DELINEATION OF MECHANISM OF ACTION OF AN AYURVEDIC ANTIHYPERTENSIVE FORMULATION AND ASSESSMENT OF ITS EFFICACY IN PREVENTION OF CARDIAC REMODELING

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DELINEATION OF MECHANISM OF ACTION OF AN AYURVEDIC ANTIHYPERTENSIVE FORMULATION AND ASSESSMENT OF ITS EFFICACY IN PREVENTION OF CARDIAC REMODELING

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Dedicated to my family

CERTIFICATE

I, **VANDANA SANKAR,** hereby certify that I had personally carried out the work depicted in the thesis entitled **"DELINEATION OF MECHANISM OF ACTION OF AN AYURVEDIC ANTIHYPERTENSIVE FORMULATION AND ASSESSMENT OF ITS EFFICACY IN PREVENTION OF CARDIAC REMODELING"**, under the direct supervision of Dr. R. Renuka Nair, Division of Cellular and Molecular Cardiology, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram except where external help was sought and is acknowledged.

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DECLARATION

This is to certify that Mrs. Vandana Sankar in the Division of Cellular and Molecular Cardiology of this Institute, has fulfilled the requirements of the regulations relating to the nature and prescribed period of research for the Ph.D. degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram. The work relating to her thesis entitled "**DELINEATION OF MECHANISM OF ACTION OF AN AYURVEDIC ANTIHYPERTENSIVE FORMULATION AND ASSESSMENT OF ITS EFFICACY IN PREVENTION OF CARDIAC REMODELING**" was carried out under my direct supervision.

Dr. R. Renuka Nair
(Guide)

The Thesis

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CONTENTS

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SYNOPSIS

INTRODUCTION

Ayurveda is an ancient Indian system of medicine. Despite therapeutic efficacy, its use is limited by the lack of scientifically validated information. Evaluating Ayurvedic drugs for their efficacy and mechanism of action using modern biological techniques and screening for toxicity will make them more acceptable and popular.

In this backdrop, the study was taken up to scientifically characterize the cardiovascular response to an Ayurvedic antihypertensive formulation. The drug, *Cardoguard* was prepared by *Nagarjuna Herbal Concentrates Ltd.*, Kerala. It is composed of crude powders of six medicinal plants namely: *Rauwolfia serpentina* (root powder), *Terminalia arjuna* (bark powder), *Boerhavia diffusa* (whole plant powder), *Terminalia chebula* (fruit powder), *Terminalia belerica* (fruit powder) and *Emblica officinalis* (fruit powder). The drug is found to be clinically effective in the reduction of blood pressure.

Hypertension is widely prevalent and is an important risk factor for left ventricular hypertrophy, coronary atherosclerotic sequelae, stroke, congestive heart failure, renal failure, etc. Sustained pressure overload imposed by hypertension leads to cardiac remodeling, resulting in alterations in size, shape, structure and function of the heart. Though initially an adaptive response, cardiac hypertrophy is a risk factor for cardiac arrhythmias and sudden death. Prolonged exposure to stress also leads to progressive decline in left ventricular performance. Pharmacological intervention in hypertension should therefore be directed to the maintenance of blood pressure as well as prevention of cardiac remodeling.

This study, aimed at ascertaining the efficacy and mechanism of action of *Cardoguard* has been designed with the following **objectives**:

1) Validate the antihypertensive potential of the drug and determine its efficacy in prevention of cardiac and vascular remodeling
2) Examine the vasorelaxant capacity of the drug and delineate its mechanism of action in vasorelaxation
3) Study the effect of the drug on myocardial mechanics
4) Determine the antioxidant capacity of the drug
5) Evaluate the toxicity of the drug
6) Examine the neurodepressive action of the drug

METHODOLOGY

Experimental studies involved the use of both *in vivo* and *ex vivo* models. The *in vivo* experiments were carried out primarily in Spontaneously hypertensive rats (SHR), whereas the *ex vivo* experimental studies were carried out in thoracic aortae and papillary muscles isolated from adult Sprague Dawley rats, cardiomyocytes and fibroblasts isolated from newborn Wistar rats and in erythrocytes from human blood samples.

SHR develop hypertension at 6-12 weeks of age, cardiac hypertrophy at 6-9 months and cardiac failure at 18-24 months. They serve as a counterpart for clinical hypertension along with the complications of hypertension comparable to that seen in man. The following studies were carried out in SHR:

1. Determination of antihypertensive potential of the drug
2. Evaluation of efficacy of the drug in prevention of cardiac and vascular remodeling

3. Evaluation of drug toxicity

Treatment was initiated at 2 months of age. The drug was orally administered daily over a period of 10 months. The therapeutic dose of the drug was calculated as 5mg/animal/day based on body surface area. The blood pressures of the untreated and treated rats at 6 and 12 months of age were determined using Noninvasive Blood Pressure Monitoring System.

Cardiac remodeling involves hypertrophy, fibrosis, electrical remodeling and functional alterations. Hypertrophy in SHR was assessed by measurement of i) hypertrophy index ii) left ventricular wall thickness iii) left ventricular cardiomyocyte cross-sectional area and iv) extent of fibrosis. The identification of signaling pathways in cardiac hypertrophy modified by the drug were determined by immmunohistochemistry and signal intensities were quantified as chromogen gray levels using *Image proplus 5.1.* Electrical remodeling was assessed by electrocardiogram. Cardiac output was determined for assessment of functional changes. Wall thicknesses and wall to lumen ratios of thoracic aortae and arteries in the stroma of kidneys of treated and untreated SHR were measured for the evaluation of vascular remodeling. Wistar rats were used as normotensive control for SHR wherever required. Toxicity of the drug was evaluated by histopathological analysis of the kidney and liver of treated SHR.

Cardoguard has *Rauwolfia serpentina* as the major constituent. Reserpine, a component of *Rauwolfia,* was widely used as an antihypertensive in the past; it became unpopular because it was found to be depressogenic in patients when used for prolonged periods. According to Ayurveda, the whole plant *(Rauwolfia)* reduces the risk of side effects that occur with the use of the isolated active principle

(reserpine). The activity of brain mitochondrial monoamine oxidase, a marker for depression was assayed in Wistar rats treated with *Cardoguard* and compared with the values in rats treated with reserpine.

The mechanism of action of the drug and the cellular response independent of the systemic changes is better understood in *ex vivo* experimental systems. Hence the following experiments were carried out in *ex vivo* models:

1) Determination of vasorelaxant potential and delineation of the mechanism of action of *Cardoguard* were carried out in isolated aortae
2) Evaluation of the effect of the drug on myocardial mechanics was assessed in left ventricular papillary muscles
3) Determination of the efficacy of the drug in prevention of cardiomyocyte hypertrophy and fibroblast proliferation was carried out in cardiac cell cultures
4) Assessment of antioxidant capacity of the drug was carried out by i) measurement of reduced glutathione levels (GSH) in stimulated human red blood cells (from healthy volunteers) ii) measurement of inotropic response of rat papillary muscle exposed to oxidative stress in the presence and absence of the drug iii) measurement of intracellular oxygen radicals in cultured fibroblasts exposed to drug and ROS generator utilizing H_2 DCF-DA (2′-7′-dihydrodichlorofluorescin diacetate). ROS generator used in all the 3 experimental models was hypoxanthine + xanthine oxidase.

Therapeutic levels of water-soluble fraction of the drug were used for all *ex vivo* experiments (64 mg/L). The therapeutic level was calculated based on average blood volume of adult. Since the drug has its constituents in the crude form and has

not been extracted with any organic solvents, water soluble fractions were preferred.

RESULTS

Antihypertensive potential and efficacy of the drug in prevention of cardiac /vascular remodeling: The drug was found to reduce systolic and diastolic blood pressure in SHR. Morphological and histological examinations established prevention of left ventricular hypertrophy in treated SHR. The differences in the hypertrophy index and left ventricular wall thickness were more significant in the 6 month old treated and untreated rats compared to the 12 month old. Histological morphometry in 6 month old SHR revealed prevention of cardiomyocyte hypertrophy and interstitial fibrosis. Immunohistochemical studies indicated the involvement of ERK and PKC ε in the modulation of hypertrophy by the drug. The differences in wall thickness and wall to lumen ratio of thoracic aortae and arteries in the kidney stroma of SHR and treated SHR were not statistically significant. Prevention of vascular remodeling by the drug is not indicated. One possibility is that treatment was initiated after the occurrence of vascular remodeling, which accompanies/ precedes the onset of hypertension in SHR. No significant ECG changes were observed in 6 month old animals. Animals with ECG changes were relatively more among untreated SHR at 12 months. Left axis deviation and ST segment changes were the main features observed. At both 6 and 12 months of age, the cardiac output was found to be higher consequent to a higher stroke volume in treated SHR compared to untreated rats, whereas no chronotropic variation was observed. A higher stroke volume without variation in heart rate is judged as a positive characteristic of the drug.

Toxicity of the drug:

No signs of toxicity were observed on histopathological analysis of the kidney and liver of treated SHR.

Neurodepressive action of the drug:

The fluorescent intensity corresponding to monoamine oxidase activity of brain samples of reserpine- treated rats was significantly higher than that of control (that received distilled water); the fluorescent intensity of the brain samples of *Cardoguard*- treated rats was found to be relatively lower than that of reserpine-treated rats and comparable to that of control.

Vasorelaxant potential:

Cardoguard induced vasorelaxation in isolated aortae preconstricted with phenylephrine as well as high K^+, with *R .serpentina* and *T. chebula* having a major role.

Mechanism of action:

Vasorelaxation was found to be endothelium dependent and nitric oxide and cyclooxygenase pathways are implicated in the endothelium- dependent relaxant effect by the drug. The drug also demonstrated Ca^{2+} antagonistic action as evident from a) the inhibition of vasoconstriction by cumulative increase in extracellular Ca^{2+} concentration on drug pretreatment b) occurrence of vasorelaxation on preconstriction with both phenylephrine and high concentration of K^+, both of which contribute to increase in intracellular Ca^{2+} concentration through Ca^{2+} entry. It was also demonstrated that the drug acts as an ATP-sensitive potassium channel activator. The drug was not effective as an angiotensin II inhibitor or a β-adrenoreceptor antagonist. The intracellular signaling mechanism associated with

vasorelaxation when examined using pathway- specific inhibitors demonstrated that vasorelaxation by the drug involved ERK1/2, p38 and calcineurin pathways, but not PKC.

Effect of the drug on myocardial mechanics:

The drug did not induce a negative inotropic response in papillary muscles at therapeutic levels.

Efficacy of the drug in prevention of cardiomyocyte hypertrophy and fibroblast proliferation:

The drug prevented cardiomyocyte hypertrophy in cultured cells stimulated with angiotensin II as well as isoproterenol. Cardiac fibroblast proliferation stimulated by angiotensin II was also found to be prevented by the drug.

Antioxidant potential of the drug:

GSH levels were significantly higher in red blood cells pretreated with the drug and subjected to oxidative stress. Negative inotropic response to oxidative stress was attenuated in rat papillary muscles pretreated with the drug. In addition, the dichlorofluorescin (DCF) fluorescence intensity of drug-pretreated cardiac fibroblasts exposed to ROS was significantly lower compared to that of ROS treated cells.

CONCLUSIONS

Cardoguard was found to be effective as an antihypertensive as observed from its hypotensive capacity in SHR. Morphological examination showed that the drug attenuated hypertrophic changes in SHR. Histological morphometry and cell culture experiments revealed the effectiveness of the drug in the prevention of cardiomyocyte hypertrophy and interstitial fibrosis. Cardiac function of treated

SHR was found to be preserved compared to untreated SHR. Since *Cardoguard* has been demonstrated to possess antioxidant properties, the hypotensive and antihypertrophic effects of *Cardoguard* is implied to be by the reduction of oxidative stress. *R.serpentina* and *T.chebula* were the major components inducing vasorelaxation. The mechanism of vasorelaxation appears to be mediated by virtue of its antioxidant capacity associated with its Ca^{2+} antagonistic action, thereby enhancing endothelium derived relaxing factor availability at the level of vascular smooth muscle. The drug is found to be nontoxic. Measurement of brain MAO activity of *Cardoguard*-treated Wistar rats indicates that the drug is less depressogenic compared to the isolated active principle, reserpine. It is suggested that lowering the concentration of *R .serpentina* and increasing the proportion of *T. chebula* may yield better results.

SIGNIFICANCE OF THE STUDY: This is for the first time; such an extensive and systematic evaluation of an Ayurvedic antihypertensive drug has been carried out. Scientific evaluation of Ayurvedic drugs is an area that needs attention. Scientific documentation of the positive and negative characteristics of clinically effective Ayurvedic formulations will go a long way in their popularization and better acceptance.

ABBREVIATIONS

ACE	Angiotensin converting enzyme
Ang II	Angiotensin II
APD	Action potential duration
AT1 receptor	Angiotensin I receptor
ATP	Adenosine triphosphate
BW	Body weight
CSA	Cross sectional area
COX	Cyclooxygenase
DAG	Diacylglycerol
DOCA	Deoxy corticosterone acetate
ECG	Electrocardiogram
ECM	Extracellular matrix
EDCF	Endothelium- dependent contracting factor
EDRF	Endothelium-dependent relaxing factor
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal regulated kinase
ET-1	Endothelin -1
FBS	Fetal bovine serum
GPCR	G protein -coupled receptor
GSH	Reduced glutathione
GSSG	Oxidized glutathione

GTP	Guanosine triphosphate
H&E	Hematoxylin and eosin
H_2DCF-DA	2′-7′-dihydrodichlorofluorescin diacetate
HX	Hypoxanthine
I P_3	Inositol-1, 4, 5 trisphosphate
JAK	Janus kinase
JNC VII	Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure
JNK	c-Jun N- terminal kinase
K_{ATP}	ATP-dependent potassium channel
LAD	Left axis deviation
L-NAME	N^G-nitro-L-arginine methyl ester
LV	Left ventricle
LVH	Left ventricular hypertrophy
LVW	Left ventricular weight
MAPKKK / MEKK	Mitogen-activated protein kinase kinase kinase
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein/extracellular signal regulated kinase kinase
MAO	Monoamine oxidase
NADPH	Nicotinamide adenine dinucleotide Phosphate
NFAT	Nuclear factor of activated T cells

NO	Nitric oxide
PBS	Phosphate buffered saline
PGI_2	Prostaglandin I_2
PE	Phenylephrine
PIP2	Phosphatidylinositol biphosphate
PKC	Protein kinase C
PLB	Phospholamban
PLC	Phospholipase C
RAS	Renin angiotensin system
RAAS	Renin-angiotensin aldosterone system
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
SERCA 2a	Sarcoplasmic reticulum Ca^{2+} ATPase
SHR	Spontaneously hypertensive rat
SHRSP	Stroke- prone spontaneously hypertensive rats
SNS	Sympathetic nervous system
VW	Ventricular weight
XO	Xanthine oxidase
WKY	Wistar Kyoto

CHAPTER 1

INTRODUCTION

INTRODUCTION

From time immemorial, plants have been used as medicines around the world and plant-based medicines have been the mainstay of traditional societies in dealing with health problems. The recent global search for alternatives to modern medicine is based on the realization of the benefits of traditional medicine especially for chronic diseases and also the relatively moderate iatrogenic effects, combined with the urge to adopt a more natural way of relating to the world and to return to nature (Gilani and Rahman, 2005). Ayurveda is a traditional Indian system of medicine which arose approximately 3,000 years ago. Ayurvedic system of medicine is now receiving more attention in countries such as the United States, Europe, and Japan. Ayurveda, in its holistic approach goes beyond the mere prescription of drugs. It has developed a system of treatment based on the inherent ability of the human body to rejuvenate, to heal and to restore its natural balance. *Ayurveda* translates to "science or knowledge of life," with *Ayur* meaning "life" and *Veda* meaning "knowledge" or "science."

Despite the fact that this traditional system of medicine has proved effective in the treatment of various diseases, its acceptance is limited by the lack of scientifically validated information on the efficacy and mechanism of action of the formulations. Use of suitable experimental systems will help to overcome this lacuna and will go a long way in better acceptance of Ayurvedic drugs. Therefore, it can be said that pharmacology is on a reverse trail. While traditional medicine has long used herbal extracts unswervingly on patients, pharmacology is now aimed at validating such extracts through science. *Reverse pharmacology* takes the reverse route of clinical safety and efficacy validation of a healthcare product originally based on traditional

knowledge and experience. *In this backdrop, the study was taken up with the objective to scientifically characterize the cardiovascular response to an Ayurvedic antihypertensive formulation, using in vivo and in vitro experimental systems.*

Hypertension, most commonly referred to as "high blood pressure", is one of the most common worldwide diseases afflicting humans. It is a medical condition in which the force exerted by the circulating blood on the walls of the blood vessels is chronically elevated. Because of the morbidity and mortality associated with hypertension, it is an important public health challenge. It will become a paramount global health problem especially in developing countries, where studies projected an increase of 80% in the number of hypertensives by the year 2025. This report was published in *The Lancet,* in 2005, and presents an overview of 30 studies, published over 13 years, which looked at blood pressure in the population of dozens of countries (Kearney et al., 2005). Hypertension is the most important modifiable risk factor for cardiac hypertrophy, coronary atherosclerotic sequelae, stroke, congestive heart failure, renal failure and peripheral vascular disease.

Though heart is not responsible for the pathogenesis of hypertension, it suffers from its consequences. Sustained hemodynamic load imposed by hypertension leads to cardiac remodeling. Cardiac remodeling refers to the alteration in shape, size, structure and function of the heart and it includes hypertrophy, fibrosis, electrical remodeling and functional changes. In particular, it has been well recognized that the presence of left ventricular hypertrophy (LVH) is an adverse feature in hypertension with affected patients having a substantially greater risk of cardiovascular

events, including mortality and morbidity from heart failure, atrial fibrillation and sudden death. LVH develops as a compensatory event initially. Due to increased systemic vascular resistance, the heart has to pump more forcefully than normal to pump blood through the systemic circulation. This results in an increase in workload to the heart. The heart, mainly the left ventricle, adapts to this increased workload imposed by hypertension, by an increase in wall thickness and a consequent increase in mass. This response is referred to as left ventricular hypertrophy. Since adult cardiac myocytes are terminally differentiated cells, they do not proliferate, instead they hypertrophy as an adaptation to increased workload. In left ventricular hypertrophy, there is a proportionate thickening of the left ventricular free wall and interventricular septum. This is the concentric pattern of hypertrophy and it is the consequence of addition of new sarcomeres in parallel to the existing sarcomeres of myocardial cells. Left ventricular hypertrophy maintains systemic perfusion despite the excessive load. However, the hypertrophied heart is often unable to maintain this increased pressure overload for an indefinite period and heart failure may eventually supervene. In the concentric pattern of hypertrophy, the myocardial compliance will be reduced progressively which causes impairment in filling. This leads to diastolic dysfunction. The transition from hypertrophy to failure is characterized by the onset of chamber dilatation with the failure of further concentric hypertrophic growth to normalize load. This is the eccentric pattern and it is the consequence of addition of new sarcomeres in series to the existing sarcomeres. This accompanies the appearance of systolic dysfunction. Though compensatory hypertrophy helps to maintain cardiac function initially, prolonged exposure to stress results in

cardiac decompensation.

