present study was chosen based on the previous studies[225, 236]. Animals were regularly weighed, and evaluated for well-being.

3.2. Transthoracic echocardiography (TTE)

All experimental rats were weighed and anaesthetised with 3 % isoflurane in a chamber, and then kept under 1.5 – 2 % isoflurane throughout the procedure. TTE was obtained at baseline and at 8 week of treatment by 2D guided M-mode and Doppler modalities with a 13-MHz probe (Vivid 9; GE Medical Systems, Milwaukee, WI) by a procedure described elsewhere[237]. Two-D M-mode parasternal short-axis view images were obtained to determine systolic functional parameters such as the LVEF and fractional shortening (FS) from end-systolic and end-diastolic volumes. The cardiac structural parameters such as IVSTs, LVPWTd and LVIDs and LVIDd and LVIDs at diastole and systole were also determined from parasternal short-axis view images. Doppler measurements included measurement of isovolumic relaxation time (IVRT) and E/A ratio. All echocardiographic images were analysed to calculate the listed parameters using EchoPAC software (GE Medical Systems, Milwaukee, WI). The values obtained for the mentioned parameters in 3 consecutive cardiac cycles were averaged to obtain the final data[182, 237].

3.3. Blood and tissue collection

All animals were anesthetised with 1-5 % isoflurane. Depth of anesthesia was assessed by pedal withdrawal reflex. The blood sample was drawn from the inferior vena cava by opening the thoracic cavity. After the blood collection, the heart was immediately excised. The whole heart was rinsed in PBS and atria, right and LVs, septum, and fibrotic scar tissue were separated, weighed and flash frozen in liquid nitrogen. Lungs and liver were also collected immediately

after excising the heart.

3.4. LV scar size (%) and lung and liver wet-to-dry weight ratio determination

Percentage of scarred (infarcted) LV tissue was calculated by dividing the weight of scarred LV tissue by whole weight of LV tissue[183]. First, LV tissue was removed from septal and left atrial tissues of the rat heart of all the animals. Then scar tissue was carefully removed from whole LV by cutting through the edges of the scar tissue and both portions were weighed. Evidence of HF was assessed by checking the presence of ascites, and by calculating the lung wet-to-dry weight ratio and the liver wet-to-dry weight ratio in both vehicle and investigational compounds received MI rats.

3.5. Oxidative stress marker assay

In order to determine MI-associated oxidant imbalance in the myocardium, the level of the lipid peroxidation product, MDA was assessed using the MDA quantification kit (Abcam, Cambridge) following the manufacturer's instructions[184].

3.6. Pro-inflammatory and cardiac fibrosis marker assays

The TNF-α level in myocardial tissue was measured by ELISA following the manufacturer's instructions (Abcam, Cambridge). The hydroxyproline level in the heart was also assayed following the manufacturer's instructions to quantify the level of collagen (Abcam, Cambridge). Collagen concentration was calculated by multiplying the hydroxyproline level by a factor 7.46 as the interstitial collagen contains an approximately 13.4 % hydroxyproline by a procedure described elsewhere[183].

3.7. NP and renal dysfunction and injury marker assays

Plasma BNP, creatinine and neutrophil gelatinase associated lipocalin (NGAL) levels were also measured from all of the groups as per the manufacturer's instructions (Abcam, Cambridge).

3.8. Statistical analysis

All values are expressed as means ±SEM. One-way analysis of variance was used to analyze variations between the means of the groups. Significant values are defined as p <0.05. If a significant difference was observed, one-way analysis of variance was followed by a Newman-Keuls post hoc test.

4. Results

4.1.General characteristics

Body weight was comparable between sham and MI rats treated with vehicle at 8 weeks after the surgery (Figure 1A). However, there was a trend towards (P > 0.05) a lower body weight in vehicle and valsartan treated MI rats compared to vehicle treated sham rats (Figure 1A). LV-to-tibia length ratio was also comparable between sham and MI rats in all groups (Figure 1B). There was a trend P > 0.05) towards a higher heart-to-tibia length ratio in vehicle treated MI rats compared to vehicle treated sham rats (Figure 1B). In sham and MI rats, pleural and abdominal cavities were devoid of effusions or ascites at 8 weeks. There was a significant increase in lung wet-to-dry weight ratio in vehicle treated MI rats compared to vehicle treated sham rats (Figure 1C. 5.07±0.07 vs 4.73±0.12, p <0.05). Sacubitril/valsartan, valsartan, resveratrol and sacubitril + resveratrol/valsartan significantly lowered lung wet/dry weight ratio in MI rats compared to vehicle treated MI rats (Figure 1C. 5.07±0.07 vs 4.77±0.82, 4.62±0.12, *p <0.05),* and 4.47±0.09,

p <0.01). Sacubitril/valsartan +resveratrol treated MI rats also had significantly lower lung wet-to-dry weight ratio (Figure 1C. 5.07±0.07 vs 4.48±0.10, *p <0.001*) compared to MI vehicle treated rats.

MI rats treated with vehicle had significantly increased liver wet-to-dry weight ratio compared to sham rats at 8 weeks (Figure 1D. 3.29±0.07 vs 2.54±0.18, *p <0.001*). MI rats administered with sacubitril/valsartan, valsartan, resveratrol had a significantly lower liver wet-to-dry weight ratio (Figure 1D. 3.29±0.07 vs 1.95±0.04, *p <0.001*, 2.93±0.15, *p <0.01)*, and 2.65±0.05, *p <0.001*) compared to MI rats treated with vehicle. Sacubitril/valsartan + resveratrol treated MI rats also had a significantly lower liver wet-to-dry weight ratio (Figure 1D. 3.29±0.07 vs 2.40±0.03, *p <0.001*) compared to MI vehicle treated rats.

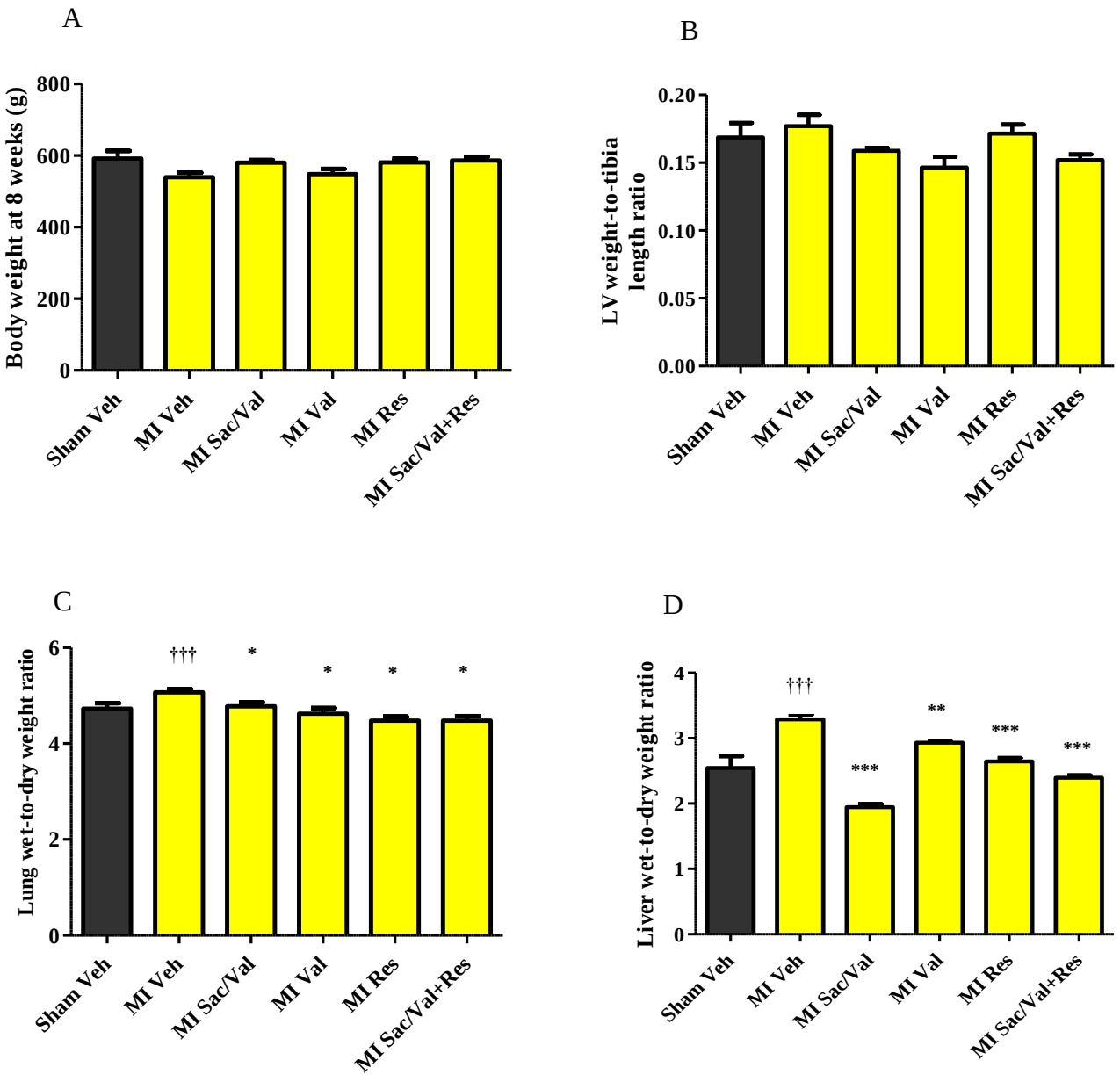


Figure 1. A. Body weight of sham and MI rats at week 8 (sham n=6-9; MI n= 10-13). B. LV weight-to-tibia length ratio of sham and MI rats (sham n=6-9; MI n= 10-13). C. Lung wet-to-dry weight ratio of sham and MI rats. D. Liver wet-to-dry weight ratio of sham and MI rats.***p <0.001 vs Sham Veh; * p <0.05 vs Sham Veh; †† p <0.01 vs MI Veh. All values are expressed as mean±SEM. Vehicle - Veh, Sac/Val - Sacubitril/Valsartan, 3. Res - Resveratrol, 4. Val - Valsartan.

All MI rats included in the study had well-defined scarred LV tissue at the anterior region due to the large anterior-infarct that resulted from LAD ligation. Scar tissue size (infarct size in %) was also compared between the MI groups alone (Figure 2). Scar size calculated as the percentage of scarred LV tissue weight versus total LV weight was also comparable between the groups (25.38±1.42, vs 21.50±0.69, 20.54±1.62, 20.51±1.08, 22.06±1.92), and there was no statistically significant difference.

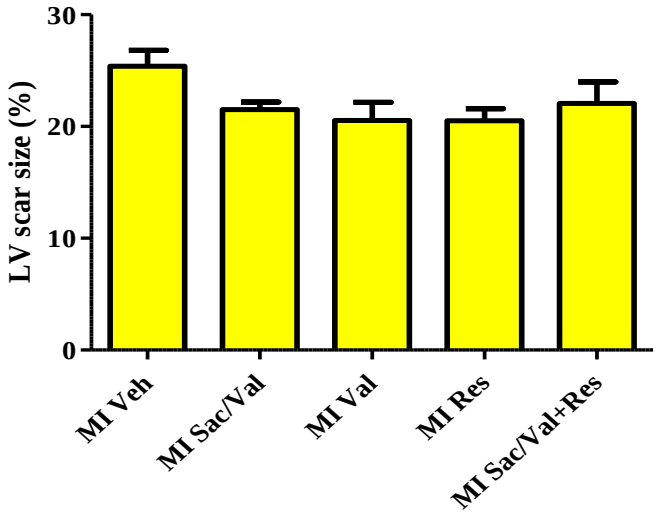
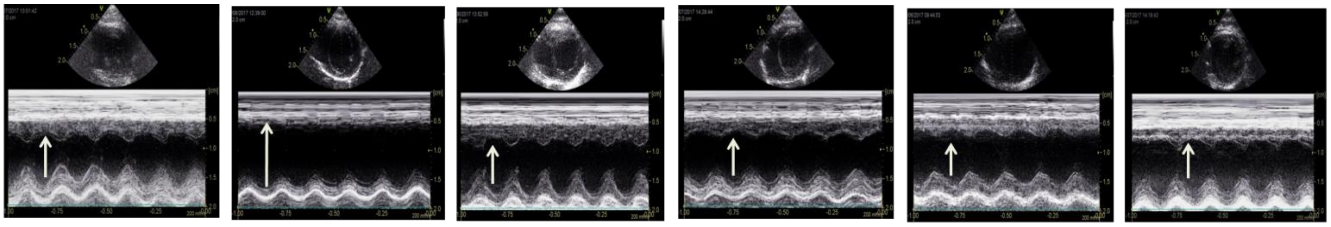


Figure 2. LV scar size (%) in MI rats at 8 weeks. n= 9-11.

4.2. Treatment with resveratrol, sacubitril/valsartan, valsartan and sacubitril/valsartan + resveratrol prevents post-MI LV dilatation

Echocardiographic analysis showed that MI rats treated with vehicle had significantly increased LV dilatation as evidenced by the increased LVID (Table. 1. LVID diastole: 10.66±0.18 *vs* 8.86±0.19, *p <0.001);* LVID systole: 7.57±0.31 *vs* 4.93±0.19, *p <0.001*) when compared with sham rats treated with vehicle at 8 weeks. As compared to MI rats treated with vehicle, treatment with sacubitril/valsartan, valsartan and resveratrol significantly prevented the LV dilatation in MI

rats (Table. 1. LVID diastole: 10.66±0.18 *vs* 9.78±0.20, 9.85±0.23, and 9.82±0.21, *p <0.05)*; LVID systole: 7.57±0.31 *vs* 6.54±0.18, *p <0.05*, 6.59±0.30, *p <0.01* and 6.53±0.27, *p <0.05*). Similarly, sacubitril/valsartan + resveratrol treated MI rats also showed significantly less LV dilatation (Table. 1. LVID diastole: 10.66±0.18 *vs* 9.01±0.31, *p <0.001*)*;* LVID systole: 7.57±0.31 *vs* 5.81±0.25, *p <0.001*). There was a significant reduction in the IVSTs in vehicle treated MI rats in comparison with vehicle treated sham rats at 8 weeks (2±0.10 vs 2.89±0.10, *p <0.001*). In comparison to vehicle treated MI rats, sacubitril/valsartan, valsartan, resveratrol and sacubitril/valsartan +resveratrol received MI rats had significantly higher IVSTs (Table. 1. 2.52±0.08, 2.44±0.07, 2.46±0.09, 2.53±0.13, *p <0.01*). LVPWTd and LVPWTs were comparable between the groups.



