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Resveratrol Attenuates the Development of Trans-Aortic Constriction (TAC) Induced Heart Failure in Mice

PRAKASH KUMAR GUPTA
University of South Carolina

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by

Prakash K. Gupta

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Accepted by:

Scott C. Supowit, Major Professor

Jay D. Potts, Chairman, Examining Committee

Ugra S. Singh, Committee Member

Sandra J. Kelly, Committee Member

Daping Fan, Committee Member

Lacy Ford, Vice Provost and Dean of Graduate Studies

DEDICATION

This dissertation is dedicated to my wonderful wife Jinyu Zhu whose unending love and support has finally brought me to this joyful moment of significant achievement. I sincerely appreciate all the sacrifices you made to provide for me and tide me through this time. I am thankful to my family, for your constant support and belief in my abilities. This would have been impossible to achieve without you.

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ABSTRACT

Heart failure (HF) still remains the leading cause of morbidity and mortality and imposes severe global affliction and enormous cost on the healthcare system. Although current pharmacological therapies have shown to slow down the progression of HF, but seems to have reached their limits in improving overall patient prognosis. Thus, an immediate call for novel alternate therapies are needed which act independently as well as in conjunction with current treatment modality. Studies were performed in the well-established transverse aortic constriction (TAC) model of chronic pressure overload (PO) in mice. In the first series of studies, Male C57BL6 mice (26-28 g) were subjected to either sham or TAC surgery. One group of TAC mice was given daily resveratrol treatment (oral gavage, 100 mg/kg/body weight (bw) for 28 days starting on day 2 after surgery. Echocardiographic, biometric, and immunohistological analyses were performed on the three groups of mice which demonstrated significantly greater adverse cardiac remodeling and dysfunction in the TAC compared to the sham operated mice. These pathological changes were significantly improved by resveratrol treatment in TAC+RSV mice. At day 28 fractional shortening was 46.4±2.4%, 26.2±1.0%, and 35±2% in the sham, TAC, and TAC +RSV mice, respectively. This was reflected by lung weight/bw ratios of 4.8±0.5 mg/g (sham), 10.2±1.4 mg/g (TAC) and 6.2±1.5 mg/g (TAC+RSV). Resveratrol treatment also significantly reduced cardiac hypertrophy as determined by the heart weight/bw ratios (sham, 4.9±0.3 mg/g; TAC, 8.8±1.1 mg/g; and TAC+RSV, 7.2±0.3 mg/g) as well as by measurement of cross-sectional area (CSA)

(sham, 108.02±12.85 μm²; TAC, 221.7±21.43 μm²; and TAC+RSV, 187±11.9 μm²). Likewise, the TAC protocol significantly increased fibrosis compared to the sham-operated mice, which was attenuated by resveratrol treatment. Pro-inflammatory cytokine infiltration of mast cells and macrophage were found up-regulated in response to PO. Similarly, markers of oxidative stress such as 4-hydroxynonenal (4HNE) and 8-hydroxydeoxyguanosine (80HdG) were up regulated while anti oxidative markers - sodium oxidase dismutase (SOD), glutathione peroxidase (GSH) were down regulated in response to PO. Similar results were obtained when hypoxia induced factor alpha (HIF1α), including apoptosis by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and activated caspase-3 were assessed in TAC hearts. These TAC-induced factors were significantly attenuated by resveratrol treatment indicating that the resveratrol was acting to inhibit the increased production of these stress inducible factors as well as able to up regulate the levels of detoxifying enzymes. In summary, these results demonstrate that resveratrol treatment significantly attenuates the adverse cardiac remodeling and dysfunction produced by the TAC protocol in C57/BL6 mice and that this activity is mediated, at least in part, by the inhibition of oxidative stress and hypoxia.

TABLE OF CONTENTS

LIST OF FIGURES

List of Abbreviations

TAC ..... Trans-aortic constriction

HF ..... Heart failure

PO ..... Pressure overload

4-HNE 4-Hydroxynonenal

8-OHdG 8-Hydroxydeoxyguanosine

GPx ..... Glutathione peroxidase

Hif1α ..... Hypoxia inducible factor 1 alpha

TUNEL ..... Terminal deoxynucleotidyl transferase dUTP nick end labeling

CHF ..... Congestive heart failure

DRG ..... Diagnosis related group

MI ..... Myocardial infarction

RAAS ..... Rennin-angiotensin-aldosterone system

GPCRs ..... G-protein-coupled receptors

RTK ..... Receptor tyrosine kinases

MAPKs ..... Mitogen-activated protein kinases

MEF2 ..... Myocyte enhancer factor 2

PDE ..... Phosphodiesterase

ACE ..... Angiotensin converting enzyme

MMPs ..... matrix metalloproteinases

IHD ..... Ischemic heart disease

LVID ..... Left ventricle internal diameter

LVPW .....Left ventricle posterior wall

EF .....Ejection fraction

FS ..... Fractional shortening

ROS..... Reactive oxygen species

NO..... Nitric oxide

DMSO ..... Dimethyl sulfoxide

SOD..... Sodium oxide dismutase

eNOS.....Endothelial nitric oxide synthase

iNOS ..... Inducible nitric oxide synthase

COX .....Cyclooxigenase

NF-κB ..... Nuclear factor kappa beta

BMMCs.....Bone marrow-derived mononuclear cells

VEGF .....Vascular endothelial growth factor

SIRT1 .....Sirtuin one

ACE..... Acetylcholine esterase

SDS-PAGE ..... Sodium dodecyl sulfate- polyacrylamide gel electrophoresis

DAPI Diamidino-2-phenylindole

WGA .....Wheat germ aglutinin

CHAPTER 1

INTRODUCTION

1.1 The Heart Overview

Heart is considered to be the main engine that drives human body and is the first organ to be formed during embryonic development. Heart is remarkably a well-tuned organ and beats about two and a half billion times in an average human lifetime. Heart constantly keeps working even while we are at rest. Heart, blood and blood vessels make up the entire circulatory system. Heart is a hollow, muscular organ and consists of four chambers that are entirely different in its structure and function and are equipped with valves. Three layers of heart muscle namely endocardium, myocardium and epicardium plays integral role in maintaining normal homeostasis of the blood supply. Heart not only pumps the blood throughout the body but also supplies oxygen and nutrients that are essential for the normal human growth and development. Valves play a significant role in maintaining the proper orientation of the blood flow and preventing the backflow. Heart maintains its pumping mechanism by contraction and relaxation, which is due to the electrical conduction system generated in Sino atrial node and relayed through purkinjee fibers. This rhythmic contraction of the heart is essential in maintaining contraction and pumping blood via series of blood vessels within the heart known as coronary circulation, which is primarily responsible for distributing, oxygenated blood and other nutrients to the working heart muscle.

Circulatory system of the heart makes use of all the four chambers where, function of the right atrium and right ventricle is to collect the deoxygenated blood returning from the organs and tissues and pump it to lungs where it gets oxygenated. This oxygenated blood is then received by left atrium and left ventricle, which then pumps it to the organs and tissues of the body to enable them, perform their normal functions. Blood is ejected out of the right and left ventricle by vigorous contraction of ventricular muscle to the body parts during ejection, which is known as systole while diastole ensues during filling phase. The amount of blood pumped through the heart each minute is called cardiac output and is the measure cardiac performance defined by ventricular ejection. Contractility, preload and afterload are the three aspects by which heart maintains its cardiac output and performance and these aspects determine the ventricular effectiveness during systolic phase. Component muscle fibers of myocardium undergo shortening in order to increase the intrinsic strength to produce work which is referred as contractility while preload is referred to pre-ejection filling of the ventricular chamber affecting preshortening stretch of its component fiber and finally afterload is referred to as the force against which myocardium has to work in order to contract and eject blood. Also, during normal physiological condition, increased cardiac preload and afterload are attributed to increased and decreased ejection of blood from the ventricles respectively. In order to complete the cardiac cycle, heart has to undergo two main steps to achieve diastolic ventricular filling which consists of an early rapid filling phase where blood stored in the atria rushes into the ventricles and second being the forceful atrial contraction pushing additional blood into the ventricles before their contraction thus contributing significantly to afterload.

1.2 Left Ventricular Hypertrophy & Congestive Heart Failure –Clinical Significance

Heart failure (HF), also known as congestive heart failure (CHF), is the clinical endpoint of several types of primary and secondary pathologies during which pumping ability of the heart becomes incapable of the supply at the rate proportionate to the demands of the body. Clinically HF is defined as a syndrome due to pathological disorder of cardiac structure or function such as ischemic heart disease, hypertension or several specific cardiomyopathies, resulting in impairment of filling and/or ejection of blood from the ventricles (Klein et al., 2003). Currently, CHF is the number one cause of death in the western world. In United States alone approximately 5 million people are suffering from HF at present and about half of a million new cases are diagnosed each year (Klein et al., 2003). Clinically patients suffering from HF with presence of obvious symptoms have poor prognosis with 5-year survival rate at 50% upon diagnosis (Klein et al., 2003). Regardless of the advancement in the diagnosis, treatment and management of HF, unfortunately 75% of the new cases die each year and the death toll is still on sharp incline (Klein et al., 2003). HF is more commonly seen in elderly people with age more than 65 and this underlying disorder accounts for staggering 12-15 million office visits and 6.5 million hospital days. In the past decade the number has increased from approximately half a million to nearly one million for HF as primary diagnosis and 1.7 to nearly 2.6 million for secondary diagnosis (Klein et al., 2003). 6-10% of these elderly patients are diagnosed with primary HF and approximately 80% of them are hospitalized (Klein et al., 2003). Treatment and management of HF is very expensive and estimated 21-50 billion dollars are annually spent in diagnosis and treatment of HF

(Klein et al., 2003). Despite all the advancement in the management and treatment of HF patients, heart transplant seems to be the last resort out (Dorn and Molkentin, 2004). Being diagnosed with HF carries poor prognosis and the mortality in the advance stages can be as high as 45% (Jessup and Brozena, 2003).

No definition of heart failure is entirely satisfactory. Depending upon the etiology of heart failure, symptoms in patients of HF such as breathlessness, fatigue and swelling ankle can vary greatly with the exception of patients suffering from myocardial infarction (MI). Onset of symptoms is usually acute in MI patients however it can be very subtle or even asymptomatic and progress over weeks or even months in patients with progressive HF (Francis, 2001). Although causes of HF can be due hypertension, different valvular disease, viral myocarditis, various drugs and toxins, genetic mutation including idiopathic cardiomyopathy however, MI due to coronary artery disease still remains the most common cause of HF (Adams, 2001). It is also possible to have a heart attack without experiencing any symptoms at all and is known as "silent" MI. Irrespective of the cause, dysregulated homeostasis due to significant deterioration in cardiac structure and function leads to attenuation in contraction and sharp decline in cardiac output which further follows into thinning of ventricular wall and dilation leading to ventricle enlargement and increased peripheral vascular resistance (Dorn and Molkentin, 2004). All of these above-mentioned causes significant decrease in the cardiac output due to the impaired contraction/relaxation eventually the heart fails to meet the metabolic demands of the body and overt HF occurs. Figure. 1.

Heart undergoes left ventricular remodeling in order to overcome the deficiency in the function of the ventricles to maintain required cardiac output

(Heineke and Molkentin, 2006). Heart does this by undergoing hypertrophy. Initially, this mechanism of ventricular remodeling can be a beneficial compensatory response resulting in enlargement of myocyte as a means of preserving cardiac output thereby decreasing wall tension (Lorell and Carabello, 2000). However, this pathological hypertrophy possesses serious risk factor for cardiovascular disease and sudden death (Haider et al., 1998; Levy et al., 1990; Schillaci et al., 2001). In order to cope with the hypertension-induced pressure-overload, the heart undergoes two main remodeling patterns of hypertrophy namely concentric and eccentric hypertrophy during which change in the ventricular chamber size and increase in fibrosis ensues (Heineke and Molkentin, 2006). During concentric hypertrophy, ventricular chamber size is increased, thinning of wall, apoptosis followed by extensive cardiac fibrosis results. Characteristics of concentric hypertrophy at cellular level is increased synthesis of sarcomeres in parallel that results in increased cellular width, while dilation is characterized by laid down of sarcomeres in series that results in increased myocyte length (Heineke and Molkentin, 2006). Due to the poor ventricular compliance and impaired relaxation, diastolic dysfunction can result if hypertrophy persists (Zile and Brutsaert, 2002). A number of other factors such as from genetic defects, MI can also directly damage the cardiac muscle and cause this type of remodeling (Heineke and Molkentin, 2006).

Despite of the fact that there are a number of key players involved in the process of progression of left ventricular remodeling and might be mediating their role through various pathways, substantial evidence points towards the activation of endogenous neurohormonal systems that may be playing an important role in cardiac remodeling and thereby in the progression of HF. During the condition of decreased cardiac output, this

activation of neurohormonal system is thought to play protective effect by vasoconstriction, salt and water retention, and adrenergic stimulation of the heart thereby compensating for the decreased cardiac output and maintaining the hemodynamics. Under certain life threatening conditions such as hemorrhage, this short-term adaptive mechanism may seem to be beneficial however long term activation is maladaptive in the heart. By retaining excess sodium and water, it causes excessive increase in the hemodynamic stress on ventricles as well as exert direct toxic effects on cardiac cells and stimulate myocardial fibrosis, which can further alter the architecture and impair the performance of the failing heart (Harris, 1983; Packer, 1992). Other factors such as elevated circulating or tissue levels of norepinephrine, angiotensin II, aldosterone, endothelin, vasopressin, and cytokines is found in HF patients, and the action of these agents working alone or in concert can further deteriorate the structure and function of the heart. Chronic stress on the myocardium causes deterioration of left ventricular function and even in the absence of new insult, it progresses further resulting in chamber dilation and decreased cardiac output. Further deterioration in observed in the function of valves, which causes back flow of blood resulting from increasing hemodynamic overload. Cardiac remodeling is an ongoing pathological process, which keeps progressing and worsening of symptoms irrespective of the continuous treatment and management of patient with HF.

Systolic or diastolic heart failure can now be well characterized with the use of advanced echocardiograms. Due to the characteristics of diastolic heart failure such as hypertension and pulmonary congestion, it still remains echo insensitive however characteristics of systolic failure is very sensitive to echocardiogram and the

characterized by left ventricular enlargement, reduced contractility and ejection fraction followed by pulmonary congestion.

Signs and symptoms are very obvious in HF patients only in later advanced stages and may exhibit as pulmonary congestion and peripheral edema due to fluid retention thereby leading to breathlessness and reduced exercise tolerance, which is manifested as fatigue. HF is not a single disease but rather a complex syndrome resulting from a manifestation of different cardiovascular disorders, therefore cannot be treated with single cure. Exhibition of chief clinical signs and symptoms and the stage of the disease should be taken into account and the treatment regimen should be tailored to their individual characteristics. Figure 1.2

According to the American heart association and American college of cardiology guideline published in 2001, prevention and treatment of patients with HF can be classified into four stages namely: Stage A- patients suffering from hypertension, coronary artery disease (CAD), or patients who recently had a myocardial infarction (MI) fall under this stage These HF do have some structural abnormality in their cardiovascular system but do not exert symptoms. Stage B- patients diagnosed with structural heart disease but do not express signs of heart failure fall under this stage. Structural abnormality of the heart is readily detectable on the echocardiogram and mild to moderate expression of symptoms. Stage C- patients showing obvious signs of heart failure with marked clinical manifestation of sign and symptoms such as dyspnea on exertion and fatigue and eventually stage D includes patients with end-stage heart failure awaiting transplantation and their mobility is very restricted and are under mechanically assisted devices to maintain CO.

1.3 Cardiac Hypertrophy And Heart Failure - The Molecular Basis

One of the major questions in hypertrophy and HF research is the mechanism by which cardiac cells sense stretch, and significant progress has been made in the recent past regarding it. Despite of the identification of the major players of hypertrophic signaling in the myocardium, it is still unclear why and how the frequent transition from hypertrophy to failure occurs. Many pathways have been identified that leads to hypertrophy however the most vital and most studied pathway is mitogen activated protein-kinase pathway (MAPK). Hypertrophic cardiomyocyte growth is due to the activation of signaling pathway that in turn causes reactivation of fetal genetic program creating a chronic condition where these responses elements further exaggerates the heart damage leading to deterioration of function (Lorell and Carabello, 2000). Initially cardiomyocytes were thought to be incapable of differentiating, however the hypothesis has chanced in the recent past. The term cardiac hypertrophy is defined as an increase in the cellular mass in response to growth stimuli, which is thought to be an adaptive response during the common disorders such as pressure or volume overload, loss of cardiac mass due to MI etc. Cardiac hypertrophy is usually seem to accompany pathological processes such as MI, valvular disease, ischemic disease which can ultimately result in heart failure however, it can also occur in absence of other cardiovascular diseases. Initially cardiac hypertrophy is considered to a compensatory stage in response to stress signals such as hypertension or myocardial infarction and protects the heart by decreasing energy consumption as well as decreasing the systolic wall stress (Grossman et al., 1975). However, if the chronic stress persists for too long, arrhythmia and sudden death followed heart failure is inevitable. A pathological

structural and functional change in the heart occurs due to persistent stress signals that cause cardiac remodeling. Remodeling involves not only the cardiomyocytes but also cardiac fibroblasts, elements of the interstitial matrix, and the entire coronary vasculature. Healthy or unaffected cardiomyocytes now has to take over the function of the affected or injured part making up for the loss as an adaptive response. This can be usually seen in condition such as MI. Cardiac remodeling at cellular and molecular level involves changes from physiology of the heart all the way to gene level.

Activation of the rennin-angiotensin-aldosterone system (RAAS) as well as adrenergic and cytokine pathways are some of the secondary changes associated along with above mentioned primary remodeling which heart has to deal with. Sarcomeres are the force generating units, which undergo production of more sarcomeres through a gene transcription modification biochemical event in the nucleus upon increased force on the cardiomyocyes. This modification of gene transcription can also be achieved through various stimuli, which activates distinct receptors in the cardiomyocyte. Figure. 1.3.

G-protein-coupled receptors, receptor tyrosine kinases and cardiotrophin receptors are some of the most studied receptor pathways which upon activation operates through signaling pathways and mostly comprises of series of phosphatases and kinases. The phosphatase pathways include calcineurin (PP2B), protein phosphatase 1 and protein phosphatase 2A (PP2A) while the kinase pathways include protein kinase C, protein kinase A, calcium-calmodulin-dependent kinase and mitogen-activated protein kinases.

It has been shown that cardiac transcription factors are responsible for the differential gene expression induced by hypertrophic stimulation. Therefore hypertrophy of cardiomyocytes occurs when there is a sudden change in the transcriptional programs

mediated by cardiac signaling pathways and convert extracellular signals into intracellular signals. GATA family, (MEF2) family, and the homeobox factor (Csx/Nkx2.5) family are some of the most studied families of cardiac transcription factors and the genes that encode regulatory or structural proteins of the cardiomyocyte by eliciting changes in other cell types in the heart are regulated by these cardiac transcription factors which are transcriptional activators or repressors that regulate the expression of cardiac genes (Kolodziejczyk et al., 1999; Liang et al., 2001; Takimoto et al., 2000; Toko et al., 2002).

1.4 Contraction, Relaxation And Regulation Of Failing Heart

Heart muscle is remarkable and beats about 70 beats per minute and about 100,000 times per day. Contraction and relaxation of the heart generate the force required to maintain the cardiac output. Cardiomyocyte contains thick and thin filaments namely actin and myosin. Sleeves of sarcoplasmic reticulum surround the myofilaments within the myocyte. Calcium plays a vital role in the maintenance of cardiac contractility and it is the second common messenger and the central regulator of cardiac contractility. Under normal physiological the exchange of sodium, potassium and calcium occurs in harmony and is well regulated to maintain the efficient contraction. The calcium release channels from heart and skeletal muscle SR are similar but not identical. Important differences distinguish the calcium release machinery in heart from that of skeletal muscle. The role of calcium as the key factor in coupling cardiac excitation (depolarization) and contraction. The calcium ion is the activator of chemically bound energy to mechanical energy in all types of contractile structures, from microfilaments to striated muscles. Strength of cardiac contraction can be varied by either altering the Ca2+ concentration or

by altering the sensitivity of the myofilaments to Ca2+ and this can be achieved by maintaining the intracellular calcium concentration by channels, pumps and exchangers. Sarcoplasmic reticulum in cardiomyocytes contains large internal store of Ca2+ and is released in large amounts following a small increase of extracellular Ca2+ into the cytosol. This phenomenon is called Ca2+ induced Ca2+ release. The myofilament proteins undergo conformational changes following the release of cytosolic calcium increase thereby achieving contraction. However, this cytosolic Ca2+ must then be removed in order to achieve a proper relaxation. Changes in the concentration of the Ca2+ homeostasis and or alterations in the contractile proteins may be one of the chief reasons for the likely cause of decreased contractility associated with heart failure (Piacentino et al., 2003). Figure 1.4

1.5 Current Treatment Modalities For Hypertensive Heart Disease And Heart Failure

HF has become increasingly prevalent as the population ages and has reached the epidemic proportion in USA as well as around the globe. HF possesses a substantial socio-economic burden on the families suffering from it as well as on the economy of the country. A wide range of pharmacological agents are employed in the prevention, management and treatment of patients suffering from HF. Popular and most widely used pharmacological agents such as vasodilators, positive inotropic agents, beta blockers, diuretics and inhibitors of neurohormonal activators have been able to address some of the cellular mechanisms of HF so far however, they fail to completely achieve their intended goal in stabilizing or regressing the disease progress and improving the quality of life by minimizing the pathological symptoms and enhancing the survival. Initially,

upon injury or stress, the feed back mechanism of the human body tries to play a compensatory role by activating a series of neurohormonal systems.

