Development of Heart Failure after Cardiac Myosin-Induced Experimental Autoimmune Myocarditis in Rats: Possible Mechanisms and Treatment Options

Thesis for the degree of Doctor of Philosophy (PhD)

By

Somasundaram Arumugam, M. Pharm.,

Department of Clinical Pharmacology, Faculty of Pharmaceutical Sciences,

Niigata University of Pharmacy and Applied Life Sciences,

Niigata City, JAPAN.

Supervised by

Prof. Kenichi Watanabe, M.D., Ph.D.,

Reviewed by

Prof. Kazayuki Ueno, Ph.D.,

Prof. Toshinari Asakura, Ph.D.,

February 2014
Dilated cardiomyopathy and its treatment options

Dedicated to my parents, my brothers and my wife, their co-operation and support has brought me upto the level where I am at present
Dilated cardiomyopathy and its treatment options

Contents

1. Acknowledgements
2. Abbreviations
3. Abstract
4. Introduction
5. Scope of the study
6. Chapter 1
7. Chapter 2
8. Chapter 3
9. Summary
10. References
11. List of original articles
12. Awards
Acknowledgements

This work has been carried out at the Department of Clinical Pharmacology, Faculty of Pharmaceutical Sciences, Niigata University of Pharmacy and Applied Life Sciences, Niigata City, Japan during the period of April 2011-March 2014. It is my privilege to express gratitude to my mentor and teacher, Prof. Kenichi Watanabe, for his gentle and never compromising approach, sparkling ideas, encouragement and thoughtful suggestions throughout my thesis work.

I am thankful to Dr. Meilei Harima, for her ever willing helping tendency, careful suggestions and possible help.

I am grateful to my reviewers Prof. Kazayuki Ueno and Prof. Toshinari Asakura for their excellent review of my thesis and cooperation.

I express my sincere gratitude to my teacher Dr. Rajarajan A. Thandavarayan and Dr. Vijayasree V. Giridharan, for their continuous support and guidance in all the aspects during my doctoral course.

I want to extend my thankfulness to Dr. Kenji Suzuki, Department of Gastroenterology, Niigata University of Medical and Dental Sciences, Niigata City, Japan for his enthusiasm, encouragement. I express my sincere thankfulness to Dr. Masaki Nagata and Prof. Ritsuo Takagi, Division of Oral and Maxillofacial Surgery, Niigata University of Medical and Dental Sciences, Niigata city for carry out RT-PCR analysis.
Dilated cardiomyopathy and its treatment options

My other collaborators, Prof. Makoto Kodama, Prof. Yoshifusa Aizawa, First Department of Medicine of Niigata University of Medical and Dental Sciences, Niigata City; Dr. Hiroyuki Yoneyama, Executive Director, Stelic Institute of Regenerative Medicine, Tokyo must be remembered for their support.

I am grateful to Dr. Masahiko Miyamoto and Dr. Akihiko Komuro, for helping me and allowing me to use many of their lab facilities. I extend my thanks to all other teachers and staffs of NUPALS for their kind cooperation.

It is nice to have wonderful lab mates such as Wawaimuli Arozal, Flori R. Sari, Vivian Soetikno, Yuhki Satoshi, Vigneshwaran Pitchaimani, Vengadeshprabhu Karuppagounder, Rejina Afrin and many other fourth grade students and I wish to express my warmth and thankfulness for their professional and personal assistance at various junctures.

I am grateful to the Department of Clinical Pharmacology, NUPALS for providing me the financial support and scholarships during my PhD. This research work was supported by grants from Yujin Memorial grant; the Ministry of Education, Culture, Sports, Science and Technology of Japan; and Promotion and Mutual Aid Corporation for Private Schools of Japan. They are thankfully acknowledged for the grant support for our research.

The sacrifice of laboratory animals is very crucial for my research work. I apologize and also thank those animals who gave their life so as to complete my research fruitfully.
At last but not least, it is heartening to remember the contribution of my family as their sacrifice, love, affection and encouragement are always instrumental. Especially my parents, Mr. Arumugam Chinnanchettiar and Ms. Sundarammal Arumugam, my wife Remya Sreedhar, my brothers - Mr. Sokkanadhan Arumugam, Mr. Sureshkumar Arumugam, Mr. Ravichandran Arumugam and my sister in law Ms. Saranya Sureshkumar and the sweet kids of our family Gangesh Sureshkumar and Vithuresh Sureshkumar.

It will not end if I thank the Nature, which provided me a healthy body, mind and environment for carrying out my research without many difficulties.
Abbreviations

ACE - angiotensin-converting enzyme;
ACE-2 – angiotensin converting enzyme-2
ANG (1-7) – angiotensin-(1-7)
ANG-II – Angiotensin-II
ANOVA - analysis of variance
ANP - atrial natriuretic peptide
ARBs - angiotensin receptor blockers
AT\textsubscript{1}R – Angiotensin-II type 1 receptor
AT\textsubscript{2}R - Angiotensin-II type 2 receptor
BSA - bovine serum albumin;
BW - body weight;
CHF - chronic heart failure;
CVP - central venous pressure;
DAB - diaminobenzidine;
DCM - dilated cardiomyopathy;
DHE – Dihydroethidium
\( \pm dP/dt \), first derivatives of left ventricular pressure;
EAM - experimental autoimmune myocarditis
Dilated cardiomyopathy and its treatment options

EDTA - disodium ethylenediamine-N,N,N',N'-tetraacetic acid

EF - ejection fraction

EIA - enzyme immuno assay

ELISA - Enzyme-linked immunosorbent assay

FS - fractional shortening

GAPDH - glyceraldehyde-3-phosphate dehydrogenase

GCM - giant-cell myocarditis

H/B - heart weight to body weight ratio

HR - heart rate

HRP - horseradish peroxidase

HW - heart weight

JNK - c-Jun-N-terminal kinase

K/B - kidney weight to body weight;

LV - left ventricular;

LVDd – left ventricular dimension in diastole

LVDs – left ventricular dimension in systole

LVDs, left ventricular dimension in systole

LVEDP - left ventricular end-diastolic pressure

LVP - left ventricular pressure

MAPK - mitogen-activated protein kinase

MAPKAPK-2 - mitogen-activated protein kinase activated protein kinase-2
Dilated cardiomyopathy and its treatment options

MBP - mean blood pressure

MMP - matrix metalloproteinase

NADPH oxidase - Nicotinamide adenine dinucleotide phosphate oxidase

OPN - Osteopontin

PI3K - phosphatidylinositol-3-kinase

RAAS - renin-angiotensin-aldosterone system;

RAS - renin-angiotensin system

RIA - radio-immuno assay

RIA - radioimmuno assay

SDS - sodium dodecyl sulphate

SEM - standard error of mean

SOLVD - Studies of Left Ventricular Dysfunction

TBS - tris buffered saline

TGFβ1 - transforming growth factor β1

Th1 - T helper 1

Th2 - T helper 2

TIMP - tissue inhibitor of metalloproteinase

VSMCs - vascular smooth muscle cells
Dilated cardiomyopathy and its treatment options

Abstract

Myocarditis is the inflammation of heart muscle which occurs after microbial infection or autoimmune diseases. Acute myocarditis may be self-limiting or progresses into dilated cardiomyopathy (DCM). Autoimmune mechanisms play an important role in the pathogenesis of myocarditis and post-infectious cardiomyopathy. To clarify the pathophysiology, many viral and autoimmune myocarditis models have been developed. Among various trial animal models, immunization with cardiac myosin in rats and mice become most suitable to study the pathophysiology because the disease course and pathology mimics human fulminant myocarditis and giant cell myocarditis. The balance between T helper 1 (Th1) cell cytokines and T helper 2 (Th2) cytokines determine the outcome of the disease. Usually during the progressive phase of myocarditis i.e. acute myocarditis Th1 cells has predominant function, later in the recovery phase Th2 cytokines become more active one. The later stage of acute myocarditis or chronic myocarditis progresses into DCM. Persistent autoimmunity is the cause of persistent myocarditis. Next important factor is occurrence of fibrosis and hypertrophy, which are collectively termed as cardiac remodeling. Activation of renin angiotensin system and elevated oxidative stress are identified to be involved in the pathogenesis of experimental autoimmune myocarditis (EAM) followed by DCM. Thus the therapeutic strategies behind the treatment of cardiac dysfunction during these
Dilated cardiomyopathy and its treatment options

conditions involves targeting renin angiotensin system or oxidative stress using one of the categories of medicines such as angiotensin converting enzyme inhibitors, angiotensin receptor blockers, aldosterone antagonists, long acting loop diuretics and natural as well as synthetic antioxidants. In this study we have performed the analysis of the effect of various antioxidant compounds such as Mulberry leaf powder, quercetin and a synthetic antioxidant edaravone against the progression of EAM to DCM. Our results identified that the post-myocarditis rats suffered from impaired cardiac function as well as elevated oxidative stress, endoplasmic reticulum stress and adverse cardiac remodeling in the form of myocardial fibrosis. After 4 weeks of immunization, the rats were fed with any one of the three different antioxidant compounds; 5% Mulberry leaf diet (group MLD), edaravone intraperitoneally at doses of 3 and 10 mg/kg/day (group Ed 3 and group Ed 10) or quercetin p.o. at a dose of 10 mg/kg body weight (Group Q10) for 4 weeks. By the end of the study, echocardiography was performed to assess the myocardial dimensions followed by hemodynamic studies to measure the cardiac function. The heart tissue was used for histopathology and Western blotting analyses. MLD supplementation suppressed the elevated oxidative stress and endoplasmic reticulum stress markers when compared with the vehicle treated rats. Azan-Mallory staining and immunohistochemical studies for collagen-III revealed the antifibrotic effects of MLD. It also improved the left ventricular ejection fraction and fractional shortening. Interestingly, the myocardial levels of endothelin-1, activated members of mitogen activated protein kinase (MAPK) pathway and vascular endothelial growth factor
Dilated cardiomyopathy and its treatment options

(VEGF) were significantly attenuated by MLD, indicating that the antihypertrophic effects of MLD are partially mediated via endothelin-1, MAPK and VEGF pathway. Collectively, these results suggest that supplementation of rats with 5% MLD has the ability to regulate cardiac remodeling and improves cardiac function and hence contributes to prevent the development of postmyocarditis DCM.

Rats treated with quercetin also showed significant cardioprotection and suppression of the adverse cardiac remodeling by reducing oxidative stress and endoplasmic reticulum stress as similar to MLD treatment, which suggest that the flavonoids present in the mulberry leaves may be responsible for the possible cardioprotective activity.

Another antioxidant, edaravone, treated DCM rats also showed better cardiac function compared with those of the vehicle treated rats. In addition, LV expressions of NADPH oxidase subunit levels were significantly down-regulated in edaravone-treated rats. Furthermore, the number of collagen-III positive cells in the myocardium of edaravone treated rats was lower compared with those of the vehicle treated rats. Our results suggest that edaravone ameliorated the progression of DCM by modulating oxidative and ER stress mediated myocardial apoptosis and fibrosis. We identified that the myocardial levels of phospho Akt and phosphoinositide 3-kinase, which are the upstream proteins of AMPK and MAPK activation and both were up-regulated in the vehicle-treated rats, whereas edaravone treatment significantly reversed these changes.

We have also measured the myocardial levels of p-AMPKα, different isoforms of protein kinase C and MAPK signaling proteins. All of these protein levels were
Dilated cardiomyopathy and its treatment options

significantly elevated in the hearts of DCM rats whereas edaravone treatment significantly reversed these changes. In viewing these results, we can suggest that along with MAPK, AMPK signaling also play a crucial role in the progression of EAM and it can be effectively blocked by the treatment with a novel antioxidant, edaravone.

**Keywords:** Experimental autoimmune myocarditis; dilated cardiomyopathy; heart failure; oxidative stress; renin angiotensin aldosterone system; antioxidants; mulberry leaves; quercetin; edaravone; AMPK; MAPK; PI3K-Akt signaling.
Introduction

Myocarditis: An overview

Myocarditis is defined as the inflammation of heart muscle and it may involve cardiomyocytes, vascular elements, interstitium or pericardium. It usually occurs during or after viral [Feldman and McNamara, 2000], bacterial [Hoefer et al., 2005] or parasitic infections [Tostes et al., 2005]. This condition may also occur due to non-infectious causes such as in the backdrop of autoimmune diseases, drug-induced hypersensitivity [Daniels et al., 2000], neoplasia [Kilgallen et al., 1998] and other systemic disorders [Frustaci et al., 2002]. The heart muscle becomes inflamed and weakened, causing symptoms of heart failure or arrhythmia, which may mimic acute myocardial infarction. The diagnosis of myocarditis is complicated, but Dallas criteria help to define it. According to Dallas criteria [Aretz, 1987], myocarditis is a process characterized by an inflammatory infiltrate of the myocardium with necrosis or degeneration of adjacent myocytes or both. The inflammatory infiltrate typically lymphocytic but also include mixed cellularity. The amount of inflammation and its distribution may be mild, moderate, severe and focal, confluent or diffuse respectively. In addition to histopathologic analysis, following diagnostic tools can aid in the diagnosis of acute myocarditis: Chest x-ray [Parada et al., 1997] and echocardiogram [Lieback et al., 1996] may show weak heart muscle, an enlarged heart, or fluid
Dilated cardiomyopathy and its treatment options

surrounding the heart. Cardiac-magnetic resonance imaging [Friedrich et al., 1998] is also helpful. Electrocardiogram is used to know the abnormalities in cardiac rhythm [Parada et al., 1997]. Biochemical analysis is to quantify the blood cell count, sedimentation rate, creatinine level and troponin-T level [Lauer et al., 1997]. Tests for antibodies against the heart muscle such as immunoassay or cardiac scintigraphic imaging has been found to be effective [Dec et al., 1990]. Once after accomplishing the difficult diagnosis, even the treatment options for autoimmune myocarditis remains elusive. Myocarditis therapy has been restricted to supportive measures to alleviate the clinical symptoms of heart failure or arrhythmias, including basic medications with angiotensin (Ang) converting enzyme (ACE) inhibitors or Ang-receptor blocking agents, diuretics, beta-blockers, calcium antagonists and amiodarone [Burian et al., 2005]. Patients with persistently impaired cardiac ejection fraction (EF) and/or life threatening arrhythmias take survival advantage from ventricular assist devices and implantable cardiac defibrillators [Starling et al., 1988; Reiss et al., 1996]. In severe and rapidly progressive cases, heart transplantation remains as the only therapeutic option [Nieminen et al., 1994; Laruelle et al., 1994].

Giant-cell myocarditis (GCM) is a rare, idiopathic and histologically distinct disease entity with a very poor prognosis, which often affects patients with latent or symptomatic autoimmune diseases, exposure to chemicals or allergic reactions to certain medications [Daniels et al., 2000]. The characteristic feature of this disease is multinucleated giant cells in the inflammatory locus of the myocardium. Patients with
Dilated cardiomyopathy and its treatment options

GCM often require the heart transplantation [Laruelle et al., 1994]. Ventricular assist devices have been used to bridge the time until patients receive heart transplantation. The acute myocarditis can be self limiting or fatal or progress into a chronic state, termed as dilated cardiomyopathy (DCM). Inflammatory changes and myocardial injury provide a background for the DCM [Starling et al., 1988]. In clinical setting, DCM due to myocarditis can be categorized as one of the three ways. Patients show symptoms of mild heart failure of short duration, patients with more prominent heart failure symptoms and rarely another group of patients with fulminant myocarditis with circulatory collapse.

As the epidemiological data suggest that myocarditis is an important cause of sudden death in the younger population [Costantini et al., 2005], finding out newer therapeutic strategies is the primary task to be met by the experimental medicine.

Experimental autoimmune myocarditis (EAM) and postmyocarditis DCM

There are two major challenges in the field of myocarditis. One is that patients with severe forms of myocarditis die from refractory heart failure because treatment of acute myocarditis is often ineffective. The other is that some of the patients who survive the acute phase develop DCM. Autoimmune mechanisms play an important role in the pathogenesis of myocarditis and post-infectious cardiomyopathy. To clarify the pathophysiology, many viral and autoimmune myocarditis models have been developed.
Dilated cardiomyopathy and its treatment options

The occurrence of organ-specific autoimmune myocarditis differs with antigens [Kaplan and Craig, 1963; Neu et al., 1990; Kodama et al., 1990], species [Kaplan and Craig, 1963; Neu et al., 1990; Kodama et al., 1990] and strains [Neu et al., 1990; Bachmaier et al., 1999]. Among various trial animal models, immunization with cardiac myosin in rats and mice become most suitable to study the pathophysiology because the disease course and pathology mimics human fulminant myocarditis and GCM [Neu et al., 1990; Kodama et al., 1990]. Especially the cardiac myosin induced myocarditis in Lewis rats showed 100% morbidity [21]. The acute myocarditis in Lewis rats is characterized by increased heart size, macroscopic changes and infiltration of inflammatory cells [Kodama et al., 1991], and typically they show the multinucleated giant cells in the lesions.