Since LVH is associated with an increased propensity for heart failure, pharmacological intervention in hypertension should aim not only at the maintenance of blood pressure but also in the prevention of hypertrophy.

Various drugs and regimens have been advocated for the control of hypertension as well as prevention of cardiac sequelae (Chobanian et al., 2003; Wing et al., 2003). In recent years, due to the revival of interest in herbal products at a global level, the use of Ayurvedic formulations will be accepted and furthermore, yield rich dividends in the future in improving health, once they are scientifically validated.

The Ayurvedic antihypertensive formulation "*Cardoguard*", used in the study was prepared by *Nagarjuna Herbal Concentrates Ltd*, Kerala, India. It is a polyherbal formulation comprised of crude powders of six medicinal plants namely *Rauwolfia serpentina, Terminalia chebula, Terminalia belerica, Emblica officinalis, Terminalia arjuna* and *Boerhavia diffusa.* The drug is being prescribed by the physicians at *Nagarjuna Herbal Concentrates Ltd.* and is found to be effective in the reduction of blood pressure in human subjects, but detailed scientific investigations have not been carried out. The aim of this study is to scientifically validate the Ayurvedic drug by delineating its mechanism of action and evaluating its antihypertensive potential and efficacy in prevention of cardiac remodeling.

Reduction of blood pressure by antihypertensives can be effected by a number of mechanisms. Antihypertensives can act as β-blockers, calcium antagonists, angiotensin-converting enzyme (ACE) inhibitors, angiotensin II type I receptor (AT1) receptor blockers etc. The ability of a drug to reduce blood pressure could be also due to its effects on the renal function / diuretic capacity. The diuretic capacity of *Cardoguard* was not tested due to nonavailability of metabolic cages.

The positive and negative properties of *Cardoguard* were assessesd in this study. The significance of this study can be attributed to the pioneering attempt to scientifically validate an Ayurvedic antihypertensive formulation in a systematic and extensive manner.

CHAPTER 2

REVIEW OF LITERATURE

REVIEW OF RELATED LITERATURE

1. HYPERTENSION AND CARDIAC REMODELING

A) Hypertension

Hypertension, commonly referred to as "high blood pressure", is one of the most common diseases afflicting humans worldwide. It is usually a slowly developing disorder of middle to old age that predisposes to cardiovascular disorders which is a leading cause of morbidity and mortality in the elderly. The incidence and sequelae of hypertension vary markedly by patient sub-group, particularly by gender and race. The prevalence of hypertension is higher in men than age-matched pre-menopausal women, but similar for 70-year old men and postmenopausal women (Hanes et al., 1996). Population studies suggest that blood pressure is a continuous variable and that there is no absolute dividing line between normal and abnormal values.

Based on recommendations of the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure [JNC VII] (Chobanian et al.,2003), the classification of blood pressure (expressed in mm Hg) for adults aged 18 years or older is cited (Table1).

Table 1. Classification of blood pressure based on JNC VII

Condition	Systolic(mmHg)	Diastolic(mmHg)
Normal	<120	<80
Prehypertension	120-139	80-89
Stage 1 Hypertension	140-159	90-99
Stage 2 Hypertension	≥ 160	≥ 100

Prevalence of hypertension: According to estimates, the worldwide prevalence of hypertension in the year 2000 was 26%; totaling 1 billion people (Kearney et al., 2005). A larger proportion of the world's population is expected to be older in 2025 and hence prevalence of hypertension has been projected to increase to 29% by that time. However, there is considerable variation between countries and geographic regions for the reported prevalence of hypertension (5% to 70%) and hypertension control rates (5% to 58%) (Kearney et al., 2004; Kearney et al., 2005). Many studies across India on the prevalence of hypertension primarily based on JNC V criteria are available. Dubey carried out one of the earliest studies in India (1954), and documented 4% prevalence of hypertension (criteria: > 160/95) amongst industrial workers of Kanpur (Dubey, 1954). In 1984, Wasir et al reported 3% prevalence of hypertension (criteria: ≥160/95) in Delhi (Wasir et al., 1984). Gopinath et al investigated in 10200 school children in Delhi (male 5709 and female 4506), aged between 5-14 years and showed that hypertension existed even among them (Gopinath et al., 1994). A study carried out in rural areas of Haryana (1994-95)

demonstrated 4.5% prevalence of hypertension (JNC V criteria) (Malhotra et al., 1999) while a study in urban areas of Delhi reported a very high prevalence of 45% during 1996-97 (Ahlawat et al., 2002). Misra et al reported 12% prevalence of hypertension in the slums of Delhi (Misra et al., 2001).

From South India, Kutty carried out hypertension prevalence study (criteria: ≥160/95 mm of Hg) in rural Kerala during 1991 in the 20 plus age group and the prevalence was found to be 18% (Kutty et al., 1993). Later studies in Kerala (Criteria: JNC VI) reported 37% prevalence of hypertension among 30-64 age group in 1998 (Kutty et al., 2002) and 55% among 40-60 age group during 2000 (Zachariah et al., 2003). Very high prevalence of 69% and 55% was recorded among the elderly aged sixty and above in the urban and rural areas respectively during 2000 (Hypertension study Group, 2001).

Few studies on prevalence of hypertension are available from eastern Indian population. In 2002, Hazarika et al reported 61% prevalence (criteria: =JNC VI) among men and women, aged thirty and above, in Assam (Hazarika et al., 2002).

The Sentinel Surveillance Project, documented 28% overall prevalence of hypertension (criteria: =JNC VI) from 10 regions of the country in the age group 20-69 (WHO-Project on sentinel surveillance of Indian Industrial population) (Reddy et al., 2006). Another study carried out in 1998 among Industrial workers of the Bharat Electronics Limited (BEL), India using the same criteria illustrated a prevalence of 30% among men (Prabhakaran et al., 2005).

Few studies were carried out comparing different socio economic groups. One study from urban Chennai, reported 8.4% prevalence of hypertension among men and

women aged 20 years and above and belonging to the low socio economic group (based on household income, occupation and dietary pattern), and the middle socio economic group had a higher prevalence (15%) during 1996-97 (Mohan et al., 2001). A study conducted in the urban areas of Chennai during 2000 (age group ≥ 40) reported a higher prevalence of hypertension (54%) among low income group (income< Rs 30000/annum) and 40% prevalence among high-income group (income ≥ Rs 60000/annum) (Ramachandran et al., 2002).

There is a widely held misconception that hypertension is a single disease that can be treated with a single recipe. On the other hand, it is a heterogeneous disorder in which patients can be stratified by pathophysiologic characteristics that have a direct bearing on the efficacy of specifically targeted antihypertensive medications. These characteristics have a direct implication on the detection of potentially curable forms of hypertension, and on the risk of cardiovascular complications.

Hypertension may be either essential/primary or secondary. Secondary hypertension is caused by altered hemodynamics associated with a specific disease, but the etiology of essential hypertension remains unresolved.

Essential hypertension

A number of factors have been implicated in the etiology of essential hypertension. Thus essential hypertension can be a heterogeneous group and develop as a consequence of a complex interplay of identifiable and unidentifiable factors. Some of the causal factors identified with essential hypertension are:

i) Cardiac output (CO) and systemic vascular resistance (SVR): An increased cardiac output may be responsible for the development of hypertension. The increase in cardiac output could logically arise in two ways: either from an increase in fluid volume (preload) or from an increase in contractility on neural stimulation of the heart. However, even if cardiac output is involved in initiation of hypertension, the increased cardiac output may only be a transient phase; since the typical haemodynamic finding in established hypertension is an elevated peripheral resistance and normal cardiac output (Cowley, 1992). The increase in peripheral vascular resistance may be related to a functional constriction, the type observed under the influence of circulating or tissue-generated vasoconstrictors, or may be the result of structural alterations in the blood vessels. In human hypertension and in experimental animal models of hypertension, structural changes in resistance vessels are commonly observed. In patients with essential hypertension, the characteristic findings include: decreased lumen diameter and increased media to lumen ratio. Vascular remodeling, can result in an increased media to lumen ratio through the rearrangement of the existing material without an increase in the cross sectional area of the vessel. In human essential hypertension, there is increasing evidence to support the view that vascular remodeling rather than growth is the predominant change occurring in resistance vessels (Izzard et al., 2006; Thybo et al., 1995).

ii) Genetics: Inherited blood pressure could be considered as the core blood pressure, whereas hypertensinogenic factors cause blood pressure to increase above the range of inherited blood pressures. The identification of variants that contribute to the

development of hypertension is complicated by the fact that the two phenotypes that determine blood pressure, namely, cardiac output and systemic vascular resistance, are controlled by intermediary phenotypes, including the autonomic nervous system, vasopressor/vasodepressor hormones, the structure of the cardiovascular system, body fluid volume, renal function; and many others. Furthermore, these intermediary phenotypes are also controlled by complex mechanisms including blood pressure itself (Williams et al., 1994).

In any hypertensive individual, risk-predisposing genes are engaged in a complex network of gene-gene and gene-environment interactions (Felder and Jose, 2006; Moore and Williams, 2002). The influence of genes on blood pressure has been suggested by family studies demonstrating correlation of blood pressure among siblings and between parents and children. There is better association among blood pressure values in biological children than in adopted children and in identical as opposed to nonidentical twins. Furthermore, genetic factors also influence behavioral pattern, which might lead to blood pressure elevation. For example, a tendency towards obesity or alcoholism will be influenced by both genetic and environmental factors. Thus, the proportion of blood pressure variability caused by inheritance is difficult to determine and may vary between populations.

Mutations in at least 10 genes have been shown to raise or lower blood pressure through common pathways by increasing or decreasing salt and water reabsorption by the nephron (Lifton, 1996). The genetic mutations responsible for 3 rare forms of Mendelian (monogenic) hypertension syndromes- gluco-corticoid remediable aldosteronism (Sutherland et al., 1966), apparent mineralocorticoid excess (New et

al., 1977) and Liddle's syndrome (Liddle et al., 1963) have been identified. Polymorphisms and mutations in genes such as angiotensin gene, angiotensin converting enzyme, β_2 adrenergic receptor, renin binding proteins, G-protein B3 subunit, atrial natriuretic factor, and the insulin receptor have also been linked to the development of essential hypertension; however, most of them show a weak association if any (Marteau et al., 2005).

iii) Renin-angiotensin aldosterone system (RAAS): RAAS plays an important role in regulating blood volume and systemic vascular resistance, which together influence cardiac output and arterial pressure. Renin may play a critical role in the pathogenesis of most hypertension (Laragh and Lewis, 1992). Renin, a proteolytic enzyme which is primarily released by the juxtaglomerular cells associated with the afferent arterioles entering the glomeruli of the kidneys, stimulates the formation of angiotensin, which in turn motivates the release of aldosterone from the adrenal cortex. The release of renin is stimulated by 1) sympathetic nerve activation (acting via β_1 -adrenoceptors) 2) renal artery hypotension (caused by systemic hypotension or renal artery stenosis) 3) decreased sodium delivery to the distal tubules of the kidney.

When renin is released into the blood, it acts upon a circulating substrate, angiotensinogen, that undergoes proteolytic cleavage to form the decapeptide, angiotensin I. Vascular endothelium, particularly in the lungs, has an enzyme, angiotensin converting enzyme (ACE), that cleaves off two amino acids to form the octapeptide, angiotensin II (AngII) although many other tissues in the body (heart, brain, vascular tissue) also can form Ang II. It has several major functions that include constriction of resistance vessels thereby increasing systemic vascular

resistance and arterial pressure; action on the adrenal cortex to release aldosterone, which in turn acts on the kidneys to increase sodium and fluid retention; stimulation of release of vasopressin (antidiuretic hormone) from the posterior pituitary, which increases fluid retention by the kidneys; facilitates norepinephrine release and inhibits norepinephrine re-uptake by nerve endings, thereby enhancing sympathetic adrenergic function; stimulation of cardiac and vascular hypertrophy, etc. A large body of evidence indicates that Ang II plays a critical role in the development of structural and functional vascular changes, mainly through an increased generation of vascular ROS (Dzau, 2001; Griendling et al., 2000; Tain and Baylis, 2006; Touyz, 2004; Touyz and Schiffrin, 2000).

iv) Baroreceptor dysfunction: Baroreceptor reflexes modulate the sympathetic nervous system and act to buffer changes in arterial pressure. When hypertension is sustained, these reflexes are reset rapidly from both structural and functional alterations so that increase in blood pressure evokes less decrease in heart rate (Chapleau et al., 1995). The decreased inhibition of the vasomotor center resulting from resetting of arterial baroreceptors may be responsible for increased sympathetic outflow and thereby in the perpetuation of hypertension (Shepherd, 1990). A study by Ormezzano et al suggested that baroreflex impairment might contribute to the risk of future cardiovascular events in hypertensive patients (Ormezzano et al., 2008).

v) Autonomic nervous system: An excess of renin-angiotensin activity could interact with the sympathetic nervous system (SNS) to mediate most of its effects. On the other hand, stress may activate the SNS directly; and SNS overactivity in turn, may

interact with high sodium intake, the renin-angiotensin system, insulin resistance, etc. Considerable proof supports increased SNS activity in early hypertension and, even more strikingly, in the still normotensive offspring of hypertensive parents, among whom a large number are likely to develop hypertension (Esler et al., 2001).

vi) Endothelial dysfunction: Clinical as well as experimental studies showed endothelial dysfunction to be frequently associated with essential hypertension (Contreras et al., 2000; Haller et al., 2002). Regulation of blood pressure is controlled, in part, by opposing actions of endothelium-derived factors [e.g., the primary endogenous vasodilator, nitric oxide (NO) (Lowenstein et al., 1994) and the vasoconstrictor endothelin-1 (ET-1)] on systemic vascular resistance (Potenza et al., 2005). Endothelial dysfunction associated with hypertension is often characterized by an imbalance between NO and ET-1 that results from both genetic and environmental causes (Ouvina et al., 2001; Perticone et al., 2001).

Several studies have reported the influence of NO on renal haemodynamics (Cowley and Roman, 1996). Inhibition of nitric oxide synthase by N-monomethyl-L-arginine in healthy human subjects acutely increased blood pressure, peripheral vascular resistance, and fractional excretion of sodium (Haynes et al., 1993). The effects of NO is tonically active in the medullary circulation, so that reducing NO production or vascular responsiveness, reportedly enhances the pressure natriuresis response, followed by renal interstitial hydrostatic pressure, and sodium excretion by almost 30%, without corresponding changes in total or cortical renal blood flow or glomerular filtration rate (Cowley and Roman, 1996).This mechanism may contribute to the blunted pressure natriuresis reported in experimental models.

vii) Oxidative stress: Experimental evidence indicates that reactive oxygen species (ROS) play an important pathophysiological role in the development of hypertension. This is due to the presence of excess of superoxide anion and decreased NO bioavailability in the vasculature and kidneys and to ROS-mediated cardiovascular remodeling (Chabrashvili et al., 2002; Kishi et al., 2004; Touyz, 2004). In human hypertension, biomarkers of systemic oxidative stress are elevated (Redon et al., 2003).

viii) Obesity: Obese individuals have higher cardiac output, and central and total blood volume and lower peripheral resistance than non-obese individuals with similar blood pressure (Oren et al., 1996). The increase in cardiac output is proportional to the expansion of body mass and may be the primary reason for the rise in blood pressure (Ferrannini, 1995). The prevalence of hypertension increased proportionately with increasing body mass index (BMI), degree of upper body obesity, and fasting insulin levels (Schmidt et al., 1996).

Studies have highlighted the key role of increased sympathetic activity in obesity-hypertension. Long-term sympathoactivation could raise arterial pressure by causing peripheral vasoconstriction and by increasing renal tubular sodium reabsorption. Elevated renal sympathetic nerve activity was reported in animal models of dietary obesity (Hall, 2003). There are a number of proposed mechanisms linking obesity with SNS activation including baroreflex dysfunction, hypothalamic-pituitary axis dysfunction, hyperinsulinemia /insulin resistance, hyperleptinemia, and elevated circulating Ang II concentrations (Davy and Hall, 2004). The evidence in support of activation of the RAS in obesity has led to the notion that blockade of RAS might be

a beneficial strategy for management of hypertension associated with obesity (Sharma, 2004). Increased vascular production of ET-1 in hypertensive patients with increased body mass has been suggested as a potential mechanism for endothelial dysfunction. There is a selective enhancement of endothelin A (ET_A) receptor-dependent vasoconstrictor tone in obese hypertensive patients (Cardillo et al., 2004).

viii) Insulin resistance and hyperinsulinaemia: Higher insulin levels are associated with hypertension, and many probable mechanisms may explain the association (Donnelly and Connel, 1992). The hypertension that is more common in obese people may arise in large part from the insulin resistance and resultant hyperinsulinaemia. However, rather unexpectedly, insulin resistance may also be involved in hypertension in non-obese people as well (Ferrannini et al, 1987). According to a recent study, hyperinsulinemia does not appear to be a major cause of obesity-hypertension (Davy and Hall, 2004).

Secondary hypertension

Secondary hypertension has an identifiable cause and accounts for approximately 5-10% of all cases of hypertension. Patients with secondary hypertension are best treated by controlling or getting rid of the underlying disease or pathology, although they may still require antihypertensive drugs. Some causes of secondary hypertension are renal artery stenosis, chronic renal disease, primary hyperaldosteronism, sleep apnea, hyper- or hypothyroidism, pheochromocytoma, preeclampsia, aortic coarctation etc. The details as given by Fauci et al follow (Fauci et al., 2008).

Renal artery stenosis: Renal artery stenosis can cause narrowing of the vessel lumen. The reduced lumen diameter augments the pressure drop along the length of the diseased artery, which reduces the pressure at the afferent arteriole in the kidney. Reduced arteriolar pressure and reduced renal perfusion stimulate renin release by the kidney that boosts circulating Ang II and aldosterone.

Chronic renal disease: A number of pathologic processes (e.g., diabetic nephropathy, glomerulonephritis) can damage nephrons in the kidney, which prevents the excretion of normal amounts of sodium; which leads to sodium and water retention, increased blood volume, and increased cardiac output. Renal disease may also result in augmented release of renin leading to a renin-dependent form of hypertension. The elevation in arterial pressure secondary to renal disease can be viewed as an attempt by the kidney to increase renal perfusion and restore glomerular filtration.

Primary hyperaldosteronism: Enhanced secretion of aldosterone generally results from adrenal adenoma or adrenal hyperplasia. Increased circulating aldosterone causes renal retention of sodium and water, thereby increasing blood volume and arterial pressure. Plasma renin levels are generally decreased as the body attempts to curb the renin-angiotensin system. High levels of aldosterone are also associated with hypokalemia.

Sleep apnea: Sleep apnea is a disorder in which people repeatedly stop breathing for short periods of time (10-30 seconds) during their sleep. This condition is often associated with obesity, although it can have other reasons such as airway obstruction or disorders of the central nervous system. These individuals have a higher incidence of hypertension and the mechanism of hypertension may be related to sympathetic

activation and hormonal changes associated with repeated periods of apnea-induced hypoxia, and from stress associated with the loss of sleep.

Hyper- or hypothyroidism: Hyperthyroidism resulting from excessive thyroid hormone secretion induces systemic vasoconstriction, an increase in blood volume, and increased cardiac activity, all of which can lead to hypertension. It is less clear why some patients with hypothyroidism develop hypertension, but it may be related to decreased tissue metabolism reducing the release of vasodilator metabolites, thereby producing vasoconstriction and increased systemic vascular resistance.