One example of such compensatory neurohormonal activation is the activation of renin angiotensin aldosterone system (RAAS). In the event of decreased cardiac contractility, the cardiac output declines significantly thereby being unable to meet up with the demands of the body. RAAS causes retention of salt and water thereby expanding the blood volume and maintaining the cardiac output. This compensatory response is thought to be beneficial in the initial stages however, long-term activation of this neurohormonal system causes severe hemodynamic pathology leading to renal function deterioration and eventual HF (Rouse and Suki, 1994; Schrier and Abraham, 1999). Increased pressure in diastolic filling due to the excessive expansion of fluid causes further severe pathological stress on the heart leading to ventricular hypertrophy and cardiac remodeling. Diuretics are the drugs of choice under condition and their action is mainly on the kidney. It causes the elimination of salt thereby preventing water retention and eventually decreasing the hemodynamic load placed on the heart. Another method by which compensatory mechanism of the heart tries to maintain the necessary cardiac output is by vasoconstriction and increasing the systemic vascular resistance. This significant increase in the peripheral vascular resistance causes increased afterload on the heart thereby imposing even greater pressure on LV against which it needs to eject the blood (Cohn and Franciosa, 1977a, b; Kass and Kelly, 1992). This vasoconstriction then becomes pathological problem and therefore needs to be relieved. Drugs that mediate vasodilation by increasing the diameter of the vessel should be employed. A number of commercially available potent vasodilators are the drug of choice. These vasodilator act

on the vascular smooth muscle cells by increasing the intracellular level of cGMP and inducing vasorelaxation thereby improving coronary blood flow as well as reducing ventricular filling pressure and wall stress (Fallen et al., 1995; Hare et al., 1995; Harrison and Bates, 1993).

Upregulation of endogenous vasodilators such as ANP and BNP have been documented in patients suffering from HF (Vesely et al., 1994; Vesely et al., 1995) and also their positive roles in improving cardiac contractility, cardiac output and clinical status have been reported (Colucci et al., 2000; Mills et al., 2002). Apart form the pathological nuerohormonal cascade activation, heart also has to deal with deteriorated contractility leading to cardiac output. A choice of positive inotropic agents that are capable of reducing the hemodynamic overload from the heart in patients suffering from HF is an ideal choice to increase the cardiac contractility. One such drug of choice is Digoxin. It is a cardiac glycoside and the mechanism by which it works is by inhibiting Na/K ATPase pump thereby increasing the intracellular sodium which can be used for the exchange of calcium thus causing the improvement in the cardiac contraction and achieving optimum cardiac output (Blaustein, 1993; Hauptman and Kelly, 1999). Another similar pharmacological agent performing through different route to improve the cardiac contractility is adrenergic agonist. It mediates its effect by increasing the intracellular calcium level by binding to G-protein coupled receptors on the sarcolemma membrane thereby improving cardiac contractility. Following the increase in the level of intracellular cAMP, PKA is activated which causes the release of voltage gated calcium ion channels thereby even more pronounced cardiac contractility is achieved.

Another strategy of increasing intracellular calcium level is by relieving the inhibition of Ca2+-ATPase in sarcoplasmic reticulum (SR). Inhibition of PDE causes upregulation of cAMP. It has been shown that in SR, conversion of cAMP into AMP by PDE III takes place thereby even more PKA activation can be observed in the presence of excess cAMP that in turn causes phosphorylation of phospholamban and finally reliving the inhibition of Ca2+-ATPase pump in SR (Forfia et al., 2007; Koss and Kranias, 1996; Leroy et al., 1996; Movsesian et al., 1991). In addition to above, inhibition of PDE III and V can also cause vasodilation and one such drug of choice is sildenafil (Forfia et al., 2007; Jiang et al., 1992). Angiotensin II has been known to cause smooth muscle proliferation, vasoconstriction thereby causing cardiac hypertrophy (Inagami, 1999). Drug of choice to inhibit such pathological effect can be achieved by using angiotensin converting enzyme inhibitor (ACEI), which prevents the formation of Ang II within the RAAS system thus preventing vasoconstriction and hypertrophy (Inagami, 1999). A need of other players such as Angiotensin receptor blocker (ARB) and aldosterone antagonist is essential because HF patients overtime develop resistance against ACEI. Thus this new drugs help to protect the heart further and improve cardiac contractility thereby increasing CO (Gottlieb et al., 1993; Havranek et al., 1999; Mazayev et al., 1998). However, every new drug has its own side effect along with its protective functions. Pathological effects such as profibrotic and arrhythmic effects of aldosterone cannot be overcome by either ARBs or ACEI, therefore an adjunct therapy using ARB is employed (Pitt, 1995; Ramires et al., 2000; Weber et al., 1993). As a compensatory response to increase the CO by increasing the cardiac contractility, the increase in the sympathetic output in response to adrenergic signaling is initiated (Haber et al., 1993). Desensitization

of this system is observed in patients with advanced stage HF as an adaptive mechanism by down regulating beta 1 however, activation of this pathway again is pathological in later stages as it causes increased overload on ventricular wall leading to ventricular dilation, contractile dysfunction and cardiomyocyte apoptosis (Bristow, 1997, 2000b; Bristow and Gilbert, 1995; Eichhorn and Bristow, 1996). Therefore by employing beta blockers, the harmful effects of NE can be attenuated significantly hence improvement in the systolic function as well as reversal of cardiac remodeling can be achieved (Abraham et al., 2002; Lowes et al., 2002). Figure 1.5

Irrespective of the significant advancement in the medical field in the areas of diagnostic tools as well as development of novel pharmacological agents that can act individually or in concert with other agents to mediate their potential positive effect in the treatment of underlying pathology of HF. None have been efficient enough to stop the progression of disease, therefore a much more potent agent is needed to combat such disease that carries dismal prognosis. A novel alternate therapy that can be used as adjunct with existing ones or that can act alone effectively in managing the neurohormonal cascades and improve overall quality of life in patients with HF is needed. One such potential drug has been recently discovered which shows promising effectiveness in the treatment and management of HF by mediating anti-oxidative

1.6 Pathogenic Effects Of Oxidative Stress In Heart Failure

Increase or decrease in the level of antioxidant protection depends on the oxidant and reactive oxygen species (ROS) generation during oxidative stress. Due to the variation of the redox condition influenced by ROS, a number of biological processes are influenced which causes changes in the signal transduction cascades upstream of nuclear

transcription factor (Palmer and Paulson, 1997). Characteristics of ROS are free radicals containing oxygen atoms and they are known to produce free radicals such as hydrogen peroxide, superoxide and peroxynitrite. A number of biological molecules such as macrophages, neutrophils and xenobiotics try to maintain the cellular defense preventing ROS by phagocytosis or by cellular respiration (Abe and Berk, 1998; Klaunig and Kamendulis, 2004; Palmer and Paulson, 1997; Pathak et al., 2005; Zangar et al., 2004). However, excessive ROS production due to any injury or stress causes the alteration in the macromolecules, essential proteins and DNA resulting in cell death. By inducing MAPK family and PKC pathway, ROS has been known to mediate a number of physiological as well as pathological stimuli such as inflammatory cytokines, angiotensin and ionization radiation. Depending upon the content of critical cysteine residues, different cell types exhibit different sensitivity to oxidation and ROS can regulate their essential biochemical events such as phoporylation/dephosporylation of proteins by changes in their structural confirmation thereby inducing growth, differentiation and even apoptosis of cell (Sundaresan et al., 1995). Another potential pathological property of ROS is shown to be able to cause chromosomal aberration leading to cellular mutation and carcinogenesis at low concentration while higher concentration causes cytotoxicity and cell death (Abe and Berk, 1998; Klaunig and Kamendulis, 2004; Palmer and Paulson, 1997; Pathak et al., 2005; Zangar et al., 2004).

Cardiac remodeling has been established as a fundamental process in the progression of HF. Cardiac remodeling is characterized by a series of alteration in the structure and function brought in by the changes in the gene and protein expression both in extracellular matrix (ECM) and individual cardiomyocyte. Despite the extensive

clinical and experimental studies to uncover the mechanisms that underlie the poor prognosis of HF patients, satisfactory treatment has not been achieved. This provides a strong reason to pursue this field to the utmost extent and eventually be able to elucidate the pathways that would be therapeutically targeted to improve the outcome of millions of HF patients. Oxidative stress and activation of matrix metalloproteinases (MMPs)- a family of enzymes capable of degrading all the matrix components of the heart, have received an increasing attention in the HF research area. Under normal physiological conditions, there exists a great balance between ECM formations and its degradation and MMP are critical in determining this balance. Inappropriate activation of MMPs and low levels of their inhibitors TIMPs have been shown to cause cardiac remodeling and influence the shape and size of the ventricular chamber. Similarly, oxidative stress due to injury of any kind on the heart has been shown to induce cardiac remodeling and HF and this has been shown in the clinical settings of HF (Sundaresan et al., 1995). Although pharmacological inhibition of MMPs by employing commercially available inhibitors such as TIMPs have been shown however, control of oxidative damage is not manageable by TIMPs and the patients with advanced HF show marked elevated levels of oxidative stress markers which again positively correlates with the role of oxidative stress in HF. It seems that an obvious mechanism of myocardial dysfunction is through oxidative stress, which causes cellular and protein function deterioration thereby leading to cardiac impairment and eventually death through apoptosis and necrosis. Figure 1.6.

Toxicity and pathogenicity of ROS depends on its concentration, the site of production as well as over all cellular redox status (Finkel, 2011). A potential hypothesis can be developed by looking at the roles of ROS in the HF as well as its potential to

influence ECM remodeling by activating MMPs (Spinale, 2002). A clinical study employing human subjects who have undergone coronary artery bypass surgery showed a strong link between oxidative stress, MMP activation and LV dilation (Kameda et al., 2003). By this study, the authors were able to successfully demonstrate a positive correlation between the level of oxidative stress, activation of MMPs and cardiac remodeling which are characterized by increased pericardial levels of 8-isoprostane, MMP-2 and 9 and increased left ventricular end diastolic volume. Another mechanism by which ROS exerts its pathological effect on the cardiovascular system is by counteracting the endogenous nitric oxide (NO) production. NO is a potent antioxidant and it mediates its effect by vasodilation. When the concentration of ROS increases excessively, the NO cannot maintain their protective role anymore thereby causing vascular endothelial dysfunction. ROS further reacts with NO causing the production of peroxinitrite, which again is a harmful reactive oxygen species. These peroxinitrite are further capable of modulating diverse intracellular signaling pathways therefore causing pathological effects (Finkel, 2011). Some of the key proteins involved in the myocardial excitation-contraction coupling are ion channels, calcium release channels from sarcoplasmic reticulum and myofilaments and these proteins are no exceptions in escaping redox signaling (Byrne et al., 2003).

Effects on cellular energetics exerted by ROS as well as chronic changes in the cellular phenotype and pathophysiology of HF has been shown to be mediated by ROS (Byrne et al., 2003). Not only activation but also increased MMP expression has been reported by ROS (Spinale, 2002). Although attenuation of ROS by chronic treatment with ROS scavengers can lead to deactivation of redox sensitive signaling pathway (MAPK,

NFkB) thereby preventing cardiomyocyte hypertrophy however, targeting the potential source of ROS may be a better approach (Byrne et al., 2003). To date, some of the most potential sources of ROS have been identified which include infiltrating inflammatory cells, mitochondria, xanthine oxidase and important of all NADPH oxidase. Contractile dysfunction in advanced HF due to mitochondria derived excessive ROS production in cardiomyocytes have been demonstrated in the experimental models of HF. Similarly, human and canine study have shown elevated levels of xanthine oxidase in advanced HF stage. These above observations were further confirmed by a study in human subjects performed by Kameda et al where they demonstrated lower level of MMP and 8-isoprostane achieved by treatment with xanthine oxidase inhibitor (Kameda et al., 2003). Apart from xanthine oxidase, an important source of ROS production is through NADPH oxidases- a family of complex enzymes. Initially they were characterized in neutrophils however, recent reports suggests its wide expression. As mentioned NADPH is a major source of ROS production and any pathophysiological stimuli in heart such as pressure overload (PO), volume overload (VO), TNF alpha, Ang II or alpha adrenergic agonist can cause serious ROS production through NADPH oxidases (Griendling et al., 2000). Several human and animal experiment studies have well implicated the pivotal role of NADPH oxidases in the ROS generation and pathogenesis of HF (Heymes et al., 2003; Li et al., 2002; Maack et al., 2003). Additional evidence is supported by the genetic knockdown of NADPH oxidase gene in rodent model and elucidating its role in Ang II induced cardiac hypertrophy and interstitial fibrosis (Bendall et al., 2002). Due to numerous pathological effects mediated by ROS, it certainly has a bad press however, it should be noted that net effect of ROS can be maladaptive by one enzymatic source but

can be adaptive through the next. Therefore ROS plays a clinical role in the CVD process and its progression to HF.

1.7 Resveratrol And Its Diverse Protective Effects

Since HF is not a single disease with a single etiology but rather a complex disorder resulting from a number of pathological conditions, therefore the treatment of HF cannot be achieved by specific targeting of certain pathways. It has to be done employing an agent that has a broad spectrum of coverage. Over the past several decades, a number of extracts from fruits and plants have been screened and studied in various experimental settings that may be beneficial to human health by responding to injury or infection as defense system. Among these phytochemicals, a non-flavonoid polyphenol resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) is a one such example. Fig. 1.7 Resveratrol has a long history and was first identified in 1940 in the roots of white hellebore, and later in the dried roots of *Polygonum cuspidatum* (Cichewicz et al., 2000; Vastano et al., 2000). However, this stilbene polyphenol was discovered only in 1976 in the leaf epidermis and the grape skin (Langcake and Pryce, 1977). Fig 1.8

By various researchers in diverse pathological conditions, resveratrol has been shown to have diverse bioactivities (Aggarwal et al., 2004; Fremont, 2000) Table 1.1 including but not limited to modulation of antioxidant, lipid profile, anti-cancer, anti-inflammatory, anti-glycemic, anti-platelet aggregation, anti-viral, anti-bacterial and including antioxidant activity, vasorelaxing activity, dyslipidemia and obesity, the ability to protect endothelial function and much more. By acting on RAAS system, resveratrol has been shown to eliminate salt and water retention thereby decreasing the pressure overload from the heart as well as inducing levels of endogenous anti-oxidant NO and

causing vasodilation thereby attenuating the hypertension and cardiac remodeling. Similarly resveratrol keeps a good check on lipid profile by various mechanisms and regulates level of HDL/LDL thereby attenuating dyslipidemia and obesity together with formation of atherosclerotic plaques. Resveratrol also increases the sensitivity of glucose to insulin in beta cells maintains check on diabetes. Resveratrol also have been shown to cause multiple other beneficial effects pertaining to CVD such as attenuating valvular disease, metabolic syndrome etc thereby ultimately leading to prevention of development and attenuation of progression of HF (Aggarwal et al., 2004; Aziz et al., 2003; Dong, 2003; Fremont, 2000; Gusman et al., 2001; Jang et al., 1997; Savouret and Quesne, 2002; Signorelli and Ghidoni, 2005).

Resveratrol has been credited for the low incidence of heart disease in France, popularly known as "French Paradox" (Pace-Asciak et al., 1995). Since the discovery of resveratrol, it has been widely used in a number of experimental settings due to the potential of resveratrol in prevention or regression of various illnesses such as CVD, cancer, and ischemic injury. This has caused the number of resveratrol citations in PubMed to increase dramatically. Moreover, resveratrol has been shown to increase the longitivity in various organisms and is thought to mediate this action by activating SIRT 1- a gene widely studied in context of ageing (Baur and Sinclair, 2006).

Fresh grape skins are the major source of resveratrol containing 50-100 mg/g while red wine contains 10 μM resveratrol on average. Long before the actual discovery of resveratrol, folk medicines in Asia have been using these plants as a part of their herb for the treatment of various illnesses such as in inflammation and arthritis. Resveratrol is produced in plants in response to radiation injury or fungal infection. Chemical structure

of resveratrol is also an important aspect of this compound to be able to bind to diverse molecules and their receptors in order to mediate its wire array of protective effects. It consists of two aromatic rings linked by a styrene double bond with two hydroxyl groups at the 3 and 5 positions of one ring and one hydroxyl group at the 4' position of the other ring. Resveratrol is more soluble in DMSO than in water and has a melting point of 253-255°C and molecular weight of 228.25. Cardiovascular disease is a serious global health issue and possesses huge socio-economic burden. Since CVD is a syndrome rather than an isolated disorder resulting from various pathological processes and multiple etiologies therefore using a pharmacological agent that has the potential to target multiple molecular mechanisms may yield better therapeutic efficacy as opposed to the one that is very selective. Since resveratrol has been shown to possess protective potentials and achieves multi-targets related to CVD, it would be logical to cut down the cost by taking as few drugs as possible while maintain the similar level of efficacy through one potential drug. (Baur and Sinclair, 2006; Opie and Lecour, 2007). A number of in vivo and vitro studies of animal models in regards to CVD have been done and resveratrol has been shown to mediate protection against ROS and preserve cardiovascular function making it a novel drug of choice for the treatment of HF.

1.8 Mechanism By Which Resveratrol Mediates Oxidative Stress

Until recently, it was highly debated as to whether resveratrol's primary protective effect is mediated by acting as a radical scavenging antioxidant or by inducing the endogenous antioxidant that are already present in the body. The reaction by which resveratrol acts as a radical-scavenging antioxidant is shown: Res-$(OH)_3$ + R· → Res-$(OH)_2O$· + RH, in this reaction, Res-$(OH)_3$ represents resveratrol while R· represents free

radical (Karlsson et al., 2000). Res -$(OH)_2O$ is the unpaired electron of resveratrol which delocalizes over aromatic ring thereby making resveratrol 's reactivity poor. Structural study mentioned above clears the methodical doubt of how resveratrol counteracts ROS and is therefore by upregulating endogenous cellular antioxidants rather than direct scavenging activity of ROS (Spanier et al., 2009). Figure 1.9. Several published studies have shown sufficient evidence regarding the inhibition of ROS by resveratrol and is shown to be primarily mediated by attenuating pro-oxidative genes (NADPH oxidases and myeloperoxidases) (Baur and Sinclair, 2006; Dolinsky et al., 2009; Spanier et al., 2009) and in addition also by inducing anti-oxidative enzymes like superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase, (Spanier et al., 2009; Tanno et al., 2010; Thirunavukkarasu et al., 2007; Ungvari et al., 2007). SOD is located in mitochondria and resveratrol has been shown to regulate the level of this anti-oxidant by acting on mitochondrial electron transport chain thereby attenuating ROS production (Tanno et al., 2010). ROS has been shown to cause oxidative stress in the cells by inducing lipid peroxidation and resveratrol counteracts this pathological effect by chelating transition metallic copper that is responsible for lipid peroxidation and generating free radicals (Belguendouz et al., 1997; Ferretti et al., 2004). Another method by which resveratrol has been shown to counteract the ROS is by upregulating stress response protein heme oxygenase (HO-1) (Thirunavukkarasu et al., 2007; Ungvari et al., 2007). By modulating the deacetylation of endothelial nitric oxide synthase (eNOS) and inducible NO synthase (iNOS), resveratrol has been shown to upregulate the level of plasma nitric oxide thereby attenuating ROS induced oxidative stress (Arunachalam et al., 2010; Csiszar et al., 2009; Wallerath et al., 2002; Zou et al., 2003). NO has been

shown to have more more affinity for O_2^- compared to SOD and during the reaction of iNOS with O_2^-, peroxynitrite is generated, NO has been shown to react with –SH group of thiol and ascorbate and eliminate O_2 therefore resveratrol/NO pathway plays vital role in elimination of O_2^- (Hattori et al., 2002). Table 2.

1.9 Anti-inflammatory Response Of Resveratrol

Traditional viewpoint regarding inflammation is depicted as a response developed to protect against infection and tissue injury and to repair damaged tissue. Depending upon the context, inflammation can be both beneficial as well harmful. Current view of inflammation describes it as an adaptive response to restore interrupted tissue homeostasis. Inflammation is mediated by tissue resident macrophages or other inflammatory cells. The inflammatory response however is a very complicated interaction of various inflammatory cells both from innate and acquired immune system. A controlled inflammation can be beneficial however, uncontrolled inflammatory response can be very detrimental and the outcome is tightly associated with various determinants. Under normal physiological conditions, inflammatory cells act as host defense. Innate immune players such as neutrophils, monocytes and macrophages all impart their protective effects by phagocytosis. Adaptive immune system however is mainly able to distinguish body's own vs foreign and eventually clear out the pathogen. These cells are B, T and dendritic cells along with macrophages. However under pathological conditions, they secrete histamine and sertonins degraded products and degranulation in cells are triggered. Similarly proteolytic enzymes such as tryptase and chymase cause degradation of ECM as well thereby initiating cardiac remodeling.