The autoimmune myocarditis heart enters into a chronic stage named as DCM [Kodama et al., 1994]. Macroscopically these hearts are dilated and diffusely discolored. The ventricle wall became thin. Interstitial fibrosis and replacement fibrosis expand into the entire myocardium. Hypertrophy and atrophy of myocytes are observed in and around fibrotic area.

Mechanism of pathogenesis of acute myocarditis

Cardiac myosin is composed of two heavy chains and four light chains. The cardiac myosin heavy chain is composed of about 2000 amino acids. In autoimmune diseases, T cells are able to recognize 10-20 amino acid residues, and B cells recognize 5-10 amino acid peptides as antigens. Therefore, knowledge of the location and structure of
Dilated cardiomyopathy and its treatment options

myocarditogenic epitopes on cardiac myosin is important to understand the pathogenesis of autoimmune myocarditis. In rat EAM, mathematical analysis of the peptide sequence, which could be presented by rat major histocompatibility (MHC) class II molecules, showed that residues 1539-1555 of the rat cardiac myosin α-chain would be the myocarditogenic epitope of EAM [Wegmann et al., 1994]. Direct sub-fragment analysis revealed that actually several myocarditogenic epitopes existed on cardiac myosin. The most effective epitope was present on the residues 1070-1165 of the porcine cardiac myosin β-chain [Inomata et al., 1995].

Initiation of autoimmune disease requires the breakdown of antigen-specific self-tolerance. Molecular mimicry of myocarditogenic epitopes to viral or foreign antigens may be one of the mechanisms for loss of self tolerance. The presence of cardiac myosin-reactive myocarditogenic T cells is basically prerequisite. The myocarditogenic T cells must be stimulated and proliferated for the initiation of EAM. Susceptibility to EAM is based on various factors such as genetic background, cytokines, chemokines and resident cells in the target organs [Liao et al., 1995; Pummerer et al., 1991; Pummerer et al., 1995]. Rat EAM is transferable to naïve syngeneic animals by in vitro-activated lymphocytes [Kodama et al., 1992], mainly by CD-4 positive T lymphocytes [Okura et al., 1998]. Subsets of infiltrating cells have been investigated in the hearts with EAM. The majority of infiltrating cells are composed of macrophages and CD-4 positive T cells [Pummerer et al., 1991; Kodama et al., 1992]. Infiltrating T cells are highly activated in the rat heart with EAM. The majority of CD-4 positive cells bear αβ T-cell receptor,
Dilated cardiomyopathy and its treatment options

lymphocyte function associated antigen-1, and interleukin-2 receptor molecules on their surface [Hanawa et al., 1993]. The infiltrating T cells in the rat heart and pericardial space have been demonstrated to be oligoclonal [Hanawa et al., 1996]. Administration of porcine cardiac myosin with complete Freund’s adjuvant, which comprises of inactivated and dried Mycobacterium tuberculosis leads to antigen presentation of cardiac myosin-specific T cells in the peripheral lymphatic organs. Freund’s adjuvant, an immunopotentiator, plays an important role in the cell-mediated immunity leads to the breakdown of self-tolerance by activation of antigen presenting cells, enhancement of the expression of MHC molecules as well as increases in vascular permeability. After break down of self-tolerance, expansion of cardiac myosin-specific T cell clones, migration of these cells to the myocardium and cardiac myosin specific T cells are activated by antigen presentation from resident dendritic cells [Smith and Allen, 1992]. Activated T cells secrete many cytokines, chemokines and other mediators which recruit and activate other inflammatory cells. These inflammatory mediators damage the myocardium and interfere with the cardiac function [Smith and Allen, 1992; Goren et al., 1998; Ishiyama et al., 1998]. Release of cardiac myosin from the damaged heart leads to further activation of specific T cells. Autoantibodies developed against the myosin bind to the injured heart and destroy them.

The balance between T helper 1 (Th1) cell cytokines and T helper 2 (Th2) cytokines determine the outcome of the disease. Usually during the progressive phase of
Dilated cardiomyopathy and its treatment options

myocarditis i.e. acute myocarditis Th1 cells has predominant function, later in the recovery phase Th2 cytokines become more active one.

The later stage of acute myocarditis or chronic myocarditis progresses into DCM. Persistent autoimmunity is the cause of persistent myocarditis. Next important factor is occurrence of fibrosis and hypertrophy, which are collectively termed as cardiac remodeling.

An animal model of chronic heart failure (CHF)

CHF is a major cardiovascular problem because of its morbidity and mortality in the population. Analysis of pathophysiology and search for the new therapeutic strategies for CHF are required. Small animal models are useful in the basic study of CHF. Rat myocardial infarction produced by coronary artery ligation during open-chest surgical procedures has been used for the purpose. However, the infarction model has significant mortality due to technical problems at operation. On the other hand, rat EAM can be used as an animal of CHF with several advantages compared to the infarction model. Rat EAM can be induced by a simple procedure: subcutaneous injection of the antigen. The morbidity is 100%. Myocardial damage is severe enough to produce CHF. Myocardial damage spreads diffusely over the entire heart [Koyama et al., 1995].

Cardiac Remodeling

Cardiac remodeling is the expression of molecular, cellular and interstitial changes in response to cardiac injury, manifesting as adverse alterations in the size, shape and
Dilated cardiomyopathy and its treatment options

function of the ventricle [Cohn et al., 2000; McKay et al., 1986]. Several factors influence this process and include hemodynamic load, neurohormonal activation, ischemia, necrosis, and apoptosis [Eaton and Bulkley, 1981; Hochman and Bulkley, 1982; Pfeffer et al., 1991]. Although etiologies for cardiac injury may differ, they all share common pathways that initiate and perpetuate remodeling, the most common and well studied of which is myocardial infarction [Eaton and Bulkley, 1981; Hochman and Bulkley, 1982; Pfeffer et al., 1991]. Cardiac remodeling is associated with the syndrome of heart failure, and many believe that heart failure results from decompensated cardiac remodeling [Cohn et al., 2000]. Nevertheless, the relationship between cardiac remodeling and heart failure remain unclear; factors influencing this relationship are still under investigation.

Cardiac remodeling is associated with a marked increase in cardiovascular morbidity and mortality Mitchell et al., 1992; White et al., 1987; St John Sutton et al., 2003]. Both the Survival and Ventricular Enlargement (SAVE) and Studies of Left Ventricular (LV) Dysfunction (SOLVD) have clearly demonstrated that cardiac enlargement and/or progressive ventricular dilatation are independently associated with adverse cardiovascular outcomes, including cardiovascular death and heart failure [White et al., 1987; St John Sutton et al., 2003]. There is great interest in not only identifying individuals who remodel, but also in evaluating various therapies that may attenuate and/or reverse the process [St John Sutton et al., 2003].

Renin-Ang-aldosterone system (RAAS)
The activity of the RAAS is central to the maintenance of water and electrolyte balance and blood volume [Kirk, 1999]. A large number of hormonal systems are activated in heart failure Francis et al., 1990]. Patients with CHF show elevated plasma levels of norepinephrine, renin, arginine vasopressin (AVP), among others. These neurohormonal vasoconstrictors mediate cell proliferation, myocyte hypertrophy and probably also interstitial growth, all of which contribute to the process of cardiac remodeling. A large part in the regulation of the synthesis and release of these hormones is played by the RAAS through its main signaling hormone, Ang II.

**Harmful effects of Ang II**

One of the most significant products of RAAS activation is Ang II. It is a potent vasoconstrictor, stimulates the formation and secretion of aldosterone from the adrenal gland, and has pleiotropic effects upon cellular growth Opie, 1999]. The majority of Ang II is derived from the precursor, Ang I, which originates from angiotensinogen, produced by the liver. The formation of Ang I from angiotensinogen is rate-limited by the protease, renin, which is produced by the juxtaglomerular cells of the kidney. Primary stimuli for renin secretion and RAAS activation are: impaired renal perfusion, salt depletion, and β1-adrenergic stimulation. Ang II inhibits renin secretion, via a negative feedback loop. While the majority of Ang I is converted to Ang II primarily through the activity of ACE, non-ACE pathways also exist and modulate the production of Ang II. The highest concentration of ACE exists within the pulmonary circulation; however, many studies have documented the existence of local tissue
Dilated cardiomyopathy and its treatment options

RAASs Campbell, 1987. Ang II mediates its actions through G-coupled protein receptors. Ang II possesses a number of characteristics that make it a potentially harmful hormone.

Ang II is involved with vascular tone and endothelial function, cardiac contractility, impulse propagation and stimulates the formation and secretion of aldosterone; it has pleiotropic effects upon cellular growth and apoptosis Timmermans et al., 1992]. The effects of Ang II upon cardiac tissue are related to two primary receptors, Ang receptor type 1 and 2 (AT1 and AT2). The AT1-receptor consists of two subtypes, AT1A and AT1B. The AT1-receptor (particularly AT1A) appears to mediate many of the pressor and growth effects of Ang II Timmermans et al., 1992]. Activation of AT1-receptors provokes the initiation of a variety of events, including phospholipase C activation, resulting in activation of protein kinase C and the phosphorylation of numerous proteins involved in cellular function and growth [Clauser, 1998]. Ligand binding of AT1-receptors also results in their internalization into intracellular vesicles, and these receptors appear to cycle continuously between endosomal vesicles and the plasma membrane; this does not occur with AT2-receptors, which remain fixed at the plasma membrane. Internalized Ang II (via AT1) is either degraded or exerts other intracellular effects, which may affect nuclear transcription, as recent evidence has shown the existence of an Ang II nuclear binding site [De Mello and Dancer, 2000]; resulting in a host of intracellular processes and responses.
Activation of the RAAS commonly accompanies myocardial dysfunction. Several clinical studies have documented significant elevations in the levels of renin, Ang II and aldosterone attending acute myocardial infarction and/or congestive heart failure [Cohn et al., 2000]. Similar to catecholamines, markedly elevated activity of the RAAS is associated with poor prognosis. Of the several pharmacological approaches to ventricular remodeling, only a few have proven efficacy in attenuating and/or reversing remodeling in large-scale clinical trials. These have been: β-adrenoreceptor antagonists (β-blockers), ACE inhibitors, Ang II receptor blockers (ARBs) and aldosterone antagonists [Swynghedauw, 2002; McMurray and Pfeffer, 2002].

**Therapeutic strategies for cardiac myosin-induced heart failure**

**ACE inhibitors**

ACE inhibitors have been shown to reduce morbidity and mortality in patients and animal models with heart failure [Francis et al., 1990; Weinberg et al., 1994]. ACE inhibitors are drugs that inhibit the formation of Ang II from Ang I, as well as the breakdown of bradykinin, by binding to ACE via its peptide-binding pocket. A large number of ACE inhibitors are commonly prescribed today, including but not limited to captopril, enalapril, lisinopril, quinapril, ramipril, trandolapril and zofenopril. Ang-II is a potent vasoconstrictor, but may also be an important immunomodulator in its own right. Some of the immunomodulatory properties of Ang-II are also briefly discussed below in the context of ACE inhibition. ACE inhibitors are commonly prescribed both as a first course of and as continued treatment for myocarditis, including autoimmune
and infectious forms of the disease. While many experimental animal studies have explored the usefulness of modulating the RAAS in heart failure and hypertension [McNamara et al., 2001]. The few published studies suggest that ACE inhibitors are immunomodulatory drugs and their use in myocarditis may aid in reducing disease morbidity. Our previous study demonstrated that, the treatment with ACEI such as quinapril, beginning from the late phase of active inflammation, decreased heart weight and LVEDP (LV end diastolic pressure), and increased intra-ventricular pressure rise and decline (\(\pm dP/dt\)), without changing heart rate or LV pressure. Remarkably, the area of fibrosis after quinapril treatment was greatly reduced in a dose-dependent manner from 32 to 22%, 13% or 6%. Moreover, quinapril treatment significantly decreases mast cell density and its degranulation in rats with DCM after EAM.

\textbf{ARBs}

The RAAS plays an important role in the pathogenesis of a variety of clinical conditions, including atherosclerosis, hypertension, LV hypertrophy, myocardial infarction, and heart failure [Ferrario and Strawn, 2006; Schmieder et al., 2007]. As a result, the RAAS represents a logical therapeutic target in the management of hypertension, renal disease, and cardiovascular disease. Inhibition of the RAAS with either ACE inhibitors which block the formation of Ang-II, the principal effector peptide of the RAAS or ARBs block the deleterious effects of Ang-II at the AT\(_1\) receptor has been shown to be effective in lowering blood pressure and reducing cardiovascular mortality and morbidity in various at-risk patient populations.
The effects of ARBs in the treatment of hypertension, congestive heart failure and myocardial fibrosis have been well analyzed in human trials, as well as animal models, and the focus of interest is now directed to its pleiotropic effects, especially on inflammatory disorders. ARBs have also been reported to suppress atherosclerotic lesions in animal models and patients with coronary artery disease or hypertension by modulating inflammatory responses [Navalkar et al., 2001; Koh et al., 2003; Fliser et al., 2004]. AT₁ receptor antagonists are reported to suppress cytokine production and the transcription of cytokine genes in vitro and in vivo [Kjeldsen and Julius, 2004; Kramer et al., 2002; Sukumaran et al., 2010]. Thus, studies of RAAS antagonists in inflammatory diseases suggested that Ang-II was involved in immune and inflammatory responses [Kjeldsen and Julius, 2004; Kramer et al., 2002; Sukumaran et al., 2010]. Our previous studies have demonstrated that Ang-II upregulated inflammatory cytokines, oxidative stress and ER stress and its marker molecule expressions in the hearts of EAM [Sukumaran et al., 200; Sukumaran et al., 2011]. These expressions were significantly lowered in the ARB-treated rats than in the vehicle-treated DCM rats. Further, cardiac mast cell density and degranulation of mast cell were significantly decreased in the ARB treated DCM rats than in the vehicle-treated DCM rats.

**Aldosterone antagonists**

Cardiac inflammation contributes to increased cardiovascular morbidity and mortality associated with activation of the RAAS. It has been demonstrated that aldosterone and/or mineralocorticoid receptor activation causes oxidative stress and vascular
Dilated cardiomyopathy and its treatment options

inflammation. Aldosterone is a mineralocorticosteroid hormone that contributes to the development of hypertension, myocardial hypertrophy, and cardiovascular morbidity [Delyani, 2000]. Aldosterone acts negatively on the cardiovascular system, and causes myocardial necrosis, vascular injury, endothelial dysfunction and catecholamine release, and produces cardiac arrhythmias [Stier et al., 2002]. Ang-II stimulates the synthesis of the mineralocorticosteroid aldosterone [Takeda et al., 1995], and many evidences indicate that aldosterone causes myocardial and aortic fibrosis in animal models, while aldosterone receptor antagonism reverses these processes [Brilla and Weber, 1992; Robert et al., 1995]. Recent study shows that eplerenone attenuates the development of ventricular remodeling and fibrosis in rats after myocardial infarction model [Delyani et al., 2001]. In addition, eplerenone has an anti-inflammatory effect by inhibiting mast-cell-derived proteineases, and improves myocardial remodeling by suppressing fibrosis [Xiao et al., 2009]. Our previous study demonstrated that eplerenone significantly downregulated the severity of disease through decreasing inflammatory cell infiltration and fibrosis in rats with myosin-induced DCM [Wahed et al., 2005]. Further, we have demonstrated that eplerenone treatment significantly reduces mast cell density and its degranulation pattern in DCM rats in addition to its anti-inflammatory and anti-fibrotic effects.