Pheochromocytoma: Catecholamine- secreting tumors in the adrenal medulla can lead to very high levels of circulating catecholamines, both epinephrine and norepinephrine. This leads to α-adrenoceptor mediated systemic vasoconstriction and β-adrenoceptor mediated cardiac stimulation, both of which contribute to significant elevations in arterial pressure.

Preeclampsia: Preeclampsia is a condition that sometimes develops during the third trimester of pregnancy that causes hypertension due to increased blood volume and tachycardia.

Aortic coarctation: Coarctation, or narrowing of aorta (typically just distal to the left subclavian artery), is a congenital defect that hinders aortic outflow leading to elevated pressures proximal to the coarctation (elevated arterial pressures in the head and arms).

Consequences of hypertension:

The main consequences of uncontrolled hypertension include cardiac hypertrophy, congestive heart failure, coronary atherosclerotic sequelae, angina, myocardial infarction, stroke, renal failure, impaired vision etc.

Hypertension can cause injury to the arteries due to the added force against the arterial walls making them more vulnerable to narrowing or plaque build up. Atherosclerosis involves the gradual deposition of plaque on the inner walls of the arteries that result in an ischemic or hindered blood flow. Obviously, if blood flow is obstructed in the coronary arteries, myocardial infarction (heart attack) occurs; if blood flow is blocked in the cerebrovasculature, stroke occurs. Long-standing hypertension may manifest as hemorrhagic and atheroembolic stroke or encephalopathy. Other cerebrovascular manifestations of hypertension include lacunar-type infarctions, and dementia.

Hypertensive nephropathy refers to damage to the kidney that has to be distinguished from renovascular hypertension. In the kidneys, as a result of benign arterial hypertension, hyaline material accumulates in the walls of small arteries and arterioles, producing the thickening of their walls and the narrowing of the lumens. Consequent ischemia will produce tubular atrophy, interstitial fibrosis, glomerular alterations and periglomerular fibrosis. In advanced stages, renal failure will occur. Functional nephrons have dilated tubules, often with hyaline casts in the lumens.

The retina is one of the "target organs" that are damaged by sustained hypertension resulting in hypertensive retinopathy. Subjected to excessively high blood pressure over prolonged time, the small blood vessels that involve the eye are damaged.

Since the present study is designed to examine the efficacy of the antihypertensive drug in the prevention of cardiac remodeling, only this sequel of hypertension is reviewed in detail.

B) Cardiac remodeling

"Cardiac remodeling" is a term that, at first reception, connotes gross pathoanatomic change but which, by extended definition, encompasses a host of alterations in homeostatic mechanisms (endocrine, autocrine and paracrine) that, under normal conditions, control vascular tone, blood volume, basal contractile state, programmed cell death (apoptosis), as well as the architectural integrity and organization of myocardial sarcomeres (Cohn et al., 2000; Hunter and Chien, 1999; Mann, 1999; Swynghedauw, 1999). These homeostatic mechanisms can be considered protective and reparative but also detrimental (Weber, 2000). Since cardiac remodeling is an important aspect of disease progression, preventing or reversing maladaptive remodeling is a vital therapeutic target.

Cardiac remodeling comprises a) structural b) functional and c) electrical remodeling. Structural remodeling includes cardiac hypertrophy, increased interstitial fibrosis and vascular remodeling.

i) Cardiac hypertrophy, fibrosis and vascular remodeling: Cardiac hypertrophy, in its physiological form, occurs during development and as an adaptation to exercise ("athletic heart") and is characterized by preserved contractile function and improved

cardiac performance (Raskoff et al., 1976). Pathological cardiac hypertrophy occurs usually as a consequence of biomechanical stress, such as prolonged arterial pressure overload, or valvular heart disease. It is a cellular process marked by the enlargement of cardiomyocytes, accumulation of sarcomeric proteins, and reorganization of the myofibrillar structure. The response of the myocardium to increased stress or load is not stereotyped. Differences have been observed in the molecular composition and performance characteristics of the heart when exposed to stress. Cardiac hypertrophy consequent to pressure overload differs from that due to volume overload.

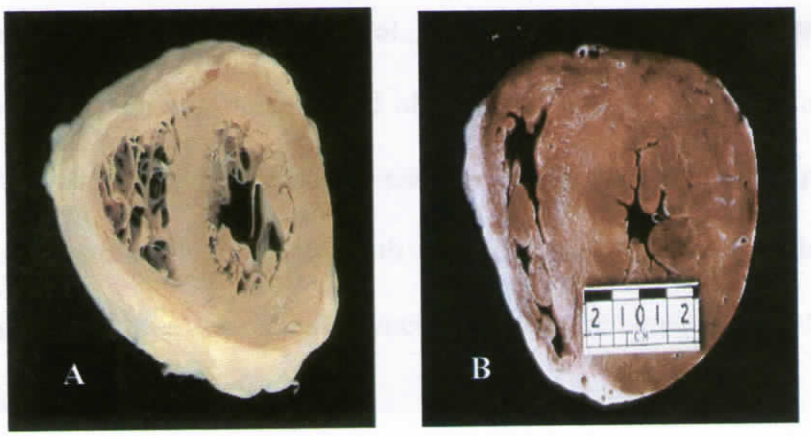

Fig. 1 Short axis section of A. the left ventricle of a normal heart and B. in a case of severe left ventricular hypertrophy (Educational Media services, Duke Medical Centre.2000.Available from www.echoincontext.com/int2/skillI2_02asp [Accessed on 10June 2008])

Cardiac hypertrophy in response to pressure overload -According to La Place's Law, the load on any region of the myocardium is given as follows: (pressure x radius) / (2 x wall thickness). Thus, an increase in pressure can be offset by an increase in wall thickness. The heart, mainly the left ventricle adapts to the abnormal haemodynamic burden imposed by hypertension by an increase in wall thickness. Hypertrophy is therefore perceived as an endeavor to normalize wall stress and stroke volume. Left ventricular hypertrophy (LVH) can be expressed as a proportionate thickening of the

left ventricular (LV) free wall and interventricular septum (Weber et al., 1991) (Fig. 1). As a result, there will be a reduction in the ventricular chamber diameter. This is the concentric pattern of LVH. Cardiac myocytes of the left ventricle are enlarged and increase in myocyte cross-sectional area is due to the in-parallel addition of myofibrillar units (Anversa et al., 1986). Myocytes of the right ventricle remain normal in size until there is chronic pressure overload due to pulmonary venous hypertension. Systolic ventricular function is commonly preserved in patients with LVH (Devereux et al., 1982) or even enhanced (Piano et al., 1998) but diastolic dysfunction may be present (Grossman et al., 1991). The heart is unable to sustain the pressure overload for an indefinite period of time and eventually, heart failure may supervene. At this stage, an eccentric pattern of hypertrophy accompanies the appearance of systolic dysfunction with LV chamber dilatation. Though the ventricular wall thickness decreases, myocardial mass remains augmented unless there is considerable myocyte necrosis.

The growth of parenchyma, or muscle mass, must be balanced with proportionate growth of stroma, or collagen if LVH is to be adaptive (Montfort and Perez-Tamayo, 1962). In hypertensive heart disease, however, there is structural remodeling of intramyocardial arteries and arterioles and their neighboring interstitial space by an abnormal accumulation of fibrous tissue. This distortion in the proportion of parenchyma and stroma (muscle mass: collagen mass) creates pathological hypertrophy. Perivascular fibrosis of intramyocardial coronary arteries and arterioles appears from which fibrillar collagen extends outward for variable distances into the interstitial space, resulting in interstitial fibrosis. This is a progressive process involving ever-increasing amounts of the interstitium (Silver et al., 1990). Individual

muscle fibers of both ventricles become encircled by thickened collagen fibers, which impair their ability to be stretched and to contract and ultimately lead to myocyte atrophy (Jalil et al., 1989 a). Experimental studies have shown that a two-to three fold rise in collagen concentration is associated with an increase in diastolic stiffness, while resting systolic stiffness and ejection fraction are preserved (Jalil et al., 1989 b). A further rise in collagen concentration (fourfold or more), particularly within the endomyocardium, raises diastolic stiffness even further and is associated with the appearance of systolic dysfunction (Capasso et al., 1990), which is seen in late hypertensive heart disease. On the other hand, myocardial fibrosis is not observed in the hypertrophy associated with athletic training (Shapiro and McKenna, 1984) or that which accompanies chronic volume overload (Michel et al., 1986).

Vascular remodeling of systemic and coronary resistance vessels may precede the appearance of hypertension and indeed may be the cause of elevated arterial pressure and impaired vasodilator reserve (Antony et al., 1993). The features of vascular structural remodeling in coronary resistance vessels include intimal hyalinization and endothelial hyperplasia besides perivascular fibrosis together with thickening of their media (Schwartzkopff et al., 1993) that collectively serve to reduce their wall thickness to lumen ratio. Hypertensive patients with LVH are at increased risk of myocardial ischemia (Otterstad, 1993). Precipitating factors may include the structural remodeling of coronary resistance vessels (Scheler et al, 1992) and prevailing coronary perfusion pressure (Mansour et al., 1993).

Myocyte shape alterations during cardiac hypertrophy parallel changes in ventricular anatomy (Grossman et al, 1975). Specifically, hypertension engenders concentric enlargement of the ventricle characterized by an increase in wall thickness and

myocyte cross sectional area (CSA) during the developing and compensated phases. Volume overload as a result of aortic regurgitation, mitral regurgitation, etc. leads to proportionate growth in chamber diameter and wall thickness, which is reflected at the cellular level by proportional growth of myocyte length and width. On the contrary, hypertrophy in pressure overload during the compensated phase is due solely to an increase in myocyte CSA (Zierhut et al, 1991). Data from patients with hypertension suggest that myocyte CSA is almost double the normal but does not change with progression to failure where cell lengthening is the predominant cellular change. The same remodeling pattern was found in Spontaneously Hypertensive Heart failure (SHHF) rats (Onodera et al., 1998)

ii) Electrical remodeling:

The most striking and consistent electrical alteration associated with LVH observed in isolated ventricular tissues and single myocytes is prolongation of the action potential duration (APD) due to the downregulation of several K^+ currents responsible for repolarization (Tomaselli and Marban, 1999). Action potential prolongation predisposes to early and late after depolarizations (Tomaselli et al., 1994).

The different phases of a cardiac action potential are as follows (Fig. 2): Electrical excitation initiates depolarization, and the flow of current opens the voltage-activated gates of the sodium (Na) channel to allow the rapid entry of Na^+, which constitutes the upstroke (Phase 0). As the Na current fades away, with the efflux of potassium ions, the overshoot is lost (Phase 1). The calcium (Ca) channel which is activated by a less negative voltage, opens. Inflow of Ca^{2+} and outflow of K^+ forms the action

potential plateau (Phase 2). The repolarizing efflux of potassium ions helps to terminate the action-potential plateau (Phase 3) and finally, resting potential is regained (Phase 4).

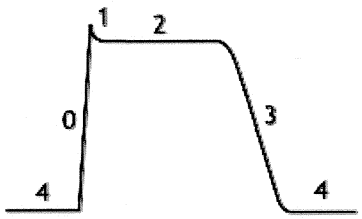

Fig. 2 Cardiac action potential

Afterdepolarizations are abnormal depolarizations of cardiac myocytes that interrupt phase 2, phase 3, or phase 4 of the cardiac action potential. Afterdepolarization underlies the propensity to arrhythmia, syncope, and sudden death. Early afterdepolarizations occur with abnormal depolarization during phase 2 or phase 3 of the action potential, and are caused by an increase in the frequency of abortive action potentials before normal repolarization is completed. Phase 2 may be interrupted due to augmented opening of calcium channels, while phase 3 interruptions are due to the opening of sodium channels. Late or delayed afterdepolarizations, on the other hand, begin during phase 4, after repolarization is completed, but before another action potential would normally occur. They are due to elevated cytosolic calcium concentrations. The overload of the sarcoplasmic reticulum may cause spontaneous Ca^{2+} release during repolarization, causing the released Ca^{2+} to exit the cell through the Na^{+} -Ca^{2+} exchanger which results in a net depolarizing current.

According to Yan et al., LVH amplifies transmural repolarization dispersion and induces early afterdepolarization (Yan et al., 2001). Increased ventricular

repolarization dispersion implies a widening in the T-wave duration in the electrocardiogram (Arini et al., 2004). In a study by Wang et al, hypertrophy-associated action potential duration (APD) prolongation was greater in subendocardial myocytes compared with subepicardial myocytes, indicating stress-induced amplification of repolarization dispersion (Wang et al., 2001). They observed that the regional variation in the electrophysiological response within the left ventricle was by way of a mechanism involving upregulated Ca^{2+} current and calcineurin. Furthermore, the studies by Wang et al revealed partial uncoupling of electrophysiological and structural remodeling in hypertrophy (Wang et al., 2001).

Left axis deviation, prolonged QRS complex, abnormal T wave etc. are some of the common electrocardiographic features of LVH in arterial hypertension (Pewsner et al., 2007).

Cardiomyocytes isolated from the left ventricle of spontaneously hypertensive rats (SHR) had a prolonged action potential compared to normotensive cells. The prolonged action potential resulted in an increased calcium content of the sarcoplasmic reticulum, which led to a greater sarcoplasmic reticular calcium release upon stimulation and an increased contraction (Brooksby et al., 1993 a).

Cardiac hypertrophy has been viewed as a pathological milestone in disease progression leading to heart failure, a syndrome in which compensatory responses such as vasoconstriction, neurohumoral recruitment and cytokine activation are maladaptive in the long term. To this list, Ca^{2+} current upregulation and action potential prolongation must be added (Nabauer and Kaab, 1998; Wickenden et al., 1998). Although an increased influx of Ca^{2+} may prove beneficial by maximizing inotropic potential in the short run, the resulting action potential prolongation

heightens dispersion of refractoriness and predisposes to after-depolarizations and triggered automaticity, reentry, and arrhythmogenesis.

iii) Functional changes: The structural changes during cardiac remodeling influence heart function with the overall goal of maintaining adequate delivery of blood to body cells while normalizing wall stress. The early remodeling during pressure overload is an adaptive response to the increased load of the heart to maintain the pumping capacity. Chronic long-term overload, however, taxes the limits of the adaptive mechanisms, forcing compromises that result in progressive deterioration of pump function. Ultimately, the left ventricle is enlarged and the diastolic and/or systolic function is impaired. Diastolic dysfunction occurs early during cardiac hypertrophy and may manifest as impairment in myocardial relaxation or an increase in passive stiffness (Hein et al., 2003).

Impairment in myocardial relaxation may be related to myocardial fibrosis and alteration of the viscoelastic properties of the ventricular myocardium. Impaired relaxation has been shown to be secondary to a decrease in intracellular calcium-handling proteins, including sarcoplasmic reticulum calcium ATPase (SERCA2a) and phospholamban (PLB) (Cory et al., 1994; De la Bastie et al., 1990; Feldmann et al., 1993; Kiss et al., 1995). Experimentally, a reduced expression of SERCA2a mRNA has been correlated to the severity of hypertrophy (De la Bastie et al., 1990) and progression of compensated hypertrophy to heart failure (Feldmann et al., 1993).

Myocardial performance remains controversial in animals with compensated cardiac hypertrophy, as mechanical activity has been reported to be increased (Capasso et al.,

1981; Cicogna et al., 2002), unchanged (Capasso et al., 1986; Cohen and Bing, 1987; Okoshi et al., 1997), and depressed (Lecarpentier et al., 1987; Matsubara et al., 1997). Furthermore, there is disagreement about myocardial function and LV performance when cardiac hypertrophy is stable. For example, Pfeffer et al have documented impaired pumping ability in hypertrophied ventricles from SHR over 12 months of age, while Conrad et al have shown that myocardial active tension development is not depressed in SHR of the same age. (Conrad et al, 1991; Pfeffer et al., 1976; Pfeffer et al., 1979)

Molecular mechanisms that underlie pressure overload cardiac hypertrophy:

The essence of hypertrophy is an increase in the number of force-generating units or sarcomeres in the myocyte. The question is, 'how the increase in force on the myocyte triggers an increase in sarcomeres?' The implication is that the mechanical input is transduced into a biochemical event that modifies gene transcription in the nucleus. Although critical proximal steps in mechanosignal transduction are not yet well understood, there is evidence that the disruption of cell-cell and cell-extracellular matrix contact is sufficient in itself to modulate both cell growth and apoptosis (McGill et al., 1997). In chronic hypertrophy, there are changes in integrin expression (Terracio et al., 1991) and possible integrin shedding into adjacent ECM (Ding et al., 2000) that raises the potential for disordered biomechanical signal transduction for growth and suboptimal myocyte-ECM coupling for force generation.

Reactive oxygen species have emerged as important triggers of the hypertrophic responses, both *in vivo* and *in vitro*, whether in response to stretch (Pimentel et al., 2001) or hypertrophic stimuli, such as Ang II (Bendall et al., 2002; Hingtgen et al.,

2006; Nakamura et al., 1998). Nuclear factor- κB (NF-κB) is known to be redox-sensitive, which translocates to the nucleus after activation by Ang II and plays a leading role in hypertrophic responses of cardiomyocytes (Freund et al., 2005; Purcell et al., 2001; Rouet-Benzineb et al., 2000). Furthermore, an association between NF-κB and cardiac remodeling was demonstrated in SHR (Gupta et al., 2005).

The first response to hemodynamic overload is the induction of proto-oncogenes (such as c-*fos*, c-*jun*, and c-*myc*) and heat shock protein genes (such as *hsp*70), therefore called 'immediate-early' genes (Izumo et al., 1988). It was shown as early as 1980's that change in gene and protein expression that reprised that of fetal heart was associated with pathologic hypertrophy of mechanically overloaded hearts (Chien et al., 1991; Izumo et al., 1988). The re-expression of immature fetal cardiac genes, include: (1) genes that modify motor unit composition and regulation (eg. the slow myosin ATPase isoform, β-myosin heavy chain relative to the fast myosin ATPase isoform), (2) genes that modify energy metabolism (eg, creatine kinase isoform, CK-B relative to CK-M; glucose transporter isoform, GLUT-1 relative to GLUT-4 isoform) and (3) genes that encode components of hormonal pathways (eg, atrial natriuretic peptide, angiotensin converting enzyme). In addition, variable or later blunted expression occurs in other genes that modify intracellular ion homeostasis (eg, downregulation of SERCA2a, with variable upregulation of the Na^+/Ca^{2+} exchanger); and downregulation of key parasympathetic and sympathetic receptors (eg, β_1-adrenergic receptors and M2 muscarinic receptors) and increase in ratio of angiotensin II (AT2) to angiotensin 1 (AT1) receptor subtypes. Some of these switches, such as the increased expression of the slow myosin ATPase isoform, β-myosin heavy chain relative to the fast myosin ATPase isoform, are adaptive and

promote a more favorable myoenergetic economy. Myocardial relaxation, which reflects the time course and extent of crossbridge dissociation after systolic contraction, is modified by the load imposed on the muscle (Zile and Gaasch, 1990). The down regulation of SERCA is nearly ubiquitous in animal models of advanced pressure overload hypertrophy, and compelling evidence shows that changes in SERCA levels have the potential to modify the time course of the calcium transient, myocardial relaxation, and the force frequency response (Feldman et al., 1993; He et al., 1997; McCall et al., 1998). The kinetics of SERCA 2 pumps and optimal calcium loading in the sarcoplasmic reticulum are modified both by the ATP dependent energy change and the phosphorylation state of the inhibitory regulatory protein, PLB (Lorenz and Kranias, 1997; Shannon and Bers, 1997).