Sham Vehicle | MI Vehicle | MI Sac/Val | MI Val | MI Res | MI Sac/Val+Res

	Sham Veh	MI Veh	MI Sac/Val	MI Val	MI Res	MI Sac/Val+Res
LVIDd (mm)	8.86±0.19	10.66±0.18†††	9.78±0.20*	9.85±0.23*	9.82±0.21*	9.01±0.31***
LVIDs (mm)	4.93±0.19	7.57±0.31†††	6.54±0.19*	6.59±0.30**	6.53±0.27*	5.81±0.25***
IVSTs (mm)	2.89±0.10	2±0.10†††	2.52±0.08**	2.44±0.07**	2.46±0.09**	2.53±0.13**
LVPWTd (mm)	1.94±0.13	2.28±0.09	2.02±0.12	2.33±0.07	2.21±0.12	2.31±0.06
LVPWTs (mm)	3.03±0.16	2.99±0.11	2.80±0.16	3.14±0.09	3.01±0.15	3.14±0.10

Table 1. Echocardiographic parameters of sham and MI rats at 8 weeks. Representative 2D M-Mode echocardiographic images showing improvement in LV dilatation. Left ventricular internal

diameter (LVIDd and LVIDs), interventricular septal wall thickness (IVSTs) and left ventricular posterior wall thickness (LVPWTd and LVPWTs) at diastole and systole of sham and MI rats. All values are expressed as mean±SEM, n=9-11.$^{\dagger\dagger\dagger}p$ <0.001 vs Sham Veh; $^{***}p$ <0.001, $^{**}p$ <0.01 and $^{*}p$ <0.05 vs MI Veh.

4.3. Treatment with resveratrol, sacubitril/valsartan, valsartan and sacubitril/valsartan + resveratrol prevents post-MI cardiac dysfunction

LVEF was significantly lower in MI vehicle treated rats compared to sham vehicle treated rats at 8 weeks (Figure 3A. 56.60± 1.70 *vs* 80.33± 1.41 (*p <0.001)*). In comparison to MI vehicle treated rats, sacubitril/valsartan, valsartan, and resveratrol received MI rats had significantly improved LVEF (56.60± 1.70 *vs* 66.82±1.43, *p <0.001*, 65.45± 2.70, and 64.82±1.02, *p <0.01*). Eight week treatment with sacubitril/valsartan +resveratrol also significantly prevented the reduction in LVEF in MI rats when compared to MI rats treated with vehicle (56.60± 1.70 *vs* 70.30±1.63, *p <0.001*).

FS was also significantly lower in MI vehicle treated rats compared to sham vehicle treated rats at 8 weeks (Figure 3B 44.44± 1.36 *vs* 26.70± 1.02 (*p <0.001)*). In comparison to MI vehicle treated rats, sacubitril/valsartan, valsartan, and resveratrol received MI rats had significantly improved FS (Figure 3B 26.70± 1.02 *vs* 33.18±0.99, *p <0.001*, 32.64± 1.94, and 31.64±0.69, *p <0.01*). Eight week treatment with sacubitril/valsartan +resveratrol also significantly prevented the reduction in FS in MI rats when compared to MI rats treated with vehicle (26.70± 1.02 *vs* 35.60±1.17, *p <0.001*). IVRT and E/A ratio were comparable between the groups (Figure 3 C and D)

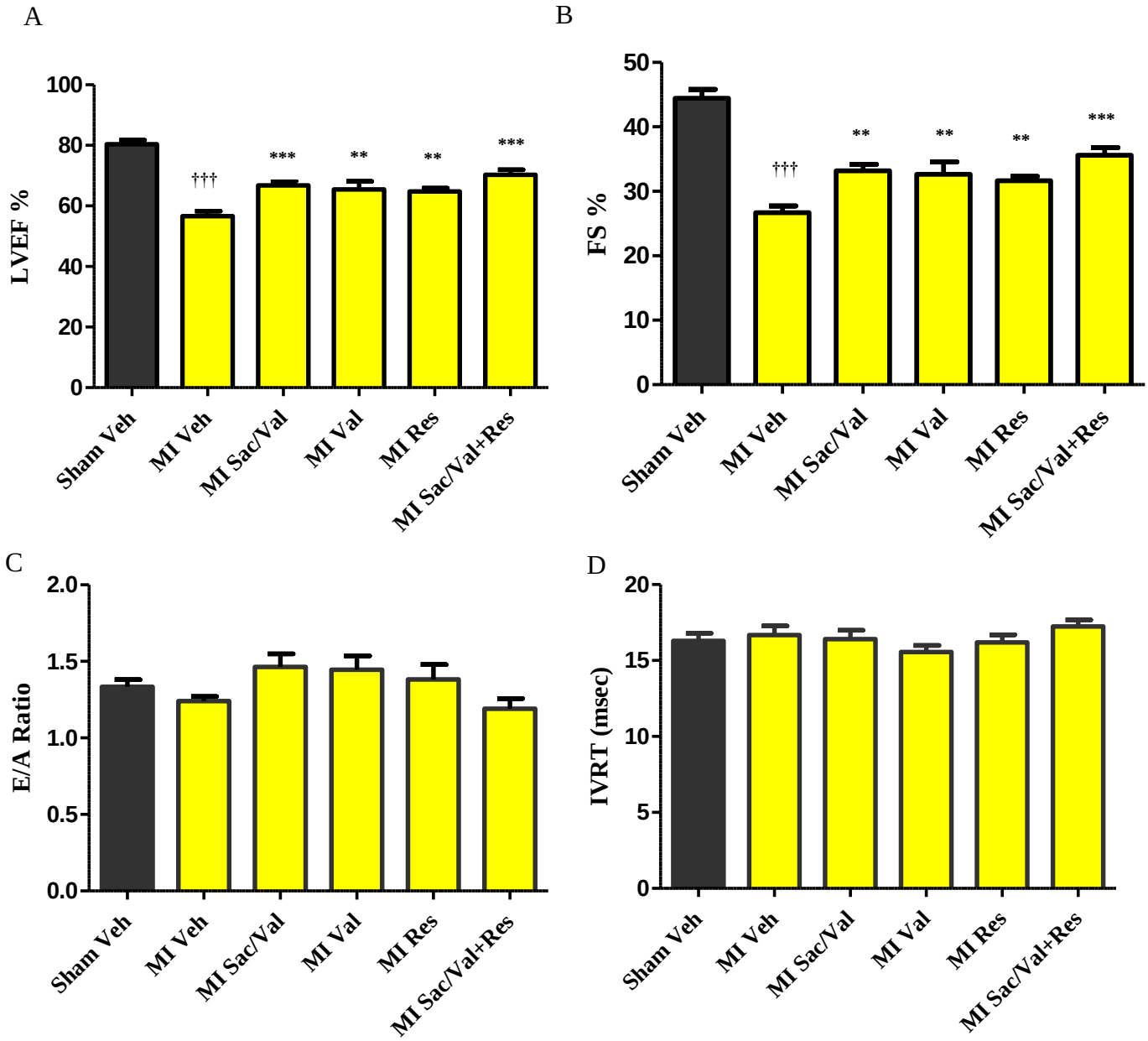
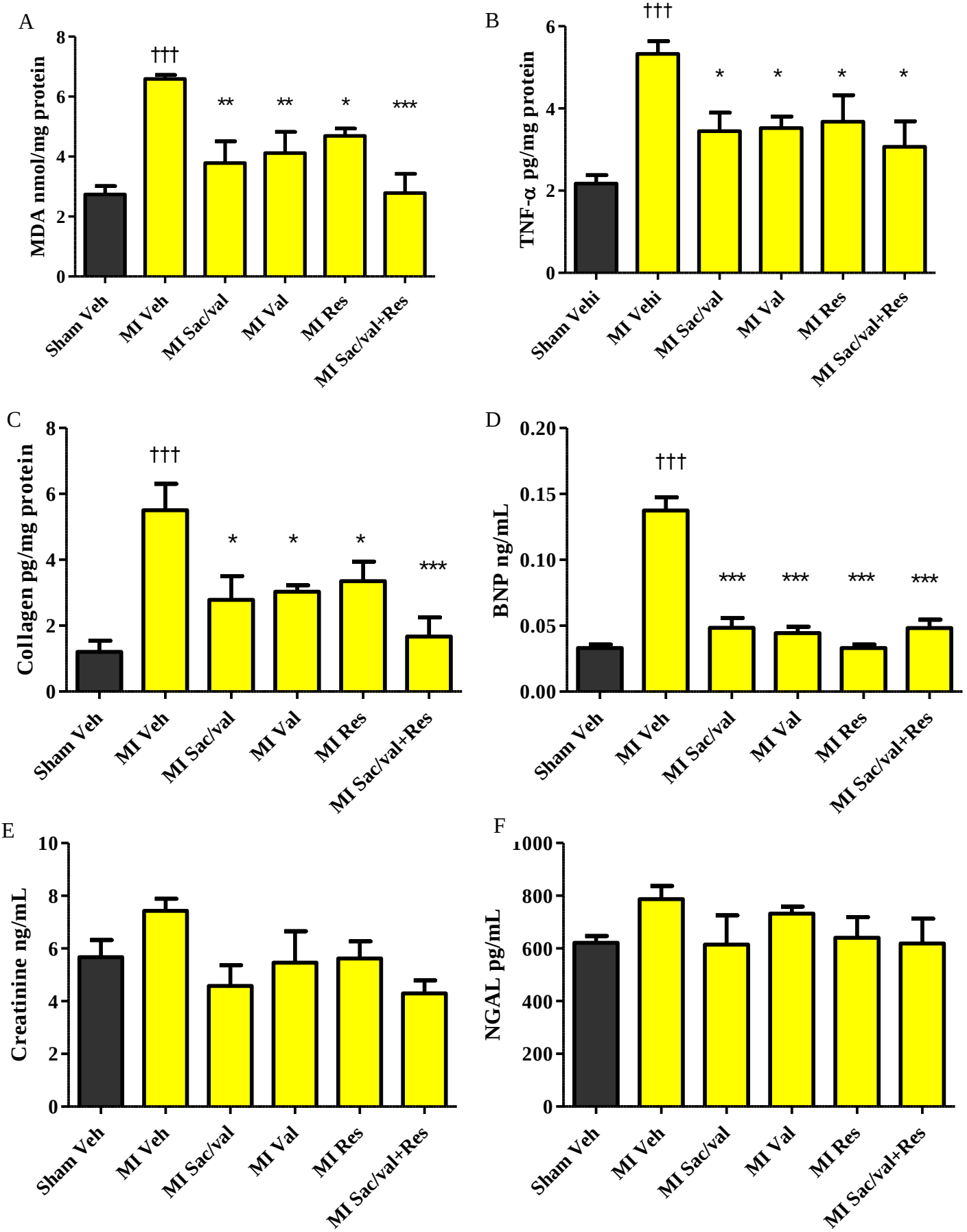


Figure 3. A. Left ventricular ejection fraction (LVEF). B. Fractional shortening (FS). C. E/A Ratio. D. Isovolumic relaxation time (IVRT) of sham and MI rats. All values are expressed as mean±SEM, n=9-11. $^{\dagger\dagger\dagger}p$ <0.001 vs Sham Veh; ***p <0.001, **p <0.01 and *p <0.05 vs MI Veh.

4.4. Treatment with resveratrol, sacubitril/valsartan, valsartan and sacubitril/valsartan + resveratrol lowers post-MI increase in MDA, TNF-α, collagen, and BNP.

At 8 weeks, vehicle treated MI rats had a significantly increased levels of MDA in heart tissue compared with vehicle treated sham rats (Figure 4A). The MI group that showed a significant improvement in cardiac remodelling and dysfunction with sacubitril/valsartan, valsartan, resveratrol and sacubitril/valsartan + resveratrol treatment had significantly lower levels of MDA compared to MI vehicle treated rats (Figure 4A 6.591±0.13 vs 3.786±0.72, 4.11±71, $p < 0.01$), 4.69±0.24, $p < 0.05$, and 2.78±0.64, $p < 0.001$). We also observed that MI rats showed significant increase in the levels of TNF-α in heart tissue when compared with vehicle treated sham rats at 8 weeks. Sacubitril/valsartan, valsartan, resveratrol, and sacubitril/valsartan + resveratrol also showed a significantly reduced the levels of TNF-α in MI rats compared to MI rats treated with vehicle (Figure 4B 5.33±0.31 vs 3.45±0.45, 3.52±0.28, 3.68±0.64, and 3.07±0.61, $p < 0.05$). At 8 weeks, vehicle treated control MI rats had significantly increased levels of collagen in heart tissue compared with vehicle treated sham rats. MI rats treated with sacubitril/valsartan, valsartan, resveratrol, and sacubitril/valsartan + resveratrol had significantly decreased levels of collagen compared to MI rats treated with vehicle (Figure 4C 5.50±0.80 vs 2.78±0.71, 3.09±0.20, 3.35±0.59, and 1.67±0.58, $p < 0.05$). Plasma BNP level was significantly increased in vehicle treated MI rats compared with vehicle treated sham rats. Sacubitril/valsartan, valsartan, resveratrol, and sacubitril/valsartan + resveratrol also significantly reduced the levels of BNP in MI rats (Figure 4D. 0.14±0.01 vs 0.05±0.007, 0.04±0.005, 0.03±0.002, and 0.05±0.006, $p < 0.001$). Creatinine and *NGAL* levels were comparable between the groups (Figure 4E and F).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MI Sac/val+Res

Figure 4. A. Malondialdehyde (MDA). B. Tumor necrosis factor-α (TNF-α). C. Collagen (left ventricular tissue). D. Brain natriuretic peptide (BNP). E. Creatinine. F. Neutrophil gelatinase associated lipocalin (NGAL) (plasma). All values are expressed as mean±SEM, n=4-6. ††† p <0.001 vs Sham Veh; ***p.<0.001, **p <0.01 and *p <0.05 vs MI Veh.