A number of studies in the heart have focused on interrelation between

inflammation and fibrosis that ultimately causes damage to cardiomyocyte and eventual death by apaoptosis. One such example of experimental study is during Ang II induced hypertension and activation of inflammatory response leading to cardiac remodeling. Under condition of pressure overload, these macrophages and mast cells have been shown to dramatically increase in their number and cause fibrosis. Due to these reasons of inflammation and the pathological mechanisms that underlie the harmful process of HF, it has gathered a significant attention in scientific community. Inflammation is not an occurrence due to a single etiology nor a single inflammatory cell type causes the entire process. A number of pathological conditions such as hypertension, diabetes, obesity, neurohormonal factors, radiation exposure as well as autoimmune all can trigger the process of pathological inflammation. Because of the significance of inflammation in these pathological processes, it has always been closely studied with heart diseases. One such example is during atherosclerosis, which is characterized by not only lipid deposition in the arterial wall but also subsequent activation of cascade of inflammatory response. These cells further causes ECM degradation and weakening of fibrous cap of the athromatous plaque which can eventually rupture to cause coronary artery disease, MI and even sudden death (DeMarco et al., 2010). Under such conditions of cellular injury potentiated by HTN, excessive ROS generation through NADPH is reported. These ROS further aggravates the pathological stimuli causing even greater adverse cardiac remodeling (DeMarco et al., 2010; Rizvi, 2010). Fortunately resveratrol has been shown to interfere with the release of inflammatory mediators, suppressing macrophages, T cells, B cells activity thereby mediating anti-inflammatory activity and protecting the heart (Fremont, 2000; Sharma et al., 2007). Resveratrol does this by inhibiting COX and

its subcomponents COX 1 and 2 that are required for the conversion of arachdonic acid into prostanoids and thromboxane. Resveratrol acts as a anti-platelet aggregation agent via COX 1 (Fremont, 2000; Jang et al., 1997; Szewczuk and Penning, 2004) and mediate inhibition of prostaglandin by COX 2 (Dave et al., 2008; Martinez and Moreno, 2000; Subbaramaiah et al., 1999). Moreover, treatment with resveratrol has been shown to attenuate MMP production thereby preventing progression of plaque formation as well as rupture of plaque cap (Dave et al., 2008).

Proteoglycan is an endogenous anti-inflammatory agent and its level is shown to be decreased during the autoimmune inflammation, however treatment with resveratrol has been shown to upregulate the level of proteoglycan thereby attenuating the pathological progression of autoimmune inflammation (Dave et al., 2008). Similarly inhibition of NFkB- a marker of autoimmune inflammation has been shown by resveratrol treatment in patients with increased risk of CVD (Kang et al., 2009; Karlsson et al., 2000; Leiro et al., 2004). With such diverse array of anti-inflammatory property packed into resveratrol, it makes resveratrol a novel therapeutic agent in the treatment and management of plaque stabilization and inhibition of thrombus formation thereby lowering the incidence of CVD significantly as well as improving the overall quality of life tremendously in patients with HF.

1.10 Effect Of Resveratrol On Endothelial Protection

Bone marrow derived mononuclear cells (BMMCs) are reported to be major source of circulating stem cells. These cells are integral in the formation and repair mechanism of vessels. During injury or oxidative stress, these cells die rapidly thereby normal functioning of the blood vessels is compromised. Fortunately resveratrol has been

shown to be a major player in mediating the level of BMMCs by activating PI3K/AKT pathway. This hypothesis was tested by a study where they induced ischemia in the hind limb of ApoE KO rodent and resveratrol was able to attenuate the ischemia by decreasing the oxidative stress as well as inducing angiogenesis (Gan et al., 2009). Another study showed that resveratrol works through VEGF and causes protection in experimental models of oxidized EPCs (Lefevre et al., 2007). In addition, resveratrol has been shown to protect endothelial cells by induction of iNOS in macrophages (Arunachalam et al., 2010; Cho et al., 2002) as well as via upregulating eNOS pathway (Klinge et al., 2008). Integrity of vascular wall is essential for the normal functioning of heart and resveratrol have successfully been shown to impart these protective effects through various mechanisms thereby making it a potential therapeutic agent for the treatment and management of HF (Lefevre et al., 2007; Wang et al., 2007; Xia et al., 2008).

1.11 Protective Effects Of Resveratrol In Cardiac Hypertrophy And Heart Failure

A number of recent studies using rodent models of PO and VO induced HF reported successful regression of cardiac hypertrophy and dysfunction by (Behbahani et al., 2010; Thandapilly et al., 2010). Resveratrol has been shown to upregulate the level of eNOS/NO thereby causing vasodilation and mediating anti-hypertrophic effect of cardiomyocyte (Juric et al., 2007). Similarly resveratrol has been shown to mediate anti-oxidative stress by activating AMPK and preventing LKB1 inhibition (Chan et al., 2004; Dolinsky et al., 2009; Langley et al., 2002). During decompensatory stage, cardiac remodeling causes excessive fibrosis, which further leads to apoptosis eventually resulting in cardiomyocyte deficiency that cannot be easily replaced. Fortunately

resveratrol has been shown to actively repair the damaged DNA and stabilize the genome. Also by activating SIRT1, resveratrol has been shown to limit premature cellular ageing thereby promoting life span in failing myocytes (Langley et al., 2002; Pillai et al., 2006). In patients with chronic HF, level of noradrenaline is increased due to decreased reuptake of catecholamine, however resveratrol has been reported to normalize the density of beta adrenoreceptor thereby restoring the sestivity of myocardium to catecholamines (Gaemperli et al., 2010). Resveratrol also has been reported to be able to reduce the infarct size in patients with MI (Burstein et al., 2007). Resveratrol has been reported to attenuate the frequency of cardiac arrhythmia by improving sympathetic neural remodeling (Xin et al., 2010). The reduction in the NE level by resveratrol also causes secondary effects on RAAS thereby improving cardiac performance and CO. A study demonstrated that Ca2+ uptake in the SR is controlled by SR Ca2+-ATPase, and this level of Ca2+ was found to be decreased in mice model of diabetic cardiomyopathy and in rats with MI. Resveratrol was able to attenuate this decline in the level of Ca2+ thereby maintain proper cardiac contractility (Schmidt et al., 2002; Sulaiman et al., 2010; Xin et al., 2010). Intriguingly, SIRT1 activation by resveratrol activates SERCA 2 level thereby further improving cardiac function. Therefore the findings of these studies suggest an invaluable role of resveratrol in mediating cardioprotection.

1.12 Potential Role Of Resveratrol On Cardiomyocytes Regeneration

After an acute or chronic insult, a number of cardiac myocytes undergo apoptosis however, for a normal functioning of the heart these losses needs to be repaired. Differentiation of these myocytes is therefore utmost essential. Although, under normal physiological conditions, endogenous anti-oxidants keep working to ward off excessive

production of ROS however, they are vital signaling molecule and activate myogenic differentiation (Ding et al., 2008; Yang et al., 2008; Yang et al., 2009). Level of antioxidant enzymes such as Nrf2 and Ref 1 has been shown to be regulated by resveratrol, enhancing the regeneration of cardiac stem cells and hence mediating the cardioprotection during oxidative insult (Gurusamy et al., 2010). One such study pretreated the cardiac stem cells with resveratrol and upon their transplantation in the damaged heart, surprisingly improved the cardiac performance. Therefore it would be of significant clinical and therapeutic value by utilizing the cardioprotective effects of resveratrol in stem cells mediated cardioprotection.

1.13 Role Of Resveratrol In Vascular Remodeling

Cardioprotective effect of resveratrol has been shown in number of ways and via various pathways. One important aspect to consider is the maintenance of normal morphology of the vascular wall. Following an insult or injury not only the cardiac myocytes but also the blood vessels are greatly affected. Decrease in the endogenous anti-oxidant levels and activation of neurohormonal cascades further deteriorates the vascular wall morphology as shown in a study (Baur et al., 2006). Factors such as IL-18 and MMP causes proliferation of vascular smooth muscle wall thereby narrowing the lumen which further causes increased systemic vascular resistance thereby causing vascular as well as cardiac remodeling. Resveratrol has been shown to cause vasodilation by increasing the level of endogenous anti-oxidant NO and attenuate the level of IL-18 as well as inhibit the activation of MMP (Ekshyyan et al., 2007; Venkatesan et al., 2009). Inhibition of DNA synthesis, cell cycle arrest and p53 induction are some of the

mechanisms by which resveratrol imparts its cardioprotective effects (Wang et al., 2006; Zou et al., 2000).

1.14 Overall Objective, Specific Aims And Hypothesis

Irrespective of the recent advance in the diagnostic tools and treatment modalities in the area of HF in the last decade, HF still remains the number one cause of morbidity and mortality in USA and imposes serious global healthcare affliction as well as socio-economic burden. Current pharmacological such as diuretics, inotropic agents, ACE inhibitors, ARBs, aldosterone antagonists, and β-blockers coupled with improved diagnostic features although slow the progression of HF, but have reached their limits in improving patient prognosis (Bristow et al., 1996; Coats, 2002; Colucci et al., 1996). Thus, novel therapies are needed that act independently as well as in combination with abovementioned agents (Bristow, 2000a; From, 1998; Sabbah and Stanley, 2002; Tang and Francis, 2003). Given the importance of oxidative injury in the development and progression of HF, anti-oxidative approach to achieve better cardiac performance sounds reasonably attractive.

Thus, the aim of this study was to determine the antioxidant efficacy of resveratrol on the stressed heart, specifically to illuminate novel pathways leading to the development of cardiac hypertrophy and failure. There were two specific aims:

1. Examine the effect of resveratrol treatment on the development of LV hypertrophy, remodeling, and contractile dysfunction in response to pressure overload.

2. To elucidate the underlying signaling cascades by which resveratrol is able to impart its cardioprotective effect.

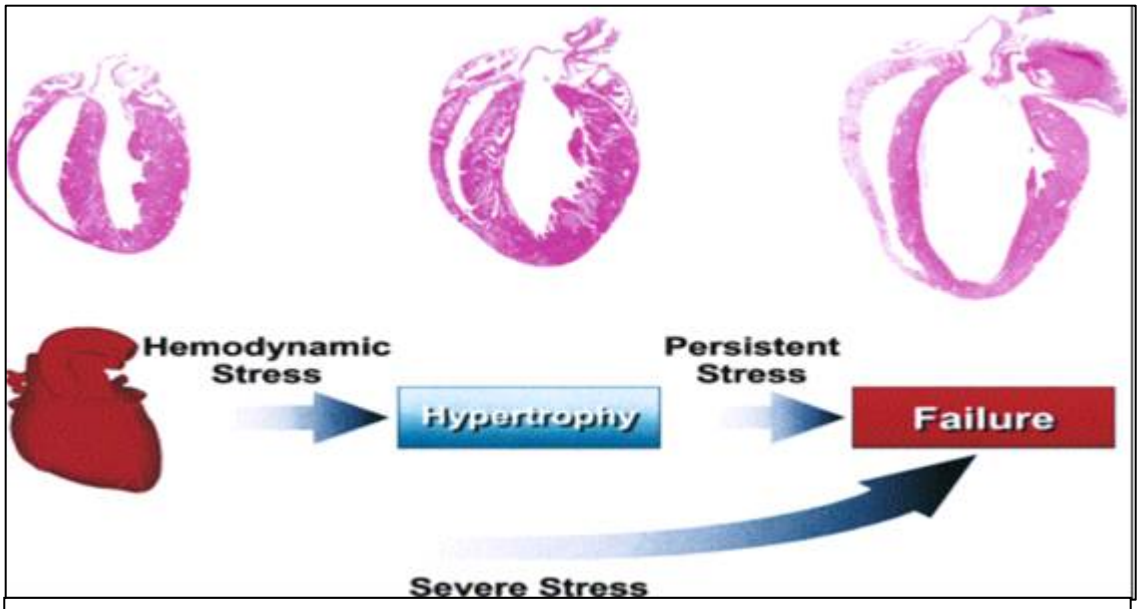

Figure 1.1. Hypothetic natural history of stress-induced heart disease. Depicted four chamber sections of normal murine heart from surgically induced pressure-overload hypertrophy and failure.
Hill, J.A. Electrical remodeling in cardiac hypertrophy. 2003 Trends in Cardiovascular Med.

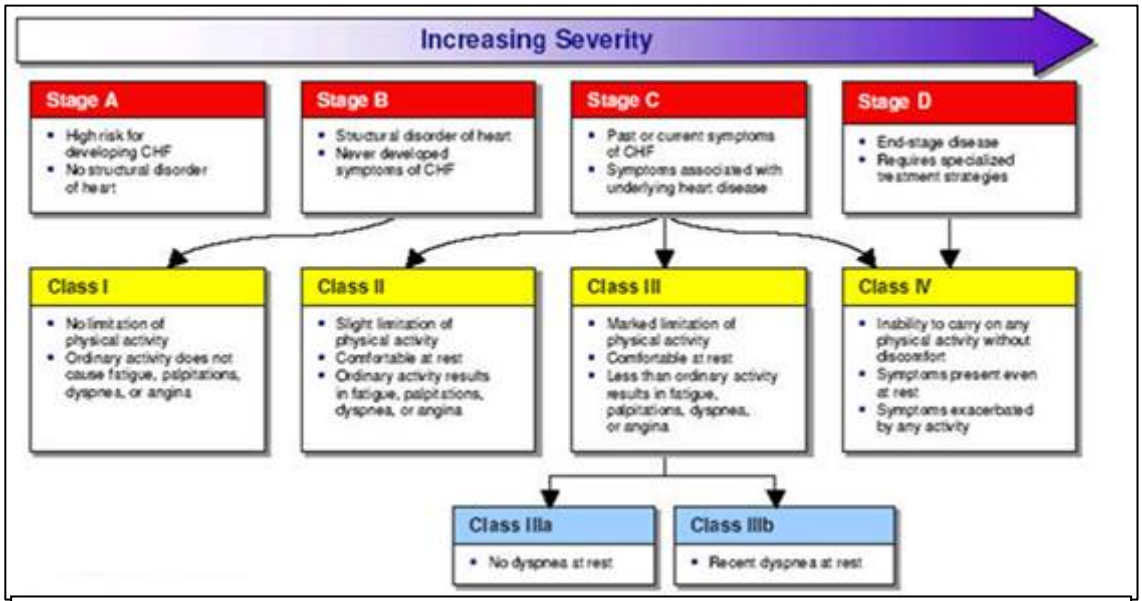

Figure 1.2. Heart Failure Staging: ACC/AHA 2005 guideline for stages of heart failure.

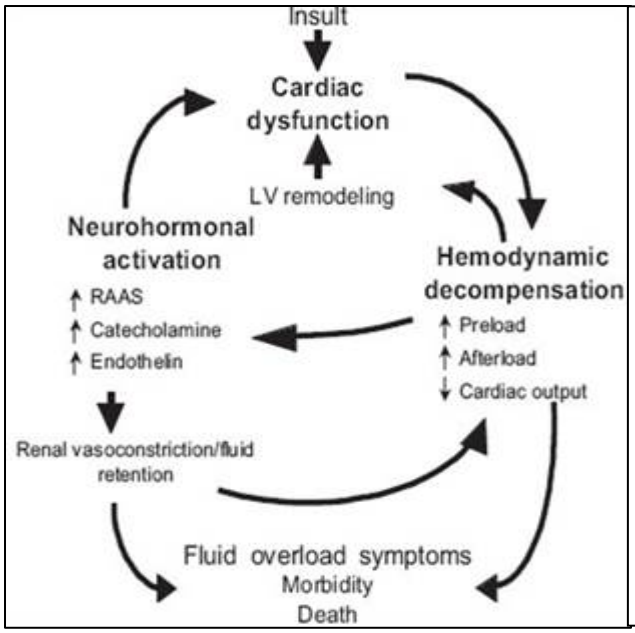

Figure 1.3. Neurohormonal systems in heart failure (HF). A review of the Renal and Neurohormonal Effects of B-type Natriuretic Peptide- Andrew J. Burger.

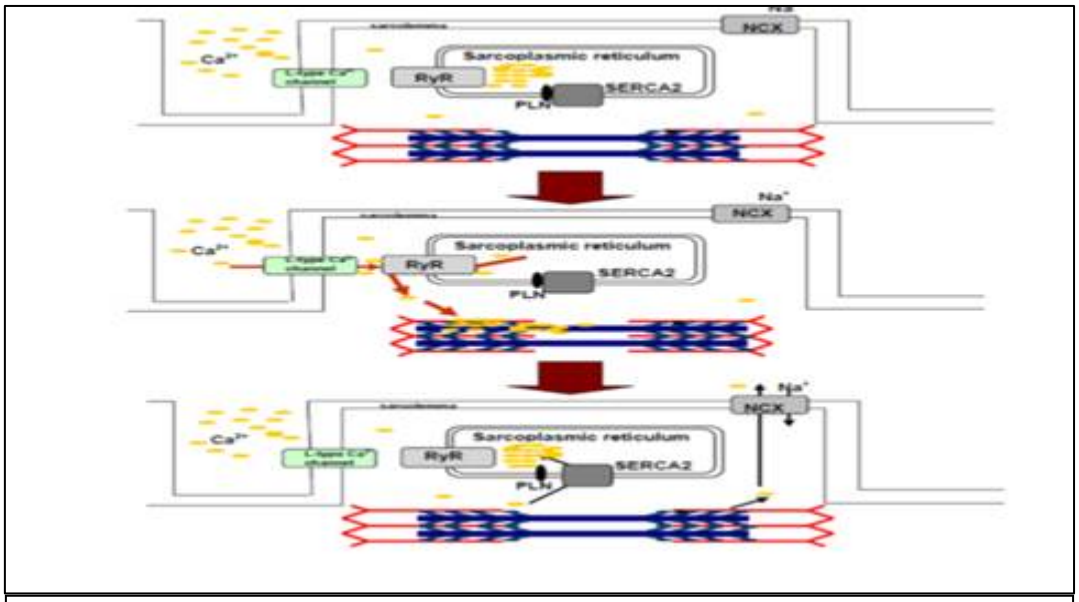

Figure 1.4. Ca2+ is the central regulator of cardiac contractility. As illustrated in the top section, Ca2+ generates signals by changing internal and external concentrations. In the heart there is a large store of Ca2+ in the SR. During contraction (middle section), a small increase of extracellular Ca2+, mediated by the L type Ca2+ channel induces a much larger release of Ca2+ into the cytosol from SR. Increased cytosolic Ca2+ initiates contraction by binding its effector TnC allowing for cross bridge formation. Ca2+ removal in relaxation (bottom section) is carried out primarily by the action of SERCAA2a and NCX, although the extent that each contributes varies between species. Solaro RJ. Regulation of Cardiac Contractility. San Rafael (CA): Morgan & Claypool Life Sciences; 2011.

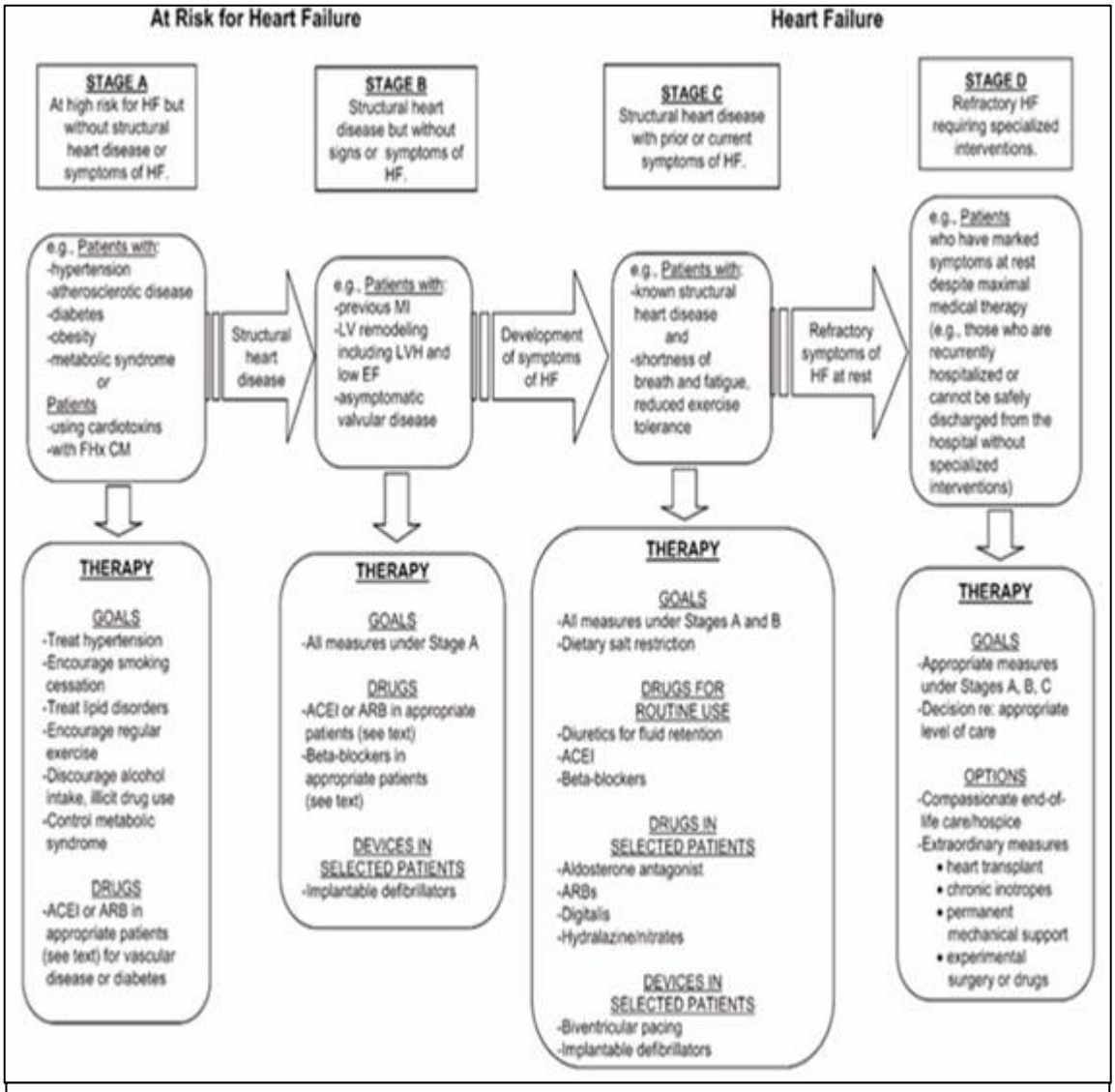

Figure 1.5. Stages in the development of heart failure and recommended therapy by stage.
ACCA/AHA 2005 Guideline for Diagnosis and Management of chronic HF in adult.