Studies have shown that pressure overload hypertrophy is induced by a number of candidate signaling pathways involving humoral growth factors (Ang II, ET-1, insulin-like growth factor-I) and inhibition of pathways of apoptosis of myocytes (gp130 ligands). There is also involvement of catecholamines, associated G proteins and downstream kinase effectors (Dorn and Brown, 1999; Hunter and Chien, 1999). The latter signaling molecules include protein kinase C (PKC), tyrosine kinases, the mitogen-activated protein kinase family, *Ras*, and the Janus kinase (JAK)/signal transducer and activator of transcription family. In addition, Ca^{2+} has been shown to be an important second messenger in cell growth and survival. In response to growth stimuli, cytosolic Ca^{2+} increases, and calcineurin, a ubiquitous phosphatase, is activated, resulting in dephosphorylation of a class of transcription factors called the nuclear factors of activated T cells (NFAT), which then regulate expression of

specific genes. Although controversial, calcineurin appears to be a requisite mediator of myocardial hypertrophy (De Windt et al., 2001; Zou et al., 2001). The major signaling pathways implicated in hypertrophy are:

a) G protein –mediated signaling pathways:

G proteins are heterotrimeric GTP- binding proteins. Multiple seven- transmembrane-spanning receptors directly couple to G proteins resulting in GDP-GTP exchange, dissociation of the Gα subunit from the Gβγ, and activation of effector proteins by both subunits. The G-proteins Gq and G11 are functionally redundant transducers of phospholipase C (PLC) signaling from prohypertrophic heptahelical receptors for angiotensin, endothelin, norepinephrine, and other neurohormones (Rockman et al., 2002). PKC- and inositol 1, 4, 5-triphosphate–mediated (IP3-mediated) calcium release is considered to be the major effectors of Gq signaling.

b) Ras signaling pathways:

Ras is a low –molecular weight GTPase and is activated through G protein –coupled receptors (GPCR), receptor tyrosine kinases (RTK), Janus kinase 1(JAK1), or increases in intracellular Ca^{2+} resulting in GDP-GTP exchange and the activation of numerous effector proteins(Molkentin and Dorn, 2001).

c) Mitogen activated protein kinase signaling pathways (MAPK):

MAPK signaling pathways are activated in cardiomyocytes by GPCRs, RTKs, transforming growth factor β receptor (TGFR), PKC, calcium, or stress stimuli. These upstream events result in the activation of mitogen-activated protein kinase kinase kinase (MEKK) factors, which leads to the activation of mitogen activated protein kinase kinase (MEK) factors, and in turn leads to the activation of the three terminal MAPK effectors, c Jun N- terminal kinases (JNK1/2/3), extracellular signal regulated

kinases (ERK1/2), and p38MAPK. Production of ROS triggers activation of ERK1/2, which is one of the first indicators of hypertrophy (Liu et al., 2004; Shih et al., 2001).

d) Protein kinase C:

PKC isoforms are a family of ubiquitous lipid - binding serine -threonine kinases that act downstream of virtually all membrane-associated signal transduction pathways (Nishizuka, 1986). Based on enzymatic properties, PKC isoforms are classified as being conventional (c PKC: α, β, γ) or calcium dependent; novel (n PKC: δ, ε, μ, η, θ) or calcium independent; and atypical (a PKC: ζ, λ), which are activated by lipids other than diacylglycerol. An important feature of PKC isoforms is that, when activated, they translocate to distinct subcellular sites. For instance, it has been observed that PKC ε is selectively translocated to the particulate ventricular fractions during acute or chronic pressure overload (De Windt et al., 2000; Gu and Bishop, 1994; Paul et al, 1997) and after Ang II stimulation (Schunkert et al., 1995). Isoproterenol-induced cardiac hypertrophy in Wistar rats was associated with an enhanced total PKC-activity and a significant increased protein expression of cytosolic PKC-α, PKC-δ and PKC-ε (Braun et al., 2003).

e) Calcineurin signaling pathway:

Calcineurin is activated by calcium bound to calmodulin, which in turn leads to NFAT dephosphorylation and its nuclear translocation. Activated calcineurin has been shown to promote activation of JNK and certain PKC isoforms (De Windt et al., 2000).It has also been reported that NFAT nuclear translocation is antagonized by JNK and p38, thereby diminishing the hypertrophic gene expression (Braz et al.,

2003; Liang et al., 2003).

f) gp130- signal transducer and activator of transcription (STAT) signaling pathway:

The gp130 transmembrane receptor associated with the leukemia inhibitory factor receptor (LIFR) is activated by leukemia inhibitory factor (LIF), cardiotrophin, and other members of the interleukin-6 cytokine family (Ancey et al., 2003; Nakaoka et al., 2003; Takimoto et al., 2002; Uozumi et al., 2001). Once activated, this receptor interacts with JAK 1 causing its activation, which in turn leads to STAT phosphorylation promoting dimerization and nuclear entry. This receptor also promotes activation of phosphtidylinositol 3-kinase (PI3K), Ras, and MEKK.

7. Insulin-like growth factor I receptor (IGF-IR) signaling pathway:

Chronic IGF-1 administration in normal rats and mice caused substantial myocardial hypertrophy (Duerr et al., 1995). IGF-IR activation leads to activation of Ras, PLC, and insulin receptor substrates (IRS-1) proteins.

It appears that no single intracellular transduction cascade regulates cardiomyocyte hypertrophy in isolation, but instead each pathway operates as an integrated component of an orchestrated response between interdependent and cross-talking networks (Adiga and Nair, 2008; Molkentin and Dorn, 2001).

2. ANTIHYPERTENSIVES

Uncontrolled high blood pressure increases the risk for problems such as LVH, stroke, aneurysm, heart failure, heart attack, and kidney damage. It is not enough to reduce merely the blood pressure in hypertensive patients; but, the fundamental goal of treatment should be the prevention of the important "endpoints" of hypertension. Anti-hypertensive treatment can at least partly normalize that risk. Recent recommendations in the Seventh Report of the US Joint National Committee on the Prevention, Detection, Evaluation and Treatment of High Blood Pressure (JNC VII) emphasize the potential importance of achieving values below 140/90 mmHg to prevent arterial stiffness and renal damage.

Different types of antihypertensives reduce blood pressure by different mechanisms, enabling the possibility of adopting different treatment strategies. Antihypertensives are generally classified under five major drug classes: diuretics, β-blockers, calcium antagonists, angiotensin-converting enzyme (ACE) inhibitors, and angiotensin II type I receptor (AT1) receptor blockers. Several classes of medications which are effective in reducing blood pressure differ in side effect profiles, ability to prevent "endpoints", and cost. Which type of medication to use initially for hypertension has been the subject of several large studies and resulting national guidelines. In the United States, the JNC VII recommends starting with a thiazide diuretic if single therapy is being initiated (Chobanian et al., 2003). This is based on a slightly better outcome for chlortalidone in *The Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT) study* versus other anti-hypertensives and because thiazide diuretics are relatively cheap (ALLHAT, 2002). A subsequent smaller study, *The Second Australian National Blood Pressure Study (ANBP2)*, published after the

JNC VII did not show this small difference in outcome and actually showed a slightly better outcome for ACE-inhibitors in older male patients (Wing et al., 2003). In the United Kingdom, the June 2006 "Hypertension: management of hypertension in adults in primary care" guideline of the National Institute for Health and Clinical Excellence, downgraded the role of beta-blockers due to their risk of provoking type 2 diabetes (Ladva, 2006).

Studies have also shown regional and racial differences in response to treatment. A significant difference was observed between the black and non-black populations in response to treatment for hypertension. The hazard ratio (losartan relative to atenolol) for the primary end point favored atenolol in black patients and losartan in non-blacks. In black patients, blood pressure reduction was similar in both groups, and regression of electrocardiographic-LVH was greater with losartan (Julius et al., 2004).

Different classes of antihypertensives:

i) Diuretics: Diuretics help kidneys eliminate excess salt and water by inhibiting the reabsorption of sodium at different segments of the renal tubular system.They include *loop diuretics, thiazide diuretics, thiazide-like diuretics and potassium sparing diuretics*. Loop diuretics include bumetanide, furosemide, etc. Chlortalidone, hydrochlorothiazide, bendroflumethiazide etc. are examples of thiazides whereas indapamide, metolazone etc. are examples of thiazide-like diuretics. Amiloride, triamterene etc. are examples of potassium sparing diuretics. *Loop diuretics* inhibit the sodium-potassium-chloride cotransporter in the thick ascending limb. This transporter normally reabsorbs about 25% of the sodium load. Consequently,

inhibition of this pump can lead to a significant enhancement in the distal tubular concentration of sodium, reduced hypertonicity of the surrounding interstitium, and less water reabsorption in the collecting duct. *Thiazide diuretics,* which are most commonly used, inhibit the sodium-chloride transporter in the distal tubule. As this transporter normally only reabsorbs about 5% of filtered sodium, these diuretics are less efficacious than loop diuretics in producing diuresis and natriuresis. Nevertheless, only the thiazide and thiazide-like diuretics have good evidence of beneficial effects on important endpoints of hypertension, and hence, recommended as the first choice when selecting a diuretic to treat hypertension. The reason why thiazide-type diuretics are better than the others (at least in part) is thought to be because of their vasodilating properties. After chronic use, thiazides cause a reduction in blood pressure by lowering peripheral resistance. The mechanism of this effect is uncertain, but it may involve effects on renal autoregulation, or direct vasodilator actions through inhibition of carbonic anhydrase (Hughes, 2004) or by desensitizing the vascular smooth muscles to the rise in intracellular calcium induced by norepinephrine (Zhu et al., 2005). Hydrochlorothiazide therapy was reported to have no beneficial effect on structural changes in precapillary resistance vessels in hypertensive patients (Dahlof and Hansson, 1993). Since loop and thiazide diuretics augment sodium delivery to the distal segment of the distal tubule, the end result is potassium loss (potentially causing hypokalemia) as there is stimulation of aldosterone-sensitive sodium pump to increase sodium reabsorption in exchange for potassium and hydrogen ion, which are lost to the urine. Unlike loop and thiazide diuretics, *potassium sparing diuretics* do not act directly on sodium transport, while they antagonize the actions of aldosterone at the distal segment of the distal tubule.

Since this class of diuretics has relatively weak effects on overall sodium balance, they are often used in conjunction with thiazide or loop diuretics to help prevent hypokalemia. The diuretic effect of thiazides may be apparent soon after administration.

ii) Adrenergic receptor antagonists: Adrenergic receptor antagonists decrease sympathetic nervous system activity. They include β-adrenergic antagonists, α- adrenergic antagonists and mixed α- and β- adrenergic antagonists.

ii a) β-adrenergic antagonists : Atenolol, metoprolol, nadolol, oxprenolol, pindolol, propranolol etc. are some of the β -adrenergic antagonists. Although these drugs lower blood pressure, they do not have as positive a benefit on endpoints as some other antihypertensives (Lindholm et al., 2005). In particular, atenolol seems to be less useful in hypertension than several other agents (Carlberg et al., 2004). In hypertensive patients, treatment with atenolol for 1 year produced no significant improvement in the media-to-lumen ratio of small gluteal subcutaneous arteries (Thybo et al., 1995).

ii b) α-adrenergic antagonists: α- adrenergic antagonists include prazosin, terazosin, indoramin, phentolamine, doxazosin etc. Despite the hypotensive activity, α-blockers have significantly poorer endpoint outcomes than other antihypertensives, and are no-longer recommended as a first line choice in the treatment of hypertension (ALLHAT, 2003).

ii c) Mixed α- and β- adrenergic antagonists: These include bucindolol, carvedilol, labetalol etc. Carvedilol is indicated in the management of congestive heart failure

(Packer et al., 2002), as an adjunct to conventional treatments (ACE inhibitors and diuretics).

iii) Calcium antagonists: Calcium antagonists decrease the intracellular calcium concentration that has entered the cell either through voltage gated calcium channels or by activation of G-protein coupled to α- adrenoreceptors. Negative inotropy, negative chronotropy, negative dromotropy and peripheral vasodilatation are the four general cardiovascular effects of calcium antagonists.

Calcium channel blockers are used extensively in clinical practice and data from several clinical studies show that they effectively lower blood pressure and reduce long-term cardiovascular risk in a wide range of patient populations (Hansson et al., 1998; Hasebe and Kikuchi, 2005; Nissen et al., 2004; Staessen et al., 1997). Calcium channel antagonists are of two types: dihydropyridines and non-dihydropyridines. Dihydropyridines include amlodipine, felodipine, nifedipine etc. and non-dihydropyridines include diltiazem and verapamil. The efficacy and safety of dihydropyridine calcium channel blockers are well established, but there are also studies supporting the benefits of non-dihydropyridine calcium channel blockers (Hansson et al., 2000; Pepine et al., 2003; Ruggenenti et al., 2004). The side effects of calcium channel blockers include edema, bradycardia, bradyarrhythmia etc. (Peterson et al., 1995).

In SHR, nifedipine, a dihydropyridine calcium channel antagonist, induced growth in the coronary capillary network (Rakusan et al., 1994) and in Goldblatt 2-kidney, 1-clip hypertensive rats, the long-acting calcium antagonist benidipine increased the

density of capillaries and reduced the wall-to-lumen ratio of arterioles in the left ventricle (Kobayashi et al., 1999).

iv) Angiotensin converting enzyme (ACE) inhibitors: ACE inhibitors inhibit the formation of the vasoconstrictor peptide Ang II. Captopril, enalapril, fosinopril, lisinopril, ramipril etc. are some of the commonly used ACE inhibitors. They reduce the degradation of bradykinin and therefore increase the circulatory bradykinin levels and hence are contraindicated in renal artery stenosis. Cough and angioedema are the side effects of these drugs due to the potentiation of tissue kinins. Hyperkalemia and acute renal failure are the other possible side effects due to decreased aldosterone and extreme hypotension with impaired efferent arteriolar autoregulation. Blockade of the renin-angiotensin system with ACE inhibitors, perhaps in combination with other therapies, including diuretics, offers considerable promise for reducing microcirculatory abnormalities in hypertension (Levy et al., 2001).

v) Angiotensin 1 receptor blockers (AT1 blockers): AT1 blockers are more specific than ACE inhibitors, since they affect the last step of the renin-angiotensin cascade. There is no bradykinin potentiation in this case. Candesartan, eprosartan, irbesartan, losartan, valsartan etc are some of the AT1 blockers. In contrast to the ACE inhibitors, cough and angioedema are rarely associated with this class. Similar to ACE inhibitors, however, hyperkalemia and acute renal failure may occur in patients at risk. (Bauer and Reams, 1995). Losartan decreased the media-to-lumen ratio of renal afferent arterioles in genetically hypertensive rats (Ledingham and Laverty, 1998).

vi) Aldosterone antagonists: Aldosterone antagonists are not recommended as first-line agents for hypertension (Chobanian et al., 2003). But spironolactone and eplerenone, belonging to this class, are used in the treatment of heart failure.

vii) Vasodilators: Vasodilators act directly on arteries to relax their walls and are used only in medical emergencies. An example is sodium nitroprusside.

viii) Centrally acting antiadrenergic drugs: Centrally acting antiadrenergic drugs lower blood pressure by stimulating α_2 receptors in the brain. These are usually prescribed when all other anti-hypertensive medications have failed. For treating hypertension, these drugs are usually administered in combination with a diuretic. Adverse effects of this class of drugs include sedation, drying of the nasal mucosa and rebound hypertension. Clonidine, guanabenz, methyldopa, etc are examples. Moxonidine and rilmenidine are examples of a new class of centrally acting antihypertensives, which cause peripheral sympathoinhibition mediated by imidazoline (I1)-receptors in the rostral ventromedulla. Their side-effect profile appears to be better than that of clonidine and α-methyl-DOPA, probably because of a weaker affinity for α_2-adrenoceptors (van Zwieten, 1999). Escalating doses of drugs in this class often give rise to salt and water retention, in which case diuretic therapy becomes a valuable adjunctive (Sica, 2007).

Some adrenergic neuron blockers are used for the most resistant forms of hypertension. They include guanethidine, reserpine etc. Reserpine exerts its antihypertensive action by the depletion of biogenic amines in peripheral sympathetic nerve endings. It impairs the storage of the amines by interfering with the uptake

mechanism. Reserpine frequently produces a disabling side effect of lethargy that resembles depression and has limited its clinical utility (Frize, 1954).

Prevention / regression of LVH in response to antihypertensives

It is well recognized that the presence of LVH is an adverse feature of untreated hypertension and is an independent risk factor for myocardial ischemia, systolic and diastolic dysfunction, arrhythmias, and cardiac mortality (Haider et al., 1998). Therefore, prevention or regression of LVH is an important therapeutic target whether achieved by pharmacological, mechanical, surgical, or genetic means (Hunter and Chien, 1999; McMullen et al., 2004; Panidis et al., 1984; Villari et al., 1995; Wettschureck et al., 2001). There has been clinical evidence that regression of LVH reduces morbidity and mortality and improves prognosis (Haider et al., 1998).

Regression of LVH in systemic hypertension has been shown to occur in both human beings and experimental animals after control of hypertension by a variety of agents, including diuretics (Idikio et al., 1983), beta-blocking agents (Sen and Tarazi, 1983), methyldopa (Pegram and Frohlich, 1983), angiotensin converting enzyme inhibitors (Pfeffer et al., 1983), clonidine (Pegram and Frohlich, 1983), hydralazine (Sen, 1983), minoxidil (Sen, 1983), combination therapy (Schlant et al., 1982) and calcium channel antagonists (Rowlands et al., 1982). Although the data are still somewhat unclear, several studies have suggested that when high blood pressure is controlled by arterial vasodilators such as hydralazine or minoxidil, regression of left ventricular hypertrophy either does not occur or occurs to a lesser degree (Drayer et al., 1983). Studies have also suggested relatively less regression of hypertrophy, particularly of

the ventricular septum, in subjects whose hypertension was treated only with thiazide diuretics (Wollam et al., 1983).

Dahlof et al performed a meta-analysis of 109 studies involving 2357 hypertensive patients. In this analysis, ACE inhibitors reduced LV mass by 15%. This was higher than that observed with diuretics (11%), β-blockers (8%), and calcium-channel blockers (8.5%). It was suggested that reduction of LV mass with antihypertensives was far less than the magnitude of early regression and the late near-normalization of mass observed after valve replacement (Dahlof et al., 1992). The relatively disappointing magnitude of regression observed in pharmacological trials in hypertensive patients is likely related to an incomplete reduction of hypertension itself rather than to inadequate targeting of downstream signal cascades (Lorell and Carabello, 2000).

Based on the diverse effects of antihypertensive agents on the various tissue compartments in the heart, three classes of antihypertensive agents may be considered (a) drugs with no effects on LVH and fibrosis (direct vasodilators); (b) drugs with effects on LVH, ie., myocyte regression (diuretics, α- and β- adrenergic receptor antagonists); and (c) agents with proven effects on regression of LVH and fibrosis (ACE inhibitors, AT1 receptor antagonists, Ca^{2+} channel blockers, and centrally acting antiadrenergic agents) (Brilla,2000).In addition, aldosterone receptor antagonists provided evidence for their antifibrotic effects (Robert et al., 1994). According to a study by Linz et al., ACE inhibitors prevent the hypertrophic effect of Ang II even at doses that do not affect the blood pressure (Linz et al., 1992).