5. Discussion

The current study demonstrated that stand-alone treatment with resveratrol, commenced immediately after MI inducing surgery and lasting for 8 weeks, significantly prevented LV remodelling and systolic dysfunction in MI rats. The resveratrol mediated cardioprotection was equal in magnitude as treatment with valsartan alone or sacubitril/valsartan alone in MI-induced rats. The combination treatment with sacubitril/valsartan + resveratrol has also been cardioprotective in MI rats. However, there was no incremental benefit with sacubitril/valsartan + resveratrol combination treatment even though there was a clear trend ($p > 0.05$) towards less adverse cardiac remodelling and better systolic function (≥4 % LVEF) than with individual treatments in MI rats.

Previously, resveratrol has been reported to be as beneficial as an ACE inhibitor, perindopril, in improving cardiac structural and functional abnormalities associated with acute MI in rats in an equal dose head-to-head comparison[225]. In this study, the doses of the treatment were not equal and doses of sacubitril/valsartan and valsartan were higher than the resveratrol dose. Although it has been unequivocally established that sacubitril/valsartan is superior to ACE inhibitor, enalapril, in HF patients, no clinical evidence is available to guide the use of sacubitril/valsartan in acute post-MI patients[234]. As the new evidence about the post-MI clinical efficacy of sacubitril/valsartan is expected to be available soon from Prospective ARNI vs ACE Inhibitor Trial to DetermIne Superiority in Reducing HF Events After MI (PARADISE-MI) trial

(NCT02924727), it is imperative to glean more information about the efficacy of resveratrol in comparison to sacubitril/valsartan[238]. To that end, this is the first study to investigate the comparative cardioprotective of resveratrol alongside sacubitril/valsartan in the pre-clinical setting of MI. A previous pre-clinical study showed that 4 weeks treatment (started after 1 week after the induction of MI in rats) with sacubitril/valsartan improved cardiac remodelling and dysfunction in the setting of MI[236]. However, the study design precluded our ability to understand the advantages of an earlier and longer term sacubitril/valsartan treatment and its effects on long-term progression of MI-related cardiac remodelling and dysfunction that leads to HF. Sacubitril/valsartan is a combination drug with a moiety of the ARB, valsartan, and the neprilysin inhibitor, sacubitril. Sacubitril is proposed to inhibit the neprilysin activity and thereby the level of NPs to aid vasodilation and reduce fluid retention. Since the previous study mentioned above did not examine the comparative effects of sacubitril/valsartan and valsartan, we also investigated the effects of both drugs (i.e., sacubitril/valsartan vs valsartan) side by side along with resveratrol. Interestingly, in this study, sacubitril/valsartan was not significantly better in improving cardiac structure and function in the setting of MI compared to resveratrol or valsartan. That being stated, in patients with HFpEF, sacubitril/valsartan reduced the level of NT-pro-BNP to a greater extent than did valsartan at 12 weeks[239]. It should also be noted that treatment with sacubitril alone showed a trend towards an increase in LVEF in MI rats compared to valsartan group, without statistical significance. The discrepancy in the findings may be further explored.

Cardiac remodelling is known to occur in 30% of anterior acute MIs[240]. In cases of non-anterior infarcts, cardiac remodelling is also found in nearly 17% post-MI patients[240]. An increase in end-diastolic volume (LV dilatation) occurs early in MI to accommodate a larger preload and

compensate for the acute decrease in contractility. Cardiac remodelling is a key indicator of a high risk of HF or cardiovascular death after an MI. For instance, in the echocardiographic sub-study of the Survival and Ventricular Enlargement trial (SAVE trial), progressive LV dilatation after MI was associated with a higher risk of both HF and cardiovascular death[241]. Similarly, the VALsartan In Acute myocardial iNfarcTion (VALIANT trial) echocardiographic study reported that patients with post-MI LV remodelling had a greater risk of the composite of cardiovascular death, MI, HF, stroke, or resuscitated cardiac arrest compared with patients without LV remodelling[242]. Cardiac remodelling is a dynamic and continuing process that goes on up to 24 months following an acute MI. Cardiac remodelling is linked to both systolic and diastolic dysfunction. LVEF deterioration due to LV remodelling is associated with an increase in end-systolic volume and less wall thickening in the non-infarcted region in some patients[243]. Patients showing LVEF improvement may demonstrate an increase in LV wall thickening both in the infarct as well as in the remote zones[243]. Consistent with previous evidence, low dose resveratrol treatment significantly prevented the LV dilatation post-MI[244]. In addition, resveratrol was able to prevent the IVS thinning in the infarcted heart[147]. The combination treatment with sacubitril/valsartan + resveratrol also afforded protection against LV dilatation and IVS thinning with higher significance than individual treatments. This finding suggests that resveratrol effectively interferes with the pathological LV remodelling in the setting of MI. Reverse cardiac remodelling has been demonstrated as a reduction in LV dilatation and recovery in cardiac function by drug therapy and device therapy in HF patients[245]. Importantly, high-dose resveratrol treatment has been shown to reverse LV dilatation (reverse remodelling) and significantly improve cardiac function in post-MI rats when treatment was started after significant remodelling had occurred[147]. Apart from the post-MI reverse remodelling efficacy, resveratrol

treatment was also able to reverse increased LV end-diastolic volume, LV end-systolic volume, LV chamber diameter, and LA dimensions in mice subjected to transverse aortic constriction[206]. Low dose resveratrol treatment has also been shown to mediate cardioprotective effects by reducing left atrial and LV remodelling in MI rats with HF[244]. Essentially, reverse remodelling describes the ability of the heart to undergo a process by which an injured LV with a dilated spherical phenotype returns towards a normal ventricular structure and function[246, 247]. None of the treatments including the combination treatment resulted in a statistically significant reduction in scar size % even though there was trend towards reduction, suggesting that anti-remodelling effects are not due to infarct sparing after MI. However, it should be noted that resveratrol has been shown to reduce the infarct size post-MI[147]. Accordingly, the current study and the studies mentioned above may suggest the efficacy of resveratrol as an anti-remodelling or reverse remodelling agent.

It is recognised that 50% of patients fail to demonstrate an improvement in LVEF following acute MI even after undergoing reperfusion and/or receiving optimal drug therapy[248-250]. The absence of LVEF recovery has prognostic significance and is associated with an increased risk of sudden cardiac arrest and all-cause mortality independent of baseline LVEF[251]. In addition, its prognostic significance, predicting LVEF deterioration may have important therapeutic implications, such as qualifying patients for additional medical or device therapies[251]. Beta blockers, ACE inhibitors/ARBs, and MRA have demonstrated potent effects on reverse remodelling and improvement in LVEF in multiple studies[252, 253,254]. The Department of Veterans Affairs Cooperative Vasodilator-HF Trials (V-HeFT) data showed that an increase of LVEF by > 5% from baseline at 6 months was the strongest predictor of mortality[255]. In the current study, low dose resveratrol prevented the early post-MI deterioration of systolic function as evidenced

by the increased LVEF in MI rats treated with resveratrol. Recently, another pre-clinical study reported the efficacy of resveratrol in improving systolic function in the setting of post- MI HF[244]. The study showed that 3 weeks after LAD ligation MI rats had signs of HF with 40% LVEF, and low dose resveratrol treatment for 2 weeks (started 3 weeks post-surgery after HF was established) was able to increase the LVEF[252]. Consistent with previous studies, sacubitril/valsartan also improved LVEF in MI rats and thereby provided protection against systolic dysfunction. In addition to LVEF improvement, stand-alone and combination treatment with resveratrol prevented a mild increase in the surrogate markers of lung and liver congestion. This finding, specifically, may suggest that resveratrol decreases the very early signs of HF post-MI. Notably, in this study, there was no evidence of diastolic dysfunction as IVRT and E/A ratio were unaltered significantly in MI rats.

ROS act as a cell signalling agents and regulators of cellular function in normal conditions[256]. ROS encompass a diverse range of free radicals and non-free radicals. The free radicals and non-free radicals include O_2^-, lipid peroxides (ROO^-), hydroxides (HO^-), NO and H_2O_2, peroxynitrite ($ONOO^-$) and hypochloric acid (HClO) respectively.[256]. ROS is a major constituent of the genesis and progression of CAD. Oxidised LDL formed by a reaction with ROS initiates vascular changes that lead to atherosclerosis progression[257]. Subsequently, ROS may also contribute to the destabilisation of atherosclerotic plaques and thrombus formation[257]. Excessive formation of ROS is also detrimental in the ischemic condition and MI contributing to cell death, as well as in reperfusion injury[256]. Pathophysiological conditions such as MI create an imbalance between ROS and antioxidants, and has a causative role in the progression of cardiac remodelling and HF. The ROS mediated direct oxidising effects on some cellular proteins, lipids and DNA in the setting of MI may cause cardiomyocyte necrosis and apoptosis. Consistent with

previous studies[258, 259], the elevated levels of MDA observed in MI rats in this study further suggest the contribution of deranged oxidant status to the cardiac anomalies in the setting of MI. The lower MDA levels in MI rats that received resveratrol, valsartan/sacubitril, valsartan and combination of sacubitril/valsartan + resveratrol suggest that the improvement in cardiac structure and function may be partly mediated through an improvement in the redox status by reducing the deleterious effects of ROS. In addition, previous studies demonstrated that treatment with resveratrol or RAAS inhibitors/blockers involves an improvement in the antioxidant status[205, 260-266].

Oxidative stress is also intrinsically linked to post-MI events such as an inflammatory response[186]. Even though the initial inflammatory response is essential for clearing the debris and initialing wound healing processes, an unchecked increase in pro-inflammatory cytokines contributes to pathological remodelling. TNF-α has been linked with a higher risk of HF by directly contributing to cardiac remodelling and dysfunction[214, 215]. TNF-α is categorised into membrane-associated TNF-α (mTNF-α) and secreted TNF-α (sTNF-α). Following an MI, cardiomyocytes and myocardial local mononuclear macrophages in the infarcted zone and infarction border zone produce large amounts of TNF-α[267]. TNF-α mediates its action via 2 TNF-α receptors (TNFRs), TNFR1 and TNFR2 which are found on the cell membranes. An increased expression of TNFR1 and 2 has been shown to increase with infarct size and is linked to reduced heart function. Ischemia/reperfusion injury or the no-reflow phenomenon has also been shown to intricately linked to TNF-α. Post-MI TNF-α levels are also associated with arrhythmias, myocardial stunning, LV systolic dysfunction, microvascular injury, and progressive myocardial necrosis[268]. Consistent with previous reports (pre-clinical and clinical)[147, 187], this study also showed that treatment with resveratrol alone as well as their combination prevented the increase

of TNF-α in MI rats[175, 216]. Overall, our findings suggest that the reduction in pro-inflammatory cytokines may also be involved in the treatment mediated improvement in cardiac dysfunction in MI rats. Altered collagen turnover, involving breakdown and synthesis post-MI, leads to ECM degradation and cardiac fibrosis. Collagen deposition is beneficial in the early phase of infarct wound repair. However, the accumulation of collagen remote from the infarct site increases myocardial stiffness and contractile dysfunction, and contributes to the progression of LV remodelling and HF[217, 218]. In addition, the infarct scar and non-infarcted fibrotic tissue may affect electrical conduction and precipitate arrhythmias. Resveratrol is also known to inhibit myofibroblast differentiation via altering the ROS/extracellular regulated kinase/TGF-β 1/periostin pathway. Emerging evidence also suggests that resveratrol decreases TGF-β 1-induced cardiac fibroblast proliferation and collagen secretion partly through the downregulation of miR-17 and Smad7 mRNA and protein expression. Therefore, our results suggest that a reduction in interstitial fibrosis may also be another mechanism underlying the resveratrol and perindopril mediated attenuation of cardiac remodelling and improvement in function.

BNP is released by ventricular cardiomyocytes due to their stretching and volume overload[269]. In patients with STEMI, a higher BNP level is an independent risk factor and a suitable prognostic marker for mortality rates. In both short-term and long-term after an acute MI, higher levels of BNP have been shown to have poorer prognosis[269]. It is well established that in post-MI patients, plasma BNP concentrations present a negative correlation with LVEF, an index of systolic function. BNP is not restricted to the detection of systolic dysfunction, but it can be extended to the identification of patients with diastolic dysfunction[269]. This study showed that treatment with resveratrol, sacubitril/valsartan, valsartan and the combination was associated with a lower level of BNP in MI rats. Previous pre-clinical studies have also reported that resveratrol treatment

effectively lowers the level of BNP[270-272]. Renal dysfunction is also considered as one of the independent risk factors for major cardiovascular events and mortality in post-MI patients[273]. Low cardiac output due to post-MI cardiac abnormalities results in reduced renal perfusion. Impaired renal perfusion consecutively leads to further overactivation of RAAS, initiating a vicious cycle of cardio-renal syndrome[274]. RAAS overactivation in turn may prove equally harmful to the heart and the kidney. However, in this study, we did not observe any structural or functional renal impairment in MI rats as evidenced by the unaltered level of NGAL and creatinine.

In light of our findings, it is clear that further studies are needed to evaluate whether a higher dose combination of resveratrol and sacubitril/valsartan could produce a statistically significant improvement in cardiac structure and function in post-MI rats. The doses of resveratrol and sacubitril/valsartan used in this study have been administered previously in the same animal model with similar results. However, it should be noted that sacubitril/valsartan was given twice daily in the PARADIGM-HF trial to ensure efficacy. In contrast, in this study, sacubitril/valsartan was given as a single dose unlike the clinical trial and it is not known whether this dosing regimen may have any bearing on the observed outcomes. Effects of neprilysin inhibitor sacubitril alone could not be assessed in this study alongside sacubitril/valsartan, valsartan and resveratrol to delineate the individual effects of sacubitril. This approach will help in obtaining further information about the mechanism of action of sacubitril/valsartan. As mentioned earlier, sacubitril/valsartan and resveratrol are known to have vasodilatory properties and hence it is reasonable to speculate that both interventions may produce similar results via the same pathways. Our results also suggest that both interventions have overlapping effects as evidenced by the comparable improvement in oxidant status,

inflammatory response and cardiac fibrosis markers. Our findings are also significant concerning the potential side effects of sacubitril/valsartan. In HFrEF patients, sacubitril/valsartan is known to cause hypotension in comparison to enalapril although it may not necessitate discontinuation of the drug. However, the concerns related to sacubitril/valsartan-induced hypotension still remains valid as it may increase the rate of adverse outcomes. In such cases, decreasing the titration of doses of sacubitril/valsartan may be required in post-MI patients to derive benefits without eliciting hypotension. In light of the pre-clinical finding in this study, adjuvant therapy such as resveratrol may help post-MI patients when the prescribed drug therapies are not tolerated.