Long-acting loop diuretics

Torasemide is a novel diuretic which has a potent and long lasting diuretic action [Uchida et al., 1991], which is achieved by inhibiting the reabsorption of water and
Dilated cardiomyopathy and its treatment options

electrolytes in the distal tubules, including loop of Henle [Uchida et al., 1991; Greger, 1988; Wittner et al., 1986]. Although torasemide is classified as a loop diuretic, like furosemide, bumetanide and piretanide [Ghys et al., 1985], have shown that i.v. injection of torasemide produces less kaliuresis than does furosemide at doses that cause an equivalent level of natriuresis and diuresis in anesthetized rats.

Numerous findings suggest that aldosterone may play an important role in myocardial fibrosis, which leads to LV remodeling and results in LV dysfunction [Delcayre and Swynghedauw, 2002; Weber and Brilla, 1991; Weber et al., 2003]. Recently, it has been reported that aldosterone is produced in the ventricle of the failing human [Mizuno Molkentin and Dorn II, 2003] and rat hearts [Sylvestre et al., 1998]. Additionally, aldosterone synthase (CYP11B2) is detected in the hearts of postmyocarditis rats [Palaniyandi et al., 2007]. Satoh et al. [2002] reported that cardiac CYP11B2 expression positively correlates with the degree of myocardial fibrosis which results in LV dysfunction in patients with CHF. Further, Torasemide reported to interferes with secretion and receptor-ligand binding of aldosterone [Goodfriend et al., 1998; Uchida et al., 1991]. In our previous study, we observed that the plasma Ang-II and aldosterone concentration were significantly increased compared with vehicle-treated rats [Veeraveedu et al., 2008]. The increase in Ang-II is due to decreased circulatory blood volume, while the increase in aldosterone concentration is due to prevention of binding of circulatory aldosterone to its receptor [Uechi et al., 2003]. Therefore, the possibility exists that the ability of torasemide to decrease myocardial fibrosis and its marker
molecules [transforming growth factor-beta (TGF-β) and collagen III] may also be related to interference with humoral profibrotic factors such as aldosterone [Delcayre and Swynghedauw, 2002; Weber and Brilla, 1991; Veeraveedu et al., 2008] and Ang-II [Gonzalez et al., 2002]. Further, we have also demonstrated that torasemide treatment significantly reduces infiltration of inflammatory cells, mast cell density and its degranulation pattern in DCM rats in addition to its anti-fibrotic effects.

Ang II is a very potent chemical that causes muscles surrounding blood vessels to contract, thereby narrowing blood vessels. This narrowing increases the pressure within the vessels and can cause high blood pressure (hypertension). ARBs are medications that block the action of Ang II by preventing Ang II from binding to Ang II receptors on blood vessels. As a result, blood vessels enlarge (dilate) and blood pressure is reduced. Reduced blood pressure makes it easier for the heart to pump blood and can improve heart failure. In addition, the progression of kidney disease due to high blood pressure or diabetes is slowed. ARBs have effects that are similar to ACE inhibitors, but ACE inhibitors act by preventing the formation of Ang II rather than by blocking the binding of Ang II to muscles on blood vessels.

**Oxidative stress during EAM and DCM**

Oxidative stress and inflammation are thought to play important roles in the progression of cardiovascular diseases. An increase in the level of oxidative stress is implicated in the pathogenesis of heart failure and various autoimmune disorders including DCM mediated by cardiac myosin [Sukumaran et al., 2011]. Investigations
Dilated cardiomyopathy and its treatment options

suggest that free radicals may be important contributors to the deterioration of the
decompensating myocardium [Prasad et al., 1996]. Excessive production of reactive
oxygen species (ROS) such as superoxide induced by inflammatory stimuli is an
important observation in failing hearts and produces myocardial injury in autoimmune
mediated heart failure [Ishiyama et al., 1997] and it has reported that the patients with
heart failure were found with excess levels of ROS in their plasma [Belch et al., 1991].
Treatments that reduce the levels of oxidative stress or inflammation have thus been
found to improve myocardial function in patients with advanced heart failure as well as
in animal models of this condition. The NADPH oxidase system, present in cardiac and
vascular tissues, is also a candidate for the source of the superoxide [Muthalif et al.,
2000].

Heymes et al., (2003) reported the presence of NADPH oxidase in human myocardium.
The increase in NADPH oxidase activity in the failing heart may be important in the
pathophysiology of cardiac dysfunction by contributing to increased oxidative stress.
Inhibition of NADPH oxidase reduces oxidative stress and myocyte apoptosis in the
remote noninfarcted myocardium and the effects are associated with the improvement
of cardiac function in heart failure after MI. Inhibition of NADPH oxidase may
represent an attractive therapeutic approach to treat heart failure (Qin et al., 2007). ROS
in cardiac hypertrophy, apoptosis, and the transition to failure cardiac hypertrophy can
be either compensatory and adaptive or a maladaptive precursor to cardiac failure.
Many extracellular factors are capable of inducing hypertrophy of cardiomyocytes, and
many of the various downstream signaling pathways that mediate the hypertrophic growth response to these factors can be activated directly or indirectly by ROS as mentioned in a review by Giordano. These include PKC; the MAPKs p38, JNK, apoptosis-signaling kinase 1 (ASK-1), and ERK1/2; PI3K; Akt; several tyrosine kinases (e.g., src and FAK); NF-κB; and calcineurin. An example in which an extracellular signal induces cardiac hypertrophy via a ROS-dependent pathway is ATII-induced hypertrophy. ATII induces cardiac hypertrophy via a G-protein–linked pathway that involves generation of ROS and ROS-associated activation of several downstream signals, including MAPKs. ATII induces ROS in large measure via NADPH oxidases, although the mechanism by which ATII activates these oxidases has remained incompletely understood. Another mechanism by which ROS can induce cardiac hypertrophy is via transcription factor–mediated alterations in gene expression. For example, ATII stimulates ROS-mediated activation of the transcription factor NF-κB. Another MAPK family member linking ROS and hypertrophy is the redox-sensitive kinase ASK-1. ASK-1 is strongly activated by ROS and in turn activates MAPKs p38 and JNK. Expression of a dominant-negative ASK-1 attenuates NF-κB activation and inhibits cardiac hypertrophy in response to ROS-generating G-protein receptor agonists, which demonstrates a role for ASK-1 in the link among ROS, NF-κB, and cardiac hypertrophy. ASK-1 also mediates TNF-α–induced apoptosis, which constitutes another link between ROS and apoptosis that contributes to the pathogenesis of heart disease. Cardiomyocyte apoptosis occurs in hypertrophied, ischemic, and failing hearts and may
Dilated cardiomyopathy and its treatment options

contribute to the development and progression of cardiac dysfunction and heart failure. Interestingly, whether or not apoptosis is induced in cardiomyocytes by oxidative stress appears to be dependent upon the level of ROS produced. For instance, in adult cardiomyocytes, relatively low levels of H₂O₂ are associated with the activation of ERK1/2 MAPK and the stimulation of protein synthesis. Conversely, a higher level of H₂O₂, while still activating ERK1/2, also activates the JNK and p38 MAPKs and Akt and induces apoptosis [Giordano, 2005].

From all of these data, we can delineate the involvement of oxidative stress and activation of various cell signaling pathways during the advancement of heart failure and also the definite role of antioxidants against this pathogenesis. This study is aimed to identify the involvement of oxidative stress and MAPK signaling pathway during the progression of EAM to DCM and to test the effect of antioxidant compounds against the cardiac dysfunction during this progression.
**Scope of the study**

An increase in myocardial oxidative stress due to excessive production of ROS may be involved in the pathophysiology of congestive heart failure and ROS are believed to play a prominent role in triggering ventricular damage, thus accelerating the progression of HF. At the molecular level, chronic exposure to ROS leads to accumulation of oxidized DNA, proteins, and lipids. This results in cardiomyocyte dysfunction and death, a determining factor in ventricular remodelling and failure. Mitochondrial oxidative damage such as DNA mutations have been related to mitochondrial dysfunction, decline in cardiomyocyte function and death. A phagocyte-type NADPH oxidase complex is a major source of ROS and recent studies have suggested an important role for myocardial NADPH oxidase in experimental models of cardiac disease. Thus, therapeutic strategies to modulate this detrimental response should be a target in the treatment of HF. Thus, studies with antioxidants for their effect on the pathogenesis and treatment of HF could offer us some effective treatment approach against HF. In our present study, our major aims are;

- Induction of rat model of heart failure in the form of EAM and its progressive condition DCM using porcine cardiac myosin
- Study of molecular mechanisms behind their pathogenesis
Dilated cardiomyopathy and its treatment options

- Identify the role of oxidative stress during the pathogenesis of EAM and its progression to DCM
- Study the effects of antioxidants such as, Mulberry leaves, quercetin and edaravone against these pathologies focusing on oxidative stress and endoplasmic reticulum stress as their targets.

This research work is divided into the following chapters

1. Mulberry leaf diet protects against progression of EAM to DCM via modulation of oxidative stress and MAPK mediated apoptosis
2. Quercetin offers cardioprotection against progression of EAM by suppression of oxidative and endoplasmic reticulum stress via endothelin-1/MAPK signaling
3. Beneficial effects of edaravone, a novel antioxidant, in rats with DCM after EAM with possible involvement of AMPK and MAPK signaling
Chapter 1

Mulberry leaf diet protects against progression of EAM to DCM via modulation of oxidative stress and MAPK mediated apoptosis
Dilated cardiomyopathy and its treatment options

Introduction

Patients with HF were reported with increased plasma biochemical markers of oxidative stress and there is a definitive correlation between oxidative stress and ventricular dysfunction. ROS are the major contributors of oxidative stress and therefore, it is likely that ROS are involved in not only the pathogenesis but also the active progression of HF [Keith et al., 1998]. Acute myocarditis is a potentially lethal disease and frequently precedes the development of acute and chronic HF. Some patients with myocarditis show a fulminant course and die of intractable cardiogenic shock and its treatment strategy is still unresolved [Fuse et al., 2000]. A model of rat EAM resembles human giant cell myocarditis, and the recurrent form of EAM leads to DCM [Mitsuma et al., 2006].

Recently, much of the attention has been focused on flavonoids, naturally occurring polyphenolic compounds, as food factors that may be beneficial for CV disease prevention. Various epidemiological studies have shown an inverse correlation between the consumption of flavonoid-rich foods and CV disease risk [Angeloni et al., 2008]. Mulberry (Morus alba L., family Moraceae) leaves (ML), which are commonly used as silkworm diet, contain various nutritional components. Among them, flavonoids and moracins are known to have effects as antioxidants or free radical scavengers. ML are known for their rich antioxidant polyphenols including quercetin, naringenin and gallocatechin gallate [Yang et al., 2011] that can reduce CV diseases [Chan et al., 2009]. There are various reports about the protective effects of ML on...
Dilated cardiomyopathy and its treatment options

various disease models and they also suggest that these effects are mainly due to its antioxidant capacity [Singab et al., 2010; Katsube et al., 2010; Naowaboot et al., 2009]. ML treatment was reported to provide significant protection against inflammatory reactions induced by stress immobilization [Lee et al., 2007]. Dietary mulberry has also been reported to have hypoglycemic, hypolipidemic, and antioxidant effects [El-Beshbishy et al., 2006]. Mulberry showed relatively high antioxidant activity on comparison with 52 kinds of edible plant products in Japan by LDL oxidation assay [Enkhma et al., 2005]. We have reported the effects of ML on the pathogenesis of EAM, where it has produced significant protection against the cardiovascular dysfunction (Arumugam et al., 2011). Because of these interesting results we have decided to examine the protective effects of dietary administration of ML on post-myocarditis DCM focusing on oxidative and endoplasmic reticulum stresses and adverse myocardial remodeling.

Experimental methods

Materials

Lewis rats (male, 8 weeks old) were purchased from Charles River Japan Inc (Kanagawa, Japan). All the chemicals and reagents were purchased from Sigma, Tokyo, Japan until otherwise mentioned.

Experimental design

All experiments were carried out using eight weeks old male Lewis rats and were performed in accordance with the guidelines of our institute.
Lewis rats were injected in the footpads with antigen-adjuvant emulsion in accordance with a procedure described previously [Kodama et al., 1990]. In brief, porcine cardiac myosin was dissolved in phosphate-buffered saline at 5 mg/ml and emulsified with an equal volume of complete Freund’s adjuvant with 11 mg/ml Mycobacterium tuberculosis H37RA (Difco Lab., Detroit, MI). EAM in rats was induced by immunization with 0.1 ml of emulsion once by subcutaneous injection into the rear footpads (0.1 ml to each footpad). The morbidity of EAM was 100% in rats immunized by this procedure.

Twenty-eight days after immunization, the surviving rats were divided into two groups namely Group ML5% and Group V and received normal powder diet mixed with or without 5% ML powder (Taimatsu Co., Niigata, Japan) respectively for 28 days. Age-matched Lewis rats without immunization were used as normal controls (Group N). Body weight (BW) of each rat was measured both before and after the experiment.

**Hemodynamic study**

By the end of study, hemodynamic measurements were performed to assess the cardiac function of each rat as described previously [Sukumaran et al., 2011]. Rats were anesthetized with 2% halothane in oxygen during the surgical procedures. A catheter-tip transducer (Miller SPR 249; Miller Instruments, Houston, TX, USA) was introduced into the left ventricle (LV) through the right carotid artery for the determination of peak LV pressure (LVP) and LV end-diastolic pressure (LVEDP), and the rates of intraventricular pressure rise (+dP/dt) and decline (-dP/dt). After instrumentation, the
concentration of halothane was reduced to 0.5% to minimize the effects of anesthesia on hemodynamic parameters.

**Transthoracic echocardiographic analysis**

Echocardiographic studies were carried out with a 7.5-MHz transducer (Aloka Inc, Tokyo, Japan). The LV dimensions in diastole (LVDd) and systole (LVDs), percentage ejection fraction (EF) and percentage fractional shortening (FS) were estimated using M-mode measurements [Thandavarayan *et al*., 2009]. After the echocardiographic analysis, all the rats were sacrificed, and their wet heart weight (HW) was measured. Then the excised myocardium was kept in formalin and the mid-ventricle sections were embedded with paraffin.

**Analysis of cardiac apoptosis by terminal transferase-mediated dUTP nick-end labeling (TUNEL) assay**

The TUNEL assay was performed as specified in the instructions for the in situ apoptosis detection kit (Takara Bio Inc., Shiga, Japan). Sections embedded in paraffin were mounted and examined using light microscopy. Digital photomicrographs were obtained by using a color image analyzer (CIA-102, Olympus, Tokyo, Japan) at X200 magnification, and 25 random fields from each heart were chosen and the number of TUNEL positive nuclei was quantified in a blinded manner. For each group, three sections were scored for apoptotic nuclei. Only nuclei that were clearly located in cardiac myocytes were considered [Thandavarayan *et al*., 2009].

**Hematoxylin-eosin (H-E) and Azan-Mallory staining**
After being embedded in paraffin, several transverse sections were prepared from the ventricle, and stained with H-E and Azan-Mallory. Infiltration of inflammatory cells was examined in the H-E stained slides viewed under a high-power light microscope. The area of myocardial fibrosis in LV tissue sections stained with Azan-Mallory was quantified using a color image analyzer (CIA-102, Olympus, Tokyo, Japan) and measured the blue fibrotic areas as opposed to the red myocardium at X200 magnification. The results were presented as the ratio of the fibrotic area to the whole area of the myocardium [Sukumaran et al., 2010].

**Immunohistochemical assay**

Immunohistochemical staining was performed [Arozal et al., 2010] with the formalin-fixed, paraffin-embedded cardiac tissue sections of the rats from different groups. After deparaffinization and hydration, the slides were washed in Tris-buffered saline (TBS; 10 mM/l Tris HCl, 0.85% NaCl, pH 7.5) containing 0.1% bovine serum albumin. Endogenous peroxidase activity was quenched by incubating the slides in 0.6% H₂O₂ in methanol. To perform antigen retrieval, the sections were pretreated with trypsin for 15 min at 37°C. After overnight incubation with the goat polyclonal anti-collagen Type III antibody (diluted 1:100) (Santa Cruz Biotechnology Inc, Santa Cruz, CA) at 4°C, the slides were washed in TBS and horseradish peroxidase-conjugated secondary antibody was then added and the slides were further incubated at room temperature for 45 min. The slides were washed in TBS and incubated with diaminobenzidine tetrahydrochloride as the substrate, and counterstained with hematoxylin. A negative
control without primary antibody was included in the experiment to verify the antibody specificity. Measurement of myocardial immunoreactivity for collagen-III was performed in 100 randomly selected fields in heart sections at X200 magnification by light microscopy.