Herbal antihypertensives

India has an established history of safe and continuous usage of many herbal drugs in the officially recognized alternative systems of medicine namely, Ayurveda, Yoga, Unani, Siddha, Homeopathy and Naturopathy. More than 70% of India's 1.1 billion population still use these non-allopathic systems of medicine (Vaidya and Devasagayam, 2007).

The recent years have witnessed resurgence of interest in herbal medicines as more and more people throughout the world are turning to use medicinal plant products in healthcare system. Herbal medicinal preparations are becoming popular in some developed countries also such as Germany, France, Italy and the United States (Calixto, 2000). The increased use of herbal medicines in developed countries is mainly due to the realization of the efficacy of these medicines in chronic diseases and also the consequence of the emergence of multi-drug resistant bacteria and parasites. Alternative medicine is used for diseases such as cancer, human immunodeficiency syndrome, diabetes, hepatitis, allergies and mental disorders. The undesirable effects of chemical drugs, their increasing costs and greater public access to information on safety and efficacy of medicinal plants have also led to an increased interest in medicinal plants (WHO, 2002). There is growing evidence to show that medicinal plants have therapeutic efficacy with relatively fewer side effects (Gilani and Rahman, 2005). The medicinal property of plants is closely related to the different classes of phytoconstituents (such as essential oils, alkaloids, acids, steroids, tannins, saponins etc.) present in the plants. Pharmaceutical companies have renewed their strategies in favor of natural product drug development and discovery (Seidl, 2002).

Screening for anti-hypertensive effects in plants and traditional plant based medicine has been performed over many years. Eventhough there are a number of reports on the mechanism of action of antihypertensive medicinal plants, there are only a few reports on the elucidation of the mechanism of action of plant based drugs/formulations in the lowering of blood pressure and prevention of cardiac remodeling. The studies dealing with both plants and plant-based drugs are reviewed in this chapter.

Among the herbal antihypertensives that have been systematically analyzed, majority are Chinese medicines. Many traditional Chinese medicines such as *Tian ma gou teng Yin*, *Shen mai injection*, *Zhen ju tablet* etc. were used to cure hypertension for a long time. The traditional Chinese medicine *Bak Foong Pills* (BFP) significantly reduced blood pressure in SHR. *In vitro* experiments demonstrated that BFP caused a concentration-dependant vasorelaxation of isolated rat aortae when contracted with phenylephrine, which was partially inhibited by nitric oxide synthase inhibitor, L-NAME. BFP was able to significantly reduce hypertension in SHR through mechanisms probably involving a combination of increased serum K^+, vasorelaxatory action, reduced serum triglyceride and altered gene regulation in the higher centers (Zhou et al., 2003). In another study, *Tong-xin-luo capsule* (TXL), a medicine consisting of traditional Chinese herbs and insects inhibited ventricular remodeling induced by hypertension. The degree of fibrosis was significantly lower when treated with TXL and there was a striking improvement of cardiac function as well. The inhibitory effect might be associated with the process of TXL increasing the expression of PPAR-γ which in turn results in the inhibition of the activation of NF-κB (Bu et al., 2008). Wen et al observed that chronic treatment of rats with an

alcoholic extract of *Corydalis yanhusuo*, a well-known traditional Chinese medicinal herb attenuated development of pressure-overloaded cardiac hypertrophy induced by transverse abdominal aorta constriction in rats (Wen et al, 2007). *Uncariae ramulus* is a Chinese plant and there have been some reports that formulations from this plant were effective for hypertension and its complications (Aisaka et al., 1985). The vasorelaxation induced by Uncariae *ramulus,* was effected by endothelial dependency with NO and endothelial independency with Ca^{2+} channel blocking *in vitro* (Goto et al., 2000). The plant appeared to protect the endothelial cell function in SHR (Goto et al., 1999). *Astragalus membranaceus,* used in Chinese medicine was reported to promote baroreflex sensitivity in SHR (Chen et al., 2003). An *ex vivo* study using human amnion showed that *A. membranaceus*, lowers interleukin-6 (IL-6) concentration (Shon et al., 2002). A possible mechanism for the cardiovascular effects of *A. membranaceus* could be due to its reduction of IL-6 (Spelman et al., 2006). Treatment with *Radix Stephaniae Tetrandrae* (root of a Chinese herb, *Stephania tetrandra*) extract returned the arterial blood pressure, cardiac compliance and coronary flow towards normal, and reduced right ventricular hypertrophy in the deoxycorticosterone acetate-salt (DOCA-salt) hypertensive rat (Yu et al.,2004).

Shichimotsu-koka-to (SKT) is a traditional Japanese herbal medicine and treatment with SKT ameliorated the histopathological damage in the kidneys of stroke prone-SHR (SHRSPs) that included dilation and degeneration of renal tubules, infiltration of inflammatory cells and hemorrhage, with partial swelling or necrotizing of glomeruli. Moreover, decreased xanthine oxidase activity and significantly increased superoxide dismutase (SOD) activity were demonstrated in the kidney of treated SHRSPs (Higuchi et al., 1998). The antioxidant action of SKT is of significant importance

because oxidative stress is found to play an important role in hypertension (Touyz, 2004).

Viscum album (Mistletoe) is a plant native to Europe, and western and southern Asia. Alcoholic extracts of Japanese and European Mistletoe showed hypotensive effects in cats when administered intravenously and orally and in other animal studies as well (Fukunaga et al., 1989; Petkov, 1979).

Crataegus oxycantha and *Crataegus monogyna* (Hawthorne), native to Europe, North America, northwest Africa and western Asia has been shown to exert a mild blood pressure lowering effect (Leuchtgens, 1993; Schussler et al., 1995) that can take up to four weeks for maximal results. *Crataegus* has significant antioxidant activity (Rakotoarison et al., 1997) and in addition, it increases coronary blood flow (Schussler et al., 1995). *Crataegus* extracts also have a positive inotropic effect on the contraction amplitude of myocytes (Popping et al., 1995). Hawthorne treatment for three weeks attenuated LV systolic dysfunction and modified cardiac remodeling in response to 1 month of aortic constriction (Hwang et al., 2008).

Solanum sisymbriifolium (Wild tomato) is used in Paraguayan traditional medicine and the crude hydroalcoholic extract from its root was found to exhibit hypotensive activity when administered intravenously in normotensive rats and hypertensive rats in which hypertension was induced by adrenal regeneration + DOCA (Ibarrola et al., 1996).

Eugenia uniflora (Brazilian cherry) is used in Northeastern Argentina and intraperitoneal administration of its aqueous crude extract decreased blood pressure of normotensive rats and it was suggested that the hypotensive effect was mediated by a direct vasodilating activity when tested on arterially perfused rat hindquarter. A weak

diuretic effect that could be related to an increase in renal blood flow was also observed (Consolini et al., 1999).

The nature and effect of the changes in mean arterial pressure of normotensive rats through intravenous injection of aqueous extracts of *Phaseolus aureus* (Green beans), *Ruta graveolens* (Common rue) and *Laminaria japonica* (Kelp) were studied by Chiu and Fung with emphasis on possible interactions among these extracts. *Common rue*, native to southern Europe and northern Africa, *showed* positive chronotropic and inotropic effects on isolated right atria. On the other hand, *Green beans* (cultivated in Philippines, India, China and Malaya) and *Kelp* (native of Japan) individually showed negative chronotropic effects on isolated right atria. A combination of *Green beans* and *Kelp* showed no additive chronotropic effect. Combinations of *Rue* and *Green beans* and of *Rue* and *Kelp* showed responses that were either positive or negative chronotropically and were less than the sum total of their individual responses. A combination of all three showed subtractive effects on chronotropy, however, no change in inotropy was observed in spite of the positive inotropic effect of *Rue*. Studies also showed that *Rue* and *Kelp* individually relaxed KCl preconstricted rat tail artery strips probably by a direct effect of vascular smooth muscle. The combination of *Rue* and *Kelp* exerted a subtractive relaxation effect. It was suggested that the active principles found in the three plants probably interacted to modify their cardiovascular effects (Chiu and Fung, 1997).

Medicinal plants used by traditional healers in South Africa were investigated for their antihypertensive properties, utilizing the angiotensin converting enzyme assay. It was observed that *Adenopodia spicata* (Spiny splinter-bean) leaves showed the highest ACE inhibition (97%) (Duncan et al., 1999). In an earlier study, traditional

medicines reported to be used as antihypertensives or diuretics from China, India and South America were investigated and it was observed that crude extracts from 7 species out of 31 species studied inhibited the ACE by more than 50% (Hansen et al., 1995).

Aqueous extracts obtained from the leaves and flowers of *Commiphora opobalsamum* (Balsam of Mecca), one of the important medicinal plants of Saudi Arabia showed hypotensive and bradycardiac effects in rats (Mossa et al., 1983). The depressor effect on blood pressure was independent of adrenaline receptors and a cholinergic involvement in the effect was suggested (Abdul -Ghani and Amin, 1997).

Bidens pilosa (Farmer's friend) widely used in folk medicine in Cameroon for the management of hypertension showed concentration –dependent relaxant effects on rat aortic smooth muscle constricted with KCl and norepinephrine. It was suggested that the relaxation effect was due to blockade of the influx of intracellular calcium (Dimo et al., 1998).

Aqueous-methanolic crude extract of the fruits of *Olea europaea* (Olive) (native to Mediterranean, Asia and parts of Africa) lowered blood pressure in normotensive anaesthetized rats and the effect remained unaltered in atropinized animals. The mechanism of action in hypotension was through calcium channel blockade, justifying its use in hypertension (Gilani et al., 2005 a). A clinical study of *Olea europaea* leaf aqueous extract was conducted on two groups of hypertensive patients, 12 patients consulting for the first time, and 18 patients on conventional antihypertensive treatment. The aqueous extract was given for three months, after 15 days of placebo supplementation. Researchers noted a statistically significant decrease of blood pressure ($p<0.001$) for all patients, without side effects (Cherif et

al., 1996). In an *in vitro* study, a decoction of olive leaf caused relaxation of isolated rat aorta and the relaxant activity was independent of the integrity of the vascular endothelium (Zarzuelo et al., 1991).

The antihypertensive properties of the aqueous extracts of the leaves of *Musanga cecropioides* (African Corkwood) in both normotensive and hypertensive rats appeared to be partly due to a direct or indirect vasodilator effect and also to α_1- and β_2-adrenergic blocking effects. The extract also exhibited significant endothelium-dependent vascular smooth muscle relaxation, accounted for by the release of NO, and induced significant ACE inhibitory effects thereby supporting its vasodilator mechanism of action (Dongmo et al., 2002).

The aqueous extract of dried leaves of *Cecropia glaziovii Sneth* (Red Cecropia) (native to South and Central America) reduced blood pressure in SHR, in L-NAME treated rats and also in rats in which hypertension was induced by renal artery constriction (Lima-Landman et al., 2007).

Alcohol-free hydroalcoholic grape skin extract (GSE) obtained from skins of *Vitis labrusca* (Fox grape) (native to the eastern United States) was found to have an antihypertensive effect in Wistar rats with DOCA-salt and L-NAME induced experimental hypertension. The antihypertensive effect of GSE appeared to be due to a combination of vasodilator and antioxidant actions. GSE was found to possess endothelium dependent vasodilatation in the isolated mesenteric vascular bed of Wistar rats that was substantially inhibited by L-NAME, but not by indomethacin, tetraethylammonium or glibenclamide. The antioxidant potential was demonstrated by the finding that lipid peroxidation of hepatic microsomes estimated as

malondialdehyde production was concentration-dependently inhibited by GSE (Soares de Moura et al., 2002).

The relaxant effects of the seeds of the hypotensive plant, *Casmiroa edulis* (Mexican apple) (native to Mexico and Central America) when investigated in rat aortic rings was found to inhibit contractions elicited by noradrenaline, serotonin and prostaglandin $F_{2\alpha}$, but did not affect responses to KCl. The fact that *Casmiroa* antagonized contractions induced by diverse receptor agonists but not by KCl indicated a lack of effect on voltage dependent channels. Also, relaxation was not exerted through release of an endothelial relaxing factor and was not affected by histamine antagonists (Magos et al., 1995).

There are also studies on the effectiveness of antihypertensives used in Ayurvedic system of medicine.

Worldwide interest in Ayurveda is rapidly growing, especially in the United States, Europe, and Japan. Ayurvedic formulations are made in the form of choorna, lehya, gutika, rasayans, ointment, oils etc. With use of modern technology in the manufacturing process, medicines are now available as capsules and tablets.

According to Ayurveda, the use of whole plant as medicine reduces the unfavorable side effects compared to the use of isolated active principles. Hence, only the studies comprising the use of plants or plant parts are presented. Studies using isolated active principles are not included in the review.

Aqueous bark and and leaf extracts of *Ficus benghalensis* (Indian banyan) exhibited negative inotropy and hypotension in dogs (Vohora and Parasar, 1969). In another study, alcoholic extract of whole plant of *Cardiospermum halicacabum* (Balloon wine) produced hypotension and bradycardia (Gopalakrishnan et al, 1976). However,

hydro alcoholic leaf extracts of *Azadirachta indica* (Neem), a major component in Ayurvedic medicine, caused a dose-dependent hypotensive effect, but did not alter the force of contraction or heart rate at low doses in isolated frog heart (Chattopadhyay, 1997). *Piper betle, Jasminum sambac, Cardiospermum halicacabum and Tribulus terrestris* (Puncture wine) have been found to possess high ACE inhibition with a reported hypotensive/cardiovascular activity (Sharifi et al., 2003; Somanadhan et al., 1999).

Intravenous administration of crude extract of rhizome of *Valeriana wallichii* (Indian Valerian) produced hypotension in normotensive anaesthetized rats and the effect was partially obstructed by glibenclamide. In rabbit aortic preparations, the plant extract also caused a selective and glibenclamide-sensitive relaxation of low K^+ induced contractions. The results indicate that hypotensive effects of *Valeriana wallichii* are mediated possibly through K_{ATP} channel activation, which justify its use in cardiovascular disorders (Gilani et al., 2005 b). *Valeriana wallichii* grows in the northwestern Himalaya and has long been used in Ayurveda and Unani systems of medicine.

Zingiber officinale (Ginger) is widely used throughout the world and is reputed for its medicinal properties. Aqueous extract lowered blood pressure in rats through a dual inhibitory effect mediated via stimulation of muscarinic receptors and blockade of Ca^{2+} channels (Ghayur et al., 2005).

Allium sativum (Garlic), native to south western Asia showed a lowering effect on the systolic blood pressure in SHR and the nitric oxide system was found to play an important role (Harauma and Moriguchi, 2006). However, the endothelium modulated vasorelaxation by the plant was found to be partly mediated through

endothelium derived hyperpolarizing factors (EDHF) and cyclooxygenase pathways and not through NO (Ashraf et al., 2004). The ACE-inhibition activity of the plant was portrayed earlier by Ebadi (Ebadi, 2002).

Aqueous-methanolic extract of *Carum copticum* seeds (Ajowan) caused a dose-dependent fall in arterial blood pressure in anaesthetized rats. In isolated rabbit aorta and jejunum preparations, *Carum copticum* caused an inhibitory effect on the K^+-induced contractions. The calcium channel blocking effect was confirmed when the extract shifted the Ca^{2+} dose-response curves to right similar to verapamil (Gilani et al., 2005 c).

Crude extract of *Hyoscyamus niger* (Henbane) (found in Europe, west and north Asia), a component of Ayurvedic formulations, caused a dose-dependent fall in the arterial blood pressure of rats under anaesthesia. In isolated rabbit aorta, it relaxed phenylephrine and high K^+ -induced contractions and suppressed phenylephrine control peaks obtained in Ca^{2+}-free medium similar to that caused by verapamil. The vasodilator effect was endothelium-independent as it was not opposed by L-NAME in endothelium-intact rat aortic preparations and was not inhibited in endothelium-denuded tissues. The data indicate that *Hyoscyamus niger* lowers blood pressure by Ca^{2+} antagonism (Khan and Gilani, 2008). The crude extract exhibited a cardio depressant effect on the rate and force of spontaneous atrial contractions measured in guinea-pig atria (Khan and Gilani, 2008). Thus the plant appears to alter the mechanical function of the heart.

Nigella sativa (Black Cumin Seeds) native to southwest Asia has a long history of use as a diuretic and hypotensive agent. In an animal study, a comparison between *Nigella sativa* and nifedipine, a Ca^{2+} antagonist, showed that mean arterial pressure

decreased by 22-and 18 percent in the *Nigella sativa* and nifedipine treated rats, respectively (Zaoui et al., 2000).

Detailed investigations of commonly used Ayurvedic antihypertensive formulations are not available, but many of the components of *Cardoguard,* the Ayurvedic drug used in the present study have been studied.

A herbal drug, *Ajmaloon*, a preparation from *Rauwolfia serpentina* produced a dose-dependent hypotensive response in anaesthetized rabbits and monkeys without any significant effect on the heart rate (Fahim et al., 1995).

Singh et al. reported that aqueous solution of 70% alcoholic bark extract of *Terminalia arjuna* produced dose-dependent decrease in heart rate and blood pressure in dogs (Singh et al., 1982). The hypotensive and bradycardiac effects of aqueous solution of *Terminalia arjuna* extract was found to be mainly of central origin. The extracts inhibited carotid occlusion response, without affecting the pressor responses induced by intravenous injection of norepinephrine and by electrical stimulation of preganglionic fibers of the abdominal splanchnic nerve (Singh et al., 1982). Hypotension and bradycardia were also observed following the injection of the extract into the lateral cerebral ventricle and vertebral artery.

Water, ethanolic and acetone extracts of fruits of *Terminalia chebula* were found to possess ACE inhibitory activity (Somanadhan et al., 1999).

The components of the drug have also been evaluated earlier for their actions on the heart. *Terminalia arjuna* was associated with increase in left ventricular stroke volume index and increase in left ventricular ejection fractions (Bharani et al., 1995). *Terminalia chebula*, showed a positive inotropic action, increasing cardiac output, without increasing heart rate (Reddy et al., 1990). The cardioprotective action of

Emblica officinalis and *Terminalia arjuna* have been reported (Chubb and Huxtable, 1978; Dwivedi et al., 1988).

Abana is an Ayurvedic herbal preparation of various indigenous drugs including *Terminalia chebula, Emblica officinalis* and *Terminalia arjuna* and its overall effect is to cause down regulation of β-receptors (Gore, 1985). Treatment with *Abana* in normotensive rats produced significant hypotensive effect. *Abana* also protected against ethinyl oestradiol- induced hypertension and increased dopamine β hydroxylase activity in the hypertensive animals, thus suggesting that it produces effects against ethinyl oestradiol- induced hypertension by its sympatholytic property (Bhatt et al., 1998). Down-regulation of the β-receptors of the myocardium and intestine of rabbits treated chronically with the preparation has been reported (Pasnani et al., 1988). Balaraman et al. has suggested that the antihypertensive effects of *Abana* might be due to its alteration in the transport of cations across the cell membrane (Balaraman et al., 1993). Cardiac contractility is improved on treatment with *Abana.* Moreover, it decreased adrenalin -induced augmentation of heart rate and was therefore found to have a negative chronotropic effect (Dubey et al., 1985; Asokan, 1986).