6. Conclusion

Stand-alone treatment with resveratrol, sacubitril/valsartan, and valsartan significantly prevented cardiac remodelling and dysfunction in MI rats. Combination treatment with sacubitril/valsartan +resveratrol also attenuated the cardiac abnormalities. Resveratrol, sacubitril/valsartan, valsartan, and sacubitril/valsartan + resveratrol mediated a prevention in cardiac remodelling and dysfunction in MI rats and this was partly mediated by a reduction in cardiac oxidative stress, inflammation and fibrosis. In this study, there was no evidence of renal dysfunction or injury post-MI and neither the stand-alone nor the combination treatments resulted in significant renal changes. Our results suggest that sacubitril/valsartan and resveratrol can be further explored for their clinical efficacy in the setting of MI in future clinical trials.

CHAPTER IV: Resveratrol improves contractile function and cell viability in adult rat cardiomyocytes exposed to NE and inhibits basal adult rat cardiac fibroblast proliferation

1. Rationale

HF is characterised by an overactivation of the SNS due to precipitating event such as MI[275-278]. Adrenergic activation is essential to counter the normal physiological stress and abnormal pathological stress of HF in patients. Conversely, chronic unchecked adrenergic stimulation forms a central part of progressive pathophysiological processes culminating in cardiac remodelling and dysfunction that precedes HF. Post-MI pathological changes necessitate a sustained elevation of catecholamines to meet the myocardial demand in contrast to normal sporadic increases associated with physiological changes[278]. In order to maintain optimal cardiac function, adrenergic nerve endings release NE which acts through beta-adrenergic receptors to increase cardiac contractility[279]. In the heart, stimulation of beta-adrenergic receptors activates the Gs protein and adenylate cyclase, and increases intracellular cyclic AMP levels, protein kinase A-dependent phosphorylation which further activates L-type Ca^{2+} channels, ryanodine receptors, and troponin I, and inhibits phospholamban (SERCA2a inhibitory protein) to increase contractility[275, 279]. However, the prolonged exposure to NE leads to deleterious pathophysiological changes that outweigh the initial beneficial effects[279].

Systemic and local cardiac increases of NE levels causes an increase in mortality in patients with HF[277, 279]. Concentrations of NE equivalent to those in the failing human heart cause dramatic changes in cell morphology, and up to a 60% loss of viability of isolated cardiac myocytes. Evidence from numerous clinical trials of beta-blocker therapy demonstrates the efficacy of blocking the beta-adrenergic receptor mediated cell signalling cascade. Excessive beta-

adrenergic stimulation results in oxidative stress and calcium overload, conditions promoting cell death through a number of mechanisms, including necrosis and apoptosis[280]. Even with the successful use of beta-blockers, the HF related mortality and morbidity remain high. Insufficient adrenergic activity in individuals with HF can also increase mortality[279]. Hence fine-tuning of the therapeutic strategy of targeting the adrenergic system is essential to achieve better clinical benefits in HF patients.

Resveratrol has been reported to be cardioprotective against NE-induced cardiomyocyte hypertrophy and it also has been demonstrate to have ionotropic effects[281, 282]. Our pre-clinical study also showed that resveratrol improves cardiac structure and function and decreases cardiac fibrosis and oxidative stress in the setting of MI[225]. Hence, it is worthwhile to understand the effects of a direct action of resveratrol on adult rat cardiomyocytes and cardiac fibroblasts from a therapeutic and a mechanistic point of view in the setting of a clinically relevant pathological trigger associated with MI such as NE. Accordingly, this *in vitro* study investigates the role of resveratrol in protecting adult rat cardiomyocytes from morphological and functional damage induced by NE and the anti-proliferative action of resveratrol on un-stimulated adult rat cardiac fibroblasts. Our previous *in vivo* study showed that resveratrol improves oxidant imbalance. Therefore, the current study also delineates the mechanism of action of resveratrol mediated improvement in redox status via SOD/FOXO1 signalling pathway in adult rat cardiomyocytes.

2. Hypotheses

1. Resveratrol will improve NE-induced contractile dysfunction, morphological changes, and oxidative stress in adult rat cardiomyocytes.
2. The resveratrol mediated reduction in cell viability will be mediated through SOD/CAT and FOXO1 in adult rat cardiomyocytes.

3. Resveratrol will inhibit the proliferation of un-stimulated adult rat cardiac fibroblasts.

3. Material and methods

3.1. Animal care

The experimental protocols involving animals in this project were approved by the University of Manitoba Office of Research Ethics and Compliance and Animal Care Committee, and were conducted in accordance with guidelines by the Canadian Council for Animal Care.

3.2. Adult rat cardiomyocyte isolation and experimental design

Adult rat ventricular myocytes were isolated from 12-week old male Sprague- Dawley rats (200-250 g)[283]. In brief, an intramuscular injection of a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg) was used to anesthetize the animal. Excised hearts were quickly transferred to a Langendorff perfusion apparatus and perfused with calcium (Ca^{2+}) free buffer containing (in mM); 90 NaCl, 10 KCl, 1.2 KH_2PO_4, 5 $MgSO_4.7H_2O$, 15 $NaHCO_3$, 30 taurine and 20 glucose for 5 min. The perfusion medium was then switched to Ca^{2+} free buffer containing collagenase (0.05%) and bovine serum albumin (0.2%). After 30min ventricles were cut into small pieces, incubated in a 37oC water bath and separated into individual cardiomyocytes by slow pipetting. Cardiomyocytes were then suspended in buffer containing Ca^{2+} and the cells were allowed to settle. The supernatant was then replaced with Ca^{2+} buffers containing a higher concentration of calcium (150 mM). This step was repeated twice to increase the extracellular Ca^{2+} concentration to 500 μM and then to 1.2 mM. Cells were finally re-suspended in Medium-199 containing 10% fetal bovine serum and transferred to laminin coated culture dishes. After 2 hours of incubation in a CO_2 incubator (5% CO_2 and 95% O_2), the existing medium was replaced with serum free M199 supplemented with 5 mM taurine, 2 mM carnitine, 1 mM creatine and 1 mM insulin. All

cells were incubated for 24 hours at 37^{o}C before starting any experimental procedure. For treatment with resveratrol, a 20 mM stock was prepared in 50% ethanol. 1 µl of resveratrol was added per milliliter of cell culture medium was used for the cell treatments, which gives a final resveratrol concentration of 30 µM/L in the medium.

Adult rat cardiomyocytes were divided into 3 groups – 1. Control group; 2. NE-exposed group (0.25 µM); 3. Both NE (0.25 µM) + Resveratrol treated group (30 µM/L). Cardiomyocytes were incubated without NE or resveratrol (control group) or with NE (NE alone group) or with NE and resveratrol (NE + Resveratrol group). In NE + Resveratrol group, cardiomyocytes were pre-treated with resveratrol for 30 min and then co-incubated with NE (0.25 µM) for 24 hours[284].

3.3. Measurement of adult rat cardiomyocyte contractility

The contractile properties of cardiomyocytes were assessed using an Ionoptix Myocam system (Ionoptix Inc., Milton, MA)[283]. Cardiomyocytes were cultured on coverslips and treated with NE, or resveratrol or both and group was left untreated. Briefly, myocytes were placed on a mounting chamber attached to an inverted microscope and superfused with a buffer containing 1mM CaCl2, 1 mM MgCl2, 4 mM KCl and 131 mM NaCl (prepared as stock and stored at 4oC). Before the experiments started, 10mM HEPES and 10mM glucose were added and pH was adjusted to 7.4 before use. To measure contractility, cardiomyocytes were stimulated at a frequency of 0.5 Hz, 10volt for 30 sec. Cell length, peak shortening, maximal velocities of shortening (+dL/dT) and re-lengthening (-dL/dT) were calculated based on cell shortening and re-lengthening of the cardiomyocyte by detecting the length of 2 edges along with the time of stimulation. Only, properly attached, long and rod-shaped cells that were not touching other were selected for assessments. Cardiomyocytes with irregular beating upon stimulation were also

excluded from measurement. At least n= 50 cells from 3 isolations were assessed from each experiment.

3.4. Measurement of adult rat cardiomyocyte cell viability

Adult rat cardiomyocytes were incubated with and without resveratrol for 30 min and further exposed to 0.25 μM/L NE for 24 hours, and control cells were left untreated[285]. After 24 hours, phase contrast images were captured using a phase contrast microscope (Olympus Canada Inc., Richmond Hill, Ontario, Canada). A total of approximately 100 or more cells for each group from independent cardiomyocyte isolations were used for the analysis. Images were taken from 6-8 random fields. Rod-shaped cells were considered healthy and viable, whereas round cells were considered unhealthy and dying. Cell viability was analysed by calculating the percentage of rod-shaped cells. Cardiomyocyte cell viability was measured by calculating the ratio of rod-shaped cells to the total number of cells (rod-shaped and round-shaped cells) using ImageJ software (National Institutes of Health, Bethesda, MD)[286]. For fluorescence microscopy, adult rat cardiomyocytes were exposed to the indicated treatments for 48 hours, and then washed twice with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde at room temperature for 1 hour. Cardiomyocytes were permeabilised with 0.1% triton X-100 in PBS for 5 minutes and blocked with 2% milk in 0.1% triton X-100 for 1 hour. Cells were then incubated with anti–α-actinin primary antibody solution (1:800 dilution) for 2 hours at room temperature. Cardiomyocytes were washed with PBS followed by incubation with an Alexa 488–conjugated goat anti-mouse secondary antibody for 1 hour at room temperature. After incubation cardiomyocytes were stained with DAPI (Thermo Fisher Scientific, MA). The fluorescent images were obtained using a fluorescent microscope (Olympus Canada Inc).

3.5. Assessment of a marker for oxidative stress in adult rat cardiomyocytes

The measurement of MDA was carried out after 24 hours of incubation with resveratrol and NE. The whole cell lysate was used for the assay. After incubation cells were scraped off the culture dish into microfuge tubes using 1mL ice cold 1X PBS. It was then centrifuged for 5 min at 3000 rpm at 4oC. 200µL lysis buffer (Sigma Aldrich, Ontario, Canada) was added and sonicated to lyse the cells. Protein estimation was done using the DC Biorad kit and MDA assay kit[225].

3.6. Treatment of adult rat cardiomyocytes with inhibitors of SOD and CAT

Cardiomyocytes were pre-treated with inhibitors of SOD and CAT, diethyldithiocarbamic acid (DDC) and 3-amino-1,2,4-triazole (AT), respectively, for 45 min followed by treatment with resveratrol for 30 min and then exposed to NE for 24 hours and control cells were left untreated[282] (Sigma Aldrich, Ontario, Canada). The effective concentrations of the SOD and CAT inhibitors (250 µM and 20 mM, respectively) were taken from previous studies carried out on adult rat cardiomyocytes[287]. After 24 hours, phase contrast images were captured using a phase contrast microscope and analysed for cell viability (Olympus Canada Inc, Richmond Hill, Ontario, Canada).

3.7. Treatment of adult rat cardiomyocytes with an inhibitor of FOXO1

In order to understand the role FOXO1 in resveratrol mediated cardiomyocyte protection, cardiomyocytes were treated with 1 µM FOXO1 inhibitor, AS1842856, for 1 hour and resveratrol followed by exposure to NE for 24 hours (Sigma Aldrich, Ontario, Canada)[288, 289]. After 24 hours, phase contrast images were captured using a phase contrast microscope and analysed for cell viability (Olympus Canada Inc, Richmond Hill, Ontario, Canada).

3.8. Adult rat cardiac fibroblast isolation and experiment design

Cardiac fibroblasts were isolated from adult male Sprague-Dawley rats (150–200 g). Isolated hearts were perfused with Dulbecco's Modified Eagle Medium (DMEM)/F12 using the Langendorff perfusion system followed by Serum MEM (SMEM). The hearts were then digested for 20 minutes at room temperature with SMEM containing 0.1% w/v collagenase II and minced for 15 minutes in diluted collagenase solution (0.05% w/v). Minced tissue was placed into a 50 mL centrifuge tube and suspended in DMEM-F12 growth medium containing 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 1 μM/L ascorbic acid and large tissue pieces were allowed to settle to the bottom of the centrifuge tube. The supernatant was transferred to another 50 mL centrifuge tube and centrifuged at 2000 rpm for 5 minutes. The supernatant was removed and cell pellets were re-suspended in growth medium and plated on to 6-well plates. Cells were incubated for 3-4 hours at 37°C and 5% CO_2, then washed 2-3 times with phosphate buffered saline (PBS) and maintained in fresh growth medium overnight. The following day, cells were washed 2 more times with PBS and fresh medium was added. Cells were allowed to grow at 37°C and 5% CO_2 for another 24 hours before further experimental procedures. To assess the potential of resveratrol in modulating proliferation of fibroblasts, cells were separated into 3 groups: untreated cells (control group), cells treated with DMSO (2 μL/2 mL medium), cells treated with resveratrol (30 μM/L in 2 μL of DMSO). Cells were treated for 24 hours. After 24 hours, phase contrast images were captured using a phase contrast microscope from 5-6 different fields and number of detached and floating cardiac fibroblasts were counted (Olympus Canada Inc, Richmond Hill, Ontario, Canada).