**In situ detection of superoxide production in hearts**

Superoxide generation was estimated by dihydroethidium (DHE) staining as previously described [Miller et al., 1998]. To evaluate in situ superoxide production from hearts, unfixed frozen cross sections of the specimens were stained with DHE (Molecular Probes, Eugene, OR, USA) according to the previously validated method. In the presence of superoxide, DHE is converted to the fluorescent molecule ethidium, which can then label nuclei by intercalating with DNA. Briefly, the unfixed frozen LV tissues were cut into 10-μm-thick sections and incubated with DHE 10 μM at 37°C for 30 min in a light-protected humidified chamber. Fluorescence images (X200 magnification) were obtained using a fluorescence microscope equipped with a rhodamine filter.

**Western immunoblotting analysis**

This analysis was carried out as described early [Thandavarayan et al., 2008]. Briefly, the myocardial tissue samples obtained from different groups were homogenized with lysis buffer. Protein concentrations in those homogenized samples were measured by the bicinchoninic acid method. For Western blots, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. According to the molecular weight of the protein of interest, we have used 7.5%, 10% and 15% sodium dodecyl sulphate-
Dilated cardiomyopathy and its treatment options

polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, USA) at 150-200 V for 35-50 min depending on the percentage of the gel used), transferred to nitrocellulose filters (semi-dry transfer at 10-12 V for 1-2h), blocked with 5% non-fat dry skimmed milk or bovine serum albumin (Sigma, St Louis, MO, USA) in 0.05% TBST (20 mM/1 Tris, pH 7.6, 137 mM/l, NaCl, and 0.05% Tween) solution and incubated with the following mono/polyclonal antibodies (goat anti-p67phox, mouse anti-osteopontin (OPN), goat anti-endothelin (ET)-1, rabbit anti-troponin I, goat anti-glucose regulated protein (GRP)78, rabbit anti-phospho Akt, rabbit anti-phospho p38-mitogen activated protein kinase (MAPK), mouse anti-phospho extracellular-ligand regulated kinase (ERK)-1/2, rabbit anti-vascular endothelial growth factor (VEGF), rabbit anti-transforming growth factor (TGF)-β1 and rabbit anti-glyceraldehyde 3 phosphate dehydrogenase (GAPDH) antibodies obtained from Santa Cruz Biotechnology or Cell Signaling, (blotting conditions and dilutions were done as per the protocol sheet of individual antibody, supplied by the manufacturer) overnight at 4°C. The blots were washed with 0.05% TBST and then incubated for 1h at room temperature with appropriate horse-radish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) and the protein bands were identified using enhanced chemiluminescence developing agents (Amersham Biosciences, Buckinghamshire, UK) and X-ray films. The band intensities were quantified using Scion Image program (GT-X700, Epson, Tokyo, Japan). The cytosolic fraction was separated from the homogenate and used for the estimation of cytochrome C level using mouse anti-cytochrome C antibody. The level of GAPDH was
Dilated cardiomyopathy and its treatment options

estimated in every sample. Finally, Western blot data were normalized with cardiac GAPDH.

Statistical analysis

All the values are expressed as means ± SEM. Statistical analysis of differences between the groups was performed by student’s ‘t’ test or one-way analysis of variance, followed by either Dunnett’s test or Tukey’s multiple comparison test wherever necessary using GraphPad Prism 5 software. A value of \( p < 0.05 \) was considered statistically significant.

Results

Effect of ML diet (MLD) on myocardial dimensions

There was a significant increase (\( p < 0.01 \)) in the HW/BW ratio of the group V DCM rats indicating the hypertrophic remodeling of the heart, whereas the rats provided MLD were significantly (\( p < 0.05 \)) protected from these changes. Although heart rate was not different among the three groups of rats, transthoracic echocardiographic studies of group V rats showed evidence for the cardiac remodeling with increased LV dimensions, reduced EF (25.07 ± 2.78 vs 84.23 ± 2.29\%, \( p < 0.01 \)) and FS (15.64 ± 7.46 vs 48.60 ± 3.17\%, \( p < 0.01 \)). These results indicate the impairment of cardiac function in the DCM rats of group V compared with that in group N. Treatment with MLD significantly decreased all these parameters compared with those in group V.
**Effect of mulberry leaf diet on hemodynamic and echocardiographic parameters of DCM rats**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group N</th>
<th>Group V</th>
<th>Group ML5%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Histopathology</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>401.1±19.6</td>
<td>316.2±34.1</td>
<td>419.2±17.1</td>
</tr>
<tr>
<td><strong>HW/BW (mg/kg)</strong></td>
<td>2.53 ± 0.02</td>
<td>6.50 ± 0.36##</td>
<td>5.66 ± 0.32##,*</td>
</tr>
<tr>
<td><strong>Hemodynamic parameters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CVP (mmHg)</td>
<td>0.5±0.3</td>
<td>10.0±0.4#</td>
<td>2.5±0.4##,**</td>
</tr>
<tr>
<td>LVP (mmHg)</td>
<td>121.4±3.1</td>
<td>60.4±6.0##</td>
<td>97.0±5.9##,**</td>
</tr>
<tr>
<td>+dP/dt (mmHg/min)</td>
<td>6522±118.5</td>
<td>1492±129.5##</td>
<td>3955±424.5##,**</td>
</tr>
<tr>
<td>-dP/dt (mmHg/min)</td>
<td>8981±518.6</td>
<td>16385±219.3##</td>
<td>3700±843.5#</td>
</tr>
<tr>
<td><strong>Echocardiography</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVDd (mm)</td>
<td>5.97 ± 0.31</td>
<td>7.42 ± 0.79##</td>
<td>6.30 ± 0.36*</td>
</tr>
<tr>
<td>LVDs (mm)</td>
<td>3.08 ± 0.22</td>
<td>6.64 ± 0.66##</td>
<td>4.66 ± 0.22##,**</td>
</tr>
<tr>
<td>FS (%)</td>
<td>84.23 ± 2.29</td>
<td>25.07 ± 2.78##</td>
<td>57.16 ± 3.05##,**</td>
</tr>
<tr>
<td>EF (%)</td>
<td>48.60 ± 3.17</td>
<td>15.64 ± 7.46##</td>
<td>26.18 ± 1.89##,**</td>
</tr>
</tbody>
</table>

N, age-matched normal rats; V, immunized rats treated with normal diet; ML5%, Immunized rats supplemented with ML5% mixed in the normal diet. ##p<0.05, ##p<0.01 vs group N; *p<0.05 vs group V (One-way ANOVA followed by Tukey Multiple comparison test)
Effect of MLD on myocardial apoptosis

The present study with TUNEL assay showed that the number of TUNEL positive apoptotic nuclei was significantly higher in myocardial tissue sections from group V rats than in group N. However, treatment with MLD significantly decreased the number of TUNEL positive nuclei compared to that in group V.

Effect of MLD on myocardial fibrosis and its marker molecules

Immunohistochemical analysis for deposition of collagen-III in the myocardial tissue sections revealed that the number of collagen-III positive cells were significantly higher in the group V rats whereas the rats under MLD showed significantly lower levels of it when compared with the DCM rats in group V.
Similarly Azan-Mallory staining of the myocardial tissue sections also confirmed the extensive fibrosis in the myocardium of group V rats. Interestingly the rats in the group ML5% showed significantly lesser staining with Azan-Mallory indicating the antifibrotic effect of MLD. In addition the hearts from DCM rats showed increased expression of OPN compared to those from group N. Treatment with MLD significantly reduced their myocardial expression than in vehicle treated rats.
Effect of MLD on inflammatory cellular infiltration

H-E staining of the myocardial sections from group V rats showed severe inflammatory cellular infiltration and damaged cellular organisation, but the rats received MLD showed significantly lower inflammatory cellular infiltration and near normal myocardial cellular architecture.

Effect of MLD on superoxide production

DHE staining revealed the involvement of superoxide mediated oxidative stress in the DCM rats as the myocardial tissue sections of group V rats showed extensive staining for superoxide whereas the rats supplied MLD showed significantly lesser staining for superoxide.
Effect of MLD on myocardial troponin I level

Western blotting analysis showed the decreased myocardial protein level of troponin I in the hearts of group V DCM rats whereas the rats supplemented with MLD had significantly improved expression of it, suggesting the role of MLD in maintenance of cardiac contractile function.

Effect of MLD on myocardial ET-1 and VEGF levels

The myocardial expressions of ET-1 and VEGF were significantly higher in the rats of group V whereas the rats supplemented with MLD had significantly reversed these changes.
Effect of MLD on myocardial p67phox level

Oxidative stress is the major hallmark of various CV disorders and has also been reported to be involved in the progression of EAM to DCM. In the present study we have measured the expression of p67phox, one of the NADP(H) subunits as they are one among the indicators of oxidative stress. The cardiac expression of p67phox was significantly increased in group V rats compared with that in group N, and these changes were significantly reversed by supplementation with MLD.

Effect of MLD on ER stress marker protein

ER stress is gaining importance as it can lead to apoptotic cell death. In order to identify its involvement in the progression of EAM, we have measured the myocardial expression of GRP78, which is an important ER stress marker. Its level was significantly elevated in group V rats compared with that in group N. But this change was significantly attenuated by the supplementation with MLD as evidenced by the
Dilated cardiomyopathy and its treatment options

decreased myocardial levels of the above-mentioned ER stress marker. Similarly the cytosolic levels of cytochrome C was also elevated significantly in the group V rats, whereas the rats provided MLD were significantly protected from this change indicating its protective effect against apoptosis mediated by cytochrome C.

Effect of MLD on MAPK signaling cascade

In order to identify the probable mode of action of MLD on DCM, we have measured the myocardial expression levels of phosphorylated forms of Akt, ERK, p38-MAPK, and TGF-β1 in both the vehicle treated and MLD supplemented rats. Group V rats showed the activation of MAPK pathway as their hearts showed significant increase in the phosphorylation of the above-mentioned members of MAPK signaling cascade. Interestingly the rats supplemented with MLD were protected from these changes as their myocardial levels of these proteins were significantly lesser when compared with the group V rats.
Dilated cardiomyopathy and its treatment options

**Densitometric ratio**

- **p-Akt/GAPDH**
  - N: 0.2
  - V: 0.8
  - ML5%: 0.2

- **p-p38 MAPK/GAPDH**
  - N: 0.0
  - V: 0.5
  - ML5%: 1.0

- **Densitometric ratio**
  - **p-ERK(1/2)/GAPDH**
    - N: 0.0
    - V: 1.5
    - ML5%: 1.5

- **Densitometric ratio**
  - **TGF-β1/GAPDH**
    - N: 0.0
    - V: 1.5
    - ML5%: 1.0

**Immunoblotting**

- **p-Akt**
  - N: Weak
  - V: Strong
  - ML5%: Weak

- **GAPDH**
  - N: Strong
  - V: Strong
  - ML5%: Strong

- **Total-Akt**
  - N: Weak
  - V: Strong
  - ML5%: Weak

- **p-p38 MAPK**
  - N: Weak
  - V: Strong
  - ML5%: Strong

- **GAPDH**
  - N: Strong
  - V: Strong
  - ML5%: Strong

- **Total-MAPK**
  - N: Weak
  - V: Strong
  - ML5%: Weak

- **p-ERK(1/2)**
  - N: Weak
  - V: Strong
  - ML5%: Weak

- **GAPDH**
  - N: Strong
  - V: Strong
  - ML5%: Strong

- **Total-ERK(1/2)**
  - N: Weak
  - V: Strong
  - ML5%: Weak

- **TGF-β1**
  - N: Weak
  - V: Strong
  - ML5%: Strong

- **GAPDH**
  - N: Strong
  - V: Strong
  - ML5%: Strong
Discussion

In the present study, dietary supplementation with flavonoids and polyphenols rich ML has provided significant protection from adverse cardiac remodeling involved in the transformation of EAM to DCM. Myocardial structural and functional parameters were improved significantly in the EAM rats provided MLD. Their cardiac contractile function was improved, as evidenced by the improved EF and FS when compared with the group V rats and which was also confirmed by the restoration of their myocardial troponin I levels.

Oxidative stress is involved in the pathogenesis of various cardiac complications among which porcine cardiac myosin-induced EAM is under extensive study. EAM can progress into post myocarditis DCM which is a form of end stage heart failure. Excessive oxidative stress can damage many biological molecules, protein and DNA [Halliwell and Chirico, 1993]. Various oxidative stress related parameters were reported to be elevated in this illness, out of which NADPH oxidase plays an important role. The NADPH oxidase complex is a cluster of proteins that promote donation of an electron from NADPH to molecular oxygen to produce superoxide, which requires recruitment of the cytosolic subunits p47phox and p67phox [Ming et al., 2007; Kyaw et al., 2004]. Our present study with DCM rats also confirmed the increased oxidative stress as evidenced by the elevated myocardial expression of its marker protein p67phox and extensive staining for superoxide in the myocardial sections by DHE staining. Several antioxidant compounds were studied for their protective role against ailments with oxidative stress.
Dilated cardiomyopathy and its treatment options

as the major pathogenic mechanism so that they can be effectively used in their treatment. ML, a natural source of various polyphenols and flavonoids, was reported to provide protection against oxidative stress in different animal models [Singab et al., 2010; Katsube et al., 2010; Kobayashi et al., 2010]. Accordingly in the present study, the expression of p67phox was significantly suppressed in the myocardium of rats treated with MLD along with significantly lesser staining for superoxide in DHE staining. Thus MLD supplementation has provided protection against activation of NADPH oxidase and production of superoxide radicals and thus reducing oxidative stress, which may be one of the mechanisms involved in the prevention of adverse cardiac remodeling in DCM rats.

Increased oxidative stress evokes many intracellular events including inflammation, ER stress and myocyte apoptosis [Kannan and Jain, 2000]. In order to confirm the effect of MLD on these events, initially we identified the inflammatory cellular infiltration in the myocardium of the rats under study using H-E staining. The sections from the hearts of group V rats showed extensive inflammatory cellular infiltration, whereas the rats received MLD showed lesser infiltration. This report has provided us the initial confirmation about the effect of MLD against the oxidative stress induced myocardial inflammation.

ER stress has been attracting considerable attention since it triggers several inflammatory disorders. In response to ER stress, there is a marked upregulation of ER chaperone like GRP78 [Pan et al., 2010]. This stress includes disruption of ER calcium
Dilated cardiomyopathy and its treatment options

homeostasis and accumulation of excessive proteins in ER. Cell apoptosis can proceed via various routes, leading to myocardial cell loss [Lai et al., 2007]. The results of the present study have also confirmed the activation of ER stress in the DCM rats as their myocardial level of GRP78 was found to be elevated significantly. Interestingly the rats supplied MLD showed significant suppression of this change. There are various reports regarding the role of antioxidants against ER stress during a variety of disorders [Shimazaki et al., 2010; Yamauchi et al., 2011]. In line with these reports, our present study has confirmed the protective effects of MLD, a rich source of natural antioxidants, against adverse cardiac remodeling induced by ER stress. Excess or prolonged ER stress leads to cell death in the form of apoptosis [Mao et al., 2007; Okada et al., 2004]. In the present study, the myocardial cellular apoptosis was significantly higher in the vehicle treated rats, whereas the rats supplemented with the MLD showed significant reduction of the apoptotic cells in their myocardium as evidenced by TUNEL staining. In addition, the cytosolic level of cytochrome C was significantly reduced by MLD supplementation. During cellular stress, cytochrome C is released from the mitochondria into the cytosol and which further activates apoptosis. Thus the present study has provided evidences for the protective role of MLD against progression of EAM to DCM by preventing oxidative and ER stresses followed by myocardial apoptosis.