In recent years there are records in literature, which suggest that *triphala* (a combination of *Terminalia chebula, Terminalia belerica* and *Emblica offficinalis:* constituents of the drug under study) possesses antioxidant activity (Naik et al., 2005). *Triphala* possesses high amount of phenols/polyphenols, accounting for its antioxidant potential. Such antioxidants can take part in electron transfer and hydrogen atom transfer reactions with many oxidizing free radicals (Naik et al., 2006). There are a number of ayurvedic formulations containing ingredients from medicinal plants that show antioxidant activities. *Abana* is one such antihypertensive

formulation (Vaidya and Devasagayam, 2007).

The herbal drugs and Indian medicinal plants are rich sources of beneficial compounds including antioxidants. Newer approaches utilizing collaborative research and modern technology in combination with established traditional health principles will yield rich dividends in the near future in improving health, especially among people who do not have access to the use of costlier western systems of medicine. (Vaidya and Devasagayam, 2007)

3. EXPERIMENTAL MODELS OF HYPERTENSION AND CARDIAC HYPERTROPHY:

In vivo and *ex vivo* experimental models are employed in studies on hypertension and cardiac hypertrophy. The use of a specific model depends on the objectives of the study.

i) In vivo models

The efficacy of *in vivo* models of hypertension is apparent from the volume of research accomplished using this research tool (Pinto et al, 1998). Human essential hypertension is a complex, multifactorial, quantitative trait under polygenic control. Apart from genetic models of hypertension, animal models have been developed by utilizing the etiological factors that are presumed to be responsible for human hypertension such as excessive salt intake, hyperactivity of RAAS, etc. An ideal animal model of hypertension/ cardiovascular disease is one that is feasible in small animals, should mimic human disease, allow studies in chronic, stable disease condition, produce symptoms which are predictable and controllable, should also allow measurement of relevant cardiac, biochemical, and haemodynamic parameters; and also satisfy economical, technical and animal welfare conditions. Animal models are available both in small and large animals. The features of commonly used rat models of hypertension and cardiac hypertrophy are presented below:

ia) Animal models of hypertension:

Spontaneously hypertensive rats:

Spontaneously hypertensive rats, the genetic strain of hypertensive rats, are the cornerstone of medical research in experimental hypertension. SHR s are descendants of an outbred Wistar male with spontaneous hypertension from a colony in Kyoto, Japan, mated with a female with elevated blood pressure. This was followed by repeated inbreeding of offspring, with selection for spontaneous hypertension, defined as a systolic blood pressure of over 150 mm Hg persisting for more than one month (Okamoto and Aoki, 1963). The various colonies of SHR are pre-hypertensive for the first 6–8 weeks of their lives with systolic blood pressures around 100–120 mmHg (Adams et al., 1989) and then hypertension develops over the next 12–14 weeks (McGuire and Twietmeyer, 1985). As in humans, hypertension develops more rapidly and becomes more severe in male than female SHR (Iams and Wexler, 1979). A major advantage of SHR is that it follows the same progression of hypertension as human hypertension, with the first stage being pre-hypertensive phase, followed by developing and sustained hypertensive phase; with each phase lasting at least several weeks (Folkow, 1993). It is a chronic stable model producing symptoms that are predictable and controllable and avoiding difficult or life-threatening technical interventions. Thus it is not surprising that SHR s have been used extensively and successfully for more than 40 years to test drug candidates for their effectiveness in lowering blood pressure, and to study the mechanisms of established hypertension

(Doggrell and Brown, 1998). The male SHR is commonly used as a model of essential hypertension. The lack of inter-individual variation is one of the major advantages of this rat model (Lindpaintner et al., 1992). However, SHR can only model one of many possible causes of human hypertension. SHR differs from human hypertension in that they reproducibly develop hypertension at a younger age compared to middle age in humans. Several hypotheses have been advanced to account for the high blood pressure in SHR. In SHR, normal cardiac output despite severe elevations of systemic arterial pressure have been observed; however, with prolongation of the pressure overload and progression of cardiac hypertrophy, ultimately left ventricular performance deteriorates (Pfeffer et al., 1979). Among the factors that have been proposed to account for the increase in arteriolar resistance in SHR are: vessel rarefaction (Struijker Boudier et al.,1992), an elevation of tone in resistance arteries (Bohlen and Lobach, 1978), an increased sympathetic activity (Lombard et al.,1984), differences in catecholamine transmitter disposition and sensitivity of smooth muscle cells (Lombard et al, 1986; Lombard et al,1990), and differences in central vascular regulation (Shah and Jandhyala,1995).

Gender difference in blood pressure in SHR: As mentioned earlier, in the SHR, males have higher blood pressure than females. The finding that androgen receptor antagonism, using flutamide, attenuated hypertension in male SHR demonstrates that androgens play an important role in the regulation of hypertension in male SHR (Reckelhoff et al., 1999).The release of cyclooxygenase-derived constricting factors appeared to be more pronounced in male than in female SHR. In addition, the relative

role of NO in endothelium-dependent arterial relaxation was higher in female than in male SHR, possibly via a hyperpolarization mechanism. Taken together, the control of arterial tone was clearly affected by gender in this model of genetic hypertension (Kahonen et al., 1998). The RAS is also found to play a role in the sex differences in blood pressure in SHR (Reckelhoff et al., 2000). Loukotova et al. observed a greater free cytosolic Ca^{2+} concentration response to Ang II in vascular smooth muscle cells of SHR male than female cells, a difference that was abolished in the absence of extracellular calcium (Loukotova et al., 2002). Hence a gender dependent difference in cell calcium handling has been observed. Extensive evidence points to the renal nerves as a link between the sympathetic nervous system and long-term blood pressure control by the kidneys and activation of the renal nerves stimulates renin release (Lohmeier, 2001; Lohmeier, 2003). A sustained activation of the baroreflex leads to a decrease in blood pressure by reducing renin release (Lohmeier et al., 2004). The male SHR has increased renal sympathetic nerve activity compared to normotensive rats (Grisk and Rettig, 2004). Furthermore, renal denervation attenuates hypertension in male SHR (Yoshida et al., 1995). According to Iliescu et al, the renal nerves contribute similarly to the hypertension in male and female SHR, but do not mediate the sex differences in blood pressure (Iliescu et al., 2006). In this particular study, baroreceptor sensitivity was found to be similar in male and female SHR. Also, despite lower blood pressure, left ventricular index was higher in females than males (Iliescu et al., 2006). This agrees with the finding that excessive cardiac hypertrophy in human hypertensive subjects is most commonly seen in women. (Lewis and Maron, 1989). A recent study demonstrates that male sex hormones influence

oxidative stress and antioxidant capacity in the SHR (Sullivan et al., 2007). Male SHR presents higher superoxide anion concentration under basal condition than does female. An AT1-dependent overexpression of the NAD (P) H-oxidase components may account for the sexual dimorphism in oxidative stress, and may play an important role in the noted gender differences on the incidence of cardiovascular disease (Dantas et al., 2004).

Stroke- prone spontaneously hypertensive rats (SHRSP):

Hypertension is an important predisposing factor for stroke (Yamori, 1974) and with a high risk of mortality in man. Stroke-prone spontaneously hypertensive rats were established by selection from among SHR (Yamori, 1974) and therefore the mechanisms of severe hypertension and stroke are better understood (Yamori et al., 1976). The SHRSP are hypertensive at 5 weeks and systolic blood pressure rises to at least 250 mmHg in males, in contrast to pressures of around 200 mmHg in SHR, with a positive correlation between the incidence of cerebral lesions and blood pressure. The similarity in the pattern of expression of SHRSP is important for further application of this model to studies of pathogenesis of stroke in man (Folbergrova et al., 1969).

Models of neurogenic hypertension:

An increased sympathetic outflow may be a key factor in essential hypertension. (Shepherd, 1990). One of the most important negative feedbacks in the control of blood pressure originates from baroreceptors located in the carotid sinus and aortic

arch. Stimulation of baroreceptors causes inhibition of vasomotor center leading to vasodilatation, bradycardia and decrease in blood pressure. Selective aortic baroreceptor de-afferentation produced persistent hypertension with increased neurogenic renal vasoconstrictor activity (Kline et al., 1983).

Continuous stimulation of stellate ganglion or continuous stretching of the aorta in dogs has resulted in hypertension being maintained as long as 7 days (Tarazi et al., 1983). Chronic electrical stimulation of the renal artery nerve or the splanchnic nerve in conscious dogs produced an increase in renal vascular resistance and results in hypertension and is maintained as long as the stimulus is continued (Katholi, 1983).

Models of mineralocorticoid hypertension:

Salt retention is characteristic of human hypertension and can be achieved rapidly in uninephrectomised rats by mineralocorticoid administration, for example by weekly subcutaneous injections of DOCA, and salt loading as 1% NaCl in the drinking water (De Champlain et al., 1967; De Champlain et al., 1969; Schenk and McNeill, 1992). It is only the combination of DOCA and NaCl that produces a major increase in blood pressure with increases in cardiac and renal weight. Both the DOCA– and aldosterone–salt models rely on impairment of kidney capacity and salt loading to rapidly induce hypertension and hypertrophy. These models progress quickly to severe hypertension and hypertrophy, and therefore are not suited for long-term studies in chronic, stable disease.

Models of nitric oxide synthase (NOS) inhibition:

Nitric oxide, a paracrine vasodilator, has been associated with regulation of vascular tone and inhibition of platelet aggregation, and therefore may be critical in the development of hypertension and atherosclerosis (Loscalzo and Welch, 1995). Chronic administration of L- NAME, a NOS enzyme inhibitor, increased systolic blood pressure and heart weight and decreased renal function; while the ACE inhibitor, ramipril was found to reverse all changes (Hropot et al., 1994).

Models of obesity induced hypertension:

In 1981, Ikeda et al developed a new model of obesity-related non–insulin-dependent diabetes mellitus, the Wistar fatty rat (WFR) (Ikeda et al., 1981). This strain was derived from crosses between obese Zucker and Wistar-Kyoto rats. The WFR developed obesity and obesity-related features, such as hyperinsulinemia and hyperlipidemia, followed by hypertension. This provides a good model to elucidate the precise relationship between hyperinsulinaemia and hypertension (Yamakawa et al., 1995).

Models of renovascular hypertension:

The chief pathophysiologic mechanism underlying renovascular hypertension involves activation of both limbs of RAAS and depends on the presence or absence of a contra lateral kidney. Anderson et al induced hypertension by the unilateral renal artery stenosis method [two-kidney, one-clip (2K1C) Goldblatt hypertension model]

where hypertension developed in response to ischemia (Anderson et al., 1990). In this model, sodium and water handling via pressure diuresis of the contra lateral kidney may be sufficient to prevent a volume component to hypertension.

The one-kidney, one clip model (1K1C) is yet another model where one kidney is removed and a clip placed on the renal artery of the remaining kidney. The chronic phase of hypertension is presumably dependent upon increased blood volume in this model (Share et al., 1982).

ib)Animal models of cardiac hypertrophy:

Cardiac hypertrophy in animal models is generally accomplished by increasing the workload of the heart that comprises pressure overloading, volume overloading and creating valvular insufficiency. Each of these techniques poses their own particular difficulties, shortcomings and limitations.

Pressure overloading as observed in hypertension has been induced by various techniques. Some of them include: the constriction of the pulmonary artery by nylon band resulting in right ventricular hypertrophy (Braunwald, 1971), aortic banding in puppies and allowing them to gradually stenose as they grow resulting in stable left ventricular hypertrophy that usually do not progress to failure unless the dogs are exercised excessively (Crawford et al., 1983).

Aortic banding leads to rapid increase in cardiac load; however, this model does not mimic a clinical condition and one cannot be certain that hypertrophy associated with this short, sharp pressure overload is similar to that observed with the more gradual process of essential hypertension in humans.

Spontaneously hypertensive rats and cardiac hypertrophy:

The SHR develop cardiac hypertrophy at 6-9 months of age (Shorofsky et al., 1999) and fulfills the criteria of an ideal animal model in that it allows studies in chronic stable disease, produces symptoms which are predictable and controllable and allows measurement of relevant cardiac, biochemical and haemodynamic parameters. Electrophysiological studies have shown that action potentials from hypertrophied SHR left ventricular slabs or myocytes are prolonged. Ion channel studies have shown impaired function of cardiac inward rectifying K^+ channels in SHR hypertrophy (Brooksby et al., 1993 b). Studies with multicellular SHR ventricular preparations usually show impaired contractility and responses to β-adrenoceptor stimulation (Nand et al., 1997) whereas hypertrophied SHR myocytes have increased contractility (Brooksby et al., 1992).

Renovascular hypertension-induced cardiac hypertrophy:

Renovascular hypertension resulting from renal ischemia as a result of renal artery occlusion leads progressively to left ventricular hypertrophy.

Catecholamine- induced cardiac hypertrophy:

When noradrenaline was intravenously infused in rats for 3 days, acute remodeling occurred in that the left ventricle developed hypertrophy. LVW/BW ratio was elevated by about 40% (Zierhut and Zimmer, 1989). The mRNA of colligin also called HSP47, which is a chaperone for collagen type I and collagen type III was increased after 12 hours and further increased progressively (Hosokawa et al., 1998).

Transgenic rats

The most commonly used species for transgenic experiments is mice but their small size limits their usefulness in cardiovascular research as few techniques are available for functional studies. Since the RAS is the key element in controlling the cardiovascular system, the murine Ren-2 gene was chosen to generate transgenic rats (Lee et al., 1996). Male rats have a sustained Ang II-dependent increase in blood pressure with low circulating renin levels thus providing convincing evidence for the physiological significance of tissue RAS. At 12–14 weeks, male transgenic rats develop concentric cardiac hypertrophy (Bohm et al., 1994). Transgenic rats expressing the human angiotensinogen gene have been used to test the functional importance of the local human RAS (Muller et al., 1995).

The use of appropriate *in vivo* models of hypertension and cardiac hypertrophy can help in the understanding of the cause and progression of the disease state as well as the response to potential therapeutic interventions.

ii) Ex vivo models of hypertension and cardiac hypertrophy:

Ex vivo models can be used to study molecular, biochemical, physiological, pharmacological and morphological characteristics associated with hypertension and hypertrophy (Sutherland and Hearse, 2000).

Cell dispersion from adult hearts and the culture of neonatal or adult cardiac fibroblasts or neonatal cardiomyocytes have replaced some animal studies since these allow studies of a single cell type in the absence of homeostatic mechanisms over a

wide range of experimental conditions. However, while these studies have allowed characterization of cellular properties, they cannot reproduce the working heart muscle.

The expression of proteins (Eble et al., 1998), regulation of their expression (Adderley and Fitzgerald, 1999) and the effect of drugs on gene expression (Wagner et al., 1998) have been studied in the neonatal rat cardiomyocyte model. An advantage of neonatal cardiomyocytes is the undemanding and easy procedure for their isolation in contrast to adult cardiomyocytes, which are very sensitive to the concentration of Ca^{2+} in the medium (calcium paradox) (Ray et al.,2000) during the whole isolation procedure. Cultured neonatal rat heart cells have been used extensively for the studies on cardiac hypertrophy and signal transduction (Simpson et al., 1982).

In vitro heart preparations, such as atrial preparations, isolated hearts, working hearts and papillary muscle preparations are also commonly used for experimental studies. The classic Langendorff preparation, ie. retrograde perfusion of the coronary vasculature using a balanced buffered solution saturated with 95/5% mixture of O_2 and CO_2 with certain modifications has been used by many investigators (Okamatsu and Lefer, 1983). The advantages of the Langendorff isolated heart preparation are that the heart is perfused through its own coronary distribution, and the heart rate, temperature and pressure can be adjusted and controlled. The method is particularly valuable for studies of myocardial metabolism since it is very easy to measure total coronary flow and oxygen consumption. Modifications of this preparation include the

use of a support animal in which case the isolated heart is blood perfused and the support animal acts as an oxygenator and CO_2 extractor, it also acts as a pH buffer and supplies all metabolic requirements as well as providing liver and kidneys for metabolic waste disposal (Serur et al., 1976). The "working heart preparation" has been used by many investigators to study the effects of changes in afterload on the efficiency of the heart as a pump. Both isolated heart preparations are extremely valuable for assessing the direct cardiovascular effects of various therapeutic agents in terms of contractile function, electrical activity or metabolic function (Hearse and Sutherland, 2000).

Spontaneously beating mouse hearts have been maintained in organ culture media, which have proven very useful for biochemical studies (Ingwall et al., 1980).

Basic electrical properties and electrophysiological mechanisms of cardiac tissue are commonly researched applying preparations of papillary muscle. Advantages of these preparations are the simplicity to satisfy their metabolic demands and the geometrical elementariness in comparison to whole heart preparations (Sachse et al., 2005). Papillary muscle contains endothelial cells and fibroblasts in addition to cardiomyocytes and is therefore representative of mechanisms operating in the whole heart. Antihypertensives can have an effect on the mechanical function of the heart. Papillary muscles are usually used to assess the inotropic response to various antihypertensive drugs.

Blood pressure is by and large determined by systemic vascular resistance. Hence the ability of a drug to reduce blood pressure could be mainly due to effects on the primary determinants of the arteriolar tone (adrenergic innervations or smooth muscle

tone) and /or on the renal function. (Karaki et al., 1997; Langer et al., 1980). There are models that provide a tool for assaying the efficacy of hypotensive agents (Bolton TB, 1979). Aortic rings prepared from thoracic aortae of adult rats have been utilized to ascertain relaxation responses to various drugs. Contractile response is ordinarily induced either, by α-adrenergic agonists or by high K^+ concentrations, both of which promote activation of calcium channels and consequent contraction (Bolton 1979; Karaki et al., 1997). In addition to the assessment of the capacity to induce vasorelaxation, isolated aortae can also be used to delineate the mechanism of action of antihypertensives. However, diuretics that reduce blood pressure by excretion of sodium and water can be assessed only in *in vivo models.* (Freis, 1983).

The introduction could have been more elaborate and more specific. It could have been cut by 35 to 40%. (62 pages)

CHAPTER 3

METHODOLOGY

DESIGN OF THE STUDY

The first step in the design of a study is the identification of an appropriate experimental model. Spontaneously hypertensive rats are a useful model for examining the cardiovascular response to the Ayurvedic antihypertensive drug because they provide a reliable model of a naturally developing pressure overload akin to essential hypertension.

Delineating the mechanism of action of the drug and the cellular response independent of the systemic changes is better understood in *ex-vivo* experimental systems. The use of *ex vivo* systems also helps to curtail the use of experimental animals.

In this study, both *in vivo* and *ex vivo* experimental models have been employed to examine the efficacy of the Ayurvedic drug in reduction of blood pressure, prevention of cardiac hypertrophy and to study the mechanism of action in vasorelaxation.

In vivo experiments carried out in SHR were:

1. Determination of antihypertensive potential of the drug
2. Evaluation of the efficacy of the drug in prevention of cardiac remodeling ie. hypertrophy, fibrosis, electrical remodeling and functional alterations.
3. Identification of the signaling pathways in cardiac hypertrophy modulated by the drug
4. Evaluation of the efficacy of the drug in amelioration of vascular remodeling
5. Screening for drug toxicity

Normotensive Wistar rats were used to evaluate the neurodepressive action of the drug.