3.9. Statistical analysis

Data were expressed as a mean ± standard error (S.E.M). Statistical analysis of data was performed by using one-way analysis of variance followed by a Newman-Keuls post-hoc test (GraphPad Prism 5). A p value <0.05 was considered statistically significant.

4. Results

4.1. Resveratrol improves adult rat cardiomyocyte contractility after NE-exposure

We first investigated the effect of resveratrol on contractility in single adult rat ventricular myocyte paced with field stimulation. Cardiomyocyte edges were continuously tracked during contraction and relaxation, displayed as a voltage signal proportional to the changes in myocyte length and analysis revealed the contractile properties. Resting cell length (sarcomere length) was comparable between the groups (Figure 1A). Adult rat cardiomyocytes treated with NE (0.25 μM/L) for 24 hours showed decreased peak shortening (Figure 1B. 7.030± 0.46 % of cell length vs 4.184 ±0.28 % of cell length, p <0.05) in comparison to control adult rat cardiomyocytes. Pre-treatment of NE-exposed adult rat cardiomyocytes with resveratrol (30 μM/L) prevented the decreases in peak shortening (4.184 ±0.28 % of cell length vs 5.694± 0.34 % of cell length, p <0.05) when compared to NE-exposed adult rat cardiomyocytes. NE treatment (0.25 μM /L) for 24 hours also resulted in decreased maximal velocities of shortening (Figure 1B. +dL/dt 31.55±3.08 μm/s vs 89.60 ±0.28 % of cell length, p <0.05) in adult rat cardiomyocytes in comparison to control adult rat cardiomyocytes. Pre-treatment of NE-exposed cardiomyocytes with resveratrol (30 μM/L) prevented the decreases in maximal velocities of shortening (Figure 1C. +dL/dt 31.55±3.08 μm/s vs 77.24±9.52 μm/s, p <0.05) when compared to NE exposed adult rat cardiomyocytes. NE treatment of adult rat cardiomyocytes (0.25 μM /L) for

24 hours also caused decreased maximal velocities of re-lengthening (-d*L*/d*t*; -65.71± 4.13 µm/s vs -133.30±11.32 µm/s, p <0.05) in comparison to control adult rat cardiomyocytes. Resveratrol (30 µM/L) pre-treatment of NE-exposed adult rat cardiomyocytes prevented the decreases in maximal velocities of shortening (-d*L*/d*t*; -65.71± 4.13 µm/s vs -92.51± 7.22 µm/s, p <0.05).

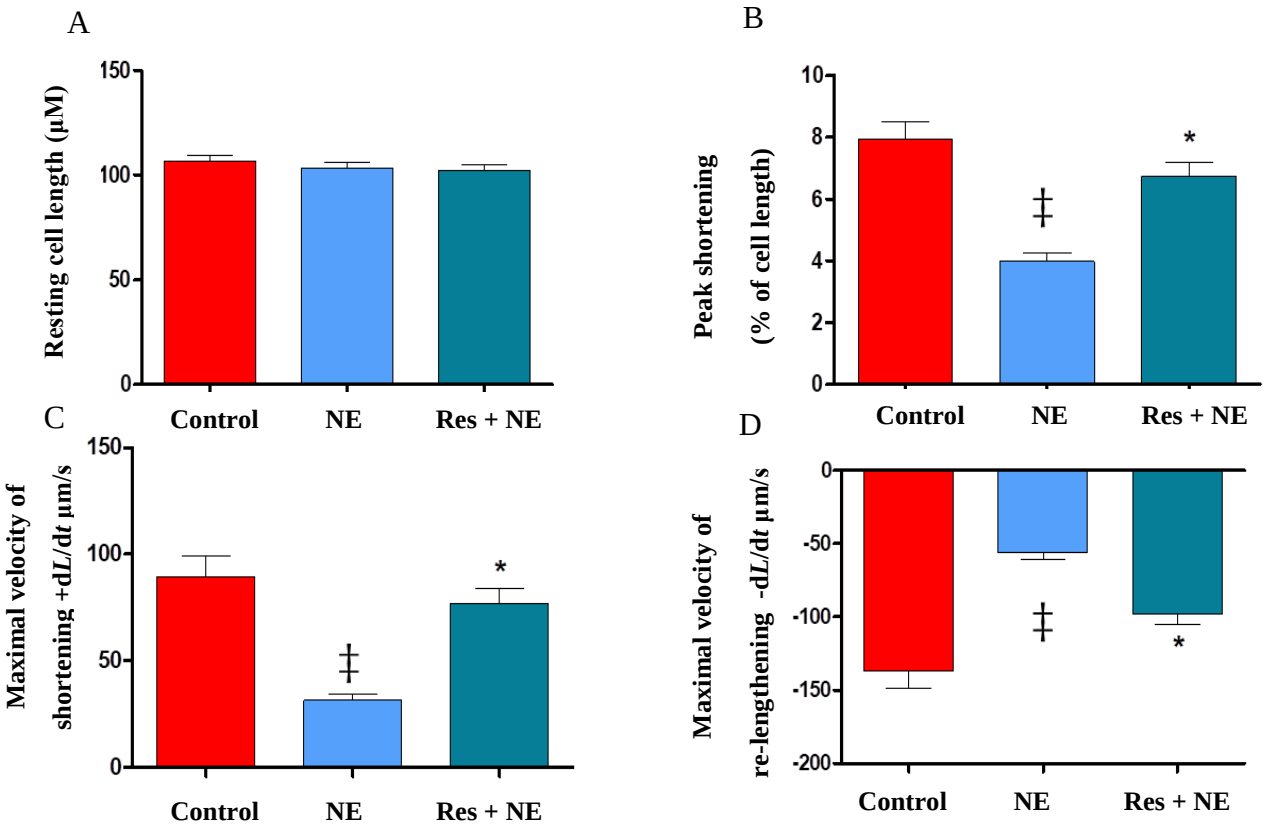
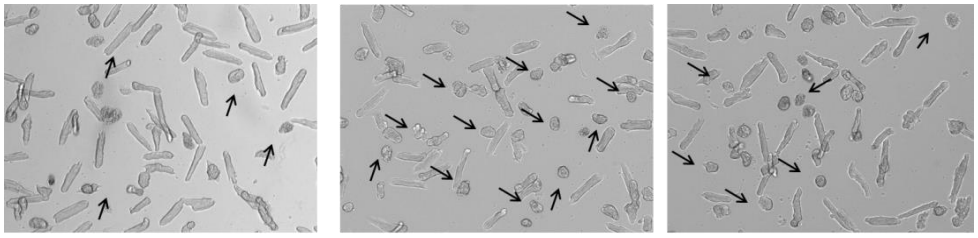


Figure 1. Effect of resveratrol on norepinephrine (NE)-induced contractile dysfunction in adult rat cardiomyocytes. A. Resting cell length. B. Peak shortening (% of cell length). C. Maximal velocity of shortening (+d*L*/d*t* µm/s). D. Maximal velocity of lengthening (−d*L*/d*t* µm/s). ‡p <0.05 vs Control cardiomyocytes *p <0.05 vs NE-induced cardiomyocytes. Values are means ± SEMs, n = 50 single cells from 3 different isolations. NE - Norepinephrine; Res - Resveratrol.

4.2. Resveratrol improves adult rat cardiomyocyte viability after NE-exposure

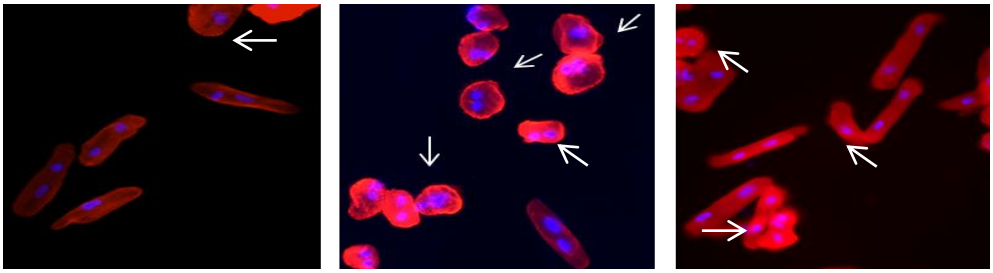
We further investigated the effects of resveratrol on adult rat cardiomyocyte viability after NE-exposure. Exposure of adult rat cardiomyocytes for 24 hours to NE (0.25 μM /L) caused a significant decrease in cardiomyocyte viability (percent of rod-shaped vs round-shaped cells), (Figure 2. 60.68±4.47 vs 31.96 ±9.32 p <0.05) as compared with control adult rat cardiomyocytes. Pre-treatment with resveratrol (30 μM/L) prevented NE-induced decrease in cardiomyocyte viability as evidenced by the increased percentage of rod-shaped vs round-shaped cells (54.99±0.55 vs 31.96 ±9.32 p <0.05) when compared to NE-exposed adult rat cardiomyocytes.

A



Control NE Res + NE

B



Control NE Res + NE

C

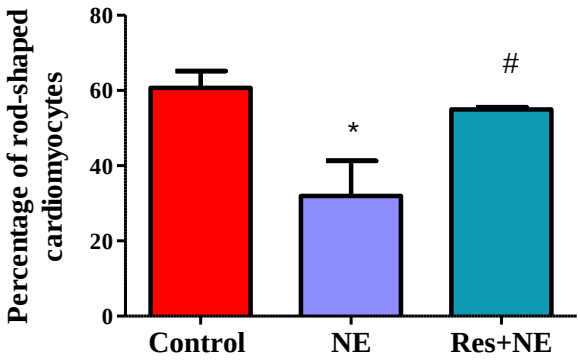


Figure 2. Effect of resveratrol on norepinephrine (NE)-induced morphological changes in adult rat cardiomyocytes. A & B. Phase contrast and α-actinin stained images of adult rat cardiomyocytes exposed to NE and treated with resveratrol (immunostained with primary antibody, anti–α-actinin, secondary antibody-Alexa 488, and nuclear stained with DAPI). The black arrows show round-shaped cardiomyocytes. C. Percentage of rod-shaped adult rat cardiomyocytes exposed to NE and pre-treated with resveratrol. Values are means ± SEMs (At

least *n* = cells from 3 different isolations). Scale bar - 400 μM *p <0.05 *vs.* Control; # p <0.05 *vs.* NE. NE- Norepinephrine; Res - Resveratrol.

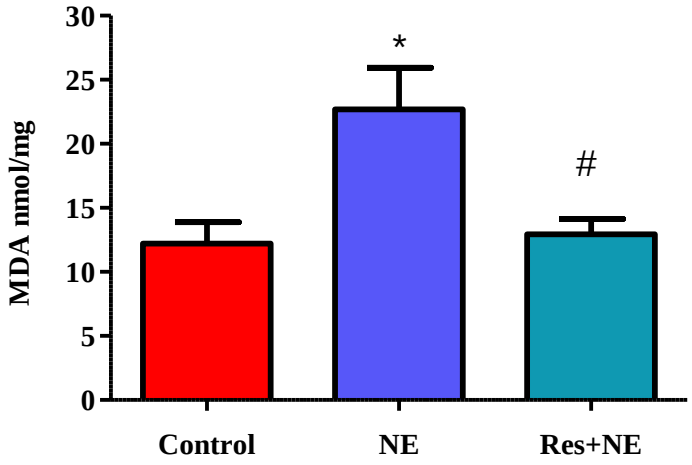


Figure 3. Effect of resveratrol on the malondialdehyde (MDA) levels in norepinephrine (NE)-exposed adult rat cardiomyocytes. Values are means ± SEMs (*n* = 5 different isolations). *p <0.05 *vs.* Control; #p <0.05 *vs.* NE. NE- Norepinephrine; Res - Resveratrol.

4.3.Resveratrol reduces adult rat cardiomyocyte oxidative stress after NE-exposure

Treatment of adult rat cardiomyocytes with NE (0.25 μM/L) for 24 hours caused a significant increase in the level of MDA (Figure 3. 12.22±1.66 vs 22.68±3.26, p <0.05) as compared with control adult rat cardiomyocytes. Pre-treatment with resveratrol (30 μM/L) prevented NE-induced increase in the level of MDA in adult rat cardiomyocytes (Figure 3. 12.94±1.19 vs 22.68±3.26, p <0.05) as compared with NE-exposed adult rat cardiomyocytes.

4.4. Inhibition of SOD with an inhibitor, DDC, blocks the resveratrol mediated improvement in cell viability in adult rat cardiomyocytes after NE-exposure

In the NE-exposed adult rat cardiomyocyte group pre-treated with resveratrol, the inhibition of SOD using its pharmacological inhibitor DDC significantly reduced the percentage of rod-

shaped cells when compared to NE-exposed, resveratrol pre-treated cardiomyocytes without DDC (Figure 4). In NE-exposed adult rat cardiomyocyte group pre-treated with resveratrol, the inhibition of CAT using its inhibitor AT did not significantly reduce the percentage of rod-shaped cells when compared to NE-exposed, resveratrol pre-treated cardiomyocytes without AT (Figure 4). In adult rat cardiomyocytes exposed to NE and pre-treated with resveratrol, inhibition of both CAT and SOD using AT and DDC at the same time significantly reduced the percentage of rod-shaped cells when compared to NE-exposed, resveratrol pre-treated cardiomyocytes without AT or DDC (Figure 4).