In the heart, endogenous ET-1, which is locally generated and secreted by cardiomyocytes, may contribute to cardiac hypertrophy via an autocrine/paracrine fashion [Haada et al., 1997; Ito et al., 1993]. The role of ET-1 in adverse myocardial
remodeling has been reported by various studies [Sun et al., 2009; Chua et al., 2008]. In the present study, supplementation of MLD has shown significant suppressive effect on the ET-1 levels in the myocardium of the DCM rats. Variety of agonists can stimulate the G-protein coupled or tyrosine kinase receptors, among which ET-1 is one of the potent agonists, which converge at MAPK playing a central role in cardiac hypertrophy. In the intact heart, p38-MAPK is activated by a variety of stresses including oxidative stress [Clerk and Sugden, 1998]. Another possibility is, ET-1 first activates the ERKs in cardiac myocytes [Bogoyevitch et al., 1993; Clerk et al., 1994; Post et al., 1996], subsequently activating SAPKs/JNKs, as these MAPKs have been implicated in cardiac hypertrophy [Gillespie-Brown et al., 1995; Ramirez et al., 1997]. In consistent with these reports, our present study has confirmed the activation of MAPK signaling cascade in DCM rats. Interestingly, the treatment of DCM rats with MLD has significantly reversed these changes. Attenuation of the increased oxidative stress by MLD supplementation might also have produced a direct inhibitory effect on p38-MAPK. In addition, the present study also showed the reduced activation of Akt in the hearts of DCM rats fed with MLD indicating the cardioprotective effects of it may be possibly via inhibiting the p38MAPK activation and subsequently the activation of Akt so that its downstream signaling can be prevented to avoid adverse cardiac remodeling.

It is also of interest to investigate the myocardial expression level of vascular VEGF in the rats treated with MLD, as ET-1-induced cardiomyocyte hypertrophy is associated with a change in expression of VEGF in rat cardiomyocytes [Shimojo et al., 2007]. VEGF
Dilated cardiomyopathy and its treatment options

is an endothelial cell-specific mitogen in vitro and an angiogenic inducer in in vivo models. Importantly, ET-1 has been documented to enhance VEGF mRNA expression via activation of ET-A receptors in rat vascular smooth muscle cells [Matsuura et al., 1998]. From these reports, we assume that VEGF may serve as a key regulator of ET-1-induced cell proliferation in CV disorders. In the present study, the myocardial levels of VEGF was significantly elevated when compared to the group V rats, indicating that the hypertrophic remodeling was induced by ET-1, which is also mediated by VEGF. Interestingly, the rats fed with MLD had significantly lower levels of VEGF in their heart, which confirms that the cardioprotective effect offered by MLD is partially mediated via the suppression of VEGF expression induced by ET-1. Thus MLD supplemented rats are protected from the adverse cardiac remodeling induced by ET-1 possibly via both the suppression of VEGF expression and modulation of MAPK signaling cascade.

Finally, the effect of MLD on end stage myocardial remodeling in the form of fibrosis was studied. The adverse myocardial fibrotic remodeling was found to be more in the group V rats, as their myocardial staining with Azan-Mallory showed extensive blue fibrotic area and immunohistochemical staining for collagen-III, a marker for myocardial fibrosis, was significantly elevated. Interestingly the rats fed with MLD showed significantly lesser fibrotic area as well as the number of collagen positive cells. TGF-β1 is a locally generated cytokine that has been implicated as a major contributor to tissue fibrosis in various organ systems [Border and Noble, 1994; Gandhi et al., 2000].
Dilated cardiomyopathy and its treatment options

MLD supplementation has significantly reduced the myocardial expression of TGF-β1, which has also confirmed the antifibrotic effects of it against DCM in rats. Flavonoids are antioxidant polyphenolic compounds ubiquitously found in plants, typically as sugar conjugates of six subgroups; flavonols, flavones, flavanones, isoflavones, anthocyanins, and catechins. Significant amounts of them are present in commonly consumed fruits and vegetables. Consumption of flavonoids, particularly the flavonol quercetin, has been associated with a reduced incidence of heart disease and cancer, hypothesized to be due to their antioxidant properties [Lean et al., 1999]. Clinical study with dietary supplementation of flavonoids also suggests that flavonoids in regularly consumed foods may reduce the risk of death from coronary heart disease in elderly men [Hertog et al., 1999]. Similarly another clinical study also suggested that dietary intakes of flavanones, anthocyanidins, and certain foods rich in flavonoids were associated with reduced risk of death due to coronary heart disease and CV disorders and all causes [Mink et al., 2007]. All these suggestions provided us the interest to study the beneficial effects of ML against DCM, a major cause of death related to CV complications. The results obtained with our present study have provided additional data to confirm the beneficial effects of dietary supplementation of ML against adverse cardiac remodeling in DCM. Our findings also provide the molecular mechanisms of MLD as a negative regulator in ET-1-induced hypertrophic response of cardiomyocytes possibly via modulation of MAPK pathway. This study may be a useful tool to allow
Dilated cardiomyopathy and its treatment options

the use of dietary supplementation of ML for either the prevention or treatment of cardiac hypertrophy and heart failure.
Chapter 2

Quercetin offers cardioprotection against progression of EAM by suppression of oxidative and endoplasmic reticulum stress via endothelin-1/MAPK signaling
Dilated cardiomyopathy and its treatment options

Introduction

Inflammation and autoimmunity are involved in many cardiac diseases among which myocarditis, an inflammatory heart disease, causes both acute and chronic heart failure as a result [Kawai, 2001]. Excessive production of ROS such as superoxide induced by inflammatory stimuli is an important observation in failing hearts and produces myocardial injury in autoimmune mediated heart failure [Ishiyama et al., 1997] and it has reported that the patients with heart failure were found with excess levels of ROS in their plasma [Belch et al., 1991]. Oxidative stress reduction induced by flavonoids has been regarded by many as the most likely mechanism in the protective effects of these compounds [Angeloni et al., 2007]. One of the strongest lines of evidence for the involvement of ROS in cardiovascular diseases is the ability of a number of structurally unrelated compounds with antioxidant properties to protect against cardiovascular pathophysiology, including myocardial ischemia-reperfusion injury, cardiomyopathy, and arterial atherogenesis [Angeloni et al., 2008]. A diet rich in flavonoids reduces the risk for oxidative-stress related chronic diseases such as diabetes, coronary heart disease and stroke. This has been associated with the antioxidant activity of flavonoids such as quercetin, one of the major representatives of flavonoids, ubiquitously present in various vegetables, fruits, seeds, nuts, tea and red wine [Coskun et al., 2005], has been widely studied, and its biological properties are consistent with its protective role in the cardiovascular system [Moon et al., 2003]. Quercetin (3,5,7,39,49-pentahydroxy flavone), an excellent free radical scavenging antioxidant, prevents oxidant injury and cell death.
Dilated cardiomyopathy and its treatment options

by several mechanisms, such as scavenging oxygen radicals, protecting against lipid peroxidation and chelating metal ions. Milenkovic et al. reported the protective effects of quercetin against inflammatory conditions in the hearts of EAM rats [Milenkovic et al., 2010]. Thus the purpose of the present study was to test the effect of quercetin treatment against porcine cardiac myosin-induced chronic heart failure model, focusing on its inhibitory effects on oxidative stress, myocardial apoptosis and fibrosis.

**Methods and materials**

**Materials**

Lewis rats (male, 8 weeks old) were purchased from Charles River Japan Inc (Kanagawa, Japan). All the chemicals and reagents were purchased from Sigma, Tokyo, Japan until otherwise mentioned.

**Experimental design**

All experiments were carried out using eight-week-old male Lewis rats and were performed in accordance with the guidelines of our institute. Lewis rats were injected in the footpads with antigen-adjuvant emulsion in accordance with a procedure described previously. Twenty-eight days after immunization, the surviving rats were divided into two groups and received quercetin p.o. at a dose of 10 mg/kg body weight (Group Q10) or vehicle alone (Group V) for 28 days. Age-matched Lewis rats without immunization were used as normal controls (Group N).

**Transthoracic echocardiographic analysis**
Echocardiographic studies were carried out with a 7.5-MHz transducer (Aloka Inc, Tokyo, Japan). The LV dimensions in diastole (LVDd) and systole (LVDs), percentage ejection fraction (EF) and percentage fractional shortening (FS) were estimated using M-mode measurements. After the echocardiographic analysis, the rats were sacrificed, and the excised myocardium was kept in formalin and the mid-ventricle sections were then embedded with paraffin.

**Analysis of cardiac apoptosis by terminal transferase-mediated dUTP nick-end labeling assays**

The transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed as specified in the instructions for the in situ apoptosis detection kit (Takara Bio Inc., Shiga, Japan). Sections embedded in paraffin were mounted and examined using light microscopy. Digital photomicrographs were obtained by using a color image analyzer (CAI-102, Olympus, Japan) at X400 magnification, and 25 random fields from each heart were chosen and the number of TUNEL positive nuclei was quantified in a blinded manner. For each group, three sections were scored for apoptotic nuclei. Only nuclei that were clearly located in cardiac myocytes were considered.

**Hematoxylin-eosin (H-E) and Azan-Mallory staining**

After being embedded in paraffin, several transverse sections were prepared from the ventricle, and stained with H-E and Azan-Mallory. Infiltration of inflammatory cells was examined in the H-E stained slides viewed under a high-power light microscope. The area of myocardial fibrosis in LV tissue sections stained with Azan-
Mallory was quantified using a color image analyzer (CIA-102, Olympus, Tokyo, Japan) and measured the blue fibrotic areas as opposed to the red myocardium at 200 magnification. The results were presented as the ratio of the fibrotic area to the whole area of the myocardium.

**Immunohistochemical assay**

Immunohistochemical staining was performed as described before with the formalin-fixed, paraffin-embedded cardiac tissue sections of the rats from different groups. After overnight incubation of the slides with the goat polyclonal anti-collagen Type III antibody (diluted 1:100) (Santa Cruz Biotechnology Inc, Santa Cruz, CA) at 4°C, the slides were washed in TBS and horseradish peroxidase-conjugated secondary antibody was then added and the slides were further incubated at room temperature for 45 min. The slides were washed in TBS and incubated with diaminobenzidine tetrahydrochloride as the substrate, and counterstained with hematoxylin. A negative control without primary antibody was included in the experiment to verify the antibody specificity. Measurement of myocardial immunoreactivity for Collagen-III was performed in 100 randomly selected fields in heart sections in 200-fold magnification by light microscopy.

**Western immunoblotting analysis**

This analysis was carried out as mentioned before. Following polyclonal antibodies were used: anti-goat p67phox, anti-mouse osteopontin (OPN), anti-goat endothelin-1, anti-goat glucose regulated protein (GRP)78, anti-mouse cytochrome C and anti-rabbit
glyceraldehyde 3 phosphate dehydrogenase (GAPDH) antibodies (Santa Cruz Biotechnology or Cell Signaling) (diluted 1:100). Membranes were blocked with 5% nonfat dry milk or 5% bovine serum albumin (Sigma, St Louis, MO) in TBS-T (20 mM/l Tris, pH 7.6, 137 mM/l NaCl, and 0.05% Tween). After incubation with primary antibody, the bound antibody was visualized with respective horseradish peroxidase-coupled secondary antibody (Santa Cruz Biotechnology) and chemiluminescence developing agents (Amersham Biosciences, Buckinghamshire, UK). The level of GAPDH was estimated in every sample. Films were scanned, and band densities were quantified with densitometric analysis using Scion Image program (Epson GT-X700, Tokyo, Japan). Finally, western blot data were normalized with cardiac GAPDH.

Statistical analysis

All the values are expressed as means ± SEM. Statistical analysis of differences between the groups was performed by student’s ‘t’ test or one-way analysis of variance, followed by either Dunnett’s test or Tukey’s multiple comparison test wherever necessary using GraphPad Prism 5.0 software. A value of $p<0.05$ was considered statistically significant.

Results

Effect of quercetin on myocardial dimensions

Although heart rate was not different among the three groups of rats, transthoracic echocardiographic studies of group V rats showed evidence for the cardiac remodeling with increased LVDd (7.42 ± 0.79 vs 5.97 ± 0.31 mm, $p<0.01$), LVDs (6.64 ± 0.66 vs 3.08 ± 0.22 mm, $p<0.01$), reduced FS (15.64 ± 5.78 vs 48.6 ± 3.17%, $p<0.01$) and EF (25.07 ± 2.79...
Dilated cardiomyopathy and its treatment options

vs 84.23 ± 2.29\%, p<0.01). These results indicate the impairment of cardiac function in the DCM rats of group V compared with that in group N. Treatment with quercetin significantly decreased all of these parameters compared with those in group V. Myocarditis was confirmed with the increased heart weights of the control group rats. The ratio (HW/BW) was found to be significantly higher when compared with group N. In the quercetin treated rats this value was significantly lesser than that of group V rats.

Effect of quercetin on echocardiographic parameters of rats with post-myocarditis DCM

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group N</th>
<th>Group V</th>
<th>Group Q10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival rate</td>
<td>100%</td>
<td>43%##</td>
<td>56%*</td>
</tr>
<tr>
<td>HW/BW (mm)</td>
<td>2.43 ± 0.04</td>
<td>6.73 ± 0.36##</td>
<td>5.29 ± 0.11**</td>
</tr>
<tr>
<td>LVDd (mm)</td>
<td>5.97 ± 0.31</td>
<td>8.37 ± 0.22##</td>
<td>6.73 ± 0.27*</td>
</tr>
<tr>
<td>LVDs (mm)</td>
<td>3.08 ± 0.22</td>
<td>7.43 ± 0.19##</td>
<td>5.17 ± 0.50*</td>
</tr>
<tr>
<td>FS (%)</td>
<td>48.6 ± 3.17</td>
<td>9.9 ± 0.87##</td>
<td>26.18 ± 1.89**</td>
</tr>
<tr>
<td>EF (%)</td>
<td>84.23 ± 2.29</td>
<td>25.07 ± 2.78##</td>
<td>51.57 ± 2.69**</td>
</tr>
</tbody>
</table>

N, age-matched normal rats; V, immunized rats treated with vehicle alone; Q10 Immunized rats orally treated with quercetin (10 mg/kg body weight). ##p<0.01 vs group N; *p<0.05, **p<0.01 vs group V (One-way ANOVA followed by Dunnett’s test)

Effect of quercetin on myocardial apoptosis

TUNEL assay showed that the number of TUNEL positive nuclei was significantly higher in myocardial tissue sections from group V rats than in group N. However, treatment with quercetin significantly decreased the number of TUNEL positive nuclei compared to that in group V.
Effect of quercetin on myocardial fibrosis and its marker molecules

Azan-Mallory staining of the myocardial tissue sections revealed the extensive fibrosis in the myocardium of group V rats; on the other hand, quercetin treatment decreased the percent area of fibrosis in the hearts of group Q10 rats.
It has also been confirmed by immunohistochemical studies for deposition of collagen-III in the myocardial tissue sections.

Similarly, the hearts from DCM rats showed massive fibrosis and increased expressions of OPN and TGF-β1 compared to those from group N. Treatment with quercetin significantly reduced their myocardial expression than in vehicle treated rats.
Effect of quercetin on myocardial inflammatory cellular infiltration

The myocardial slices of group V rats showed extensive inflammatory cellular infiltration as evidenced by H-E staining, whereas the rats treated with quercetin showed reduced number of them indicating its protective effect against the involvement of inflammatory cells during the progression of EAM to DCM.

Effect of quercetin on oxidative stress

The sections from the hearts of group V rats showed excessive production of superoxide as evidenced by DHE staining, whereas the sections from the rats treated with quercetin showed lesser staining for superoxide indicating the role of quercetin against oxidative stress.
Dilated cardiomyopathy and its treatment options

In addition, we have carried out Western blotting for identification of other oxidative stress marker molecule. The cardiac expression of NADP(H) oxidase subunit p67phox was significantly higher in group V rats compared with that in group N, and these changes were significantly reversed by the treatment with quercetin.

Effect of quercetin on ER stress marker proteins and cytosolic cytochrome C level

The myocardial expressions of GRP78 and GADD 153 levels were significantly elevated in group V rats compared with that in group N.
But this stress was significantly attenuated by the treatment with quercetin as evidenced by the decreased myocardial levels of these above-mentioned markers. Prolonged ER stress stimulates apoptotic signaling and in the present study with quercetin has provided significant suppression of myocardial apoptosis via blockade of ER stress. Similarly the cytosolic level of cytochrome C was also significantly reduced by the treatment with quercetin, which has further confirmed its protective role against apoptotic cell death.