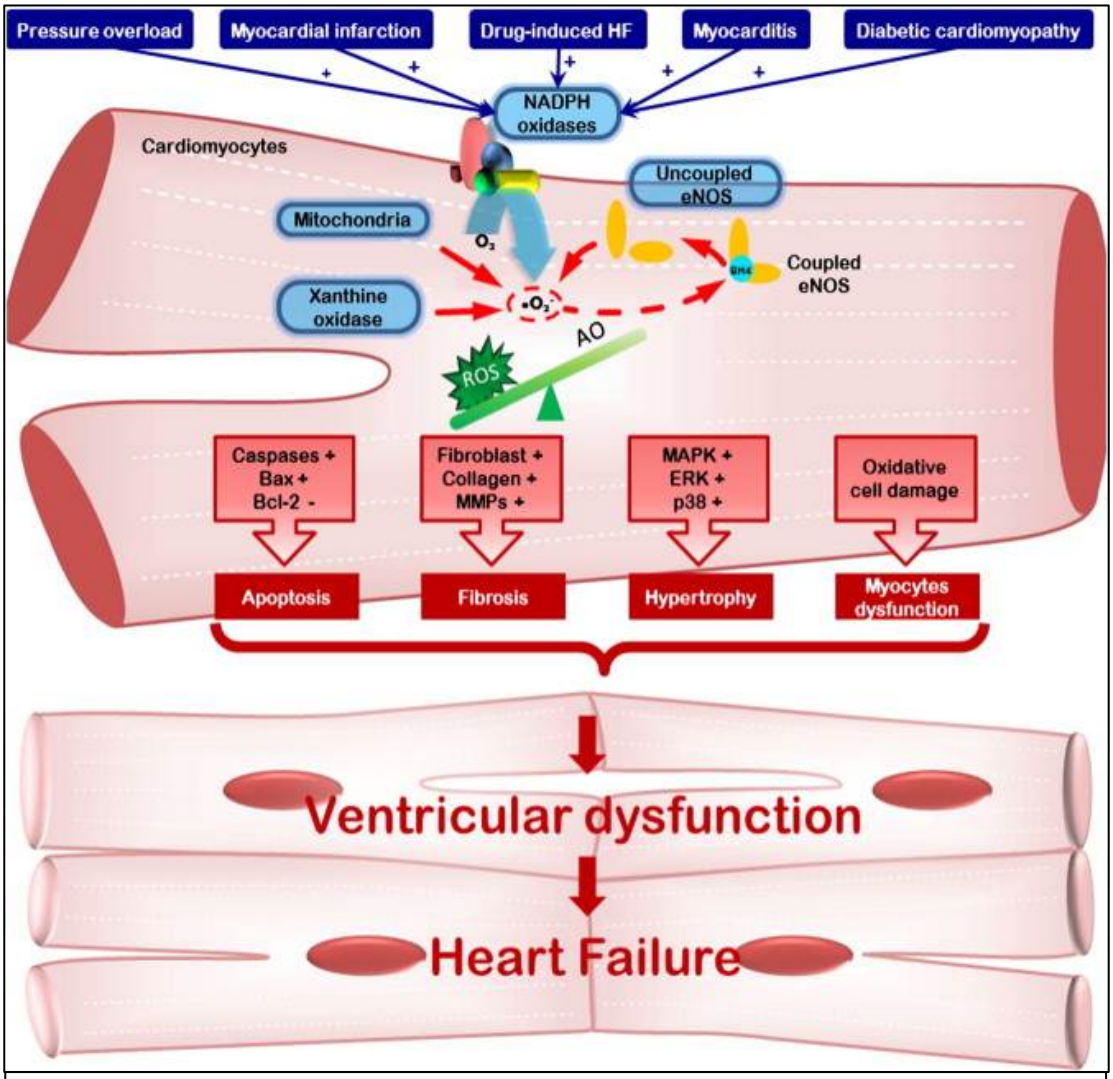

Figure 1.6. NADPH oxidase-dependent oxidative stress in failing heart. From pathogenic roles to therapeutic approach. NADPH oxidases serve as a major contrin=butor in producing reactive oxygen species (ROS) in myocardium and fibroblast. Oxidative stress is caused by increased ROS.
Yanti Octavia et al. Free Radical Biology and Medicine Volume 52, Issue 2. 2012 291-297

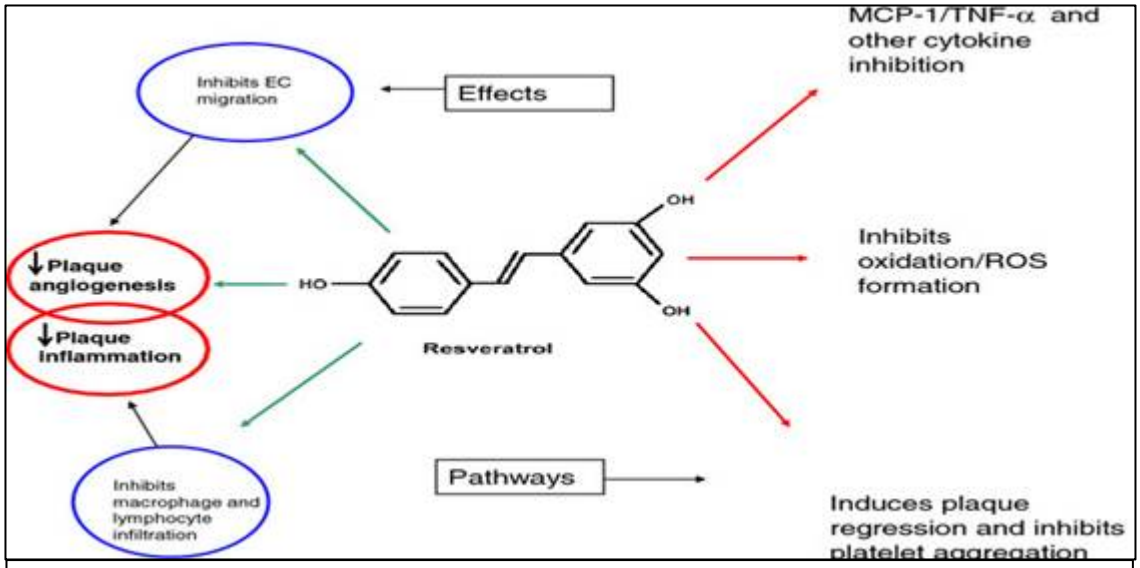

Figure 1.7. Schematic showing structure of resveratrol and some of its most potent effects.
Slevin et al. Vascular Cell. 2012

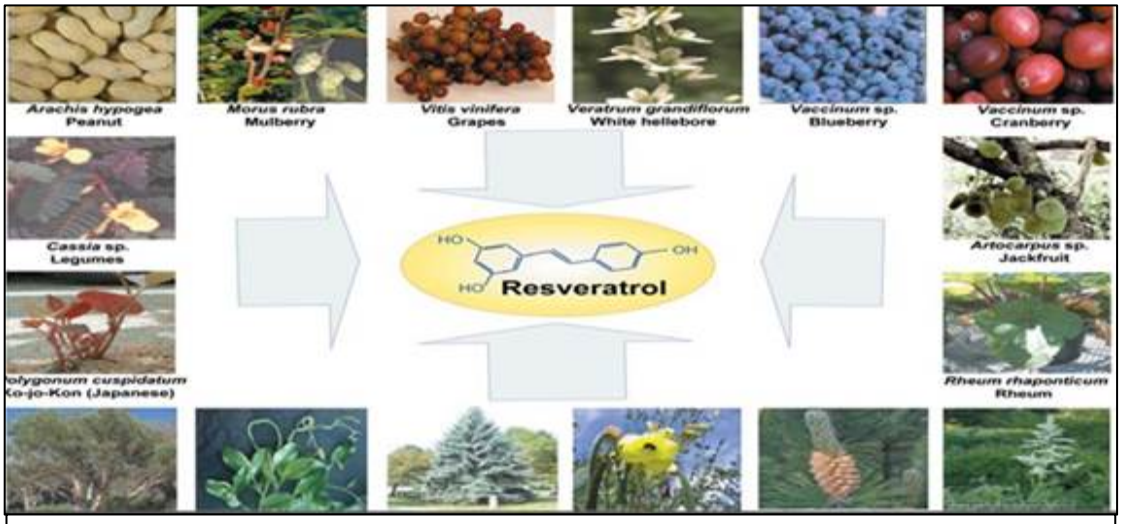

Figure 1.8. Sources of resveratrol. Resveratrol is a natural phytoalexin found in a number of different plants, including grapes, peanuts and mulberries. Resveratrol is produced by these plants in response to stress and fungal infection.
Aggarwal et al. Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies. Anticancer Res. 2004 Sep-Oct;24(5A):2783-840

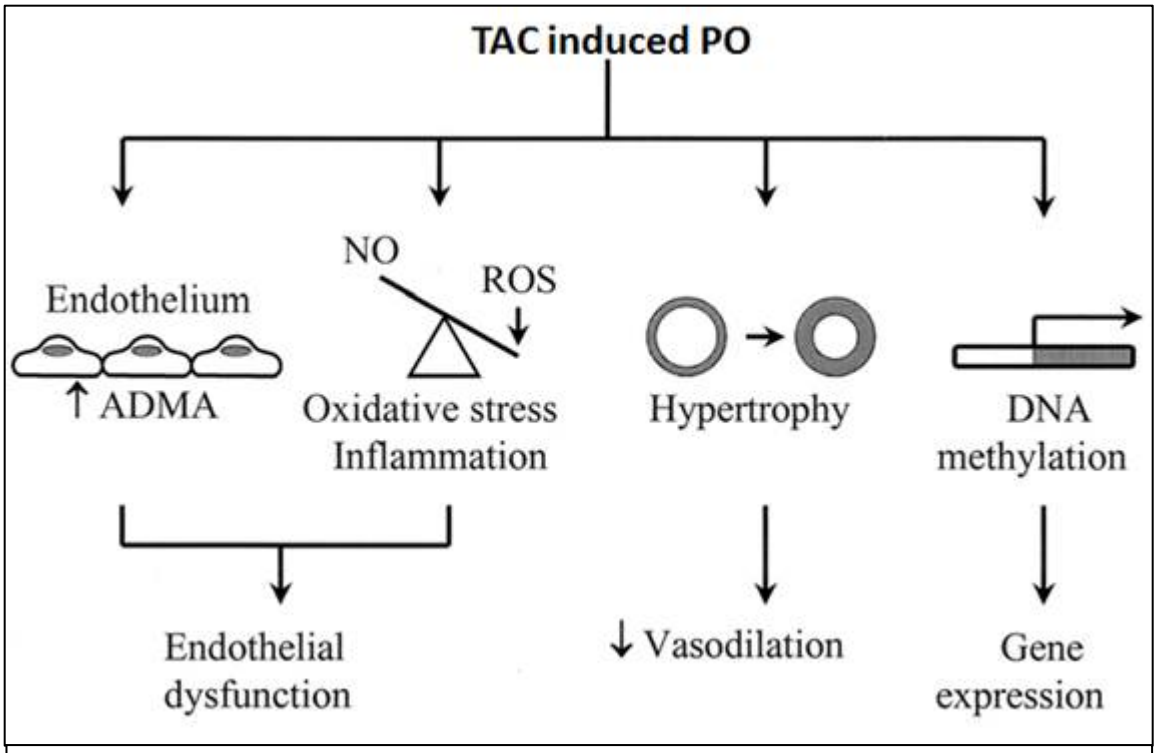

Figure 1.9. A schematic representation of ROS formation and its pathogenic effects. These effects include increased levels of asymmetric dimethylarginine (ADMA), increased formation of reactive oxygen species (ROS), oxidative stress, reduced bioavailability of NO, inflammation, hypertrophy of vascular muscle, and changes in DNA methylation. The resulting changes include impairment of signaling by NO, endothelial dysfunction, reductions in maximum vasodilator capacity, and alterations in gene expression.
Neil Baldwin et al. Advances in stroke. 2003.

Table 1. Effects of resveratrol on different cell signaling pathways.
Huige Li et al. Cardiovascular effects and molecular targets of resveratrol. Nitric Oxide. 2012

Effects of Resveratrol on Different Cell Signaling Pathways	
Activation of adenyl-cyclase pathway	Modulation of metabolism of carcinogens
Activation of ceramide pathway	Modulation of NO/NOS pathway
Activation of p53 pathway	Radioprotective and radiosensitive
Antioxidant effects	Regulation of Egr-1 pathway
Chemosensitization	Suppression of adhesion molecules
Effects on bone cells	Suppression of androgen receptors
Effects on normal cells	Suppression of angiogenesis, invasion and metastasis
Estrogenic/antiestrogenic effects	Suppression of cell cycle proteins
Immunomodulatory effects	Suppression of COX2 and lipooxygenase
Induction of cellular differentiation	Suppression of growth factors and associated kinases
Inhibition of AP-1 signaling pathway	Suppression of inflammatory cytokines and inflammation
Inhibition of MAPK pathway	Suppression of mutagenesis
Inhibition of mitochondrial pathway	Suppression of protein kinases
Inhibition of NF-kappa B signaling pathway	Suppression of PSA
Inhibition of Rb/E2FDP pathway	Suppression of tranformation
Inhibition of the expression of cytochrome p450	Up-regulation of Fas pathway
Inhibition of tubulin polymerazation pathway	

Table 2. Effects of resveratrol in different animal model of cardiovascular disease along with concentration used.

Huige Li et al. Cardiovascular effects and molecular targets of resveratrol. Nitric Oxide. 2012.

Animal model	Effective dose	Resveratrol effects (and possible mechanisms)
Oxidative stress		
Rat trauma hemorrhage	30 mg/kg (single dose, i.v.)	Aortic O_2^-↓, NOX1↓, NOX2↓, NOX4↓, p22phox↓, p47phox↓, HO-1↑ (all preventable with ER antagonist ICI 182, 780)
ApoE$^{-/-}$ mice	30 or 100 mg/kg/d (7 days, p.o.)	Cardiac O_2^-↓; eNOS uncoupling↓ (SOD1↑, SOD2↑, SOD3↑, catalase↑, GPx1↑, GCH1↑, BH_4↑; NOX2↓; NOX4↓)
T2DM db/dbmice	20 mg/kg/day, p.o., for 4 weeks	Mitochondrial DNA content↑; PGC-1α↑; Tfam↑ (aorta)
T2DM mice (Lepr^{db})	20 mg/kg/d (4 weeks, p.o.)	Endothelial function↑; TNF-α↓; NOX2↓; eNOS-P↑
Rats exposed to cigarette smoke	25 mg/kg/d (9 days, p.o.)	Endothelial function↑ (ROS↓)
Ischemic heart disease		
I/R in isolated rat heart	2.5 mg/kg/d (7 days, p.o.)	Myocardial infarct size↓, cardiomyocyte apoptosis↓ (NO↑, HO-1↑; p38 MAPK- and Akt-dependent)
I/R in isolated rat heart	2.5 mg/kg/d (14 days, p.o.)	Myocardial infarct size↓, cardiomyocyte apoptosis↓ (SIRT1↑, SIRT3↑, SIRT4↑; resveratrol > red wine > hydroxytyrosol > white wine > tyrosol)
LAD occlusion-induced MI in rats	1 mg/kg/d (14 days, p.o.)	Infarct size↓ (Trx-1↑ → HO-1↑ → VEGF↑ → neovascularization↑)
LAD occlusion-induced MI in rats	2.5 mg/kg/d (14 days, p.o.)	Cardiac function↑, Stem cell survival and function↑ (oxidative stress↓)
Hypertension and cardiac hypertrophy		
Hypertensive rats (partially nephrectomized)	50 mg/kg/d (4 weeks, p.o.)	Reduction in systolic blood pressure by ~20 mm Hg (NO↑; ET-1↓; Ang II↘)
Hypertensive rats (Ovariectomy)	5 mg/kg/d (3 weeks, p.o.)	Reduction in systolic blood pressure by 15% (endothelial function↑)
Fructose-fed rats	10 mg/kg/d (45 days, p.o.)	Blood pressure↓; cardiac hypertrophy↓; vascular eNOS activity↑; TBARS↓
Spontaneously hypertensive rats	2.5 mg/kg/d (2 weeks, p.o.)	Left ventricular hypertrophy↓ (4-HNE↓; LKB1↑; AMPK↑; mTOR/p70S6↓; blood pressure↘)
Rat aortic banding	2.5 mg/kg/d (2 weeks, p.o.)	Centric remodeling↓, contractile function↑ (eNOS↑; iNOS↑; ref-1↑)
Rat aortic banding	2.5 mg/kg/d (26 days, p.o.)	Pressure overload-induced concentric hypertrophy↓
Rat aortocaval shunt	2.5 mg/kg/d (26 days, p.o.)	Volume overload-induced eccentric hypertrophy↔
TO-2 hamster heart failure	145 mg/kg/d (35 weeks, p.o.)	Cardiac fibrosis↓, cardiac function↑, survival↑ (cardiac SOD2↑)
Mouse diabetic cardiomyopathy	100 mg/kg/d (1 or 3 months, p.o.)	Cardiac function↑; survival↑ (SERCA2a↑; SIRT1 expression↑; blood glucose↘)
Atherosclerosis and intimal hyperplasia		
Hypercholesterolemic rabbits	3 mg/kg/d (12 weeks, p.o.)	Atherosclerotic lesions↓ (endothelial function↑; no effects on plasma lipid levels)
ApoE$^{-/-}$ mice	0.02% or 0.06% w/w in chow (20 weeks)	Atherosclerotic lesions↓ (total cholesterol↓; LDL↓; HDL↑; plasma PON activity↑; hepatic HMG-CoA reductase activity↓; ICAM-1↓, VCAM-1↓
Rabbit intimal hyperplasia	4 mg/ kg/d (5 weeks, p.o.)	Intimal hyperplasia↓ (DNA synthesis↓)
Mouse intimal hyperplasia	50 mg/kg, p.o., three times a week for 21 days	Neointima formation↓ (HO-1↑)
Mouse intimal hyperplasia (♀)	50 mg/kg/d (2 + 2 weeks, p.o.)	Neointima formation↓ (ERα- and NO-dependent); eNOS↑, iNOS↑, NO↑, BH_4↑, GCH1↑

CHAPTER 2

Material and Methods

2.1 Pressure Overload Model

For the pressure-overload model, eight to ten weeks old (26-28 g) C57/ BL6 male mice were used in this study. (Harlan Sprague Dawley, USA). Surgical details of the TAC procedure are described elsewhere (Wojciechowski et al., 2010). Briefly, mice were kept in a temperature- and humidity-controlled room with a 12-h light:12-h dark cycle for 1 week before creation of the PO model. Standard mice chow and tap water were available ad libitum. All mice were anesthetized for surgeries with 5% isoflurane carried by oxygen at a flow rate of 2 l/min. Mice were then maintained in surgical plane of anesthetic with 2% isoflurane. Hair on the surgical area was removed using hair remover lotion and incision site was disinfected using 100% alcohol and iodine.

A midline thoracotomy was performed at the level of the suprasternal notch. Gently the ribs were retracted and isthmus was separated allowing direct visualization of the transverse aorta without entering the pleural space thereby avoiding the need for mechanical ventilation. A fine needle and 7-0 silk suture was used to pass under and around the transverse aorta between the right innominate and left common carotid arteries. A piece of 27-gauge needle piece was placed over the transverse aorta and quickly a knot was secured to the diameter of a 27-gauge needle yielding a 70-80% constriction. The needle and 7-0 silk suture was the retracted. Successful bands were

snug while blood flow to the brain and body was maintained. The sternum, thoracic musculature and the skin incisions were closed by standard techniques with absorbable suture and auto clips.