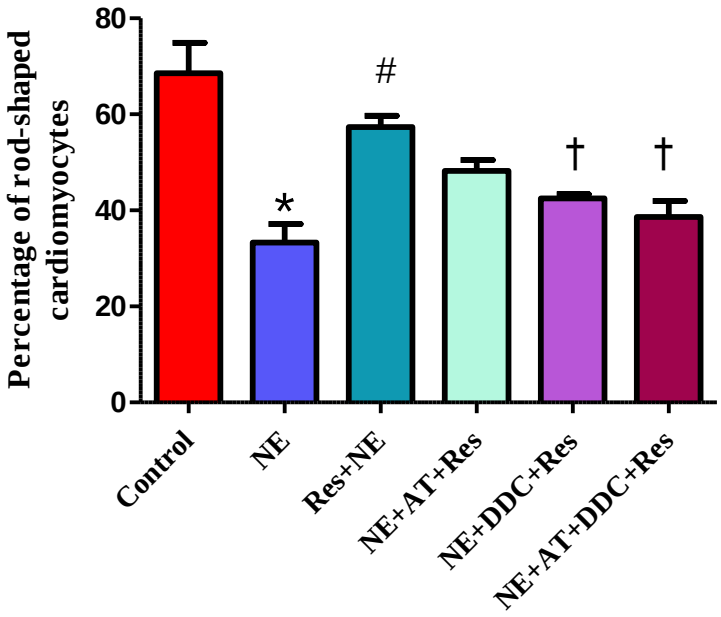


Figure 4. Effect of superoxide dismutase (SOD) and catalase (CAT) inhibitors on the cytoprotective action of resveratrol in norepinephrine (NE)-exposed adult rat cardiomyocytes. Values are means ± SEMs (*n* = cells from 3 - 4 different isolations). * *p* <0.05 *vs.* Control; #*p* <0.05 *vs.* NE, †*p* <0.05 vs Res + NE. NE- Norepinephrine; Res – Resveratrol, DDC - Diethyldithiocarbamic acid and AT - 3-amino-1,2,4-triazole.

4.5. Inhibition of FOXO1 with inhibitor, AS1842856, blocks the resveratrol mediated improvement in cell viability in adult rat cardiomyocytes after NE-exposure

In NE-exposed adult rat cardiomyocytes pre-treated with resveratrol, inhibition of FOXO1 using its pharmacological inhibitor, AS1842856, significantly reduced the percentage of rod-shaped cells when compared to NE-exposed, resveratrol pre-treated cardiomyocytes without exposed to AS1842856 (Figure 5).

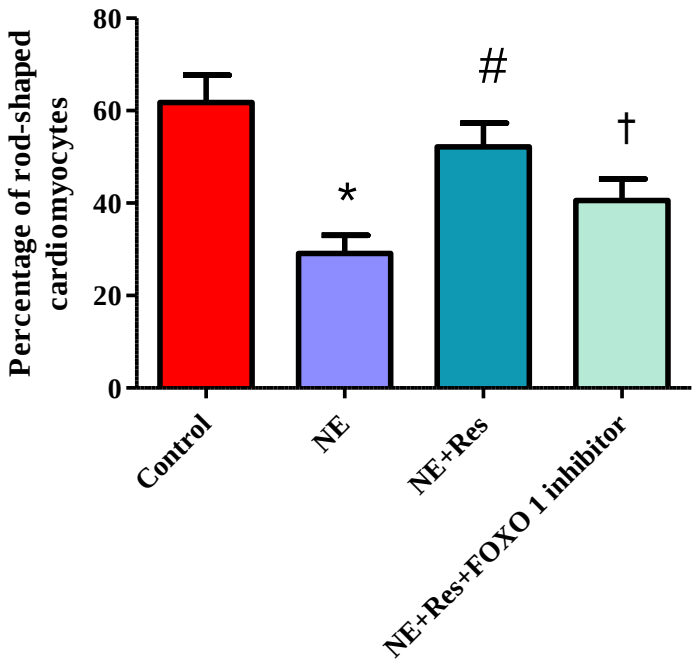


Figure 5. Effect of FOXO1 inhibitor on the cytoprotective action of resveratrol in norepinephrine (NE)-exposed adult rat cardiomyocytes. Values are means ± SEMs (*n* = cells from 5 different isolations). * p <0.05 *vs.* Control; $^{\#}p$ <0.05 *vs.* NE, †p <0.05 vs R+NE. NE- Norepinephrine; Res– Resveratrol.

4.6. Resveratrol reduces un-stimulated adult rat cardiac fibroblast proliferation

Adult rat cardiac fibroblasts treated with resveratrol (30 μM/L) for 24 hours had a significantly increased detached (lower attached) cell number compared with untreated control cells or cells treated with DMSO (Figure 6).

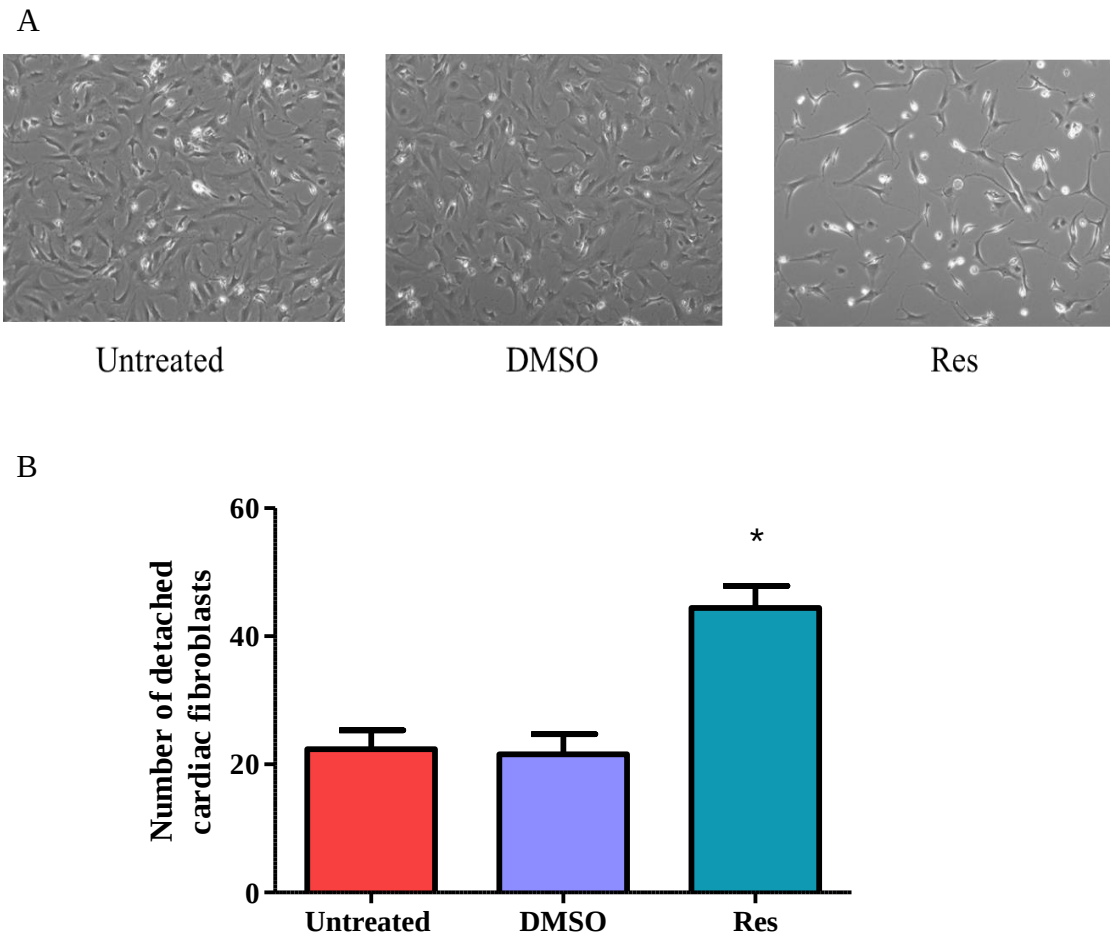


Figure 6. Effect of resveratrol on un-stimulated cardiac fibroblast proliferation. A. Representative images of cardiac fibroblasts from untreated, DMSO treated and resveratrol treated groups. B. Number of detached cardiac fibroblasts. Values are means ± SEMs (*n* = cells from 4 different isolations). * $p < 0.05$ *vs.* Untreated and DMSO. Res – Resveratrol, DMSO – Dimethyl Sulfoxide

5. Discussion

The first and foremost finding of this study is that pre-treatment with resveratrol prevents NE-induced contractile dysfunction in adult rat cardiomyocytes. Secondly, the resveratrol mediated improvement in contractile dysfunction was associated with preservation of cellular viability and morphology of adult rat cardiomyocytes. Resveratrol also prevented the NE-induced increase in the level of oxidative stress biomarker in adult rat cardiomyocytes. In addition, the resveratrol mediated improvement in cell viability and morphology of adult rat cardiomyocytes was diminished by the inhibition of the antioxidant enzyme activity of SOD. Inhibition of the activity of transcription factor FOXO1 also abolished the protective effect of resveratrol against NE in adult rat cardiomyocyte.

Primary cardiomyocytes from male adult rat hearts were used to create an *in vitro* model system for cardiac injury in our current study. In order to mimic the clinical condition of overactive sympathetic system and increased catecholamine levels which causes cardiac damage, NE was used as a pathological trigger to induce direct cardiomyocyte abnormalities. Specifically, the concentration of NE used to elicit direct cardiomyocyte abnormalities has been derived from previous studies using a similar *in vitro* model[285]. It should also be noted that primary cardiomyocyte viability is drastically reduced overtime in *in vitro* models (by 30-40%) even without any pathological agent present and this effect precludes the studies from being conducted for a longer time period in the presence endogenous agents such as NE[290]. The optimal time period for an *in vitro* study using adult cardiomyocytes is generally considered to be 48 hours[281, 285]. NE is known to reduce the viability and induce cardiomyocyte hypertrophy and apoptosis[280, 290-295]. The NE mediated cell toxicity is significantly blocked by beta-adrenoceptor antagonists in *in vitro* models. The NE-induced toxic effect is mainly due to PKA

mediated calcium overload and apoptosis[17-22]. The apoptotic effect of NE can also be achieved by the beta-adrenergic receptor agonist isoproterenol[296]. In addition, NE treatment in rats and transgenic overexpression of the beta-adrenergic receptor in the myocardium of mice lead to cardiac hypertrophy, dilatation and contractile dysfunction[286, 292, 297].

A previous study showed that resveratrol prevents NE-induced adult cardiomyocyte hypertrophy (increase in surface area and protein synthesis induced by NE)[281]. Resveratrol also inhibits Ang II-induced cardiomyocyte hypertrophy in neonatal cardiomyocytes[298]. These studies may suggest that resveratrol demonstrates protective action in both neonatal and adult rat cardiomyocytes by blocking SNS and RAAS signalling. The current study demonstrates that resveratrol helps to preserve the peak shortening % and the maximal velocity of shortening, and maximal velocity of re-lengthening *in vitro* in adult rat cardiomyocytes. Peak shortening % and the maximal velocity of shortening *in vitro* are considered as representative of systolic function *in vivo* whereas maximal velocity of re-lengthening *in vitro* is considered as a representative of diastolic function *in vivo*. This is consistent with a large number of *in vivo* studies showing resveratrol mediated cardiac functional protection in the setting of heart disease[92, 299, 300]. Maximum velocity of shortening is thought to reflect cross-bridge cycling rate and force generation[301]. Studying the contractile properties in isolated individual cardiomyocytes has certain advantages because of the absence of potential confounding factors such as perfusion limitation, deficiency of cardiac substrate, vagal influences as well the constraining presence of ECM[301]. NE is an ionotropic agent which enhances the cardiac contractility[292]. However, prolonged exposure to NE precipitates adverse effects that limit its ionotropic action[275]. Interestingly, previous study showed that resveratrol enhances the ionotropic action in rat ventricular myocardium without any adverse effects[282]. The positive effect of resveratrol on cardiomyocyte contractility also includes

the preservation of contractile properties and improved calcium handling in myocytes isolated from the hearts of streptozotocin-induced type-1 diabetic rats[302]. The current study and the studies mentioned above suggest that resveratrol may be able to provide positive ionotropic benefit (improved contractility) without any adverse effects as opposed to the positive ionotropic action of NE, which becomes deleterious overtime by causing maladaptive changes. That being said, it is yet establish whether long-term treatment with resveratrol may result in a loss of its ionotropic effects.

NE is known to cause catecholamine toxicity and cardiac oxidative stress[303]. Catecholamines are characterised by the ability to auto-oxidise and produce catecholamines-O-quinones, which are quite reactive. Unstable catecholamines-O-quinones lead to the production of adrenochromes and oxygen free radicals like O_2^- radicals[303]. In addition, beta-adrenergic receptor stimulation is associated with increased production of ROS in rat cardiomyocytes. Our previous study also reported that NE induces oxidative stress in adult rat cardiomyocytes[285]. Resveratrol is considered as an antioxidant due to its polyphenolic structure and ability to improve antioxidant enzyme activity[92]. Resveratrol has been reported to reduce the deleterious oxidant effects of H_2O_2 in adult rat cardiomyocytes[287]. In addition, resveratrol protects neonatal cardiomyocytes against high glucose-induced NADPH oxidase derived ROS[24]. Resveratrol has also been shown to protect against hypoxia/reperfusion injury by decreasing mitochondria-mediated oxidative stress injury and structural impairment in neonatal cardiomyocytes[304, 305]. Treatment with resveratrol also prevents phenylephrine-induced oxidative stress and alteration of the LKB1/AMPK signalling[306]. Resveratrol mediated blockade of pro-hypertrophic p70S6 kinase signalling is known to inhibit phenylephrine-induced cardiomyocyte hypertrophy[306]. Resveratrol also prevents the iron overload related oxidative stress in human ventricular cardiomyocytes.

This study also showed NE induces oxidative stress in adult rat cardiomyocytes. Consistent with the previous study, our current data show that NE-induced cardiomyocyte oxidative stress is attenuated by resveratrol[287]. We also observed that the inhibition of antioxidant enzyme activity of SOD with its pharmacological inhibitor resulted in a significant reduction of resveratrol mediated improvement in cardiomyocyte cell viability, which suggests that resveratrol reduces oxidative stress partly through preserving/increasing the activity of SOD. Our previous study also showed that resveratrol significantly improves the activity of SOD and CAT in adult rat cardiomyocytes treated with H_2O_2[287]. Our previous study showed that the SOD and CAT inhibitors AT and DDC, respectively, alone or in combination do not have any effect on cell viability in the normal adult rat cardiomyocytes[287]. However, in adult rat cardiomyocytes exposed to H_2O_2, inhibition of SOD and CAT activity by inhibitors AT and DDC resulted in significantly more cell death than that resulted by H_2O_2 alone[287]. In light of this previous finding, the blocking of SOD and CAT activity in adult rat cardiomyocytes exposed to NE in the presence of inhibitors AT and DDC may have caused further reduction in cell viability. SOD activity has been shown to be elevated in resveratrol treated ischemia/reperfused cardiomyocytes[307]. In addition, resveratrol increases the mRNA and protein level of SOD and SOD siRNA blocks resveratrol mediated reduction of the ROS levels in cardiomyocytes[205].