Ex vivo experiments were performed in tissues and cells isolated from normotensive rats. Cultures of cardiac myocytes and fibroblasts were prepared from newborn rat hearts. Aortic rings and ventricular papillary muscles were isolated from adult rats.

Ex vivo experiments:

1. Mechanism of vasorelaxation by the drug was delineated using aortic rings
2. Effect of the drug on myocardial mechanics was evaluated in ventricular papillary muscles
3. Efficacy of the drug in prevention of cardiomyocyte hypertrophy and fibroblast proliferation was determined in cultured cardiac cells.
4. Antioxidant capacity of the drug was assessed in cultured cardiac cells, papillary muscle and human red blood cells

The Investigations conform to the guidelines laid down by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA); and the Institutional Animal Ethics Committee approved the study.

MATERIALS

Ayurvedic antihypertensive drug (Cardoguard):

The drug, *Cardoguard* was prepared at *Nagarjuna Herbal Concentrates Ltd.* It is a combination of six medicinal plants. The plants were identified, collected at a specific season of the year, dried, powdered separately, weighed and mixed homogenously and made into capsules. The capsule has the following composition:

Rauwolfia serpentina	(Sarpagandha/Indian Snakeroot)	(120mg)
Terminalia arjuna	(Arjuna /Myrobalan)	(40mg)
Boerhavia diffusa	(Punarnava/Spreading Hogweed)	(40mg)
Terminalia chebula	(Hareethaki/Chebulic myrobalan)	(40mg)
Terminalia belerica	(Vibheetaki /Beleric myrobalan)	(40mg)
Emblica officinalis	(Amlaki/Indian gooseberry)	(40mg)

Fine chemicals:

Phenylephrine, Acetylcholine, L-NAME, Indomethacin, Glibenclamide, Cyclosporin A, Bisindolylmaleimide, PD98059, Verapamil, Hypoxanthine, Xanthine oxidase, 5, 5'-dithiobis (2-nitrobenzoic acid), Medium-M199, RPMI-1640, Bovine serum albumin (Fraction V, fatty acid free), Collagenase Type I, Trypsin, Fetal bovine serum, Deoxyribonuclease, immunohistochemistry kits for Desmin and Vimentin, Amphotericin B, Angiotensin II, Isoproterenol, Reserpine, 2′-7′- dihydrodichlorofluorescin diacetate, Direct Red 80, Ketamine, Xylazine and

MATERIALS

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Monoclonal Anti-Goat and Anti-Rabbit, peroxidase conjugated secondary antibodies were purchased from Sigma, USA. Penicillin (Alembic, India), Gentamycin (Ranbaxy, India), Heparin (Troikaa, India), Fluorescent Monoamine Oxidase Detection Assay Kit (Cell Technology, USA), Primary antibodies to p-ERK, p-JNK, p-p38, PP2B-B1(calcineurin), dystrophin and p-PKC ε (Santa Cruz Biotechnology).

Routine chemicals:

Sodium chloride, Potassium chloride, Sodium bicarbonate, Disodium hydrogen phosphate, Sodium dihydrogen phosphate, Magnesium sulphate, Magnesium chloride hexahydrate, Hepes, Glucose, Calcium chloride, Phenol red, Potassium dihydrogen phosphate, Hematoxylin, Eosin, TCA(trichloroacetic acid), Ether, Ethanol, Methanol, Xylene, Formalin, Chloroform.

Routine chemicals were purchased from Sigma (USA), Sisco Research Laboratories, Nice chemicals and Merck (India).

Cell culture ware:

35mm culture dishes and cell culture flasks ($25cm^2$) were purchased from Nunc, USA and Falcon, USA respectively. Cell culture filter ware was from Milipore, USA.

Composition of buffers and solutions:

Hank's Balanced Salt Solution with Calcium and Magnesium (pH 7.4):

NaCl 137mM, KCl 5.4mM, KH_2PO_4 0.44 mM, $NaHCO_3$ 4.17mM, Na_2HPO_4 0.63 mM, Glucose 5.55 mM, $MgCl_2.6H_2O$ 0.49 mM, $MgSO_4$ 0.83 mM, $CaCl_2$ 1mM, Phenol red 10 mg/L

Hank's Balanced Salt Solution without Calcium and Magnesium (pH 7.4):

NaCl 137mM, KCl 5.4mM, KH_2PO_4 0.44 mM, $NaHCO_3$ 4.17mM, Na_2HPO_4 0.63 mM, Glucose 5.55 mM, Phenol red 10 mg/L

Buffer for differential trypsinization of cardiomyocytes (pH 7.4):

NaCl 117mM, KCl 5.36mM, $MgSO_4$ 0.83 mM, Glucose 5.55 mM, KH_2PO_4 0.44 mM, Na_2HPO_4 0.34 mM, Hepes 20mM.

Phosphate buffered saline (pH 7.4):

NaCl 137mM, KCl 2.7mM, Na_2HPO_4 10.14mM, KH_2PO_4 1.76 mM.

Krebs Ringer Hansleit buffer (pH 7.4):

NaCl 133mM, KCl 3.6mM, KH_2PO_4 1.1mM, $CaCl_2$ 1.8mM, $MgCl_2$ 1.2mM, glucose 16mM, Hepes 3mM

10% buffered neutral formalin (pH 7.4)

Distilled water-900ml

NaH_2PO_4 (anhydrous) - 3.5g

Na_2HPO_4 (anhydrous) - 6.5g

Formalin- 100ml

Elastin Van Gieson's (EVG) stain:

Ingredients:

Crystal violet- 2.5g

Basic fuchsin-2.5g

Dextrin- 1g

Resorcinol- 10g

Preparation of Van Gieson's counter stain:

Saturated aqueous solution of Picric acid (9 parts)

1% Acid fuchsin (1 part)

Instruments used:

Laminar Flow chamber (CLAS, India), CO_2 water-jacketed incubator (Sanyo, USA), High speed refrigerated centrifuge (Hitachi, Japan), Eppendorf centrifuge 5415 R, Incubators (Beston India; Kemi, India), Weighing balance (Sartorius, USA and Ohaus), Homogenizer (IKA, Labortechnik, Germany), Deepfreezer -20°C (Vestfrost), Deepfreezer -80°C (Sanyo), Steam distillation unit (Beston), Microwave oven (IFB) Water bath (LKB, Sweden), Ice machine (Hoshizaki, Japan), pH meter(Labindia),

Phase contrast microscope with camera (Nikon, Japan), Phase contrast microscope (Olympus KX4, Philippines) with camera (Evolution LC, model no:PL-A662, Media Cybernetics), Low speed magnetic stirrer (Remi, India), Hot air oven (Tempo, India), EASY pure UV/UF compact reagent grade water system (Barnstead, USA), Eye-piece micrometer (Labomed, India) UV- visible Spectrophotometer (Shimadzu), Spectroflurometer (Molecular Devices, USA), Stimulator (Biodevices, India), Platinum electrode (fabricated), Thermostat (Power lab, AD instruments), Bridge Amp (Power lab, AD instruments), Organ Bath (Letica, Spain), Micropositioners (Letica, Spain), Force displacement transducers (AD Instruments, Australia), Noninvasive Blood Pressure Monitoring System for small animals (NIBP200A) (Biopac Systems, USA), EBI100C and ECG100 to obtain cardiac output and ECG data respectively (Biopac Systems), BIOPAC Data Acquisition Unit (MP35/MP30), Noninvasive Cardiac Output Sensor (SS31L) and BIOPAC Electrode Leads (SS2L).

Software used:

Chart5 for recording physiological data (AD Instruments, Australia)

Image-Pro Plus 5.1 for image analysis (Media Cybernetics, USA)

BSL PRO for blood pressure, stroke volume, heart rate and ECG (Biopac Systems, USA)

EXPERIMENTAL METHODS

1. In vivo experiments:

1.1. Assessment of antihypertensive potential of the drug:

Spontaneously hypertensive rats (SHR) were used as the *in vivo* experimental model to determine the efficacy of the preparation in the reduction of blood pressure.

The SHR develop hypertension at 6-12 weeks of age (Adams etal., 1989; McGuire and Tweitmeyer, 1985), hypertrophy at 6-9 months (Shorofsky et al., 1999) and cardiac failure at 18-24 months (Bing et al., 1995). They serve as a counterpart for clinical hypertension along with the complications of hypertension comparable to that seen in man.

The animals were purchased from *Animal Resource Centre, Perth, Australia.* They were housed in an isolated experimental room in the Division of Laboratory Animal Science of the Institute.

Treatment was initiated at 2 months of age. The drug was orally administered daily (by mixing with the routine feed) over a period of 10 months (long term) with the dosage based on body surface area (BSA).Animals were examined at 6 months of age (mid-age) and also at 12 months (adult). The body weight varies according to the size of the individual, whereas the fundamental physiological processes of an organism are constant in terms of body surface area. The ratio of body surface area of man to rat is approximately 70:1 (Freireich et al., 1966). The therapeutic level of the drug was therefore calculated to be 5mg/animal/day.

The blood pressures of the untreated and treated rats at 6 months and 12 months of

age were determined using Noninvasive Blood Pressure Monitoring System for Small Animals (Fig.3).. The measurements were taken in conscious rats after restraining them.The blood pressure of 2-month old SHR was not taken, mainly due to the marked lability in blood pressure that precedes the established phase of hypertension in SHR (Okamoto, 1969). Moreover, it is difficult to measure blood pressure in very young conscious rats.

The tail cuff was placed proximally on the tail to occlude the blood flow. Upon deflation of the cuff, blood pressure was determined which coincided with the restoration of caudal artery pulse. This was achieved by using a piezo electric pulse transducer placed distal to the cuff, which was coupled to the Data Acquisition System. For accurate noninvasive blood pressure measurement, the tail of the animal was pre-warmed to 32°C. At least four determinations were made in every session of blood pressure measurements and the average was obtained. The systolic blood pressure value coincided with the point of appearance of first pulse when the cuff is deflated and the point of the first maximum peak corresponds to diastolic blood pressure value.

In treated animals, the blood pressure measurements in each session were taken 2 hours after drug administration.

In order to examine the dose - dependent effect on blood pressure,12-month old SHR were treated with double the concentration of the therapeutic dose and the blood pressure was recorded following short term treatment (2 weeks).

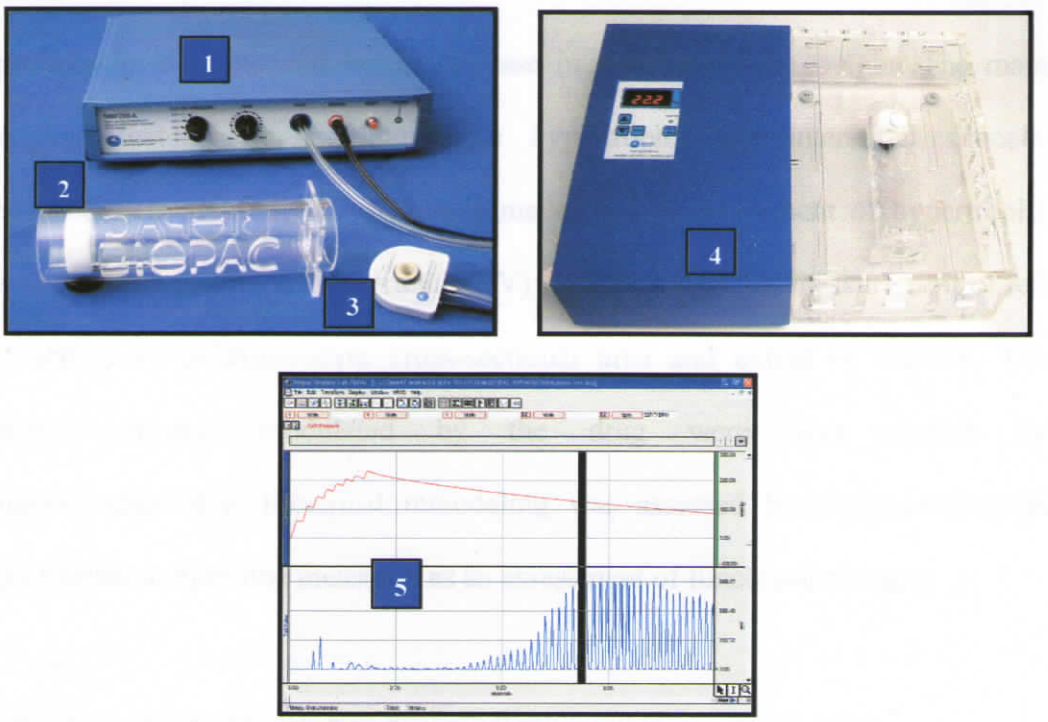


Fig.3 Set *up for blood pressure measurement*

1 NIBP200A Front Panel
2 Restrainer
3 Piezo electric pulse transducer (distal to cuff)
4 Animal heating chamber
5. A typical recording using BSL PRO software for NIBP.

1.2. Evaluation of the efficacy of the drug in prevention of left ventricular remodeling:

Both morphological and functional features of left ventricular remodeling were evaluated.

Hypertrophy is characterized by an increase in left ventricular weight. The main histological features are cardiomyocyte hypertrophy and interstitial fibrosis. Assessment of hypertrophy in SHR was made by i) measurement of hypertrophy index ii) measurement of left ventricular(LV) wall thickness, iii) measurement of left ventricular (LV) cardiomyocyte cross-sectional area and extent of fibrosis. The signaling pathways modulated by the drug were also studied by immunohistochemistry. Electrical remodeling was assessed by electrocardiogram (ECG). Cardiac output was measured as an assessment of functional change.

a. Assessment of hypertrophy by morphological and histological changes

Hypertrophy was assessed by measurement of hypertrophy index and LV wall thickness in both 6-month old and 12-month old rats, whereas histologic myocyte size and fibrosis were assessed in the 6-month old rats.

Measurement of hypertrophy index: The rats were ether anaesthetized for excision of the hearts. The atria were removed and the ventricular weights obtained. Though hypertrophy is mainly of the left ventricle, to avoid the possible experimental variation arising on separating the left and right ventricles, the hypertrophy index was

determined as the ratio of total ventricular weight (VW) to body weight (BW) [VW (mg) /BW (g)]. The hearts were then fixed in 10% formalin for histological studies.

Measurement of LV wall thickness: For measurement of LV wall thicknesses, the ventricles which were fixed in 10% buffered formalin for 1 week were processed and paraffin embedded sections of 5-6μm thickness prepared. They were stained with hematoxylin and eosin (H&E) and measurements taken by means of eye piece micrometer.

Measurement of cardiomyocyte cross-sectional area: This forms an assessment of cardiomyocyte size. The cross sectional areas were measured after immunohistochemical staining of the paraffin embedded ventricular sections (5-6μm thickness) with antibody to dystrophin. Mid ventricular cross sections from heart equator were taken. Cross sectional area of 50 cross–sectioned cardiac muscle fibers were measured in at least 5 images obtained from each ventricle using digital camera attached to the microscope.

Dystrophin is a rod-shaped cytoplasmic protein that forms the vital part of a protein complex that connects the cytoskeleton of the muscle fiber to the surrounding extracellular matrix through the cell membrane. For immunohistochemical staining, the sections were dewaxed in xylene, rehydrated in descending grade series of alcohol, treated with 3% hydrogen peroxide for blocking endogenous peroxidase, target antigen retrieved by immersing the slides in 0.01M freshly prepared sodium

citrate buffer (pH-6.0) followed by heating for 5 minutes in microwave oven at power 5, blocked unspecific binding with 3% BSA and incubated with primary antibody overnight at 1:50 dilution at 4°C. After washing off unbound primary with phosphate buffered saline (PBS), incubation with horse-radish peroxidase (HRP) conjugated secondary antibody (dilution-1:400) was carried out. After washing off unbound secondary antibody, the sections were treated with the substrate, diaminobenzidine with 3% hydrogen peroxide. Dystrophin provided a good outline of the cardiomyocytes that enabled measurements to be carried out using image analysis software *(Image-Pro Plus 5.1).*

Measurement of fibrotic changes in left ventricular wall: Fibrosis is characterized by excessive deposition of interstitial collagen. Extent of fibrosis was determined by morphometric measurement following Sirius red staining of interstitial collagen in the sections of left ventricular wall.

Paraffin embedded ventricular sections (5-6μm thickness) were deparaffinized in xylene and incubated at room temperature for 2 hours in the dark under continuous, mild agitation with a saturated solution of picric acid in distilled water containing 0.1% fast green FCF which stained noncollagenous proteins and 0.1% Sirius red which stained collagen. *Image-Pro Plus 5.1* was used to analyze the sections and fibrosis was quantified from 5 different fields (x 4 objective) of vision randomly chosen. Fibrosis was expressed as percentage area stained red (collagen) in a particular microscopic field, without any gap areas. The perivascular regions were excluded from the measurements.

Identification of signaling pathways modified by the drug: The major pathways associated with cardiac hypertrophy in response to pathophysiological stress are MAPK, calcineurin and PKC. Paraffin embedded sections (5-6μm thickness) of the ventricular tissues of 6-month old SHR were immunostained with antibodies to the different signaling proteins using standard protocol. The sections were counterstained with hematoxylin. All sections used for quantitation were fixed, processed, sectioned and immunolabelled at the same time and under the same conditions to limit variability. The proteins analysed were, *phospho-ERK, phospho-p38 and phospho-JNK* representing the three major subfamilies of the MAPK pathway, *calcineurin* and *phospho-PKC ε.* Studies on genetically manipulated mice suggest a role for PKC ε isoform in cardiac hypertrophy and in heart failure. Hence the specific expression pattern of p PKC ε isoform was tested. Immunostaining was visualized using diaminobenzidine. Staining intensity was quantified as chromogen gray levels using *Imag –Pro Plus 5.1.* For quantitation 5-6 fields (x 20 objective) per ventricular section were randomly selected. The gray scale intensities in the ventricular sections of treated SHR were expressed as percentage of the mean value of untreated control (SHR).Negative controls were included by omitting the primary antibodies.

b. Assessment of electrical remodeling

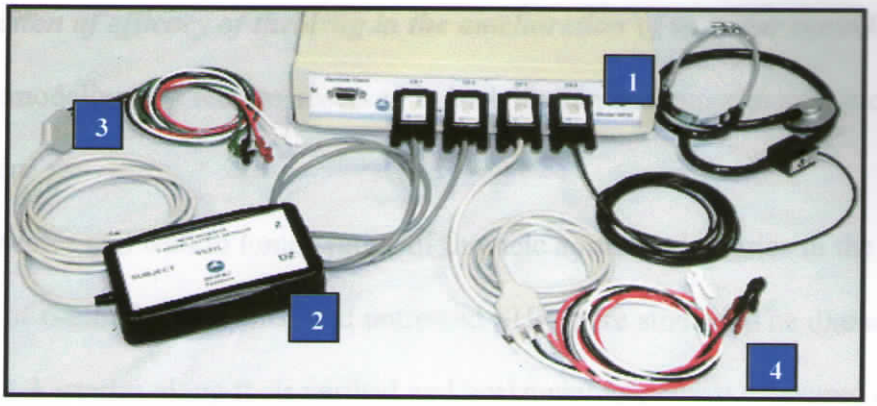
Electrical remodeling was assessed by electrocardiographic changes in 6-month old and 12-month old SHR.