**Effect of quercetin on MAPK signaling cascade**

In order to identify the probable mode of action of quercetin on DCM, we have measured the myocardial expression levels of ET-1 and phosphorylated forms of Akt, ERK and p38-MAPK in both the vehicle and quercetin treated rats. Group V rats showed the activation of MAPK pathway as their hearts showed significant increase in the phosphorylation of the above-mentioned members of MAPK signaling cascade. Interestingly the rats treated with quercetin were protected from these changes as their
myocardial levels of these proteins were significantly lesser when compared with the group V rats.
Discussion

Cardiomyocytes could be better prepared to subsequent toxic insults after quercetin nutritional intake. In particular, it may have a role in the counteraction and prevention of cardiac stress related to diseases [Angeloni et al., 2008]. Here in the present study cardiac stress was induced by injection porcine cardiac myosin, which acutely resulted in EAM and chronically led to post myocarditis DCM. There are reports indicating the protective effects of quercetin against EAM [Milekovic et al., 2010], whereas its progression to DCM has not been reported. Thus to test the hypothesis that treatment with quercetin can ameliorate the progression of EAM to post myocarditis DCM, we have carried out the measurement of parameters related to adverse cardiac remodeling in the DCM rats treated with quercetin at a dose 10 mg/kg/day orally. Myocardial structural and functional parameters were improved significantly in the DCM rats treated with quercetin. Rats received quercetin treatment were found to be with improved cardiac contractile function, as evidenced by the improved EF and FS when compared with the group V rats. With this initial confirmation regarding the protective effects of quercetin against DCM, we have carried out the measurement of various stress-related parameters involved in adverse cardiac remodeling.

Oxidative stress is involved in the pathogenesis of various cardiac complications. Excessive oxidative stress can damage many biological molecules, protein and DNA [Halliwell and Chirico, 1993]. Various oxidative stress related parameters were reported to be elevated in post myocarditis DCM, a form of end stage heart failure. Our present
Dilated cardiomyopathy and its treatment options

A study with DCM rats also confirmed the increased oxidative stress as evidenced by extensive staining for superoxide in the myocardial sections by DHE staining. Several antioxidant compounds were studied for their protective role against ailments with oxidative stress as the major pathogenic mechanism so that they can be effectively used in their treatment. There are various reports regarding the protective effect of quercetin on the illnesses dependent on oxidative stress. Quercetin provided protection against oxidative injuries of H9c2 cardiomyoblasts, possible via modulation of mitochondrial dysfunction and inhibition of caspase activity [Park et al., 2003]. It was also reported that quercetin prevents and protects against ethanol-induced oxidative stress in mouse liver [Molina et al., 2003] and streptozotocin-induced oxidative stress and beta-cell damage in rat pancreas [Boots et al., 2007]. In line with these reports, our study with DCM rats also confirmed that treatment with quercetin prevents the oxidative stress-induced tissue injury as evidenced by the reduced superoxide production in the hearts of the quercetin treated DCM rats.

Increased oxidative stress evokes many intracellular events including inflammation, ER stress and myocyte apoptosis [Kannan and Jain, 2000]. H-E staining revealed that DCM rats in group V suffered from extensive inflammatory cellular infiltration, whereas the rats treated with quercetin showed significantly lesser infiltration. From these results we have confirmed the protective effect of quercetin against the oxidative stress induced myocardial inflammation. ER stress has been attracting considerable attention since it triggers several inflammatory disorders. In response to ER stress, there is a
Dilated cardiomyopathy and its treatment options

marked upregulation of ER chaperone like GRP78 [Pan et al., 2010]. This stress includes disruption of ER calcium homeostasis and accumulation of excessive proteins in ER. Cell apoptosis can proceed via various routes, leading to myocardial cell loss [Lai et al., 2007]. In the present study, the myocardial levels of GRP78, GADD153 and cytochrome C were found to be elevated significantly in the DCM rats of group V, whereas the rats under quercetin treatment showed significant suppression of these changes. There are various reports regarding the role of antioxidants against ER stress during a variety of disorders [Shimazaki et al., 2010; Yamauchi et al., 2011]. Interestingly, quercetin treatment provided protection against ER stress caused by calcium dynamics dysregulation in intestinal epithelial cells [Natsume et al., 2009] and down-regulated the expression levels of ER stress proteins, GRP78, activating transcription factor 6α, X-box binding protein 1, inositol-requiring protein-1, phospho-eukaryotic initiation factor 2α, and C/EBP-homologous protein in H9c2 cardiac cells. In line with these reports, our study with quercetin also confirmed its protective effect against adverse cardiac remodeling induced by ER stress during DCM. Excess or prolonged ER stress leads to cell death in the form of apoptosis [Mao et al., 2007; Okada et al., 2004]. In the present study, the myocardial cellular apoptosis was also significantly lesser the myocardium of the quercetin treated rats as evidenced by the reduced number of apoptotic cells using TUNEL staining.

In addition, Azan-mallory staining for myocardial fibrosis and immunohistochemical staining for collagen-III, a marker for myocardial fibrosis, were significantly elevated in
Dilated cardiomyopathy and its treatment options

the DCM rats belonging to group V. Interestingly the rats treated with quercetin showed significantly lesser fibrotic area as well as the number of collagen positive cells. TGF-β1 is a locally generated cytokine that has been implicated as a major contributor to tissue fibrosis in various organ systems [Border and Noble, 1994]. Similarly high expression levels of OPN in acute myocarditis are associated with the development of extensive fibrosis [Szalay et al., 2009]. Quercetin treatment has significantly reduced the myocardial expressions of TGF-β1 and OPN, which has also confirmed the antifibrotic effects of it against DCM in rats. These data confirmed that treatment with quercetin has provided protection against the adverse cardiac remodeling that leads to DCM.

The role of ET-1 in adverse myocardial remodeling has been reported by various studies [Ito et al., 1993; Sun et al., 2009]. Our present study has also identified that the myocardial levels of ET-1 in the hearts of vehicle treated rats were found to be significantly elevated. Interestingly, quercetin treatment has shown significant suppressive effect on the ET-1 levels in the myocardium of the DCM rats. ET-1 is an agonist at the G-protein coupled or tyrosine kinase receptors which lead to the activation of MAPK pathway. The p38 MAPK is a subfamily of the MAPK superfamily and is stress responsive. Activation of p38 MAPK is associated with accumulation of reactive oxygen species generated under stress conditions [Clerk et al., 1998]. Various antioxidants were reported to inhibit p38 MAPK activation [James, 2006; Kyaw et al., 2004]. In the present study, the myocardial levels of phosphorylated forms of p38 MAPK, ERK1/2 and Akt are significantly lesser in the quercetin treated rats when
Dilated cardiomyopathy and its treatment options

compared with the group V rats suggesting that quercetin treatment not only prevented the oxidative and ER stress in the DCM rats, but also avoided the activation of MAPK signaling cascade through which it has prevented the adverse cardiac remodeling involved in post-myocarditis DCM. Thus the present study provides evidences for the cardioprotective effects of MLD possibly via the modulation of ET-1/p38 MAPK/Akt pathway in the DCM rat hearts.

Flavonoids are antioxidant polyphenolic compounds ubiquitously found in plants, typically as sugar conjugates of six subgroups; flavonols, flavones, flavanones, isoflavones, anthocyanins, and catechins. Consumption of flavonoids, particularly the flavonol quercetin, has been associated with a reduced incidence of heart disease and cancer, hypothesized to be due to their antioxidant properties [Lean et al., 1999]. Quercetin, a natural flavonoid antioxidant, has provided mitochondria-targeted cardioprotection in aldosteronism [Shahbaz et al., 2011]. Ginkgo biloba extract, which contain quercetin as the major flavonoid component, inhibits mitochondria-dependent caspase pathway and prevents apoptosis in hypoxia-reoxygenated cardiomyocytes [Shen et al., 2011]. Similarly, quercetin was reported to provide protection against adriamycin-induced cardiotoxicity in mice [Peri et al., 2007]. In H9c2 cardiomyoblast cells, quercetin protected the hydrogen peroxide-induced apoptosis via inhibition of mitochondrial dysfunction [Park et al., 2003]. All of these protective effects of quercetin against cardiac complications provided us the interest to carry out this study. The
Dilated cardiomyopathy and its treatment options

results obtained with our present study have provided further evidences confirming the beneficial effects of quercetin against cardiac complications.
Beneficial effects of edaravone, a novel antioxidant, in rats with DCM after EAM and possible involvement of AMPK and MAPK signaling
Introduction

Cardiovascular disorders are more prevalent all over the world as compared to other diseases and it is a fact that severe heart failure is more predominant than most of the cancers. Also, cardiovascular disease is one of the leading causes of deaths worldwide, accounting for 16.7 million deaths per annum (Rohini et al., 2010). Myocarditis is an inflammation of heart muscle and it is most often due to infection by common viruses, with an inflammatory infiltrate and damage to the heart (Baughman, 2006). It can also occur due to autoimmune reactions (Cooper, 2009). Myocarditis can be induced in rats by immunization with cardiac myosin (Kodama et al., 1990), which resembles the human giant cell myocarditis and well characterized by the extensive myocarditis necrosis, congestive heart failure and appearance of multinucleated giant cell (Kodama et al., 2007 and 2006). Inflammation and autoimmunity are involved in this condition causing both acute and chronic heart failure as a result [Kawai, 2001]. Neurohumoral factors such as cytokines and chemokines, and myocardial remodeling including myocardial apoptosis play important roles in the progression of EAM [Okura et al., 1997; Ishiyama et al., 1998]. Excessive production of ROS such as superoxide induced by inflammatory stimuli is an important observation in failing hearts and produces myocardial injury in autoimmune mediated heart failure [Ishiyama et al., 1997]. Myocardial fibrosis probably has an important role in both diastolic and systolic dysfunction and has adverse clinical consequences that result in increases in mortality because of progressive heart failure [Yamaguchi, 1981]. Many kinds of cytokines, such
Dilated cardiomyopathy and its treatment options

as basic fibroblast growth factor, Angiotensin (Ang)-II, transforming growth factor (TGF)-β1, and collagen-III, have been suggested to have an important role in structural remodeling of the nonmyocyte compartment of the myocardium after heart failure [Schelling et al., 1991].

The heart is capable of utilizing a variety of substrates to produce the necessary ATP for cardiac function. Many cardiovascular-related disorders, such as pathological cardiac hypertrophy, heart failure, myocardial ischemia, diabetic cardiomyopathy, and lipotoxic heart disease are associated with alterations in cardiac energy metabolism (Dolinsky and Dyck, 2006). Fatty acids are the primary energy substrate of the adult heart. Switches in myocardial substrate utilization and energy production rates have been shown to occur in various cardiomyopathies, as well as in any subsequent heart failure (Taha and Lopaschuk, 2007). It has been found that the AMPK, a master sensor of cellular energy balance in mammals plays significant role in maintaining the cardiac metabolic function through activation of energy producing pathways and suppressing energy consuming processes (Sambandam and Lopaschuk, 2007). Since AMPK is central to the regulation of cardiac energy metabolism, the regulation of AMPK may be important in these various pathological settings (Dolinsky and Dyck, 2006). AMPK activation may be essential for adaptation of cardiac energy metabolism to acute and/or minor metabolic stresses, it is unknown whether AMPK activation becomes maladaptive in certain chronic disease states and/or extreme energetic stresses. However, alterations in cardiac AMPK activity are associated with a number of
Dilated cardiomyopathy and its treatment options

cardiovascular-related diseases such as pathological cardiac hypertrophy, myocardial ischemia, glycogen storage cardiomyopathy, and Wolff-Parkinson-White syndrome, suggesting the possibility of a maladaptive role. There are various reports states that the activation of AMPK has both pro- and anti-apoptotic role in heart of various experimental animal models (Meisse et al., 2002, Hickson-Bick et al., 2000). Therefore, in this study we studied the possible role of AMPK in chronic heart failure induced by EAM.

Edaravone, a novel free radical scavenger, may be an effective agent for myocardial inflammation by combating oxidative stress (Tada et al., 2003). Various reports suggest the protective effect of edaravone against myocardial complications during cardiovascular disorders. Reduced myocardial infarct size and improved cardiac function and left ventricular (LV) remodeling were reported with 14 days edaravone treatment after myocardial infarction (Onogi et al., 2006). Interestingly, edaravone treatment significantly attenuated pressure overload–induced cardiac hypertrophy mediated through its antioxidative function (Tsujimoto et al., 2005). From our lab, we have also reported the protective effects of edaravone against EAM induced by cardiac myosin, focusing on oxidative stress, endoplasmic reticulum stress, inflammatory cytokines and myocardial apoptosis (Shimazaki et al., 2010). But it is now of interest to identify whether treatment with edaravone can inhibit the progression of EAM into DCM, if so, the involvement of AMPK signaling cascade and other signaling proteins during this protection.
Dilated cardiomyopathy and its treatment options

Methods

Materials

Edaravone was generously provided by Mitsubishi Research, Japan and Lewis rats (male, 8 weeks old) were purchased from Charles River Japan Inc (Kanagawa, Japan). All the chemicals and reagents were purchased from Sigma, Tokyo, Japan until otherwise mentioned.

Monoclonal antibodies of anti-rabbit phos pho Akt, anti-rabbit phos pho p38-mitogen activated protein kinase (MAPK), anti-rabbit phos pho MAPKAPK2, anti-rabbit phos pho c-Jun NH2 kinase (JNK), anti-mouse phos pho extracellular ligand regulated kinase (ERK)-1/2, anti-rabbit phos phoinositide 3-kinase (PI3K), anti-rabbit phos pho AMPK\(\alpha\), anti-rabbit AMPK\(\alpha\) and anti-rabbit glyceraldehyde 3 phosphate dehydrogenase (GAPDH) were obtained from Cell signaling. Other monoclonal antibodies used in this study include: anti-rabbit protein kinase C (PKC)-\(\alpha\), anti-rabbit PKC-\(\beta_1\), anti-rabbit PKC-\(\beta_2\) and anti-rabbit PKC-\(\delta\), which were purchased from Santa Cruz biotechnology.

Experimental design

All experiments were carried out using eight-week-old male Lewis rats and were performed in accordance with the guidelines of our institute. EAM in Lewis rats was induced by porcine cardiac myosin as described before. Twenty-eight days after immunization, the surviving Lewis rats were divided into three groups and received intraperitoneal injection of edaravone (3 and 10 mg/kg/day; Group Ed 3 and Group Ed
Dilated cardiomyopathy and its treatment options

10) or vehicle (Group V) for 28 days. Age-matched Lewis rats without immunization were used as normal controls (Group N). The doses used in the experiments were determined on the basis of the previous report suggesting its cardioprotective effects (Tsujimoto et al., 2005).

**Hemodynamic study**

On day 49 after immunization, cardiac function of each rat was measured by hemodynamic study and cardiac pressure changes were recorded as described previously [Watanabe et al., 2001].

**Transthoracic echocardiographic analysis**

Echocardiographic studies were carried out with a 7.5-MHz transducer (Aloka Inc, Tokyo, Japan). The LV dimensions in diastole (LVDd) and systole (LVDs), percentage ejection fraction (EF) and percentage fractional shortening (FS) were estimated using M-mode measurements [Thandavarayan et al., 2009]

**Histopathology**

The body weight (BW) of rats was noted just before the surgical procedure. After the echocardiographic analysis, the rats were killed, and the wet heart was isolated and weighed to calculate the ratio of heart weight (HW) to BW. The excised myocardium was kept in formalin and the mid-ventricle sections were then embedded with paraffin.

**Hematoxylin-eosin (H-E) staining**

After being embedded in paraffin, several transverse sections were prepared from the ventricle, and stained with H-E. Infiltration of inflammatory cells was examined in the
H-E stained slides viewed under a high-power light microscope at 200x magnification. The cardiomyocyte diameter was measured from the stained slides at 400x magnifications by measuring 100 cells per slide and calculating the average diameter of each cardiomyocyte using light microscopy (CIA-102, Olympus, Tokyo, Japan).

**Analysis of cardiac apoptosis by terminal transferase-mediated dUTP nick-end labeling assays**

The transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed as specified in the instructions for the in situ apoptosis detection kit (Takara Bio Inc., Shiga, Japan). Sections embedded in paraffin were mounted and examined using light microscopy. Digital photomicrographs were obtained by using a color image analyzer (CAI-102, Olympus, Japan) at X400 magnification, and 25 random fields from each heart were chosen and the number of TUNEL positive nuclei was quantified in a blinded manner. For each group, three sections were scored for apoptotic nuclei. Only nuclei that were clearly located in cardiac myocytes were considered [Thandavarayan *et al.*, 2010].