Even though our previous study reported that resveratrol increases the activity of SOD and CAT in H_2O_2 treated cardiomyocytes, the mechanism of action was not identified. FOXO1 is a transcription factor that helps to prevent oxidative stress by upregulating the expression and thereby, the activity of SOD and CAT[308-310]. Phosphorylation by p-Akt and acetylation by p300/CBP result in FOXO1 nuclear export, cytoplasmic sequestration and inhibition of its transcriptional activity. In contrast, deacetylation of FOXO1 by SIRT1 (deacetylase) increases

the nuclear localisation of FOXO1 and thereby an increase in its transcriptional activity[308-312]. In addition, FOXO1 plays an important role in regulating cardiomyocyte viability and cardiac function in the setting of MI. FOXO1 improves cardiomyocyte survival in a positive manner when subjected to oxidant imbalance[313]. In the current study, inhibition of FOXO1 activity resulted in a significant blockage of the resveratrol mediated improvement in cardiomyocyte viability in NE-exposed adult rat cardiomyocytes. Inactivation of FOXO1 under stress situations such as MI can contribute to decreased ROS elimination and cause cardiac remodelling and dysfunction. Our finding suggests that resveratrol may act through the FOXO1 signalling pathway to mitigate cardiomyocyte abnormalities. An earlier study showed that resveratrol increases the FOXO1 activity in the myocardium of a mice model of iron-overload cardiomyopathy[314]. In streptozotocin-induced diabetic rats, resveratrol has been shown to increase the activity of FOXO1 in the kidney[315].

Resveratrol possesses significant anti-fibrotic properties in the setting of cardiac diseases[316]. Cardiac fibroblasts contribute to LV remodelling via their conversion to myofibroblasts. Cardiac fibroblasts undergo dynamic changes involving proliferation, and differentiation and produce excessive amount of ECM proteins in response to cardiac injury. In an *in vitro* setting, cultured primary cardiac fibroblasts are capable of proliferation even without any stimulation. Adult rat cardiac fibroblasts were used in this study to test our hypothesis that resveratrol will inhibit the rapid proliferation of cultured primary cardiac fibroblasts. To that end, we examined whether resveratrol has any direct effects on the proliferation of adult rat cardiac fibroblasts. Interestingly, resveratrol inhibited the proliferation of cardiac fibroblasts *in vitro* as evidenced by the change in number of cardiac fibroblasts in the resveratrol treated group. This finding suggests that resveratrol may offer beneficial effects against cardiac fibrosis by limiting the overactivation

of resident cardiac fibroblasts. Similarly, earlier studies showed that resveratrol prevents basal, Ang II- and TGF-β 1-induced adult rat cardiac myofibroblast differentiation[316, 317]. Further studies are needed to clearly establish the mechanism of action of the resveratrol mediated reduction of the number of adult cardiac fibroblasts *in vitro*. It should be noted that this study used adult male rat cardiomyocytes and cardiac fibroblasts. The findings may not be generalised to female rat cardiomyocytes or cardiac fibroblasts because we did not evaluate the sex-specific differences of resveratrol treatment against NE-induced anomalies. No studies have been carried out till date to examine the direct effects of resveratrol on beta-adrenergic receptor signalling in cardiomyocytes. Hence it is worthwhile to study if resveratrol mediated protection against NE-induced abnormalities is possibly due to its action as a beta-adrenergic receptor antagonist.

6. Conclusion

In this study, we investigated the cardiomyocyte protective role of resveratrol in adult rat cardiomyocytes treated with NE. We delineated the potential mechanism of action in reducing NE-induced oxidative stress and revealed its role as an anti-fibrotic agent in proliferating adult rat cardiac fibroblasts. The findings suggest that resveratrol protects against NE-induced cardiomyocyte contractile dysfunction and morphological changes that affect the cell viability. We also concluded that resveratrol mediated cardiomyocyte protection is partly achieved through reducing oxidative stress via a mechanism of action contingent upon the SOD and FOXO1 signalling pathway. In addition, the current study revealed that resveratrol has the ability to inhibit cardiac fibroblast proliferation. Overall, this study suggests that resveratrol may help in preventing contractile dysfunction and cardiac fibrosis and warrants further clinical evaluation in future studies.

CHAPTER V: Overall conclusions, Future directions and Significance

1. Overall conclusions

CVD continues to be a major healthcare burden around the world. Ischemic heart disease is identified as one of the most critical contributors to this burden that need immediate attention. HF significantly affects the quality of life and the long-term prognosis of HF patients remains grim even with advanced therapies. Novel therapies are needed to address these burdens more effectively. To that end, many preclinical studies and some emerging early clinical trial data support the cardioprotective efficacy of resveratrol. The stand-alone cardioprotective ability of resveratrol has been reported widely in different animal models of CVDs. However, data regarding the comparative efficacy of resveratrol is lacking because no study has attempted to investigate the question of what is the true worth of resveratrol by comparing it with the current front-line medications in relevant animal models of MI and HF.

Our study for the first time compared resveratrol alongside an ACE inhibitor which is a drug that forms the cornerstone of post-MI treatment. The head-to-head comparison of the same of dose resveratrol and the ACE inhibitor, perindopril, revealed that these 2 drugs are equally potent in protecting against MI related complications in an animal model that recapitulates clinical post-MI complications. LAD ligation in male rats resulted in significant LV dilatation and a decrease in LVEF by 8 weeks. Individual treatments with resveratrol and perindopril were able to prevent LV cavity dilatation in MI-induced rats even though they did not completely block the remodelling. Consistent with cardiac structural improvement, both stand-alone treatments afforded cardiac functional improvement as evidenced by the preservation of LVEF in MI-induced rats. The combination treatment did not produce significantly superior benefits in comparison to the individual treatments.

In addition to the impairment in cardiac structure and function, there was an increase in the oxidative stress biomarker MDA in MI rats. Resveratrol and perindopril prevented the increase in oxidative stress in MI-induced animals. Post-MI animals also demonstrated a significant increase in the level of the pro-inflammatory marker TNF-α. Treatment with resveratrol and perindopril was effective in blocking the increased pro-inflammatory response. Post-MI cardiac remodelling also included a significant increase in LV interstitial fibrosis. MI-induced animals when treated with resveratrol and perindopril had significantly less LV interstitial fibrosis. Increased oxidative stress in MI-induced animal was also associated with a decrease in the activity of antioxidant enzymes namely SOD and CAT. The treatment with resveratrol and perindopril was effective in preventing the decrease in the activity of SOD and CAT in MI-induced animals.

A recent finding from a landmark clinical trial established that ARNI, sacubitril/valsartan is superior to an ACE inhibitor in HFrEF patients[234]. In light of our findings that resveratrol is comparable to an ACE inhibitor in the setting of MI, we further investigated the comparative efficacy of resveratrol alongside sacubitril/valsartan. Similar to previous data, resveratrol improved cardiac structure (reduced LV dilatation) and systolic function (LVEF and FS) in MI-induced animals comparable to sacubitril/valsartan and valsartan. In this study, the combination treatment with resveratrol and sacubitril/valsartan showed a trend towards an incremental improvement in cardiac structure and function in MI-induced rats. Consistent with the first study, our results demonstrated that the improvement in cardiac structure and function was associated with a decrease in a cardiac HF biomarker, oxidative stress, pro-inflammatory status and cardiac fibrosis. Our data also showed that there was a significant improvement in lung and liver congestion with the treatments.

Cardiac remodelling and dysfunction can in part involve changes at the cardiomyocyte level due to an increase in circulating catecholamines. The third study further investigated the direct effect of resveratrol on adult rat cardiomyocytes exposed to NE. NE-induced morphological changes that reduce viability and also caused contractile dysfunction in cardiomyocytes. NE-induced anomalies were blocked by resveratrol as evidenced by the improvement in viability and contractile function with resveratrol treatment. NE also resulted in an increase in the levels of an oxidative stress marker in cardiomyocytes whereas resveratrol was able to reduce its levels. Our study also revealed that resveratrol-mediated cardiomyocyte protection was lost when the antioxidant enzyme activity of SOD was pharmacologically blocked, which further established that resveratrol decreases NE-induced abnormalities partly through positively modulating SOD activity. In addition, the cardioprotective effect of resveratrol was found to be contingent upon antioxidant enzyme transcription factor FOXO1 as the blockade of FOXO1 resulted in the negation of resveratrol-facilitated beneficial effects. Resveratrol also significantly reduced adult cardiac fibroblast cell numbers *in vitro*, indicating a potential anti-fibrotic mechanism of action of resveratrol.

Taken together, resveratrol appears to be a cardioprotective molecule in the setting of MI as it improves cardiac structure and function in MI-induced adult male rats at a comparable level to ACE inhibitor, ARB and ARNI tested in our studies. Our study further shows that resveratrol pleiotropically modulates cardiac oxidative stress, inflammation and fibrosis *in vivo*. Resveratrol also improves adult rat cardiomyocyte viability and contractile function and reduces adult rat cardiac fibroblast proliferation, the former through a mechanism depending on SOD and FOXO1.

2. Future directions

Our studies showed that low dose resveratrol is cardioprotective in MI related cardiac remodelling and systolic dysfunction. In our studies, the MI-induced rats did not have severe systolic dysfunction (LVEF <40%) or any diastolic dysfunction at 8 weeks. By contrast, LVEF cut off value of patients included in major landmark HF clinical trials ranged from <40% to <30%. In our 8 week study, MI-induced animals showed a significant reduction in LVEF, however, it was not lower than 55% in MI-induced vehicle treated animals. Despite this, our study supports the protective role of resveratrol in systolic dysfunction, and further study may be needed to identify the potential of resveratrol in conditions where LVEF continues to drop to significantly lower values (LVEF <40%). Since 50% of the HF patients are HFpEF patients and MI patients develop diastolic dysfunction as well, in future studies, it may be worthwhile to examine the cardioprotective roles of resveratrol in the setting of MI with diastolic dysfunction.

Our study used permanent LAD ligation for 8 weeks without reperfusion as an animal model of MI. Even though this animal model of MI has been used historically for drug development (including ACE inhibitors), it should be noted that an animal model of ischemia/reperfusion may be more valuable to study ischemia/reperfusion injury and subsequent cardiac remodelling. As stated earlier, the single low dose resveratrol treatment regimen employed in this study did not allow us to evaluate the dose dependent effect. The choice of the dosage for this study was based upon previous evidence that demonstrated efficacy with low a dose of resveratrol. Nonetheless, our study provides a stepping stone to establish the precise dosing regimen for future studies in an MI model. It is not clear whether a higher dose of resveratrol throughout the study or starting with a low dose and titrating up to higher doses as the study progresses will have a differential and improved cardioprotective effects in the setting of MI. The need to establish a distinction

between different doses is essential as ineffective treatment outcomes may be due to inadequate dosage regimens and may be interpreted as a lack of efficacy. HF clinical trials are generally designed with mortality as the primary endpoint to assess the efficacy of the test drugs. The mortality rate tends to be very low during 8 week or 16 week studies in MI-induced animals. In order to generate similar pre-clinical data, long-term studies are also needed to establish the mortality reduction benefits with resveratrol treatment. The PARAGIDM-HF trial used the combination drugs, sacubitril and valsartan, as a single equimolar entity in an encapsulated form, not as 2 separate pills. Presently, it is not known whether combining resveratrol with the 2 established drugs in a similar approach will produce better favourable results than with a separate combination treatment. Future studies may identify how resveratrol works when resveratrol and other combination drugs are administered in the form of one encapsulated pill.

In terms of *in vitro* studies, our study used NE as the pathological trigger to induce cardiomyocyte abnormalities based on our previous studies. Due to the multifactorial nature of the HF pathophysiology in the *in vivo* setting, it is also imperative to use multiple pathological agents in *in vitro* models using cardiomyocytes. To that end, a combination of NE and Ang II may be used as disease trigger to alter the myocyte biology and the direct effects of resveratrol on cardiomyocytes may be established in that setting. Alternately, cardiomyocytes isolated from MI-induced animals may also be used to understand the direct effects of resveratrol on structural alterations and contractile dysfunction *in vitro*. Resveratrol is a pleiotropic agent that acts extracellularly and through multiple intracellular pathways. Our current study focused on delineating the potential molecular mechanisms of its antioxidant action through SOD/CAT/FOXO1. However, resveratrol has also been shown to improve oxidant status and inflammatory status via other transcription factors such as Nrf2 and NFkB, respectively. The role

of other transcription factors in resveratrol facilitated cardiomyocyte protection may also be studied. Moreover, further studies are also needed to understand the potential role of resveratrol in improving contractile function via excitation and contraction coupling machinery via calcium cycling mediators in cardiomyocytes. In addition, the molecular mechanism of action of resveratrol in decreasing the proliferation of adult rat cardiac fibroblasts also needs further investigation.

3. Significance

Based on the current evidence, resveratrol alone or in combination with existing drug therapies appears to offer a promising potential therapeutic strategy for the management of ischemic heart disease. However, to translate the pre-clinical findings to clinical practice, appropriate clinical studies are warranted. To that end, our group has been recruiting non-ischemic HFrEF patients for a randomised, double blinded, placebo controlled, one year clinical trial (RCT) to test the efficacy of resveratrol in HFrEF (NCT01914081). Importantly, our pre-clinical studies in the ischemic heart disease setting which have been reported here provided further supportive evidence to amend the protocol to include ischemic HF patients to our ongoing RCT. The study design, protocol and current status of our resveratrol RCT are described in the next section in detail. This bench to bedside translational approach involving pre-clinical and clinical studies that investigate the cardioprotective role of resveratrol may reduce the existing gap in the literature regarding the efficacy of resveratrol.

CHAPTER VI: Resveratrol: A potential anti-remodelling agent in heart failure (RES-HF) – Trial design and protocol

1. Introduction

This section describes the design and the implications of our ongoing RCT with resveratrol in HFrEF patients (NCT01914081). This trial involving HFrEF patients has been designed based upon our previous and current pre-clinical findings in ischemic and non-ischemic heart disease.