Sham operations on sex- and age-matched mice underwent exact same procedure as TAC with the exception of actual aortic banding and served as a control for all experimental groups. At 24 hours after surgery aortic-banded mice were randomly divided into two groups. One group of aortic-banded mice were administered resveratrol (100 mg·kg body wt−1·day−1) by oral gavage for a period of 4 wk while sham operated and the other group of aortic banded mice were given vehicle treatment (0.5ml water). All the three groups (sham-operated, aortic-banded; resveratrol- treated,) were maintained for a total of 4 wk. (Figure 2.1)

2.2 Echocardiographic Assessment of Cardiac Structure and Function

Two-dimensional-guided (2D) M-mode echocardiography of the mice was performed on days 0, 7, 14, 21 and 28, using Vevo 770 High-Resolution Imaging System with a 37.5-MHz high-frequency linear transducer (VisualSonics Inc. Toronto, ON, Canada) as previously described (Xing et al., 2012). Briefly, mice were anesthetized with 3% isoflurane and maintained with 1.5% isoflurane in room air supplemented with 100% O2. After the anterior chest was shaved, the animals were placed on a warming pad to maintain normothermia. Echocardiographic gel was warmed prior to use in order to avoid hypothermia. Care was taken to avoid excessive pressure on the thorax, which can induce bradycardia and result in severe deterioration of functional parameters and or even death. Two-dimensionally (2D) long axis images of left ventricle (LV) were acquired at the level of the aortic and mitral where the LV cavity is largest allowing adequate

visualization of the LV apex. Echocardiographic recording of anterior and posterior LV wall was done in M-mode at the speed of 21 frames per second. Images were acquired at the level of the papillary muscle tips, and measurements were then performed to obtain the LV internal dimension during systole (LVIDs; in mm), the LV internal dimension during diastole (LVIDd; in mm), LV posterior wall thickness during systole (LVPWs; in mm) and LV posterior wall thickness during diastole (LVPWd; in mm) according to the leading-edge method of the American Society of Echocardiography. LV percent ejection fraction (EF) and fractional shortening (FS) was calculated via VisualSonics Measurement Software.

2.3 Isolation Of Mice Heart.

After the 4-wk echocardiographic assessment, mice from all groups were weighed and anesthetized by using isoflurane before being euthanized. Toe pinch was performed to make sure the mice were totally unresponsive. Hearts and lungs were quickly isolated and washed in ice-cold saline and the wet weight of the heart (HW) and wet weight of the lung (LW) were measured as indices of cardiac hypertrophy and lung edema. Tissue was separated, flash-frozen in liquid nitrogen, and subsequently stored at −85°C until further experimentation.

2.4 Preparation Of The Homogenate.

LV tissue was pulverized and homogenized in a buffer containing 10 mM NaHCO3, 5 mM NaN3, and 15 mM Tris·HCl at pH 6.8 (10 ml/g tissue). This was aliquoted and frozen in liquid nitrogen before storage at −85°C. The buffer used for LV tissue homogenization also contained a cocktail of protease inhibitors consisting of (in

μM) 1 leupeptin, 1 pepstatin, and 100 phenylmethylsulfonyl fluoride to prevent protein degradation during the procedure.

2.5 Histological And Immunochemical Analysis

Hearts were exercised and washed with ice-cold 0.9% saline, fixed in 4% paraformaldehyde, and were embedded in paraffin. 5 μm thick paraffin sections were prepared using (Leica RM2030, rotary microtome) and stored at room temperature until further staining. Immunohistochemistry involved the use of microwave based antigen retrieval process. Using three changes of xylene and five chances of alcohol at varying concentration, sections were deparaffinized and the Texas Red-X conjugated wheat germ agglutinin (WGA) (Invitrogen Corp., Carlsbad, CA) staining was done to stain the membranes to acquire left ventricular cardiomyocyte cross-sectional area (CSA) as described elsewhere (Xing et al., 2012). Images were acquired by observing slides under the fluorescence microscope (Nikon Eclipse E600; Nickon In, Melville, NY) at 400 × magnification. By using Q capture software (MAG Corp., Pleasanton, CA), twenty fields of each section were randomly photographed and cardiomyocyte area was measured using Image-Pro Plus software (Media Cybernetics, Inc., Bethesda, MD).

PO induced myocardial fibrosis was assessed by staining the sections for collagen with Masson's Trichrome Kit (Poly Scientific, Bay Shore, NY). Myocardial sections from LV were deparaffinized, rehydrated and stained with products supplied in the kit according to the protocol provided by the manufacturer. Images were acquired under the light microscope (Nikon Optiphot-2; Nikon Inc., Melville, NY) at 200 × magnification. The relative fibrotic area (% of total area) was averaged from 20 fields of each section that were randomly photographed.

To access the extent of damage caused by inflammatory response, sections were stained for macrophage. Sections were deparaffinized, rehydrated and rat anti-mouse macrophage antibody Mac-2 primary antibody (1:200 dilution) (cedarlane,NC) along with staining kit (Immunocruz ABC staining system, santa cruz) and corresponding secondary antibody was used according to the protocol provided by the manufacturer. Sections were observed under light microscope (Nikon Optiphot-2; Nikon Inc., Melville, NY) at 200 × magnification. Twenty fields of each section were randomly photographed using Axio Vision 3.1 software (Carl Zeiss Inc., Maple Grove, MN). The number of (the brown stained cells) was counted by Image-Pro Plus software (Media Cybernetics, Inc., Bethesda, MD), and quantitative assessment of macrophage density was performed by counting the number of Mac-2 immunopositive cells and expressed as percentage change relative to control.

Similarly staining for mast cells as a marker of inflammatory response was done using Toluidine blue O (Sigma Aldrich). The sections were stained with 0.1% toluidine blue for mast cell identification. Paraffin sections were deparaffinized, hydrated, and rinsed in deionized water according to the protocol provided by the manufacturer. Briefly, sections were placed in 1% acid alcohol for 3–5 min, followed by a transfer to 0.5% toluidine blue solution for 15 min. The sections were then rinsed in deionized water, dehydrated in acetone, and cleared in acetone-xylene. Finally, the sections were mounted in permount and coverslipped. Images were acquired by observing under light microscope (Nikon Optiphot-2; Nikon Inc., Melville, NY) at 200 × magnification. Twenty fields of each section were randomly photographed using Axio Vision 3.1 software (Carl Zeiss Inc., Maple Grove, MN). The number of (the blue stained cells) was

counted by Image-Pro Plus software (Media Cybernetics, Inc., Bethesda, MD). Mast cell density was assessed by counting all mast cells identified per section and expressed as average mast cell number in percentage change relative to control.

Staining of 4-Hydroxy-2-Nonenal (4-HNE), a marker of lipid peroxidation, was performed with a mouse anti 4-HNE antibody (ab48506, Abcam Inc., Cambridge, MA) according to the protocol recommended by the manufacturer. Elaborate procedure is described elsewhere (Xing et al., 2012). Briefly, 5 µm thick paraffin sections were prepared using (Leica RM2030, rotary microtome) and stored at room temperature until further staining. Using three changes of xylene and five chances of alcohol at varying concentration, sections were deparaffinized. Immunohistochemistry involved the use of microwave based antigen retrieval process for 30 minute in citrate buffer. Sections were blocked using 5% serum at 20 degree for 20 minutes followed by incubation with primary antibody (1/25 dilution) for overnight at 4 degree and corresponding secondary antibody was used. Cardiomyocytes were stained with rabbit anti-tropomyosin I(ab55915, Abcam Inc, Cambridge, MA) and nuclei were stained with DAPI. Images were acquired by observing slides under the fluorescence microscope (Nikon Eclipse E600; Nickon In, Melville, NY) at 400 × magnification. By using Qcapture software (MAG Corp., Pleasanton, CA), twenty fields of each section were randomly photographed and quantification was done using Image-Pro Plus software (Media Cybernetics, Inc., Bethesda, MD). Relative fluorescent intensity was semi-quantified and represented as integrated optical density (IOD) with respect to the area measured.

Staninig for 8- hydroxydeoxyguanosine (8-OHdG), a marker of DNA oxidization, was performed with a mouse anti 8-OHdG antibody (sc-660369, Santa Cruz

Biotechnology, Inc., Santa Cruz, CA), according to the protocol of the manufacturer. Detailed method is described elsewhere (Xing et al., 2012). Briefly, 5 µm thick paraffin sections were prepared using (Leica RM2030, rotary microtome) and stored at room temperature until further staining. Using three changes of xylene and five chances of alcohol at varying concentration, sections were deparaffinized. Immunohistochemistry involved the use of microwave based antigen retrieval process for 30 minute in citrate buffer. Sections were blocked using 5% serum at 20 degree for 20 minutes followed by incubation with primary antibody (1/25 dilution) for overnight at 4 degree and corresponding secondary antibody was used. Cardiomyocytes were stained with rabbit anti-tropomyosin I(ab55915, Abcam Inc, Cambridge, MA) and nuclei were stained with DAPI. Images were acquired by observing slides under the fluorescence microscope (Nikon Eclipse E600; Nickon In, Melville, NY) at 400 × magnification. By using Qcapture software (MAG Corp., Pleasanton, CA), twenty fields of each section were randomly photographed and quantification was done using Image-Pro Plus software (Media Cybernetics, Inc., Bethesda, MD). Relative staining area was quantified and represented as integrated optical density (IOD) with respect to the area measured.

TUNEL staining on tissue sections by using In Situ Cell Death Detection Kit was done to access apoptosis, TMR red (Roche Applied Science, Indianapolis, IN) according to the protocol provided by the manufacturer. Detailed procedure is described elsewhere (Xing et al., 2012). Briefly, the sections were deparaffinized, rehydrated, microwaved for 30 minutes for antigen retrieval and incubated with 50 µl TUNEL Reaction Mixture at 37oC for 1 hr. A section was digested for 30 minutes with DNase (RNase-Free DNase Set, QIAGEN Inc., Valencia CA) for positive control and a negative control section was

only incubated with labeling solution. The apoptotic nuclei were labeled with TUNEL (red) all nuclei were counterstained with DAPI (blue) (Invitrogen Corp., Carlsbad, CA), and the F-actin was stained by Alexa Fluor® 488 phalloidin (green) (Invitrogen Corp., Carlsbad, CA). Images were acquired by observing slides under the fluorescence microscope (Nikon Eclipse E600; Nickon In, Melville, NY) at 400 × magnification. By using Qcapture software (MAG Corp., Pleasanton, CA), twenty fields of each section were randomly photographed and quantification was done using Image-Pro Plus software (Media Cybernetics, Inc., Bethesda, MD). TUNEL positive cells were quantified as a percent of all nuclei in the section of LV.

2.6 Western Blotting And Protein Activity

The protein content of sodium oxide dismutase (SOD) was measured by SOD assay kit (Sigma, Switzerland) and Glutathione synthase (GSH) was measured by GSH-Glo Glutathione assay kit (Promega, Madison, WI) according to the protocol provided by the company. 20/20 luminometer (Turner BioSystems, Sunnyvale, CA) was used to detect the total GSH activity while spectra max plus (Sunnyvale, CA) was used to detect the total SOD activity. Myocardial protein content of cleaved caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA), hypoxia inducible factor 1alpha (Hif-1α) (Novus Biologicals, Littleton, CO) from LV homogenates were determined by Western blot analysis as described previously (DiPette et al., 1989; Latronico et al., 2008; Zhang et al., 2007). Briefly, total protein from LV tissue was extracted by T-PER tissue protein extraction reagent (Thermo Scientific, Rockford, IL). Protein samples (25-30 μg) were fractionated by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to nitrocellulose membranes. The membranes were probed with corresponding primary antibodies. Appropriate HRP-conjugated secondary antibodies

were used and the antibody-antigen complexes in all membranes were detected by the ECL PLUS Detection Kit (Thermo Scientific, Rockford, IL). The expression of these proteins was quantified with Scion Image (NIH) and adjusted to β-actin.

2.7 Statistical Analysis

Data were expressed as mean ±SD. Differences among groups were tested by one-way ANOVA followed by Bonferroni's multiple comparison post hoc test. A value of $p<0.05$ was considered significantly different.

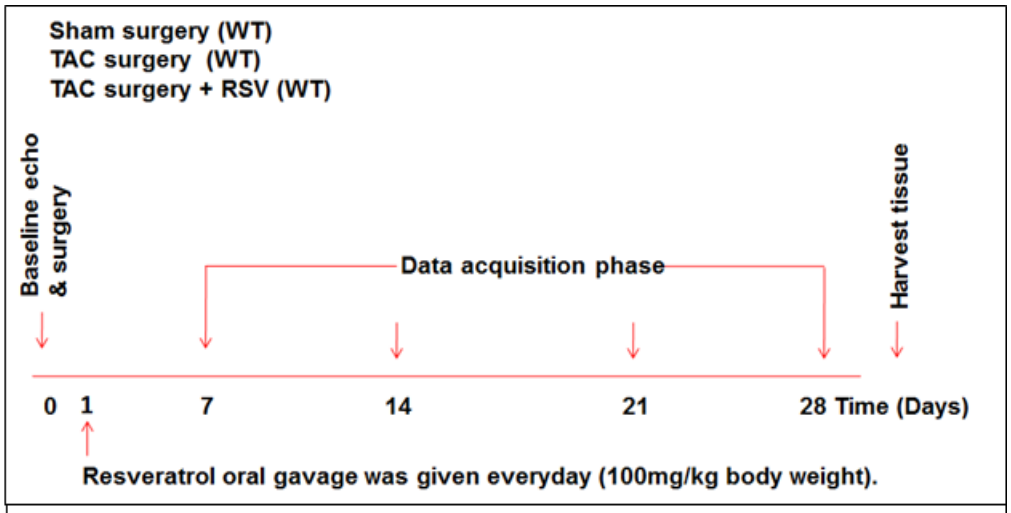

Figure 2.1. Protocol layout of resveratrol treatment and echocardiographic assessment following TAC.

CHAPTER 3

RESULTS

3.1 Hemodynamics, Survival Rate, Body Weight, Heart Weight And Heart Rate Prior To TAC

There were no operative deaths within 24 h after TAC or SHAM operation. Baseline blood pressure was taken by tail cuff method (MC4000 Blood pressure analysis system). No significant difference in the systolic blood pressure was observed among the three groups. Similarly no significant difference in the heart rate was found among the three groups. There was no significant increase in mortality of any of the group. However autopsy revealed that mice that died before 4 weeks of TAC had apparent signs of heart failure (data not shown).

3.2 Assessment Of Body, Heart And Lung Weights After TAC.

Analysis of surviving mice after 4 weeks of chronic pressure overload showed that heart weight (HW) in ratio of body weight (BW) were significantly greater in TAC mice compared to sham-operated mice (4.9 ±0.3 vs 8.8 ±1.1). Similarly the ratio of lung weight to body weight (LW/BW) was also found to be significantly higher in TAC mice compared to sham. and (4.80 ±0.53 vs 10.28 ±1.4). The increase in (HW/BW) and (LW/BW) ratios after TAC were significantly attenuated in the TAC+RSV treatment group (7.2 ±0.3) and (6.2 ± 1.8) . Figure 3.1

3.3 Resveratrol Treatment Improves Cardiac Function

Echocardiography clearly demonstrated ventricular hypertrophy and LV dysfunction induced by pressure overload (PO) in (TAC) mice. Figure 3.2 shows typical representation of the echocardiogram showing cardiac dimensions whereas Figure 3.3A-F illustrates representative echocardiograms from the three groups taken over the course of 4 weeks.

Cardiac structure assessment involved measurements of LVID, LVPW, IVS, %EF and %FS at both systole and diastole. After 4 weeks of TAC, LV internal dimensions during diastole (LVID;d) was significantly increased in TAC mice (5.16±0.26) compared to sham operated (4.08 ±0.10). Resveratrol treatment was able to significantly attenuate this adverse parameter (4.39 ±0.12). Similarly LV internal dimension during systole (LVIDs) were significantly increased in TAC mice compared to sham-operated. (3.4±0.03 vs 2.46±0.09). Remarkably, diastolic impairment was significantly attenuated with resveratrol treatment in TAC+RSV group (2.72±0.08). LV systolic function, as assessed by LV ejection fraction (%EF), fractional shortening (%FS) were significantly decreased in the TAC mice to a greater extent relative to sham-operated mice.(64.2 ±2.0 VS 33.13 ±1.87 and 46.46 ±2.41 vs 26.15 ±1.97) Interestingly, resveratrol treatment significantly attenuated the sharp decline of both parameters (52.4 ±4.37 and 35 ±2.63) and maintained cardiac performance.

3.4 Attenuation Of Cardiac Hypertrophy And Fibrosis By Resveratrol.

LV hypertrophy assessed by WGA staining was significantly greater in TAC mice (221.7 ±21.43) compared to sham treated (108.02 ±12.85). However the degree of hypertrophy was significantly attenuated in RSV treated mice (187 ±11.9) as compared to

TAC alone. Figure 3.4A. Similarly LV interstitial and perivascular fibrosis as determined by Masson's trichrome staining was found to be significantly higher in TAC mice (2.23 ±0.3 and 0.87 ±0.1) respectively as compared to sham treated mice (0.28 ±0.05 and 0.10 ±0.06). However this increase in fibrosis was significantly attenuated in RSV treated group as compared to TAC alone (1.29±0.20 and 0.65±0.13). Figure 3.4 B

3.5 Anti-inflammatory Activity Mediated By Resveratrol

Macrophage infiltration was found to be significantly increased after 4 weeks of TAC (14.8 ± 0.06) compared to sham operated (1.71 ± 0.01). Treatment with resveratrol was found to attenuate macrophage infiltration in the heart of TAC+RSV (10.43 ± 0.03) mice as compared to TAC alone. Figure 3.5 A

Similarly pressure overload induced increase in the number of cardiac mast cells were significantly higher in TAC group (12.5 ± 0.03) compared to sham operated (2.56 ± 0.02). However treatment with resveratrol was found to attenuate mast cells infiltration as well in the heart of mice as compared to TAC alone (7.96 ± 0.03). Figure 3.5 B

3.6 Resveratrol Attenuates Oxidative Damage In TAC Induced Heart.

Anti-oxidative property of resveratrol in hearts of TAC induced PO mice was assessed by measuring myocardial biomarker expression of 4-hydroxynonenal (4-HNE) which is a marker of oxidative index of lipid peroxidation, and 8-hydroxydeoxyguanosine (8-OHdG), a marker of DNA damage induced by oxidative stress. These myocardial biomarkers of oxidative stress 4HNE were significantly increased in the mice that underwent TAC procedure (18.4±3.1%) relative to sham surgery (3.38±1.0%). Resveratrol treatment however, was able to attenuate the upregulation of 4-HNE in the TAC+RSV group (14.4±1.9%). Figure 3.6 A

Similarly, the oxidative marker 8-OHdG level was sharply upregulated in the TAC mice (22.7±3.1%) compared to sham counterparts (2.8±1.0%). Again, treatment with resveratrol was able to significantly attenuate this pathological damage in TAC+RSV group (16.22±0.9%). Figure 3.6 B

3.7 Resveratrol Prevents Cardiomyocyte Apoptosis

Decrease in the LV function with chronic pressure overload induced LV hypertrophy may be mediated by excessive apoptosis resulting in a reduction in total contractile units and LV mass. TUNEL immunohistochemical shaining was performed to access the cardiomyocyte apoptotic death. Treatment with resveratrol may prevent myocyte apoptosis thereby maintaining LV mass. As expected, the number of apoptotic nuclei in the heart of TAC mice (0.13±0.01) was significantly greater than in sham operated mice (0.01±0.007). Resveratrol treatment was able to successfully attenuate the ongoing apoptosis to a significant level (0.09±0.01) relative to untreated group. Figure 3.7

3.8 Resveratrol Protects Heart Failure By Inhibiting Hypoxia And Inducing Antixoidative Enzymes.

Interestingly, in line with the maintained attenuation of elevated TUNEL positive nuclei, myocardiac level of cleaved caspase-3 was significantly also found to be significant up-regulated in TAC mice (177±3.2) compared to sham operated (154±3.1). attenuated in the TAC+RSV group(166±7.9) Figure 3.8. The results between western blot and immunostaining show a strong positive correlation.