**Azan-Mallory staining for myocardial fibrosis**

The area of myocardial fibrosis in LV tissue sections stained with Azan-Mallory was quantified using a color image analyzer (CIA-102, Olympus, Tokyo, Japan) and measured the blue fibrotic areas as opposed to the red myocardium at 200 magnification. The results were presented as the ratio of the fibrotic area to the whole area of the myocardium [Sukumaran *et al.*, 2010; Watanabe *et al.*, 2011]
**Dilated cardiomyopathy and its treatment options**

**Immunohistochemical assay**

Immunohistochemical staining was performed as described earlier with the formalin-fixed, paraffin-embedded cardiac tissue sections of the rats from different groups. After overnight incubation with the goat polyclonal anti-collagen Type III antibody (diluted 1:100) (Santa Cruz Biotechnology Inc, Santa Cruz, CA) at 4ºC, the slides were washed in TBS and horseradish peroxidase-conjugated secondary antibody was then added and the slides were further incubated at room temperature for 45 min. The slides were washed in TBS and incubated with diaminobenzidine tetrahydrochloride as the substrate, and counterstained with hematoxylin. A negative control without primary antibody was included in the experiment to verify the antibody specificity. Measurement of myocardial immunoreactivity for collagen-III was performed in 100 randomly selected fields in heart sections in 200-fold magnification by light microscopy.

**Western immunoblotting analysis**

This analysis was carried out as per the earlier method by Thandavarayan et al., [2008]. Following polyclonal antibodies were used: anti-rabbit p47phox, anti-goat p67phox, anti-goat gp91phox, anti-goat Nox4, anti-mouse osteopontin (OPN), anti-rabbit TGF-β, anti-goat glucose regulated protein (GRP) 78, anti-mouse growth arrest and DNA damage inducible gene (GADD) 153, anti-mouse Cytochrome C and anti-rabbit cleaved caspase-3 antibodies, phospho AMPKα, AMPKα, PI3K, phospho p38 MAPK, phospho ERK1/2, phospho Akt, phospho JNK, phospho MAPKAPK2, PKC-α, PKC-β1, PKC-β2, PKC-δ and anti-mouse anti-rabbit glyceraldehyde 3 phosphate dehydrogenase Membranes.
Dilated cardiomyopathy and its treatment options

were blocked with 5% nonfat dry milk or 5% bovine serum albumin (Sigma, St Louis, MO) in TBS-T (20 mM/l Tris, pH 7.6, 137 mM/l, NaCl, and 0.05% Tween).

After incubation with primary antibody, the bound antibody was visualized with respective horseradish peroxidase-coupled secondary antibody (Santa Cruz Biotechnology) and chemiluminescence developing agents (Amersham Biosciences, Buckinghamshire, UK). The level of GAPDH was estimated in every sample. Films were scanned, and band densities were quantified with densitometric analysis using Scion Image program (Epson GT-X700, Tokyo, Japan). Finally, western blot data were normalized with cardiac GAPDH.

Statistical analysis

All values are expressed as the means ± SEM. Statistical analysis of differences between the groups was performed by one-way ANOVA, followed by Tukey’s or Bonferroni’s method and the two tailed t-test when appropriate. P < 0.05 was considered as significant. For statistical analysis, GraphPad Prism 5 software (San Diego, CA, U.S.A) was used.

Results

Effect of edaravone on myocardial dimensions

Although heart rate was not different among the four groups of rats, transthoracic echocardiographic studies of group V rats showed evidence for the cardiac remodeling with increased LVDs (7.42 ± 0.67 vs 3.44 ± 0.19 mm, \( p < 0.01 \)), and reduced FS (15 ± 0.68 vs 49.2 ± 1.9\%, \( p < 0.01 \)) and EF (28.8 ± 3.71 vs 85.1 ± 1.6\%, \( p < 0.01 \)).
These results indicate the impairment of cardiac function in the DCM rats of group V compared with that in group N. Treatment with edaravone significantly decreased all of these parameters compared with those in group V.
Myocarditis was confirmed with the increased heart weights of the control group rats. The ratio (HW/BW) was found to be significantly higher when compared with group V. In the edaravone treated rats this value was less than that of group V rats.

Changes in hemodynamic and echocardiographic parameters in rats with DCM after treatment with edaravone.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group N</th>
<th>Group V</th>
<th>Group Ed 3</th>
<th>Group Ed 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>329 ± 5**</td>
<td>338 ± 7**</td>
<td>337 ± 8**</td>
<td></td>
</tr>
<tr>
<td>HW (g)</td>
<td>1.24 ± 0.03**</td>
<td>1.06 ± 0.03</td>
<td>1.08 ± 0.01*,#</td>
<td></td>
</tr>
<tr>
<td>HW/BW (g/kg)</td>
<td>3.62 ± 0.14**</td>
<td>3.20 ± 0.11**</td>
<td>3.27 ± 0.12**</td>
<td></td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>378 ± 19</td>
<td>329 ± 8</td>
<td>349 ± 14</td>
<td>364 ± 7</td>
</tr>
<tr>
<td>Mean BP (mmHg)</td>
<td>99 ± 6</td>
<td>82 ± 4*</td>
<td>75 ± 2**</td>
<td>75 ± 3**</td>
</tr>
<tr>
<td>CVP (mmHg)</td>
<td>1.0 ± 0.4</td>
<td>4.2 ± 0.4*</td>
<td>3.3 ± 0.9</td>
<td>3.2 ± 0.8</td>
</tr>
<tr>
<td>LVP (mmHg)</td>
<td>128 ± 5</td>
<td>121 ± 4*</td>
<td>118 ± 5*</td>
<td>106 ± 1**</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>19.7 ± 1.4</td>
<td>40.4 ± 2.3**</td>
<td>33.8 ± 3.7*</td>
<td>35.9 ± 4.4*</td>
</tr>
<tr>
<td>+dP/dt (mmHg/min)</td>
<td>644 ± 881</td>
<td>4205 ± 77*</td>
<td>4769 ± 211</td>
<td>4530 ± 404</td>
</tr>
<tr>
<td>-dP/dt (mmHg/min)</td>
<td>7291 ± 1124</td>
<td>3933 ± 413**</td>
<td>4054 ± 191**</td>
<td>3730 ± 274**</td>
</tr>
<tr>
<td>LVDd (mm)</td>
<td>6.7 ± 0.31</td>
<td>7.62 ± 0.32</td>
<td>7.56 ± 0.22</td>
<td>7.5 ± 0.1</td>
</tr>
<tr>
<td>LVDs (mm)</td>
<td>3.44 ± 0.19</td>
<td>7.42 ± 0.67**</td>
<td>5.44 ± 0.12**,##</td>
<td>5.48 ± 0.11**,##</td>
</tr>
<tr>
<td>FS (%)</td>
<td>49.2 ± 1.9</td>
<td>15 ± 0.68**</td>
<td>32.7 ± 1.49**,##</td>
<td>25.8 ± 1.4**,##</td>
</tr>
<tr>
<td>EF (%)</td>
<td>85.1 ± 1.6</td>
<td>28.8 ± 3.71**</td>
<td>64.4 ± 1.58**,##</td>
<td>56.3 ± 2.29**,##</td>
</tr>
<tr>
<td>E/A ratio</td>
<td>2.94 ± 0.05</td>
<td>0.32 ± 0.01**</td>
<td>1.73 ± 0.10**,##</td>
<td>2.05 ± 0.16**,##</td>
</tr>
</tbody>
</table>

Group N, age matched intact rats; Group V, rats with DCM treated with vehicle; Group Ed3, rats with DCM treated with edaravone (3 mg/kg); Group Ed10, rats with DCM treated with edaravone (10 mg/kg); BW, body weight; HW, heart weight; CVP, central venous pressure; LVP, left ventricular pressure; LVEDP, left ventricular end-diastolic pressure; dP/dt, rate of intra-ventricular pressure rise and decline; LVDd, left ventricular dimension in diastole; LVDs, left ventricular dimension in systole; FS, fractional shortening; EF, ejection fraction. *p<0.01 and *p<0.05 vs Group N, ##p<0.01 and ##p<0.05 vs Group V, †p<0.05 vs Group E3. Values are mean ± SEM. (One way ANOVA followed by Tukey’s multiple comparison test)
Effect of edaravone on oxidative stress

The rats of group V suffered from the excess oxidative stress as identified by Western blotting for its marker molecules. The cardiac expressions of NADP(H) oxidase subunits like $p_{47}^{\text{phox}}$, $p_{67}^{\text{phox}}$, $\text{gp91}^{\text{phox}}$ and Nox4 were significantly higher in group V rats compared with that in group N, and these changes were significantly reversed by the treatment with edaravone.

![Graphs showing the densitometric ratio of different subunits](image-url)
Effect of edaravone on myocardial fibrosis and its marker molecules

The hearts from DCM rats showed massive fibrosis and increased expressions of OPN and TGF-β1 compared to those from group N. Treatment with edaravone significantly reduced their myocardial expression than in vehicle treated rats.

Similarly Azan - Mallory staining of the myocardial tissue sections also revealed the extensive fibrosis in the myocardium of group V rats. In line with the reduction in the expression levels of fibrosis markers, edaravone treatment also decreased the percent area of fibrosis.
It has also been confirmed by immunohistochemical studies for deposition of collagen-III in the myocardial tissue sections.

**Effect of edaravone on Endoplasmic reticulum (ER) stress marker proteins**

The myocardial expressions of GRP78 and GADD 153 and cytochrome C levels were significantly elevated in group V rats compared with that in group N. But this stress was significantly attenuated by the treatment with edaravone as evidenced by the decreased myocardial levels of these above-mentioned markers. Prolonged ER stress stimulated apoptotic signaling via caspases and in the present study with edaravone has provided significant suppression in the myocardial levels of caspase-3, which is the downstream of the caspase mediated apoptotic signaling when compared with the group V rats.
Dilated cardiomyopathy and its treatment options

Effect of edaravone on myocardial apoptosis

TUNEL assay showed that the number of TUNEL positive nuclei was significantly higher in myocardial tissue sections from group V rats than in group N. However, treatment with edaravone significantly decreased the number of TUNEL positive nuclei compared to that in group V.
Effect of edaravone on inflammatory cellular infiltration and cardiac hypertrophy

The myocardial sections from the vehicle treated rats showed extensive inflammatory cellular infiltration whereas the edaravone treated rats were significantly protected from these changes. Similarly the cardiomyocyte diameter was significantly increased in the group V rats indicating the adverse cardiac remodeling in the form of hypertrophy, but the myocardial sections from edaravone treated rats showed significantly lesser cardiomyocyte size, which confirms its action against progression of EAM.
Effect of edaravone on the activation of AMPK protein during the progression of EAM

AMPK signaling has been reported to be involved in several cardiovascular disorders but its role in the progression of EAM is not studied. In order to identify its role, we have measured the myocardial levels of p-AMPKα and AMPKα levels in vehicle- and edaravone-treated DCM rats by western blotting. Interestingly, the myocardial level of p-AMPKα was significantly increased in the DCM rats whereas edaravone treatment significantly suppressed it.
Effect of edaravone on the upstream proteins for activation of AMPK during the progression of EAM

Further to identify the involvement of upstream proteins, we have measured the levels of activated Akt and PI3K in the experimental animals. It has confirmed the involvement of both of these proteins in the progression of EAM and also the activation of AMPK via these upstream proteins. These results clearly indicate that the AMPKα, could play a significant role in the adverse cardiac remodeling during the progression of EAM to DCM and edaravone treatment significantly attenuated the activation of myocardial AMPK in improving the cardiac function.
Effect of edaravone treatment on different subunits of PKC in DCM hearts

Increased expression of PKC isoforms may be important markers of heart failure. To reveal their involvement in the progression of EAM in the present model, we have carried out the measurement of its various isoforms: PKC-α, -β₁, -β₂ and δ in the hearts of both vehicle and edaravone treated DCM rats by Western blotting. Interestingly all of these subunits were found to be elevated in the hearts of vehicle treated DCM rats. Edaravone treated rats showed significant suppression of their myocardial levels when compared with the vehicle treated rats.

![Graphs showing densitometric ratios of PKC-α and PKC-β₁](image)
Effect of edaravone treatment on MAPK signaling cascade in DCM hearts

In order to identify the probable mode of action of edaravone on DCM, we have measured the myocardial expression levels of phosphorylated forms of p38-MAPK, MAPKAPK2, ERK1/2 and JNK in both the vehicle and edaravone treated. Group V rats showed the activation of MAPK pathway as their hearts showed significant increase in the phosphorylation of the above-mentioned members of MAPK signaling cascade. Interestingly the rats treated with edaravone were protected from these changes as their myocardial levels of these proteins were significantly lesser when compared with the group V rats.
Dilated cardiomyopathy and its treatment options

![Graph showing densitometric ratio of p-p38 MAPK/GAPDH, p-ERK/GAPDH, p-JNK/GAPDH, and p-MAPKAPK2/GAPDH with corresponding images of Western blots.](image)

- **p-p38 MAPK**
  - N: 0.5, V: 2.0, Ed 3: 1.0, Ed 10: 1.0
  - Significance: ##

- **p-ERK**
  - N: 0.0, V: 1.5, Ed 3: 1.0, Ed 10: 0.5
  - Significance: *

- **p-JNK**
  - N: 0.0, V: 1.0, Ed 3: 0.5, Ed 10: 0.0
  - Significance: *

- **p-MAPKAPK2**
  - N: 0.0, V: 0.6, Ed 3: 0.4, Ed 10: 0.0
  - Significance: ##
Dilated cardiomyopathy and its treatment options

Discussion

The present findings clearly suggest that edaravone, a novel free radical scavenger, reduced the severity of EAM and also prevented its progression to DCM. The cardioprotection offered by edaravone treatment may be partly due to the suppression of oxidative stress.

Edaravone treatment has protected the hearts from functional deterioration as evidenced by hemodynamic study. There was a significant improvement in the cardiac hemodynamics of the edaravone treated rats, as the LV Pressure parameters were significantly improved when compared to those in the group V. Similarly, echocardiography also confirmed the improved cardiac performance in the edaravone treated rats as their % FS and % EF values were improved significantly when compared to the group V rats. There was no significant change in the HR was observed, however, the HW/BW ratio of the edaravone treated rats were significantly lesser when compared with the group V rats. Thus our present study confirmed the effect of edaravone in maintaining the cardiac function against DCM derived from EAM induced by immunization with porcine cardiac myosin.

Oxidative stress and inflammation are thought to play important roles in the progression of cardiovascular diseases. An increase in the level of oxidative stress is implicated in the pathogenesis of heart failure and various autoimmune disorders including DCM mediated by cardiac myosin [Sukumaran et al., 2011]. Investigations suggest that free radicals may be important contributors to the deterioration of the
Dilated cardiomyopathy and its treatment options

decompensating myocardium [Prasad et al., 1996]. Treatments that reduce the levels of oxidative stress or inflammation have thus been found to improve myocardial function in patients with advanced heart failure as well as in animal models of this condition. The NADPH oxidase system, present in cardiac and vascular tissues, is also a candidate for the source of the superoxide [Muthalif et al., 2000]. Our study has confirmed that the DCM rats suffer from oxidative stress as evidenced by the increased levels of myocardial NADPH oxidase subunits like $p47^{phox}$, $p67^{phox}$, $gp91^{phox}$ and Nox4. In order to confirm the role of edaravone against oxidative stress we have measured the myocardial levels of these NADPH oxidase subunits in the DCM rats treated with edaravone. The results showed that the edaravone treated have been protected from myocardial oxidative stress as observed with their reduced myocardial levels of the above-mentioned NADPH oxidase subunits. This effect was expected as edaravone has been reported to provide protection against oxidative stress in various animal models. Zhang et al. [Zhang et al., 2008] reported the protective effect of edaravone against acute myocardial ischemia/reperfusion injury where they have suggested its antioxidative role in reducing the infarct size. It was also reported that edaravone at 10 mg/kg dose provided significant cardioprotection and reduced the ROS production in pressure overload-induced cardiac hypertrophy [Tsujimoto et al., 2005]. In line with these reports, our study with DCM rats also confirmed the protective role of edaravone against oxidative stress. From these results we can suggest that apart from improving the
antioxidant system, edaravone treatment can also provide significant cardioprotection in rats with DCM, at least in part via the inhibition of oxidative stress.