2. Rationale

It is apparent that there is a potential for additional reductions in mortality and hospitalisations in HF patients to decrease the economic burden on the healthcare system by augmenting the existing HF therapies. Although pre-clinical studies have reported the benefits of resveratrol in HF, clinical studies that evaluated the potential cardiac benefits of resveratrol in patients with HFrEF have not been previously reported. The purpose of this study is to examine the clinical benefits of resveratrol in HFrEF patients and provide preliminary data to support future large scale clinical trials which would eventually provide the evidence for the recommendation of resveratrol as an adjunct to current HFrEF therapies.

3. Hypothesis

Resveratrol will improve clinical, echocardiographic, patient reported outcome and experience measures including exercise capacity, quality of life and biochemical parameters in patients with HFrEF.

The specific objectives of the RES-HF trial are to:

i. Conduct the first long-term clinical trial of resveratrol therapy in patients with HFrEF.

ii. Examine the effects of resveratrol on clinical and echocardiographic parameters in patients with HFrEF.

iii. Track patient reported outcome and experience measures including quality of life.

iv. Examine the mechanisms associated with resveratrol action in HFrEF patients

4. Trial design

4.1.Protocol

This is a randomised, double blinded, placebo controlled study of 40 adult HF patients (≥18 to 90 years old). The patients will be followed in the HF clinic at St. Boniface Hospital. After screening, in total, 20 subjects will be randomly allocated to the placebo arm, and 20 to the treatment arm (Figure 1). Patients will receive either one uncoated immediate-release caplets containing 500 mg resveratrol manufactured by Biotivia LLC (New York) or placebo, twice daily for the 12 month intervention period. The dose (1 g, 2 x 500 mg) of study drug and intervention period has been selected on the basis of the safety and pharmacokinetic profile reported in humans earlier[98].

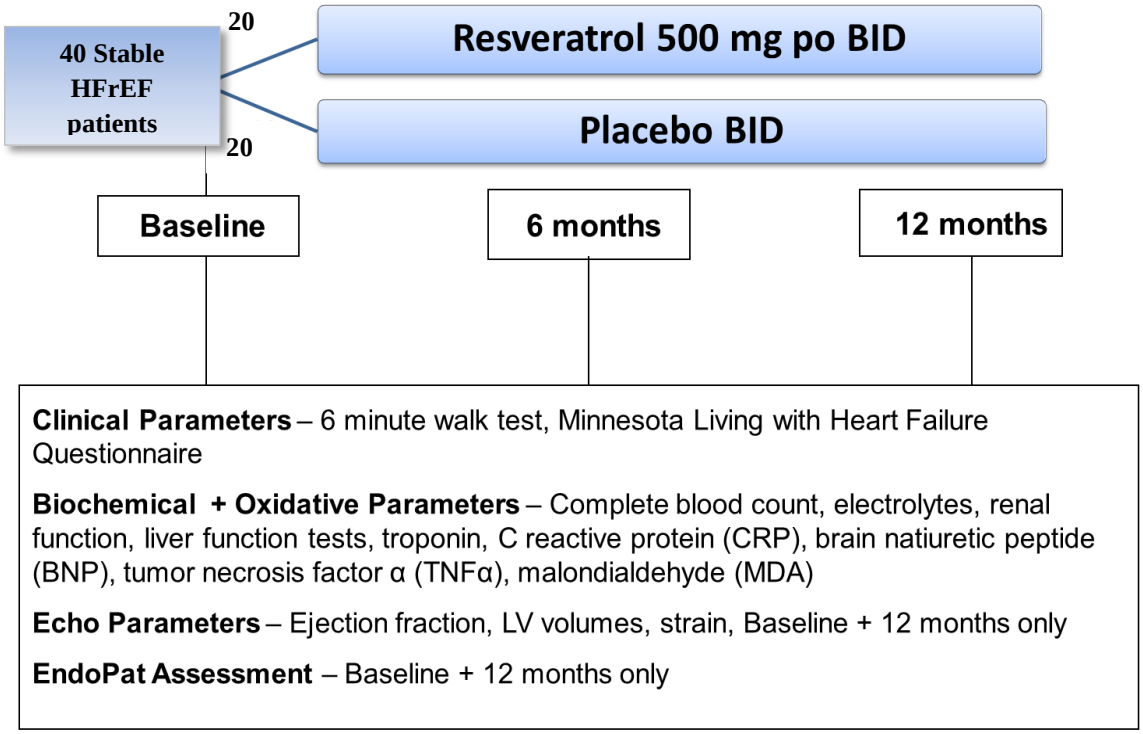


Figure 1. RES-HF trial design

4.2. Primary outcomes

Primary outcomes include the effects of resveratrol on clinical and echocardiographic parameters including patient reported outcome measures in patients with HFrEF.

4.3. Secondary outcomes

Secondary outcome includes the evaluation of the effects of resveratrol on biochemical parameters and its potential mechanism of action in patients with HFrEF.

4.4. Selection of subjects

Study subjects will be recruited from the St. Boniface Hospital HF clinic. Patients will be given both written and verbal information describing the nature of the study. All original signed consent forms will be stored with the study documentation at the respective sites. Patients to be recruited to the study will be stable in their clinical status prior to enrolment.

4.5. Inclusion criteria

• Subjects (between 18-90 years of age) with cardiomyopathy, HFrEF (HFrEF defined as LVEF ≤40%, based on a most recent assessment)

• NYHA Functional class II-III (mild to moderate HF symptoms)

• On optimal medical management for 6 months as per standard care

4.6. Exclusion criteria

• Severe valvular cardiomyopathy

• Surgical intervention planned or in past 6 months

• Subjects on diltiazem (or any other calcium channel blocker)

• Patients with a history of serious hypoglycemia requiring hospitalisation or hyperglycemic emergencies requiring hospitalisation in the past 6 months

• Subjects on anticoagulants, coumadin, dabigatran

• Subjects on HIV protease inhibitor (saquinivir), immunosupressants (cyclosporine, tacrolimus)

• Subjects on terfenadine, midazolam, and triazolam

• Subjects on sildenafil or any other drugs used to treat erectile dysfunction

• Chronic renal failure (defined as estimated glomerular filtration rate (eGFR) ≤30 mL/min per 1.73m2)

• Known liver cirrhosis or other significant comorbidity e.g. cancer affecting the ability to complete study

• Pregnant or lactating women

• Subjects on hormone replacement therapy and estrogen containing birth control

4.7. Treatment of subjects

After screening and randomisation, 20 patients each will receive either one uncoated immediate-release caplets containing 500 mg resveratrol manufactured by Biotivia LLC (New York) or placebo, twice daily for the 12-month intervention period. The dose (1 g, 2 x 500 mg) of study drug and intervention period has been selected on the basis of the safety and pharmacokinetic profile reported in humans by Brown et al[318]. One gram resveratrol has been deemed safe in humans by the report mentioned above[318].

4.8. Transthoracic echocardiography (TTE)

All subjects will undergo resting TTE assessment – at baseline and 12 months. All TTE assessments will be performed based on standardised protocols. RES-HF trial will use a single blinded echocardiographer to assess the heart structure and function in all patients (T. Imaging protocol will include Echo-Doppler, Biplane Simpson's method for measures of LV volumes and

EF and Pulsed Doppler and TDI evaluation of LV diastolic function, Myocardial Strain Measurements, M-Mode Data: The following echocardiographic endpoints will be recorded. **a)** A change in LV end diastolic volume and LV end systolic volumes from baseline to follow-up. We expect that LV diastolic and systolic volumes will decrease. **b)** A change in LVEF (by biplane Simpson's measurement) with associated improvement in strain and strain rate parameters. We also expect that LVEF will increase.

4.9. Biochemical analysis

All biochemical measurements will be done with blood plasma collected from HF patients in the study. These samples will be stored for up to one year before being destroyed.

i) Measurement of oxidative stress and total antioxidant status (TAS) - Lipid peroxidation levels will be estimated by measuring the amount of MDA using the Oxiselect MDA Assay Kit (Cell Biolabs, California) for assessment of oxidative stress.

ii) Inflammatory marker measurements - ElISA kits (Thermo Scientific, Illinois) will be used to measure plasma levels of IL-6 and TNF-α according to manufacturer's instructions.

iii) NO determination – Total NO assay kit (Stressgen, Michigan) levels will be done to determine total nitric oxide levels in blood plasma samples according to the manufacturer instructions

iv) Routine Chemistry measurement will include (on site)

Creatinine , Electrolytes (Sodium, Potassium, Chloride, Bicarbonate), Digoxin (for patients who are taking this medication)

v) Other Biochemistry measurement will include (on site)

Hemoglobin A1c (HbA1c), CK-MB, cTn, Alanine transaminase (ALT), BNP

4.10. EndoPAT2000 testing

Endothelial dysfunction has been shown to be an independent predictor of cardiovascular events (reviewed by [319]). Endothelial dysfunction occurs in the early stages of atherosclerosis and is present in HF patients, which can be measured by peripheral arterial tonometry using the EndoPAT 2000 (Itamar Medical, Franklin, MA)[320]. The test involves measuring hyperaemia-induced pulse volume changes at the fingertips. A Reactive Hyperemia Index below 1.67 indicates endothelial dysfunction[320].

Visit Schedule	**Baseline**	**1 Month (phone call)**	**3 Months**	**6 Months**	**9 Month (phone call)**	**12 Months (EOS Visit)**	**13 Month EOS (phone visit)**
Evaluation of Inclusion Criteria	☑						
Informed Consent	☑						
Randomisation	☑						
Demographics	☑						
Medical History	☑						
Height	☑		☑	☑		☑	
Weight	☑		☑	☑		☑	
Vitals Signs	☑*		☑*	☑*		☑*	
Blood work (Routine Chemistry)	☑*		☑*	☑*		☑*	
Blood work (Other Chemistry)	☑		☑	☑		☑	
Blood work (Additional Biochemistry Analysis)	☑		☑	☑		☑	
Urine Sample (pregnancy)**	☑		☑	☑		☑	
Minnesota Living with Heart Failure questionnaire	☑		☑	☑		☑	
Dispensing of Study Medication	☑		☑	☑		☑	
Concomitant Medication	☑		☑	☑		☑	
Review of patient logs (if applicable)			☑	☑		☑	
Medication Compliance Check			☑	☑		☑	
EndoPAT 2000	☑					☑	
2D Echocardiography	☑*					☑*	
6-Minute Walk	☑		☑	☑		☑	
Physical Examination (including NYHA class determination)	☑*		☑*	☑*		☑*	
Untoward Medical Event (AE)		☑	☑	☑	☑	☑	☑

Table 1. Visit Schedule

4.11. Assessment of safety

Should any participant experience adverse effects as a consequence of participation in the study, they may be required to stop the study medications immediately. The study team will continue to follow these patients as per the protocol. All serious adverse events as well as death due to any cause or cardiovascular death will be reviewed by the principal investigator within 24 hours and will be reported to the sponsors, institutional Ethics Boards as well as regulatory agencies. Additional lab

work and follow-up may be required depending on the occurrence and nature of reported Adverse Events. Any patient that is enrolled into the study will be advised to avoid pregnancy while on HF therapies and will be advised to use acceptable methods of contraception.

4.12. Statistical analysis

The statistical plan was developed after consultation with the St. Boniface Hospital biostatistician. The sample size of the study was determined by a statistical power analysis. At a 5% significance level (p <0.05), a sample size of 40 patients (20 in each of the placebo and resveratrol groups) would provide 80% power to detect an increase in LVEF if the underlying increase in LVEF (in the population) is 4.0%, assuming that the SD of LVEF values is 5%. The same sample size would provide 90% power to detect an increase if the underlying increase in the population is 4.6%. Differences between groups for continuous variables will be analysed by Friedman tests (non-parametric version of repeated measures ANOVA). The between subjects factor is group (experimental or control) and the within subjects repeated measure factor is time (baseline and 6 months). The relationship between resveratrol ingestion and cardiac function will be further analysed by point-biserial correlation coefficients and regression analysis with continuous cardiac function variables as outcomes. The level of statistical significance for all analysis will be p <0.05 and one-tailed tests will be employed, since we have an a priori hypothesis as to the direction that we expect of any differences in treatment groups. The statistician will also help assess the quality of the study data. Please note that we do not anticipate any dropouts, however, in the unlikely event that this happens, we will recruit additional participants into the study in order to maintain the power of the study that we provided above (80-90% power, depending on the true underlying increase in LVEF values due to the treatment).

5. Ethical approval

This study is already approved by the University of Manitoba Biomedical Research Ethics Board and the St. Boniface Hospital Research Review Committee. It is also Health Canada approved as a Natural Health Product Directorate protocol (NHP-MF #172501, NPN/dIN/DIN-HM380033777) and ClinicalTrial.gov (NCT01914081). Any necessary amendments will be submitted for review and approval prior to implementation. Study status will be reported annually. A final study notification will be forwarded at the completion of the study or in the event of early termination. The tenets of Good Clinical Practice will also be followed. Subjects are free to withdraw from the study at any time without affecting their right to ongoing medical care.

6. Current status and discussion

Two HF patients from the St. Boniface Hospital HF clinic have been recruited after obtaining their informed consent and randomised to study intervention groups in double-blinded method till date and are being followed-up as per the RES-HF study protocol. RES-HF trial is the first study to investigate the cardioprotective effects of resveratrol in HF patients. Even though there are reports from a few clinical trials involving CVD patients, no study has tested the efficacy of resveratrol in HF patients. The findings obtained from RES-HF trial will provide compelling novel information and expand knowledge on the therapeutic value of resveratrol as an adjunct to the current standard of care for HF patients. The study outcomes will also justify the development of more elaborate long-term multi-centre trials with resveratrol in HF patients and substantiate our claims for future grant funding for those larger scale HF trials. Our trial could also become another important study in terms of the future of nutraceuticals as a potential therapeutic strategy in CVDs as not many nutraceuticals have been tested in HF trials before.

The success of resveratrol trials in HF patients may impact upon the HF management and provide significant benefits to HF patients and reduce the economic burden.

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