In addition, we analyzed the cardiac expression of the angiogenic factor HIF-1α. It has been reported that VEGF and HIF1α is up-regulated in the pressure overload-

induced hypertrophic WT hearts (Hilfiker-Kleiner et al., 2005; Sano et al., 2007). In the TAC WT hearts there was a significant increase in HIF-1α (176±10) levels as compared to sham-operated mice (83.2±4.4). The increase in HIF-1α may be due to high demand of oxygen to hypoxic heart and may play a role in the increase in capillary density through VEGF. Treatment with resveratrol however attenuated the HIF-1α level in TAC+RSV group (115±7.4) suggesting resveratrol may be mediating anti-hypoxic activity through other pathway. Figure 3.8

Likewise the levels of antioxidative enzymes such as SOD and GSH was found to be sharply declined in the TAC mice (7.3±0.9 U/mg and 5.2±0.2 nM/mg) compared to sham surgery(10.7±1.3 U/mg and 8.5±0.27 nM/mg) . Resveratrol was able to successfully attenuate this decline to a significant extent in RSV treatment group relative to TAC alone (9.4±0.4 U/mg and 6.69±0.32 nM/mg). Figure 3.9

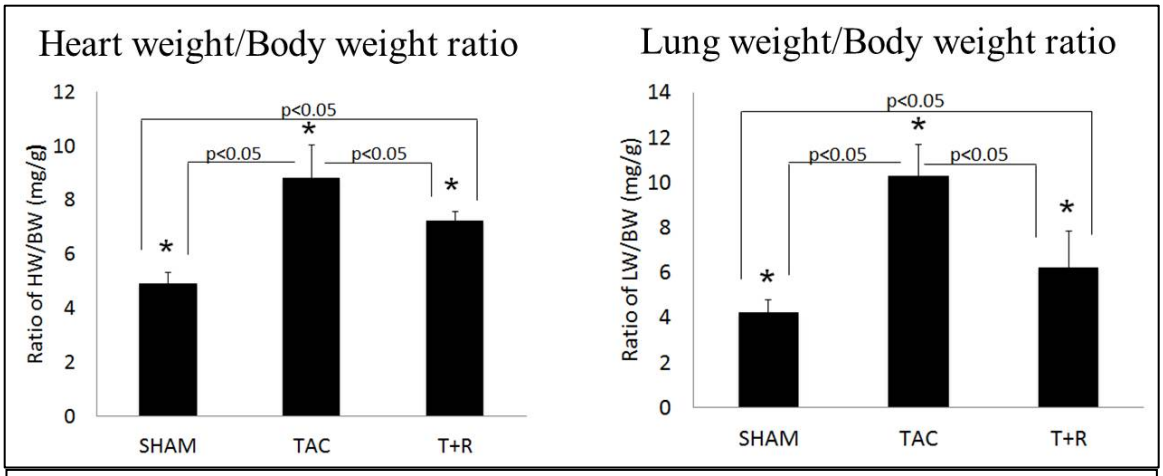

Figure 3.1. Assessment of body, heart and lung weight after TAC. Groups (n=7/group) of WT mice were subjected to either SHAM or TAC surgery. Wet weight of heart and lung was recorded as an index of cardiac hypertrophy and heart failure at day 28 post surgery. (A), Heart weight/body weight (HW/BW) ratio (B) Lung weight/body weight ratio (LW/BW). Both HW/BW and LW/BW ratio were found to be significantly increased in TAC mice compared to SHAM. Resveratrol treatment however attenuated this pathological change. Values are expressed as mean±SD. $p<0.05$ was considered statistically significant.

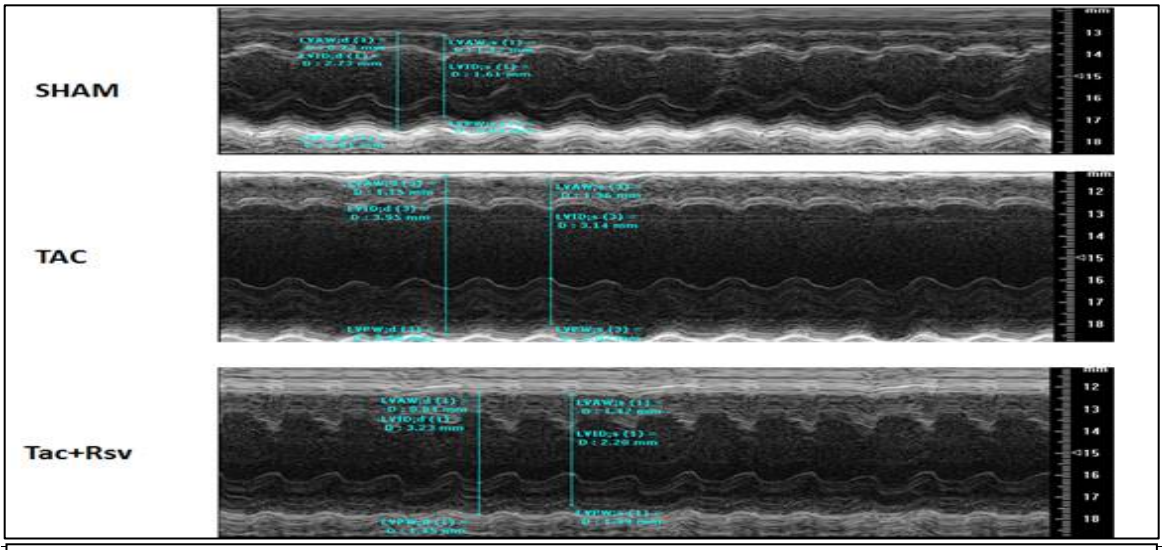

Figure 3.2: Representative image acquired from 2D echocardiogram (Vevo 770) showing the internal dimentions of LV. LVAD, LVPW, LVID parameters were compared between sham operated,TAC and TAC+RSV treated at 0,7,14, 21 and 28 day respectively.

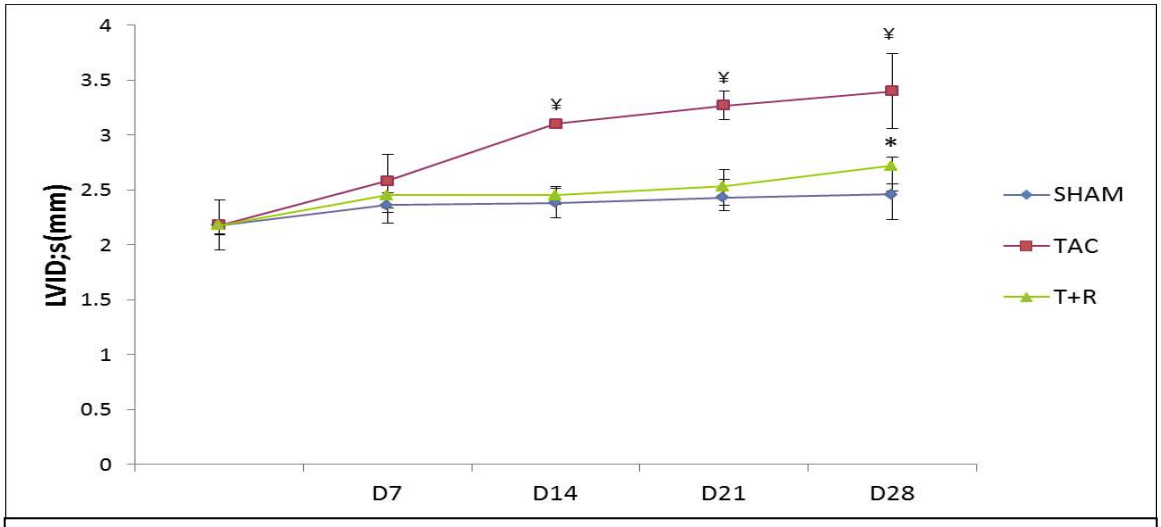

Figure 3.3. A. Resveratrol treatment attenuates cardiac contractile dysfunction in PO mice. Groups (n=7/group) of WT mice were subjected to either SHAM or TAC protocol and parameter for left ventricular inter diameter during systole was obtained at given time points. Values are expressed as the mean ± SD. * p<0.05 vs SHAM, ¥ P<0.05 vs T+R.

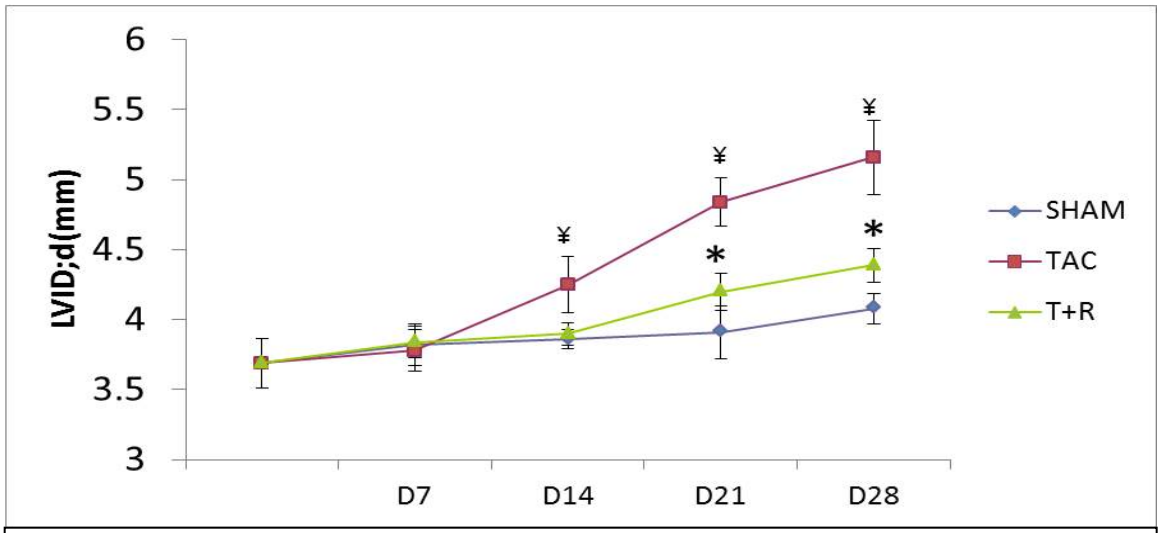

Figure 3.3. B. Resveratrol treatment attenuates cardiac contractile dysfunction in PO mice. Groups (n=7/group) of WT mice were subjected to either SHAM or TAC protocol and parameter for left ventricular inter diameter during diastole was obtained at given time points. Values are expressed as the mean ± SD. * p<0.05 vs SHAM, ¥ P<0.05 vs T+R.

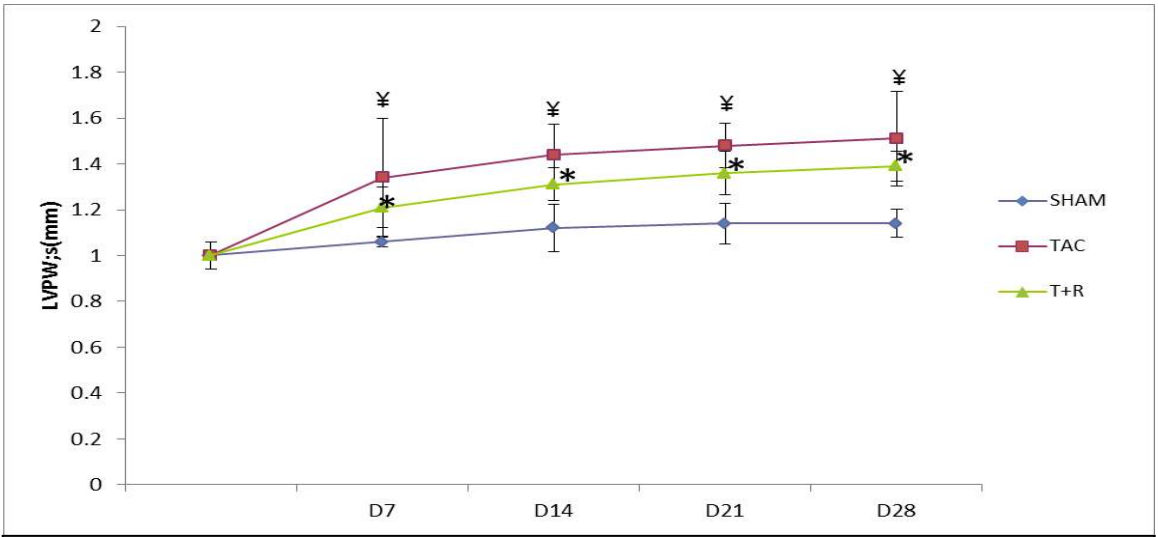

Figure 3.3. C. Resveratrol treatment attenuates cardiac contractile dysfunction in PO mice. Groups (n=7/group) of WT mice were subjected to either SHAM or TAC protocol and parameter for left ventricular posterior wall thickness during systole was obtained at given time points. Values are expressed as the mean ± SD. * p<0.05 vs SHAM, ¥ P<0.05 vs T+R

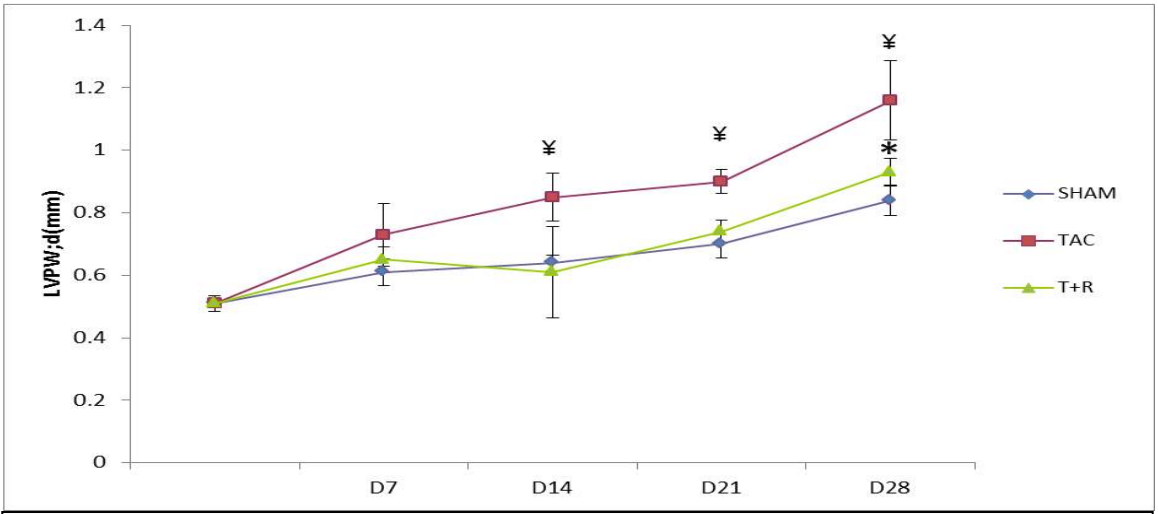

Figure 3.3. D. Resveratrol treatment attenuates cardiac contractile dysfunction in PO mice. Groups (n=7/group) of WT mice were subjected to either SHAM or TAC protocol and parameter for left ventricular posterior wall thickness during diastole was obtained at given time points. Values are expressed as the mean ± SD. * p<0.05 vs SHAM, ¥ P<0.05 vs T+R.

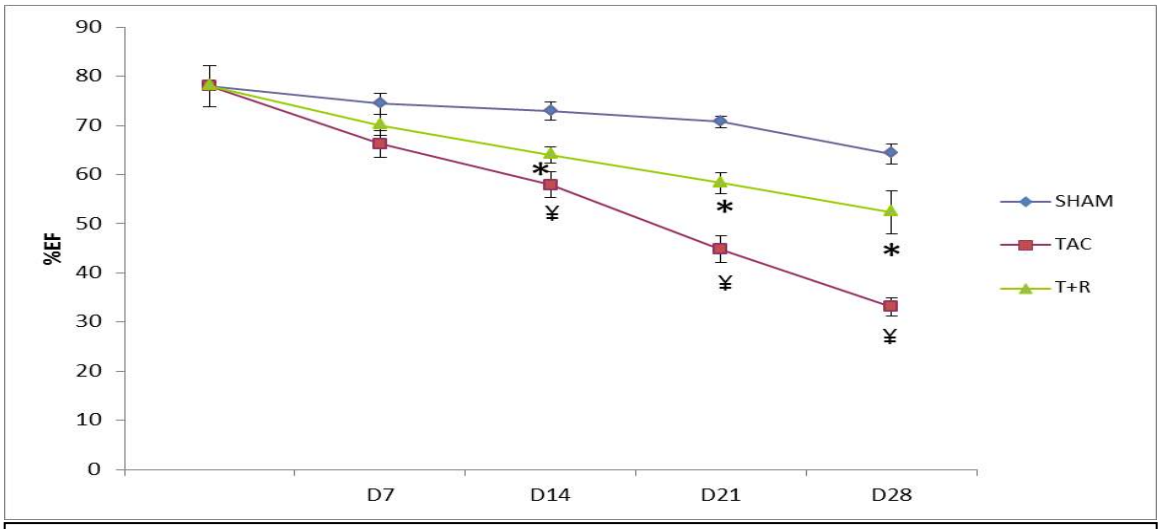

Figure 3.3. E. Resveratrol treatment attenuates cardiac contractile dysfunction in PO mice. Groups (n=7/group) of WT mice were subjected to either SHAM or TAC protocol and parameter for percent change in ejection fraction was obtained at given time points. Values are expressed as the mean ± SD. * p<0.05 vs SHAM, ¥ P<0.05 vs T+R.

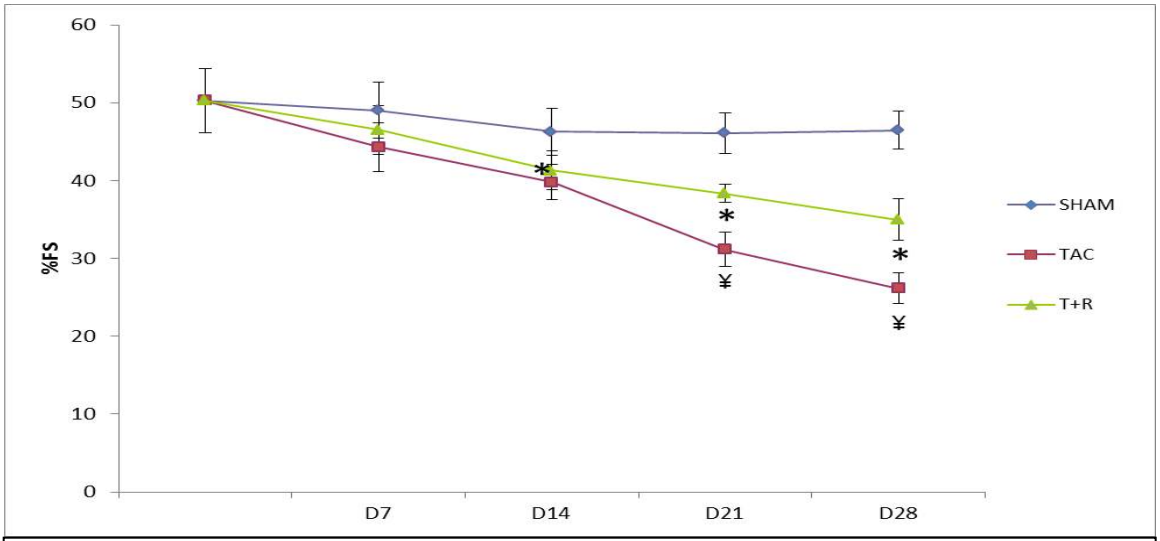

Figure 3.3. F. Resveratrol treatment attenuates cardiac contractile dysfunction in PO mice. Groups (n=7/group) of WT mice were subjected to either SHAM or TAC protocol and parameter for percent change in fractional shortening was obtained at given time points. Values are expressed as the mean ± SD. * p<0.05 vs SHAM, ¥ P<0.05 vs T+R.

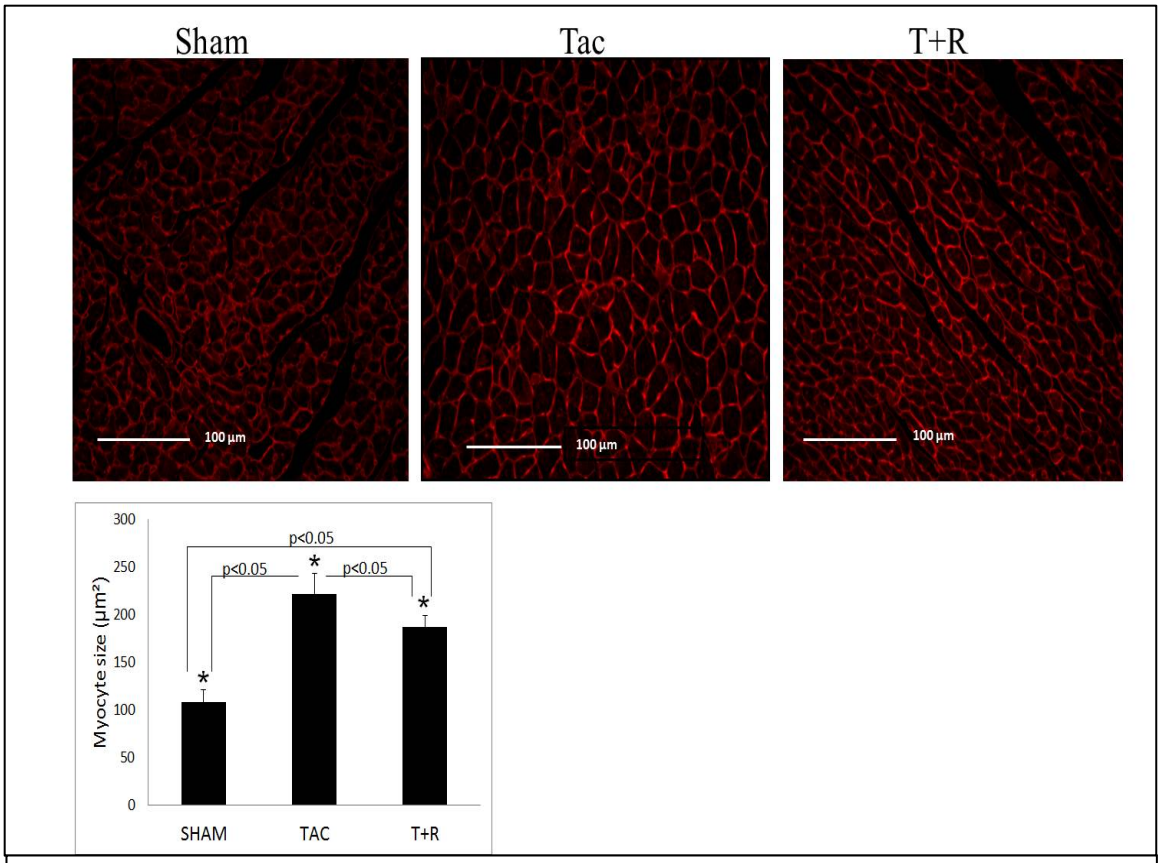

Figure 3.4 (A) TAC induced PO causes exacerbation of cardiac hypertrophy. WT mice were subjected to TAC or sham-surgery. Upper panel representative WGA staining of LV sections (400x magnification) in sham control, aortic banded mice treated with or without resveratrol at day 28 post surgery). Quantitation of myocyte surface area is shown in lower left panel. Values are expressed as the mean ± SD. * $p<0.05$ was considered statistically significant. The number of hearts (n=6/group).