Oxidative stress is known to induce cardiomyocyte apoptosis, an important contributor to hypertrophic remodeling and cell dysfunction [Cesselli et al., 2001] in a variety of cell types by activating intracellular cell death signaling cascades [Martindale and Holbrook, 2002]. Apoptosis is the key contributor to cell loss during heart failure and death by any or many mechanisms is a loss of contractile muscle mass, and largely irreplaceable [Chandrashekhar and Narula, 2003]. The endoplasmic reticulum (ER) is classically characterized as an organelle that participates in the folding of membrane and secretory proteins. Stimuli such as ischemia, hypoxia, heat shock, genetic mutation, oxidative stress, and increased protein synthesis that cause ER dysfunction are collectively known as ER stress [Oyadomari et al., 2002]. Any disturbance in its function causes ER stress, leading to upregulation of ER chaperones such as glucose regulated protein 78. GRP78 serves as the master modulator of unfolded protein response network by binding to various ER stress sensors. Increased GRP78 was reported in ER stress-associated apoptosis of cardiocytes in the heart failure [Bhimji et al., 1986]. In our present study, the protein levels of GRP78, GADD 153 and cytochrome C were markedly upregulated in the group V rats confirming the ER stress whereas the treatment with edaravone significantly attenuated this change suggesting its protective effect against ER stress. When ER stress is excessive and/or prolonged, however, apoptotic signals are initiated by the ER, proceed through various routes involving
Dilated cardiomyopathy and its treatment options

caspases and finally induces apoptosis by activation of caspase-3 [Oyadomari et al., 2002; Mao et al., 2007]. Similarly, we have also observed the elevated myocardial level of cleaved caspase-3, which has confirmed the activation of apoptosis in the group V rats that can be mediated via oxidative stress and/or ER stress. Interestingly, the rats treated with edaravone showed significant suppression of caspase-3, suggesting its antiapoptotic role in DCM. Apart from this, the antiapoptotic role of edaravone is also confirmed by the reduction in the number of TUNEL positive apoptotic cells in the myocardial sections of the DCM rats treated with edaravone. From these results we can suggest that treatment with edaravone is effective in preventing myocardial apoptosis possibly via modulation of oxidative and ER stresses.

Cardiac adaptation in response to intrinsic or external stress involves a complex process of chamber remodeling and myocyte molecular modifications and growing evidence highlights oxidative stress as important mechanism for this maladaptation [Takimoto and Kass, 2007]. ROS generated by NADPH oxidase plays a key role in cardiac remodeling [Bendall et al., 2007]. Myocardial fibrosis, the replacement of fibrous tissue in place of damaged myocardium is the hallmark of DCM, was observed in DCM hearts as measured by Azan–Mallory staining and increased concentrations of its marker molecules (TGF-β1 and OPN). Myocardial fibrosis has an important role in contractile and diastolic dysfunction of the heart. Excessive expression of TGF-β1 and collagen III are involved in the myocardial fibrosis which promote the synthesis of extracellular matrix constituents such as proteoglycans and fibronectin. In addition, they also
Dilated cardiomyopathy and its treatment options

suppress the degradation of extracellular matrix. In this experiment using a rat model of DCM, we examined the anti-fibrotic effects of free radical scavenger edaravone. It was reported that, in a rat model of dimethylnitrosamine-induced liver cirrhosis, treatment with 10 mg/kg of edaravone produced significant reduction in the fibrotic area [Tanaka et al., 2009]. Similarly, in a pressure overload induced LV hypertrophic rat model, cardiac fibrosis was significantly attenuated by the treatment with the same dose of edaravone [Tsujimoto et al., 2005]. Consistent with those reports, in the present study, treatment with edaravone has reduced the percentage area of fibrosis in the hearts of DCM rats when compared with the group V rats. This antifibrotic effect of edaravone was also confirmed by the reduced myocardial levels of TGF-β1, OPN and collagen III in the edaravone treated rats. From these results we can confirm that edaravone, a novel antioxidant is effective in preventing the myocardial fibrosis in the rats with DCM.

Pathological hypertrophy occurs in response to pathological stress signals (e.g. neurohormonal activation, aortic stenosis, inflammation or cardiac injury). Initially, it may be adaptive to normalize wall stress and to preserve contractile performance but can proceed to decompensation and heart failure (Rohini et al., 2010). In the present study we have confirmed the involvement of inflammatory cellular infiltration and cardiomyocyte hypertrophy during the progression of EAM to DCM as identified by the increase in cardiomyocyte diameter of the DCM rats. Interestingly, when the rats were treated with edaravone, they are significantly protected from these adverse cardiac remodeling.
Dilated cardiomyopathy and its treatment options

The deregulated cardiac energy substrate metabolism in various cardiovascular disorders plays a key role in the pathogenesis of heart failure. The utilization of carbohydrate substrates becomes the predominant metabolic pathway in the diseased heart, because it can provide greater efficiency in producing high energy products per oxygen consumed compared to fatty acids (Nagoshi et al., 2011). AMPK has emerged as a key regulator of cellular energy homeostasis and coordinates multiple catabolic and anabolic pathways in the heart. AMPK has been reported to be activated by various reasons, such as hypoxia, low glucose and heat shock and it has been demonstrated to play a critical role in myocardial hypertrophy (Kudo et al., 1995; MacRae et al., 1995; Jones et al., 2005). When cellular energy levels are depleted, and the AMP:ATP ratio rises, AMPK is activated via allosteric binding of AMP to the AMPKc subunit, which has been proposed to induce a conformational change in the complex, improving the ability of AMPKa-subunit to serve as a substrate for upstream kinases (Dolinsky and Dyck, 2006). AMPK activation may be essential for adaptation of cardiac energy metabolism to acute and/or minor metabolic stresses, it is unknown whether AMPK activation becomes maladaptive in certain chronic disease states and/or extreme energetic stresses. In order to identify its role in the pathogenesis of heart failure, we have used chronic heart failure rats induced by EAM.

A number of kinases have been shown to phosphorylate AMPK directly. It has been reported that activated Akt directly phosphorylates AMPK on a site separate from Thr-172 (Dolinsky and Dyck, 2006). In the present study, the myocardial levels of activated
Akt was significantly elevated in the DCM rats, suggesting that these upstream kinases are essential to activate AMPK signaling in order to maintain normal energy supply. In line with this result, the activation level of AMPK was also found to be significantly elevated in the DCM rats when compared to the control rats. This increase may be due to the fact that activation of AMPK supplies the required energy via change in the metabolic reactions. We have also studied the change in AMPK signaling in the DCM rats treated with edaravone. It was interesting that the activation level of AMPK was significantly decreased in the DCM rats treated with edaravone. This interesting result indicates that the protective effect offered by edaravone is partly via attenuation of AMPK via blockade of Akt activation, but there may be some other mode also through which it can prevent AMPK activation.

In order to identify the other modes of the action of edaravone we have carried out the measurement of PKC levels in the hearts of DCM rats treated with vehicle or edaravone. PKC has received increasing attention in the pathogenesis of cardiomyopathy as it has been shown to function as an important intracellular signaling pathway for modulating cardiac myocyte development, inotropic function, and cellular growth (Meier and King, 2000). We have measured PKC-α, -β₁, -β₂, and -δ in this study as these isoforms have all been implicated in the pathogenesis of cardiac hypertrophy and ventricular dysfunction in cell culture as well as transgenic animal models (Hahn et al., 2003; Jalili et al., 1999). PKC-β₁ and β₂ has been implicated in the development of pathological cardiac hypertrophy and heart failure (Bowling et al., 1999; Rigor et al., 2009) and ventricles
Dilated cardiomyopathy and its treatment options

from patients with end-stage heart failure show increased expression of PKC-β2 (Bowling et al., 1999). There are other reports regarding the role of PKC-α as a necessary mediator of cardiomyocyte hypertrophic growth through an ERK1/2-dependent signaling pathway (Jalili et al., 1999; Braz et al., 2002). In addition, PKC-δ is also associated with myocardial ventricular hypertrophy and impairment of myocardial contractility (Venema and Kuo, 1993). In the present study, the myocardial levels of these isoforms of PKC have been increased in the DCM rats, whereas the edaravone treated rats were protected from these changes, indicating that edaravone can protect the hearts from adverse cardiac remodeling via blocking the upstream kinases involved in those processes.

Similarly, involvement of PI3K in cardiac hypertrophy has been reported. Activation of PI3K occurs downstream to the stimulation of tyrosine kinase receptors such as TGF as well as G protein coupled receptors. One of the principal targets of PI3K signaling is serine/threonine kinase Akt (Zhu et al., 2001). PI3K/Akt activation typically leads to pathological (maladaptive) hypertrophy, which may explain the increased PI3K/Akt activity observed in various models of cardiac pathological hypertrophy induced by pressure-overload, Fas-ligand, and chronic β-adrenergic stimulation (Gruson et al., 2010). In the present study, the vehicle treated rats showed significant increase in the myocardial levels of PI3K, whereas treatment with edaravone significantly reversed this change suggesting the upstream blockade of the activation of AMPK as well as PKC in DCM rats.
Previous reports have demonstrated that activation of PKC triggers the MAPK cascade (Schonwasser et al., 1998; Yamaguchi et al., 1995). Of these, ERK and p38MAPK were shown to be crucial in cell proliferation and differentiation. Through its signaling cascades, the p38MAPK as well as ERK1/2 modulate genes that regulate cellular hypertrophy, cardiac fibrosis, and cardiac cytokine-mediated inflammation (Li et al., 2005; Riad et al., 2007) leading to downstream activation of several transcription factors which is important in regulating cell growth, such as NF-κB and MEF-2 (McKinsey et al., 2001; Purcell et al., 2001; Han and Molkentin, 2000). MAPK signaling cascades are usually divided into three parallel pathways: ERK, JNK and p38 MAPK pathways (Watanabe et al., 2010). Subsequently, SAPKs/JNKs can be activated, and these MAPKs have also been implicated in cardiac hypertrophy (Ramirez et al., 1997). In consistent with these reports, our present study showed the increased myocardial levels of phospho p38-MAPK and phospho ERK1/2 in the DCM rats of group V. Interestingly, the treatment of DCM rats with edaravone has significantly reversed these changes. In addition, the present study also showed the reduced activation of Akt in the hearts of DCM rats fed with edaravone indicating the cardioprotective effects of it may be possibly via inhibiting the p38MAPK activation and subsequently the activation of Akt so that its downstream signaling can be prevented to avoid adverse cardiac remodeling. Considering all these findings, it is suggested that the induction of HF by EAM activates the master sensor of cellular energy balance in mammals, AMPK through the stimulation of various upstream kinases leading to myocardial dysfunction and
Dilated cardiomyopathy and its treatment options

treatment with edaravone, a novel antioxidant significantly improves the cardiac function by attenuating AMPK activation and MAPK signaling cascade. Various studies have reported the cardioprotective effects of edaravone based on its free radical scavenging property and antioxidant activity (Onogi et al., 2006; Tsujimoto et al., 2005). This is the first study which describes the involvement of AMPK signaling in the cardioprotective effects of edaravone in rat model of HF induced by EAM. The possible mechanism of edaravone in this study might also be because of its antioxidant activity with the involvement of AMPK and MAPK signaling.

In conclusion, there are several reports regarding the protective role of edaravone against cardiac impairment in various immune mediated cardiac inflammatory conditions [Nimata et al., 2005; Okabe et al., 2004] and ischemia-reperfusion injury [Watanabe et al., 2007], but its effect on progression of EAM to chronic cardiac complications like DCM is not available. The present study has provided evidences for the protective effects of edaravone against the deterioration of cardiac architecture during EAM mediated DCM.
Summary

Pathogenesis of EAM and its chronic form DCM in rats involves oxidative stress, inflammation and activation of RAAS and other cellular signaling mechanisms including p38MAPK pathway. Therapeutic approach for its treatment may target one or more of these targets with drugs among the following categories; antioxidants, ACEIs, ARBs, aldosterone antagonists and diuretics. In this research work we have performed studies using few antioxidant compounds and identified their protective roles against the progression of EAM to DCM. The following conclusions can be made with the aid of this research work,

1. Progression of EAM to DCM involves oxidative and ER stresses.
2. Tissue loss occurs following these stresses possibly by apoptosis via MAPK signaling cascade.
3. Natural antioxidant compounds; Mulberry and Quercetin as well as synthetic novel antioxidant edaravone produced significant cardioprotection against the progression of EAM to DCM.
4. Central behind the actions of all these studied compounds involves reduction of oxidative stress, ER stress and modulation of MAPK signaling cascade.

Future prospective

There is a significant change in the lipid/fatty acid metabolism during the pathogenesis of DCM after EAM. It needs further detailed studies as well will be our interest to identify the effect of these antioxidant compounds against these biochemical changes.
Dilated cardiomyopathy and its treatment options

References


Dilated cardiomyopathy and its treatment options

Circulation 1999; 99: 1885-1891 [PMID: 10199887]


Dilated cardiomyopathy and its treatment options


[22] Chua S, Sheu JJ, Chang LT, Lee FY, Wu CJ, Sun CK, Yip HK. Comparison of
Dilated cardiomyopathy and its treatment options


Dilated cardiomyopathy and its treatment options


[37] Eaton LW, Bulkley BH. Expansion of acute myocardial infarction: its relationship
Dilated cardiomyopathy and its treatment options

114


Friedrich MG, Strohm O, Schulz-Menger J, Marciniak H, Luft FC, Dietz R.
Dilated cardiomyopathy and its treatment options

Contrast media-enhanced magnetic resonance imaging visualizes myocardial changes in the course of viral myocarditis. *Circulation* 1998; 97: 1802-1809 [PMID: 9603535]


Dilated cardiomyopathy and its treatment options


Dilated cardiomyopathy and its treatment options


Dilated cardiomyopathy and its treatment options


Dilated cardiomyopathy and its treatment options


Dilated cardiomyopathy and its treatment options


Dilated cardiomyopathy and its treatment options


Dilated cardiomyopathy and its treatment options


Dilated cardiomyopathy and its treatment options


[118] Mizuno Molkentin JD, Dorn II GW. Cytoplasmic signaling pathways that regulate
Dilated cardiomyopathy and its treatment options


Dilated cardiomyopathy and its treatment options

2009;258:164-175.


Dilated cardiomyopathy and its treatment options


[137] Pan C, Giraldo GS, Prentice H, Wu JY. Taurine protection of PC12 cells against


Dilated cardiomyopathy and its treatment options

histocompatibility antigen expression and immunopathogenic mechanisms in cardiac myosin-induced myocarditis. Lab Invest 1991; 65: 538-547 [PMID: 1753703]


Dilated cardiomyopathy and its treatment options


[157] Schonwasser DC, Marais RM, Marshall CJ, Parker PJ. Activation of the mitogen-
Dilated cardiomyopathy and its treatment options


Dilated cardiomyopathy and its treatment options


[169] Sukumaran V, Watanabe K, Veeraveedu PT, Gurusamy N, Ma M, Thandavarayan RA, Lakshmanan AP, Yamaguchi K, Suzuki K, Kodama M. Olmesartan, an AT1 antagonist, attenuates oxidative stress, endoplasmic reticulum stress and cardiac...


Dilated cardiomyopathy and its treatment options


[182] Thandavarayan RA, Watanabe K, Ma M, Veeraveedu PT, Gurusamy N, Palaniyandi SS, Zhang S, Muslin AJ, Kodama M, Aizawa Y. 14-3-3 protein...
Dilated cardiomyopathy and its treatment options


Dilated cardiomyopathy and its treatment options


Dilated cardiomyopathy and its treatment options


[201] Weinberg EO, Schoen FJ, George D, Kagaya Y, Douglas PS, Litwin SE, Schunkert H, Benedict CR, Lorell BH. Angiotensin-converting enzyme inhibition prolongs survival and modifies the transition to heart failure in rats with pressure overload
Dilated cardiomyopathy and its treatment options

hypertrophy due to ascending aortic stenosis. *Circulation* 1994; 90: 1410-1422


Dilated cardiomyopathy and its treatment options


As first author


Dilated cardiomyopathy and its treatment options


As co-author

Dilated cardiomyopathy and its treatment options


Dilated cardiomyopathy and its treatment options


11. Watanabe K, Sukumaran V, Veeraveedu PT, Thandavarayan RA, Gurusamy N,
Dilated cardiomyopathy and its treatment options


Awards

1. **Young Investigator Award**: 2nd International Conference on Cardiovascular Disorders 2011, New Delhi, India.

2. **Young Investigator Award for International students**: 78th Annual Scientific Meeting Japanese Circulation Society, March 21-23, Tokyo, Japan.