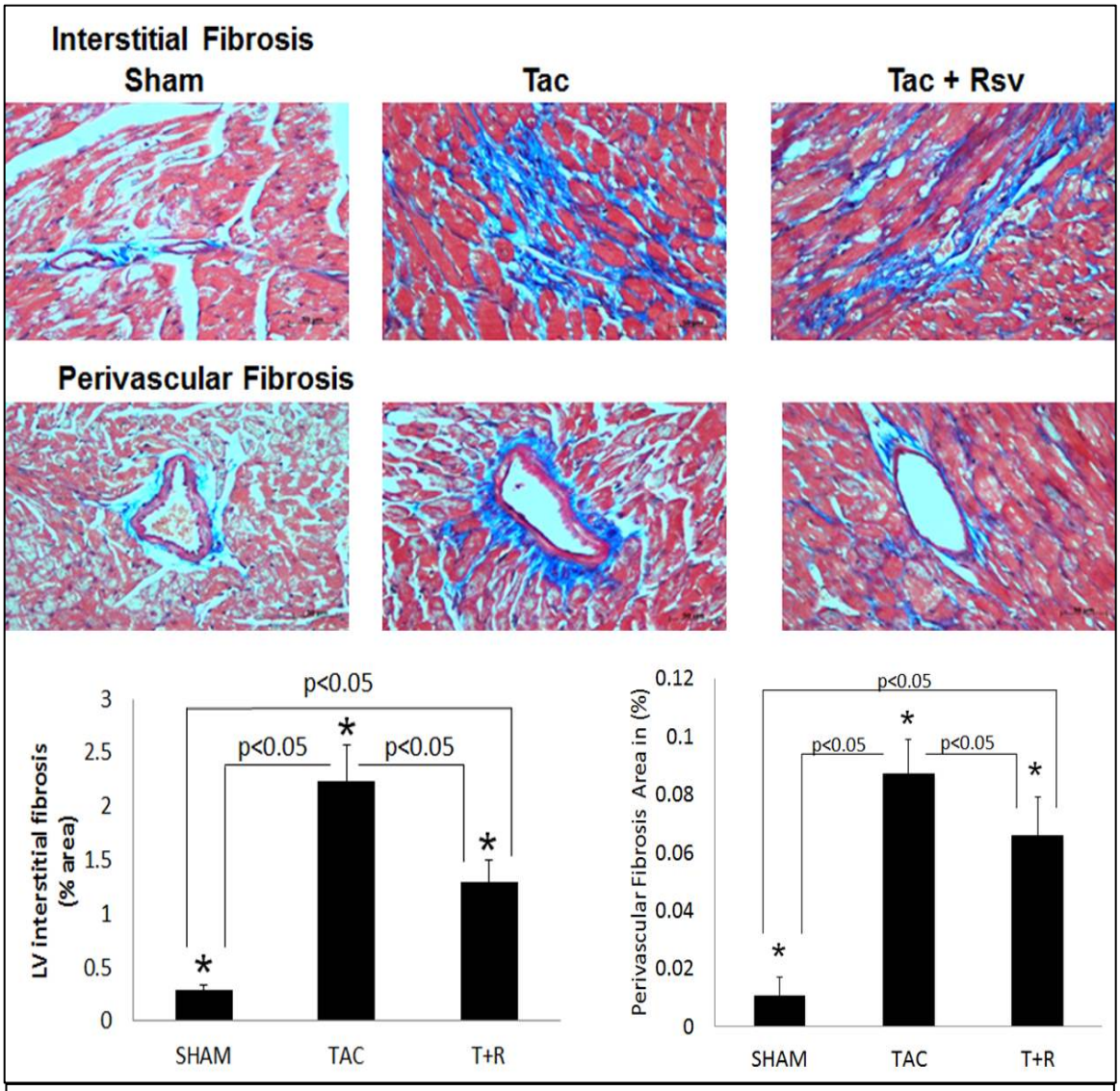

Figure 3.4 (B): Resveratrol treatment significantly attenuates exacerbation of cardiac fibrosis following TAC. A and C. Myocardial fibrotic area was measured from sham, TAC and Tac+Rsv treated mice and analysis of digital images of sections stained by Masson's trichrome kit was done at day 28. In the upper panel representative photomicrographs of fibrotic areas of LV interstitial fibrosis and lower panel showing LV perivascular fibrosis. (200x magnification). Values are represented as a percentage of total microscopic area per heart. The numbers (n=6/group) represents the number of sham and TAC hearts analyzed. Values are expressed as the mean ± SD. * $p<0.05$ was considered statistically significant.

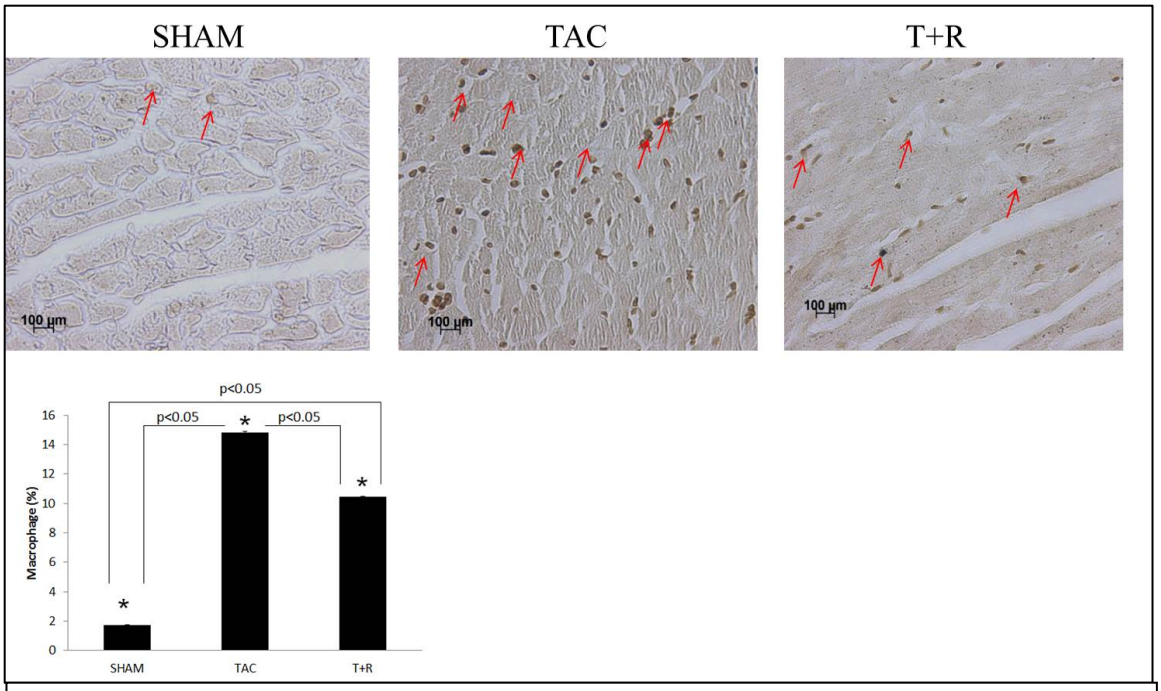

Figure 3.5(A): Resveratrol attenuates exacerbation of inflammation following TAC. Groups (n=6/group) of WT mice underwent either SHAM or TAC protocol. Upper panel, representative immunohistochemical staining of macrophage with anti-macrophage antibody Mac-2 (brown color) from LV sections of sham, TAC and TAC+RSV treatment mice at day 28 (200x magnification). Lower panel, slides were quantified and depicted as percent macrophage cells with respect to control group. Values are expressed as the mean ± SD. * $p<0.05$ was considered statistically significant.

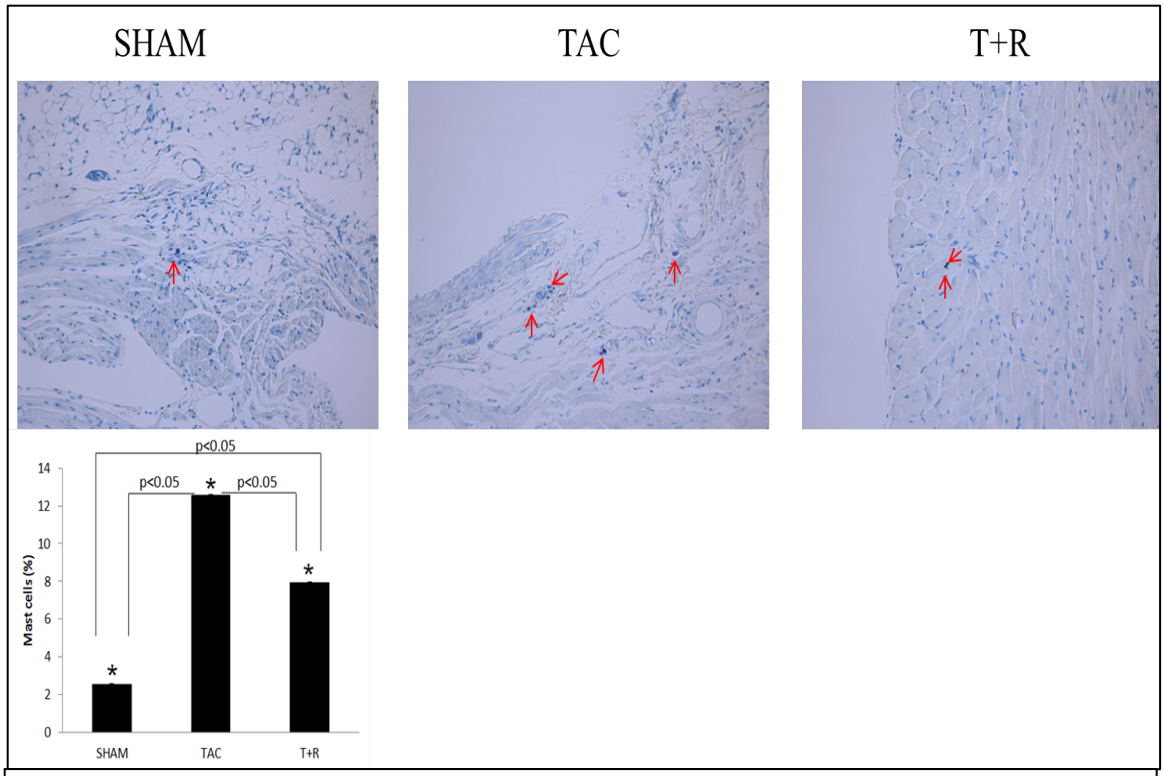

Figure 3.5 (B): Resveratrol attenuates exacerbation of inflammation following TAC. Groups of mice (n=6/group) underwent SHAM or TAC protocol. Upper panel, representative staining of mast cells with toluidine blue (blue color) from LV sections of sham, TAC and TAC+RSV treatment mice at day 28 (100x magnification). Lower panel, slides were quantified and depicted as percent mast cells with respect to control group. Values are expressed as the mean ± SD. * $p<0.05$ was considered statistically significant.

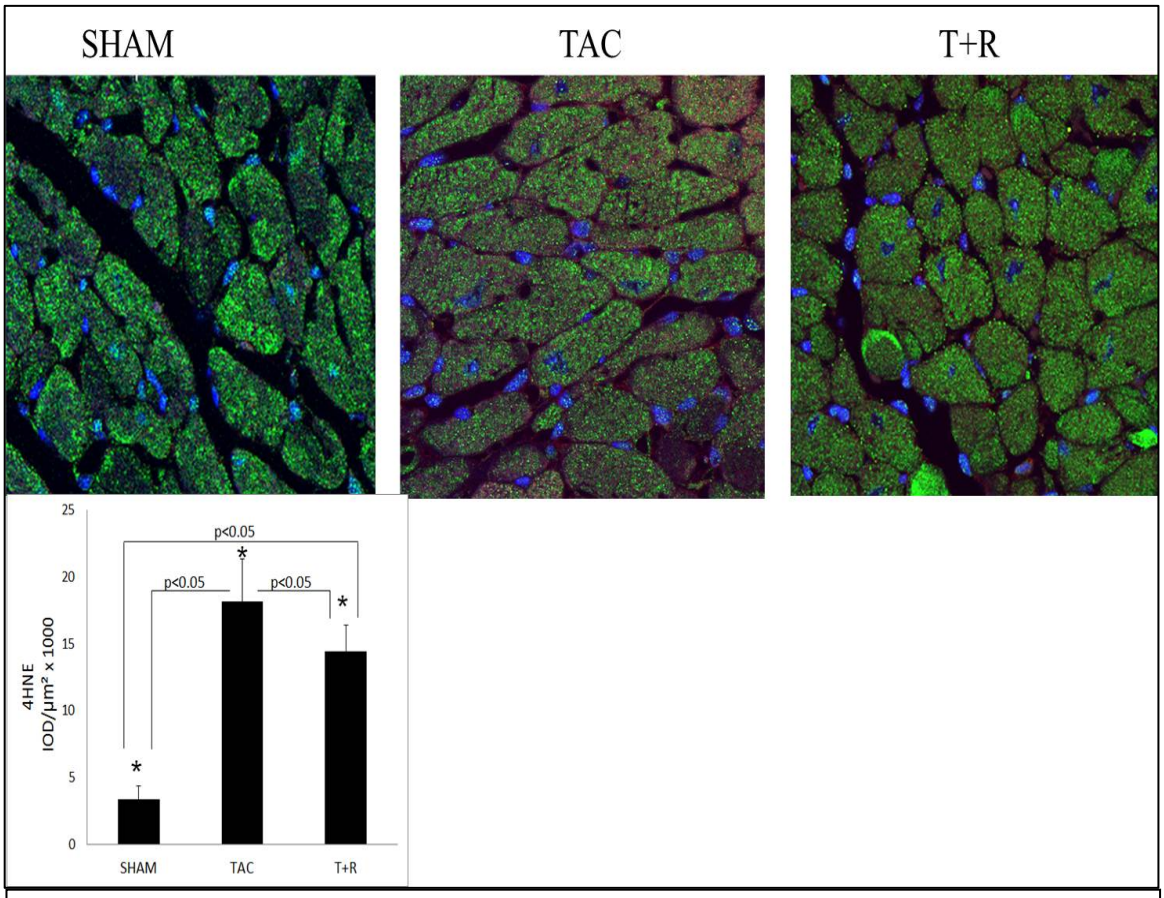

Figure 3.6 (A): Exacerbation of oxidative stress in TAC induced PO mice heart. Upper panel, representative images of 4-HNE staining of LV sections at day 28 from TAC, sham mice and Rsv treated mice. Lower panel- level of 4-HNE was semi-quantification by measuring IOD of six randomly chosen fields in each myocardial tissue section. Areas shown in red are positive for 4-HNE. Cardiomyocytes is stained green using anti-Tropomyosin I and nuclei in blue were labeled with DAPI. (n=6/group).* $p<0.05$ was considered statistically significant.

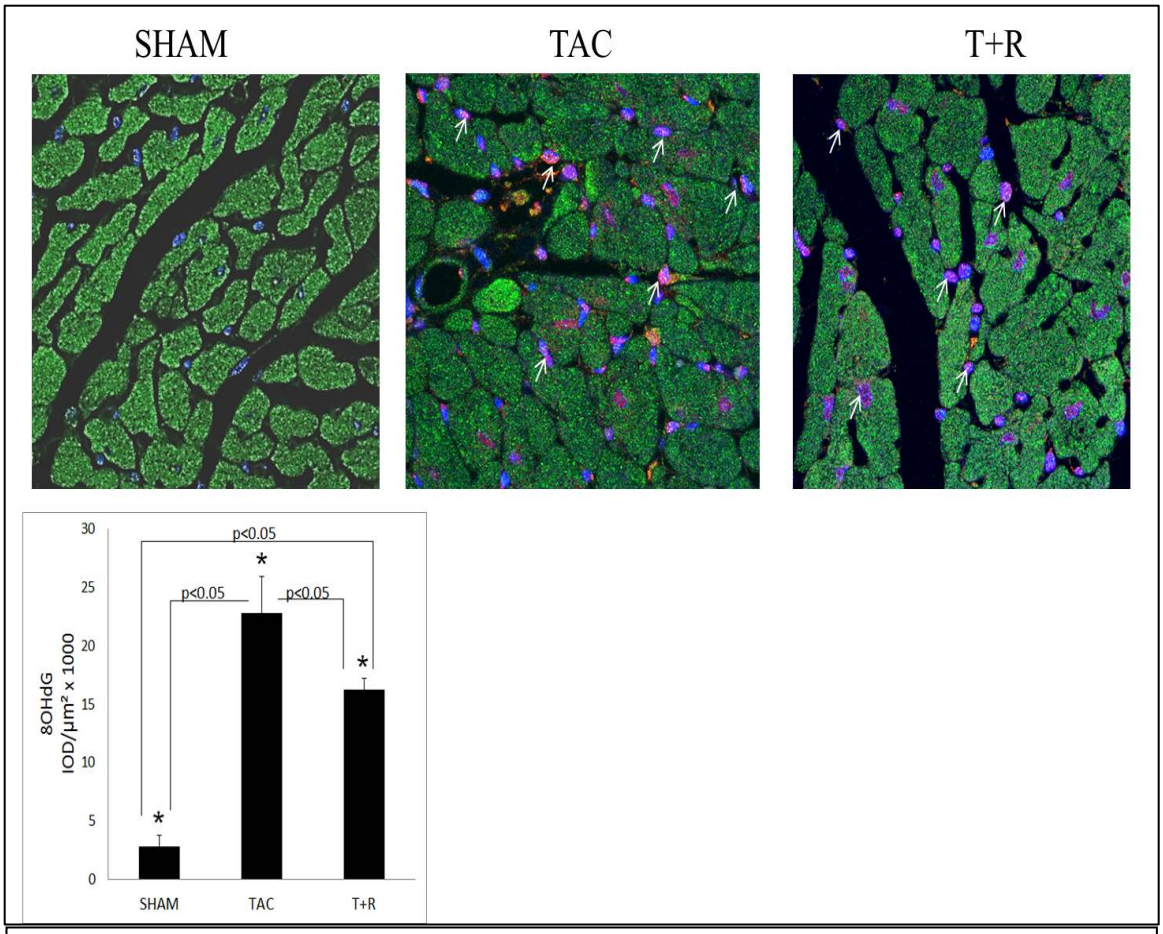

Figure3.6 (B): Exacerbation of oxidative stress in TAC induced PO mice heart. Upper panel representative images of 8OHdG staining of LV sections at day 28 from TAC, sham and Tac+Rsv treated mice. Lower panel- level of 8-OHdG was semi-quantification by measuring IOD of six randomly chosen fields in each myocardial tissue section. Areas shown in red are positive for 8OHdG. Cardiomyocytes is stained green using anti-Tropomyosin I and nuclei in blue were labeled with DAPI. (n=6/group). * $p<0.05$ was considered statistically significant.

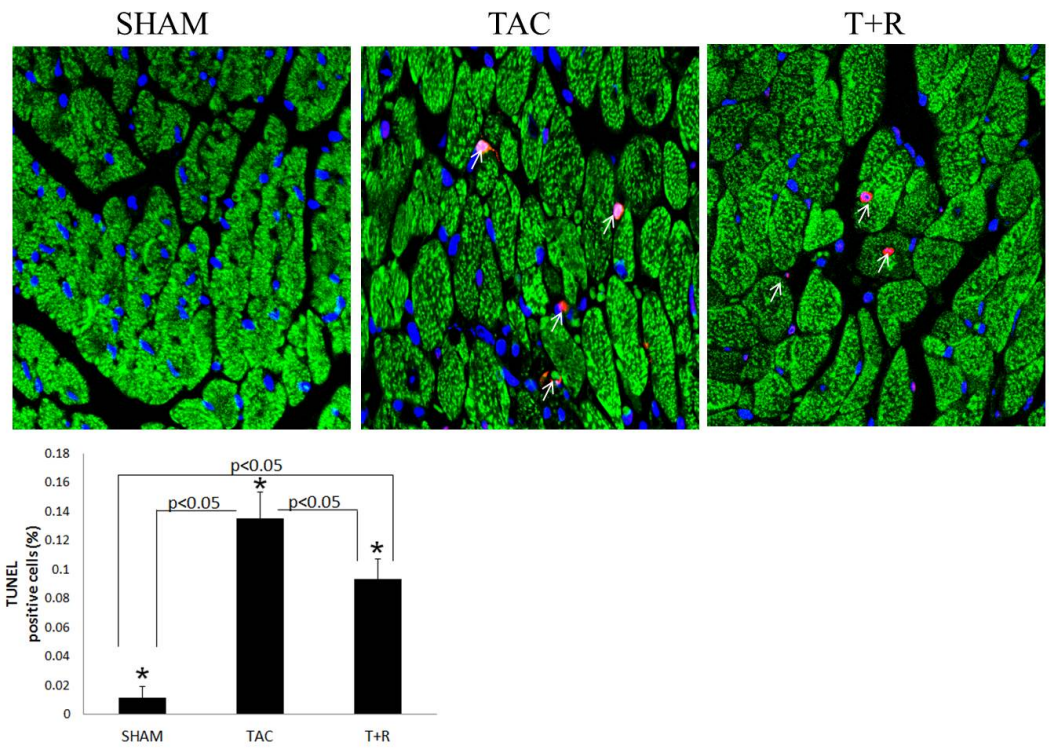

Figure 3.7: Effect of resveratrol on myocardial apoptosis in TAC induced PO mice heart. Representative images of TUNEL staining of left ventricular are shown in upper panel. Quantification of TUNEL positive cells are shown in lower panel. Apoptotic nuclei in TAC hearts were quantified by using image pro plus and represented as percent of total nuclei in the section of LV. Apoptotic nuclei shown in red, myocardium green and nuclei in blue. (400x magnification). (n=6/group). * $p<0.05$ was considered statistically significant.

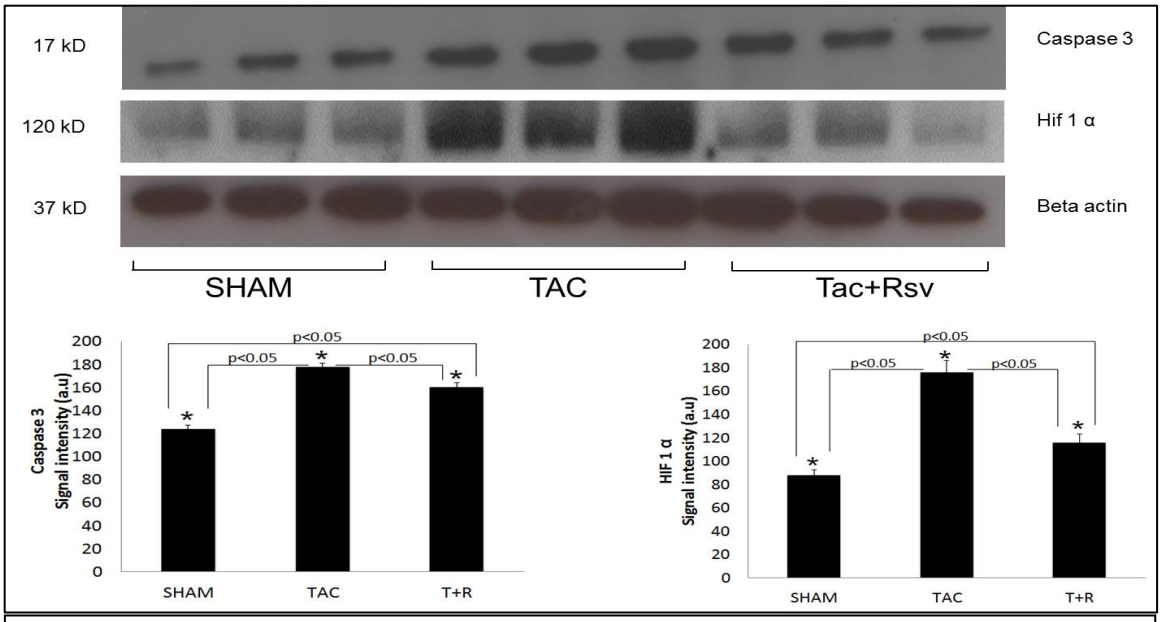

Figure 3.8: Resveratrol protects heart failure by inhibiting apoptosis and hypoxic damage. Group (n=7/group) of WT mice were subjected to either sham or TAC surgery and one group was treated with resveratrol for 4 weeks. LV tissue was extracted and processed for western blot analysis for caspase 3 and HIF1-α, normalized to β-actin as control for equal loading . Each lane contained 30 µg of protein. Lower panel-representative quantitation by densitometric tracing of protein bands (n= 3/group). * p<0.05 was considered statistically significant.

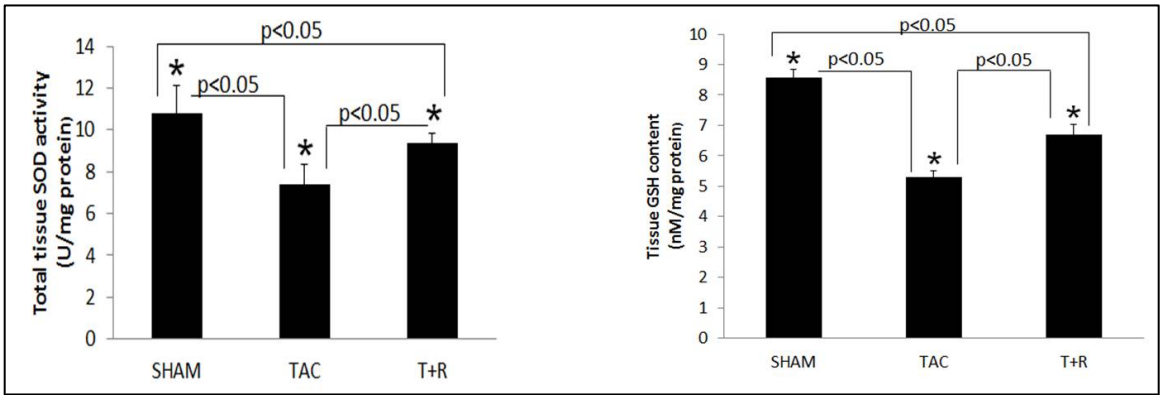

Figure 3.9: Treatment with resveratrol causes upregulation of anti-oxidative proteins in the heart. Group (n=7/group) of WT mice were subjected to either sham or TAC surgery and one group was treated with resveratrol for 4 weeks. Tissue was extracted and processed for total activity measurement at day 28. A: Quantitation of total tissue SOD activity (U/mg protein). B: Quantitation of total GSH content in the cardiac tissue (nM/mg protein). * p<0.05 was considered statistically significant.

CHAPTER 4

DISCUSSION

Prevention of cardiac structural and functional alterations by resveratrol treatment in experimental model of pressure overload created by TAC has been reported (Juric et al., 2007). Similarly regression of PO–induced cardiac hypertrophy and its deleterious consequences on heart function have also been reported previously in resveratrol-treated abdominal aortic-banded rats. (Li et al., 2005). Thus, it appears that resveratrol is beneficial in treating pathological pressure overload conditions that include hypertension.

The main purpose of this study was to investigate the use of resveratrol as a potential alternative therapy for PO-induced HF. It has been firmly established that sustained chronic PO causes the activation of various oxidases which in turn causes increased ROS formation to induce cardiac hypertrophy, including cardiac myocyte hypertrophy, myocardial fibrosis and apoptosis leading to heart failure (El Hasnaoui-Saadani et al., 2013). Given the critical role of resveratrol in antioxidant defenses, it was not surprising to find that resveratrol is cardio protective by decreasing oxidative damage in the heart.

The significant findings of this study were that resveratrol treatment resulted in 1) significant attenuation of myocardial dysfunction; 2) prevention of cardiac myocyte against oxidative stress damage including attenuation of cardiac hypertrophy, fibrosis, inflammation and apoptosis, 3) upegulation of anti-oxidative proteins thereby combating

the oxidative injury. These beneficial effects of resveratrol occurred despite the continued presence of cardiac hypertrophy and hypertension in this model and if left untreated would ultimately result in LV dilation and overt heart failure.

4.1 TAC-induced PO Mice Presents Signs Of Heart Failure.

Four weeks after TAC surgery, mice developed PO induced concentric cardiac hypertrophy and decreased cardiac performance characterized by significant change in LV wall thickness, %EF, %FS leading to diastolic dysfunction. These results are consistent with previous studies and validate the experimental models used in this study (Juric et al., 2007; Li et al., 2005; Wojciechowski et al., 2010).

4.2 Treatment With Resveratrol Improves Cardiac Function And Enhances Survival.

Echocardiographic analysis revealed that that the WT hearts developed adverse structural remodeling and progressive dilation following induction of PO. This led to a significant reduction of EF and FS as well as a significant increase in the LW/BW ratio, a strong indication that these hearts were beginning to fail. The adverse remodeling and dilation was markedly exacerbated in the TAC hearts resulting in an even more pronounced decline in FS and a sharp increase in the LW/BW ratio by day 28 compared to the RSV treated. Additional evidence for the increased vulnerability of the TAC hearts to PO-induced heart failure is provided by the increased HW/BW ratio and cardiomyocyte size (CSA) in the TAC mice compared to their WT counterparts. Resveratrol treatment attenuated concentric remodeling by normalizing LVPW thickness both at systole and diastole. These beneficial effects of resveratrol in attenuating PO-induced cardiac hypertrophy are consistent with other recent study that demonstrated

anti-hypertrophic effects of RSV in a rat model of PO-induced cardiac hypertrophy (Wojciechowski et al., 2010). In this model, chronic RSV administration (2.5mg/kg bw) for 28 days was ensued. Echocardiographic and histopathological studies clearly showed marked deterioration in the cardiac function characterized by significant increment in the LV wall thickness and reduction in the cardiac function parameters as compared to sham or vehicle treated rats. Treatment with RSV was found to regress cardiac hypertrophy and dysfunction significantly.

4.3 Resveratrol Treatment Induces Anti-oxidative Enzymes And Protects Against Oxidative Damage In Pressure Overloaded Mice Heart.

Reactive oxygen species play an integral role in the development of inflammation and promote oxidative stress during hypertension (DeMarco et al., 2010). Another characteristic of resveratrol is anti-inflammation. Resveratrol not only modulates biochemical responses of polymorphonuclear leukocytes by interfering with the release of inflammatory meaditors but also suppresses the activity of macrophages (Sharma et al., 2007). The inflammatory response of the heart to pressure overload, as determined by macrophage infiltration was significantly higher levels in the TAC mice compared to their WT counterparts. This response is likely initiated by multiple pathways through increased oxidative stress, inhibition of cyclooxygenase (COX) and activation of the potent pro-inflammatory NF-κB system. Our study and other have shown that RSV attenuates the generation of ROS and inhibits macrophage infiltration (Kang et al., 2009; Karlsen et al., 2010; Leiro et al., 2005). Macrophage infiltration in TAC+RSV group dropped significantly indicating that in the absence of control over ROS production, there

is a marked acceleration of the pathophysiological mechanisms that underlie the development of heart failure.

Similarly, mast cells have been implicated in the pathogenesis of HF by degranulating myocardial collagen and inducing fibrosis in response to infectious and inflammatory stimuli (Matsumori et al., 1994). Although found mainly in the skin, gastrointestinal tract, and airways, they are normally known to reside in cardiac tissue (Mina et al., 2013). Interestingly, levels of mast cells show a similar increase following the TAC procedure. This increase in mast cell number was strikingly higher in TAC compared to sham treatment. However, treatment with RSV was able to attenuate this mast cell infiltration significantly as compared to TAC alone.

4.4 Resveratrol Exerts Marked Anti-fibrotic Property In TAC Induced PO Mice Heart.

Consistent with the enhanced inflammatory response in the TAC mice compared to their WT counterparts, we also found striking increase in both interstitial and perivascular fibrosis in the pressure overloaded TAC hearts. This is most likely the primary cause of the significant deterioration of cardiac function. These data clearly indicates that the PO-induced fibrosis seen in the TAC mice is markedly increased compared to sham surgery. This may be in part, by necrotic cell death. There is a significant increase in LV apoptosis in the TAC WT mice compared to the sham mice. While myocyte apoptosis is well documented in heart failure and can reduce the force-generating capacity of the myocardium (Juric et al., 2007), apoptotic cells are scattered across the wall of the chamber and are usually found as single cell losses. Indeed, replacement fibrosis in heart failure is the result of multiple diffuse foci that contain a

much larger number of dead cells than seen in apoptosis. While necrosis is not as well studied as apoptosis, it is now clear that this is a tightly regulated process. In heart failure, it appears that necrotic cell death is triggered at the level of the mitochondria by multiple factors including sympathetic and Ca^{2+} overload, oxidative and metabolic stress, and hypoxia (Schmidt et al., 2002). Several lines of evidence indicate that RSV has significant anti-apoptotic activity, both in vivo and in vitro, that is mediated by the inhibition of NF-κB, p53, Enos and PGC-1α via activation of SIRT1 pathways which stimulate survival pathways (Rodgers et al., 2005; Vaziri et al., 2001; Yeung et al., 2004).

4.5 Exaggeration Of Cardiomyocyte Hypoxia In TAC Mice Is Attenuated With Resveratrol Treatment

In addition, we analyzed the cardiac expression of the angiogenic factors HIF-1α. Accumulating evidence has demonstrated that during development of cardiac hypertrophy, a mismatch between the number of capillaries and the size of cardiomyocyte develops, leading to myocardial hypoxia and subsequent cell death (Maeda et al., 2013). It has been reported that HIF1α and VEGF were up-regulated in the pressure overload-induced hypertrophic WT hearts (Hilfiker-Kleiner et al., 2005; Sano et al., 2007). In the present study, we found that TAC resulted in significant increase in HIF-1α level which might have predisposed cardiomyocytes vulnerable to pressure overload induced cardiomyocyte apoptosis. However treatment with RSV protected cardiomyocytes against hypoxia induced injury and death. The angiogenic effect of RSV found in this study was consistent with other observations that RSV increased angiogenesis during ulcer and wound healing (Razban et al., 2012).

Past and present studies have shown a growing consensus pointing towards NO as an endogenous anti hypertrophic molecule (Kempf and Wollert, 2004). It would be therefore beneficial to explore its potential in combating cardiac hypertrophy. Endothelial dysfunction (characterized as an impairment of endothelium-dependent relaxation) is known for structural changes in vasculature leading to endothelial dysfunction and ultimately hypertension. Several studies have shown that treatment with resveratrol results in enhancement of agonist-stimulated , endothelium dependent relaxation (Mizutani et al., 2000; Silan, 2008; Zhang et al., 2009). This improvement is largely attributable to NO derived from endothelial NO synthase (Enos) and is mediated through multiple mechanisms (Li and Forstermann, 2009a, b). eNOS serves as a vital role in regulating beta-adrenergic transduction system and therefore cardiac function where chronic activation of beta-adrenergic receptor results in cardiac hypertrophy (Buys et al., 2007). Given the role of resveratrol in mediating oxidative stress by upregulating endothelial nitric oxide in PO mice model of HF (Juric et al., 2007). Our results fit well with the anti-hypertrophic and cardioprotective roles of NO as we observed that resveratrol treatment significantly attenuated cardiac hypertrophy and as well as prevented contractile dysfunction as compared to TAC alone.

Moreover it should be noted that resveratrol may also indirectly improve cardiac energy metabolism through its vasorelaxing effect, which could ameliorate perfusion and enhance oxygen and substrate delivery to the heart and the periphery. Finally, the beneficial cardiovascular and metabolic effects of resveratrol could also result from its phytooestrogenic properties. As a polyphenolic compound, resveratrol has been shown to be scavenger of hydroxyl, superoxide (Hung et al., 2002). Resveratrol induces

antioxidant enzymes in cardiovascular tissue (Cao and Li, 2004). SOD catalyze the dismutation of superoxide into hydrogen peroxide, which is further inactivated by glutathione (Forstermann, 2010). However it remains largely unclear as to how resveratrol causes induction of these antioxidant enzymes. Recent studies have demonstrated that overexpression of SIRT 1 leads to upregulation of SOD (Ungvari et al., 2009). Consistent with above study, our results show that PO causes myocardial levels of SOD and GSH to decrease dramatically relative to sham operated. Treatment with resveratrol however was able to induce the level of antioxidants and protect cardiomyocyte from oxidative injury. Resveratrol has also been reported to reduce oxidative stress by inhibiting ROS production through NADPH oxidase (NOX) system (Forstermann, 2010).

4.6 Mode Of Action Of Resveratrol In Mediating Anti-oxidative Property

Attenuation of hypertrophic response and also delaying the transition from hypertrophy to heart failure has been shown to be mediated by LKB1/AMPK pathway (Dolinsky et al., 2009). LKB1 is an upstream kinase of AMPK and it inhibits cardiac remodeling by preventing angiotensin II –induced myocardial fibrosis. Oxidative stress has been reported to regulate LKB1/AMPK pathway. Lipid peroxidation product such as 4-HNE is elevated during oxidative stress (Dolinsky et al., 2009). 4-HNE forms covalent adducts with LKB1 leading to inhibition of LKB1/AMPK signaling and activation of Mtor/p70s6 kinase mediated protein synthesis and cardiac myocyte growth. Treatment with resveratrol has been shown to prevents 4HNE modification of LKB1/AMPK signaling by blunting prohypertrophic p70S6 kinase response thereby attenuating left ventricular hypertrophy (Dolinsky et al., 2009). Consistent with the above mentioned

study, our results show a sharp induction of 4-HNE level in TAC mice compared to sham operated. However this upregulation of 4-HNE level was attenuated sharply with resveratrol treatment.

4.7 Anti-apoptotic Potential Of Resveratrol.

In failing heart, decompensation due to the chronic PO and cardiomyocyte deficiency due to fibrosis and apoptosis turns to be the predominat problem. Under such condition, resveratrol has been thought to potentiate an active DNA repair process instead of an inhibited protein synthesis, which preserves the genomic stability of cardiomyocyte. Oxidative DNA damage in cardiomyocyte was assessed to to determine extent of oxidative stress in PO hearts. 8OHdG and TUNEL positive stained nuclei were determined as an indicator of oxidative DNA damage in the heart. The induction of 8OHdG and immunopositively stained TUNEL nuclei indicated the presence of oxidative DNA damage. Consistent with others findings, (Sin et al., 2013) our results demonstrated a significant oxidative damage in TAC hearts compared to sham. However this induction was significantly attenuated with resveratrol treatment. The content of 4HNE was positively correlated with the number of 8OHdG and TUNEL immunopositive nuclei.

4.8 Clinical Relevance

Irrespective of numerous protective roles mediated by resveratrol in various diseases and experimental settings, it has not yet achieved the clinical significance through mass human clinical trials. Variety of fruits and food has been shown to contain resveratrol and is expected to keep people healthy by scavenging free oxygen radicals. However due it its short half-life and rapid metabolizing property, the potency of resveratrol cannot be maintained for long in the body thereby ways to improve its

bioavailability either by blocking its rapid metabolism or developing analogs which can potentiate similar yet long lasting effects is needed (Baur and Sinclair, 2006). Although there are various ongoing research whose primarily focus have been the structure of resveratrol (Lagouge et al., 2006), yet great deal of difficulties and challenges are encountered in the process of screening the effective elements of resveratrol in vivo and determining the differences between in vivo and in vitro results (Baur and Sinclair, 2006). Another challenge is to figure out the effective yet safe dose in humans because the dose at which resveratrol shows its potential to inhibit or treat certain disease might not apply on humans simply by extrapolating the figure or weight conversion (Reagan-Shaw et al., 2008). There has been significant increase in the number of publications involving protective effects of resveratrol in diverse disease and experimental settings, while on the other side numerous clinical trials are ongoing regarding the safety and efficacy of this molecule. Fortunately, several phase one study have been completed among which one showed potential decrease in circulating IGF-1 as well as IGF- binding protein 3 in normal human subjects as compared to pre-dosing values (Patel et al., 2011). Another study involving resveratrol in human subjects showed marked attenuation of reactive oxygen species leading to downregulation of tumor necrosis factor-α as well as inflammation markers interleukin-6 and C-reactive protein (Ghanim et al., 2010). So far resveratrol has not been shown to cause significant side effects (Boocock et al., 2007; Camins et al., 2010; Ghanim et al., 2010; Patel et al., 2011) however, some increase in blood bilirubin and alanine aminotransferases were noted in healthy volunteers receiving high doses (Almeida et al., 2009). Some degree of headache, dizziness, myalgia and epididymitis were also reported in a rising multiple-dose study in healthy volunteers

providing the evidence that resveratrol is well tolerated under conditions of repeated administration (Almeida et al., 2009).

With such diverse protective effects, resveratrol is expected to do much more wonders in treatment and management of HF. In the setting of cardiovascular disease, by correcting the oxidative stress, resveratrol sharply regulates the pathogenic factors like inflammation, hyper coagulation, obesity, dyslipidemia, atherogenic plaque formation, hyperglycemia, apoptosis, fibrosis and cardiac dysfunction thereby providing ample evidence of being prime candidate for treatment of heart failure. However, much research is needed to finally achieve targeted therapy, treat cardiovascular diseases and increase the quality of life in HF patients.

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