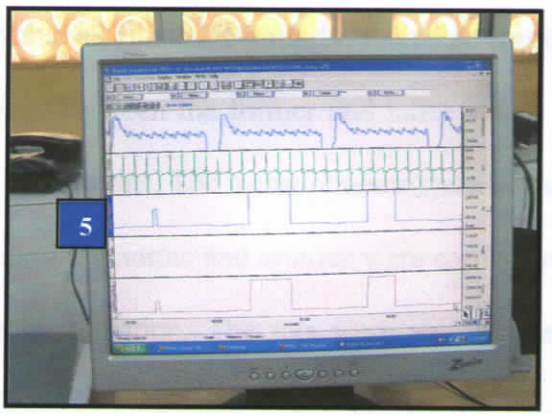
Electrocardiogram (ECG): ECG data were obtained non-invasively in rats under ketamine (30mg/kg) and xylazine (5mg/kg) anaesthesia administered intraperitoneally. The equipment for measurement of ECG includes *Biopac Student Lab data acquisition system*, SS2L Electrode Lead Set and button electrodes. A 3 lead ECG was recorded using electrodes connected from the ECG amplifier module to the left arm, right arm and sternum (Fig.4).

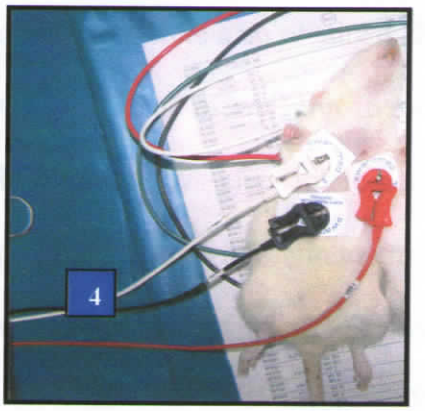
c. Determination of cardiac output

Cardiac output was determined as an index of the functional status. It was assessed in 6-month old and 12-month old SHR. Stroke volume, together with heart rate, determines cardiac output, which is the blood volume that flows out from the ventricle towards the vascular bed in a given period.

Stroke volume and heart rate were determined, non-invasively, by employing electrical bio-impedance measurement technique. The process of determining impedance involves placing electrodes on the thorax and neck and then applying high- frequency, low- amplitude alternating current through the electrodes (Fig.4). The change in the voltage between the sensing and delivering electrodes is expected to be directly proportional to changes in measured impedance of the tissue volume. The measurements were taken after anaesthetizing the rats using ketamine (30mg/kg) and xylazine (5mg/kg) intraperitoneal injections.







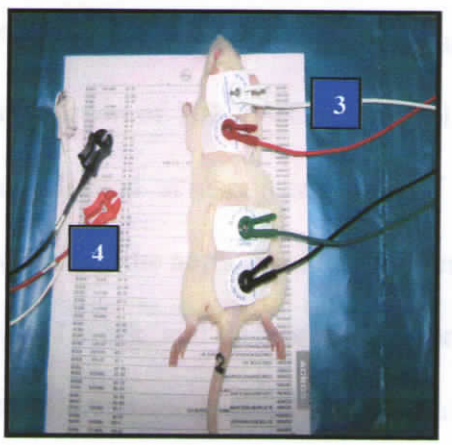


Fig.4 *Set up for ECG and cardiac output measurement*

1. BIOPAC Acquisition Unit (MP35/MP30)
2. BIOPAC Noninvasive Cardiac Output Sensor (SS31L)
3. SS31L leads for cardiac output
4. SS2L leads for ECG
5. ECG and cardiac output using BSL PRO software

1.3. Evaluation of efficacy of the drug in the amelioration of vascular remodeling:

Vascular remodeling is a feature observed in both human and experimental models of hypertension.

Wall thicknesses and wall to lumen ratios of thoracic aortae and arteries in the stroma of kidneys of 6-month old treated and untreated SHR were studied. The diameters of the aortae and arteries along their vertical and horizontal axes were measured and the mean value calculated. Similarly, the mean lumen diameters were also obtained. The difference between the former and latter values gave the value of wall thickness of aortae and arteries. The measurements were taken by means of eye-piece micrometer. Fixation of the aortae and arteries were carried out by perfusion of formalin to ensure maximum dilatation. The rats were anaesthetized with diethyl ether and perfused through the left ventricle with a 0.9% NaCl solution containing 20 IU heparin. The solution was maintained at 37°C and perfusion lasted for 5 minutes .The first solution was then replaced by a second solution of 10% buffered neutral formalin (pH 7.4) at 25°C. Perfusion pressure was adjusted at a constant rate of 1ml/min/100g body weight. After 30 minutes of perfusion, the aorta and kidneys were removed and fixed in the same perfusion fixative for 1 week, and then processed for paraffin embedding. 5-6 μm thick sections of aortae were stained with H&E for morphometric analysis. For arterial morphometry in kidney stroma, the sections of kidneys were stained with Elastin Van Gieson's stain. For this, the sections were deparaffinized, washed in absolute alcohol and kept in Elastin Van Gieson's solution overnight. The sections were differentiated with 95% alcohol till the elastic fibers attained a blue-black color.

This was then counter stained with Van Gieson's stain for 1-3 minutes, dehydrated quickly in absolute alcohol, cleared and mounted. The elastic fibers appear blue black, muscle fibers yellow and collagen red.

1.4. Screening for drug toxicity:

Drug toxicity was assessed in SHR following 10 months of treatment. The presence of drug toxicity was evaluated by histopathological analysis of the kidney and liver of treated SHR. This is because adverse drug toxicity effects can transiently disrupt the functions of these organs. Six animals each from treated and untreated group were used for the study. The kidney and liver were fixed in 10% buffered formalin and sections 5-6 μm thick were taken. They were stained with H&E and analyzed under the microscope for toxicity changes. Toxicity in kidneys was assessed by examining the presence of histological alterations like tubular degeneration, interstitial inflammation, interstitial oedema, glomerular fibrosis and vascular damage. Liver toxicity was assessed by examining the presence of lobular necrosis, vacuolation of hepatocytes, Kupffer cell hyperplasia, enlarged sinusoids and portal tracts, mononuclear cell infiltration in the parenchymal tissue and microvesicular steatosis.

1.5. Evaluation of neurodepressive action of the drug:

The Ayurvedic antihypertensive drug has *Rauwolfia serpentina* as the major constituent. Reserpine is the chief alkaloid principle of *Rauwolfia, with* antihypertensive potential. Doctors began using reserpine-based antihypertensives in

the 1950's, but the drug went out of favor because of its unfavorable side effect. Reserpine was found to be depressogenic in patients, when used for the management of hypertension for prolonged period (Frize, 1954). Reserpine inhibits the vesicular monamine transporter pump, normally responsible for the sequestration of biogenic amines such as *norepinephrine, dopamine and serotonin* into the storage vesicles located in the presynaptic neuron. Administering reserpine causes the amines to remain exposed within the cell and break down by monoamine oxidase (MAO).

According to Ayurveda, the whole plant *(Rauwolfia)* reduces the risk of side effects that occur with the use of the isolated actve principle (reserpine). Hence, the activity of brain mitochondrial monoamine oxidase, a marker for depression was assayed in Wistar rats treated with *Cardoguard* and compared with the values in rats treated with reserpine.

Male Wistar rats were divided into three groups comprising six animals each. One group served as the control. The second and third groups were treated respectively with reserpine (10μg/day/animal) and *Cardoguard* (5mg/day/animal) for 28 days. Wistar rats were chosen for the experiment because this short-term study does not involve the assessment of cardiovascular parameters. The mode of administration was oral gavage using gavage needles. The control rats were given distilled water. The rats were sacrificed for detection of brain MAO activity. MAO is localized in the outer mitochondrial membrane. The brains were quickly removed and placed on ice in 0.32M sucrose solution. They were homogenized in 10 volumes of 0.32 M sucrose solution (previously adjusted to pH 7.4 with 0.5 M $NaHCO_3$). The homogenates were

centrifuged at 600 g for 10 min at 2^0C and the supernatant retained and the residue discarded. The supernatant was centrifuged again at 14,000 g for 15 min at 2^0C, and the residue retained (crude mitochondrial pellet) and the supernatant discarded .The crude mitochondrial pellets from brain tissue were then resuspended in 500 µL of 0.32 M sucrose and stored at -80°C until use.

MAO activity was detected using Fluorescent Monoamine Oxidase Detection Assay Kit. Prior to the MAO assay, homogenates were thawed and diluted 1:20 using reaction buffer. The detection kit utilized a non - fluorescent substrate, 10-acetyl-3, 7-dihydroxyphenoxazine (ADHP) to detect H_2O_2 released from the conversion of a substrate to its aldehyde via MAO-A/B. The released H_2O_2 oxidized ADHP in a 1:1 stoichiometry to produce a fluorescent product resorufin. This oxidation was catalyzed by peroxidase. The excitation and emission wavelengths were 530-570 nm and 590-600nm respectively. The assay was carried out in black 96 well plates and fluorescence measured using fluorescence plate reader. The fluorescence intensities of the brain samples of the different groups were compared.

2. Ex vivo experiments:

Ex vivo experiments include assessment of vasorelaxant potential of the drug and delineation of mechanism of action ; examining the effect of the drug on myocardial mechanics; examining the effect of the drug in prevention of cardiomyocyte hypertrophy and fibroblast proliferation and assessment of antioxidant potential.

The *ex vivo* experiments were carried out in tissues and cells isolated from rats supplied from the Division of Laboratory Animal Science of the Institute.

Since the drug has its constituents in the crude form and has not been extracted with agents such as petroleum ether, ethanol etc., the water-soluble fraction was preferred for all the *ex vivo* experiments.

Therapeutic level of water-soluble fraction of the drug used for all *ex vivo* experiments (64 mg/L) was calculated based on average blood volume of adult.

The three *ex vivo* experimental models used are as follows:

2.1. Aortic ring model:

Rat aortic rings were used to examine the vasorelaxant capacity of the drug and also to delineate the mechanism of action in vasorelaxation.

Aortic rings were prepared from thoracic aortae of adult Sprague Dawley rats weighing 150-200g. The thoracic aortae were cleared of fat and connective tissue and ring preparations of 3-4mm length were made. The rings were suspended in *Krebs Ringer Hansleit buffer* with pH 7.4 in 25ml vessels of a thermostated organ bath. They were suspended between a stationary clip and a force-displacement isometric transducer by means of two stainless steel wires inserted into the vascular lumen (Fig.5). The vessels were continuously oxygenated and temperature of the bath was maintained at 37^{0}C. The rings were incubated to equilibration at a resting tension set at 2g (20mN) for 60 minutes. Changes in isometric forces were recorded using *Chart5* software. The presence of functional endothelium was assessed in the preparations by determining the ability of acetylcholine (1μM) to induce more than

50% relaxation of rings precontracted with phenylephrine (PE) (3μM). The concentration of PE was adjusted to generate a force of 7mN. After confirmation of the presence of endothelium, the rings were washed and returned to base line tension for the experiments to be carried out.

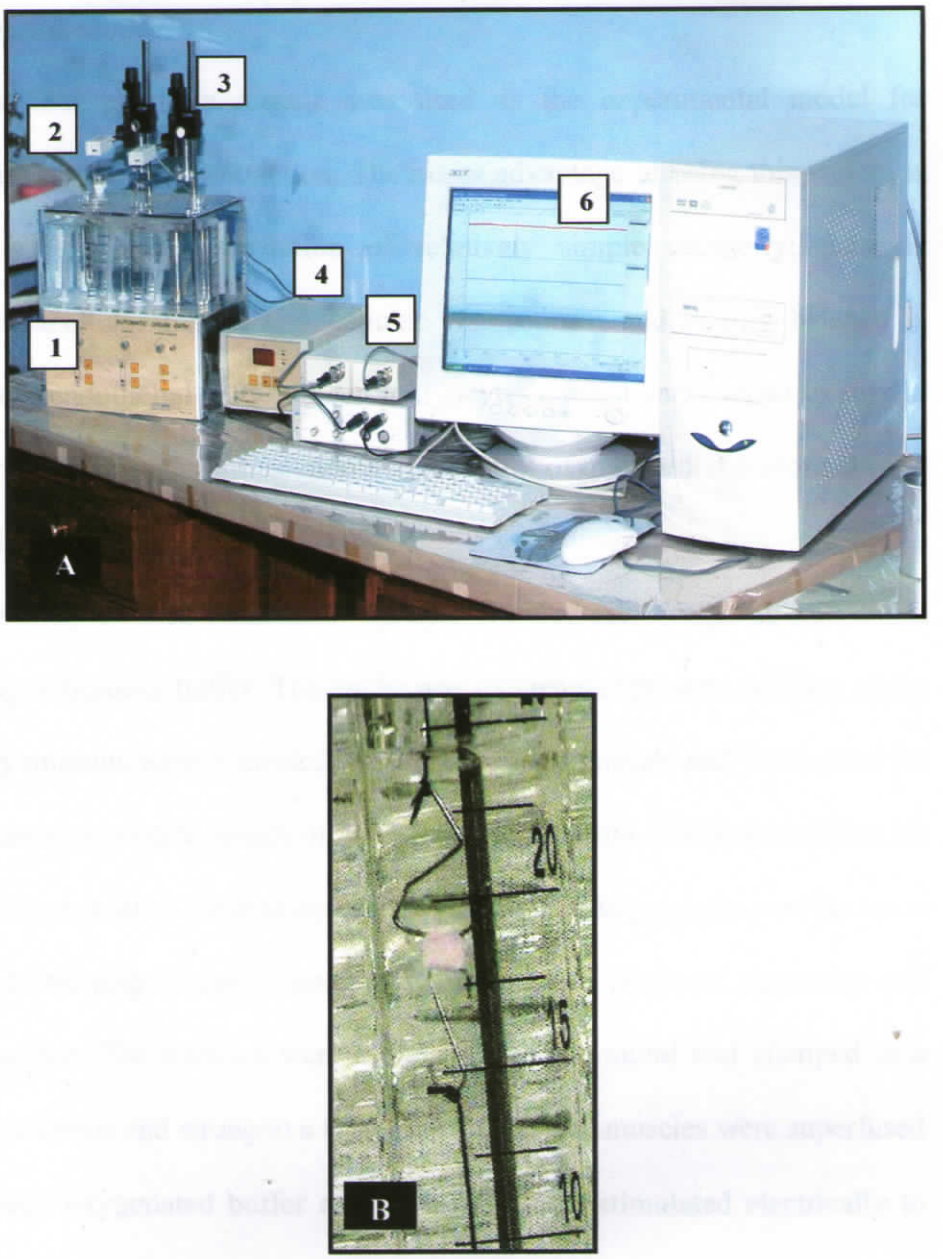


Fig .5 .Set up used for isometric force measurements

A. *Electrophysiology recording system* 1.Organ bath 2. Force transducer

3. Micropositioner 4.Thermostat 5.Bioamplifer 6.Chart software

B. Aortic ring suspended between 2 hooks in the organ bath chamber

2.2 Papillary muscle model:

Rat left ventricular papillary muscle was used as the experimental model for measurement of myocardial contraction. The major advantage of using this system is that, in this multicellular preparation of relatively simple geometry, there is undisturbed extracellular matrix and cellular connections; and energy balance is maintained. Here endothelial cells and cardiomyocytes coexist and interact to sustain optimal performance. Hence cardiac endothelial control of myocardial performance is well preserved.

Hearts were quickly excised from adult Sprague Dawley rats (weighing 150-200g) into *Krebs Ringer Hansleit* buffer. The buffer was oxygenated prior to excision of the heart. Papillary muscles were dissected out from the left ventricle and those used for the study had an approximate length of 4mm and width <1mm. The experimental set up used for measurement of force of contraction of the papillary muscle was the same as that of the aortic ring model except for the presence of platinum electrodes and electrical stimulator. The muscles were mounted with the mural end clamped to a hook and the tendinous end strung to a force transducer. The muscles were superfused with continuously oxygenated buffer at 32°C. They were stimulated electrically to contract isometrically at 0.5 hertz at a voltage 10% above the threshold by pulses of 5ms duration delivered through 2 platinum electrodes. The muscles were gradually stretched in a stepwise manner to give 95% of the maximum contraction after an initial equilibrium period of 1 hour in the buffer. The muscles were allowed to stabilize and force of contraction recorded using *Chart5* software. This was taken as

the baseline contraction. Steady state contraction in response to the treatments was recorded and calculated as percentage variation in developed force. This procedure allows each muscle preparation to serve as its own control by eliminating differences in initial contractility. Muscle preparations remained viable for more than 6h.

2.3. Cell culture model:

A cell culture model permits the analysis of specific variables directly at the cellular level, bypassing complicated systemic interactions that usually affect the interpretation of *in vivo* experiments.

2.3 a) Isolation and culture of cardiomyocytes:

The hearts were isolated from 1-3 day old newborn Wistar rats after ether anaesthesia. The hearts were collected in Hank's balanced salt solution containing calcium and magnesium with penicillin (150U/ml), gentamycin (50µg/ml) and amphotericin (2.5µg/ml). Atria were removed and ventricles were minced into bits of $1mm^3$ sizes and enzymatically dissociated in medium containing collagenase (0.3mg/ml), trypsin (0.2 mg/ml) and deoxyribonuclease (5.5µg/ml). Products of first digestion were discarded as it contained red blood cells and dead cells. Cells from subsequent digestions were collected. Trypsin present in the cell suspension was inactivated with Medium- M199 supplemented with 20% foetal bovine serum (FBS). Generally 5-6 digestions were carried out. The cell suspensions were centrifuged at 1000rpm for 10minutes and the pelleted cells were plated at a density of $1x10^6$cells/ml in cell culture flasks containing Medium- M199 with 10%FBS. The flasks were incubated at 37°C with 5% CO_2 in air and 99% humidity. Cell suspension thus obtained was a

mixture of myocytes and non-myocytes (Nair and Gupta,1989). Myocytes were separated from non-myocytes by selective adhesion technique. After 90 minutes of plating, majority of fibroblasts adhered to the culture surface and a myocyte rich suspension was obtained. The suspension was centrifuged at 900rpm for 6 minutes and plated in 35mm dishes and incubated at 37°C in a CO_2 incubator. The cell density was adjusted to $2.5x10^5$cells /35mm dish. The experiments were carried out in the primary culture on the fourth day of isolation, after 24 hours of serum-deprivation. Cardiomyocytes were morphologically identified under phase contrast optics and also by immunocytochemistry.

2.3 b) Culture of fibroblasts:

The non-myocytes, mainly the fibroblasts obtained during the preplating procedure for isolation of myocytes were sub cultured twice in a ratio of 1:3 to obtain pure fibroblast cultures. The fibroblasts were enzymatically detached using trypsin-EDTA (0.05% trypsin and 0.02% EDTA) dissolved in PBS and the cells were plated at a concentration of $1x10^5$ cells/ 35mm dish. The experiments were carried out in cells that were serum deprived for 24 hours. Cardiac fibroblasts were morphologically identified under phase contrast optics and also by immunocytochemistry.

2.1. Determination of vasorelaxant potential and delineation of mechanism of action

Vasorelaxatory potential of a drug contributes to its antihypertensive action.

a) Vasorelaxant capacity of the drug:

To determine the vasorelaxant capacity of the drug, the aortic rings with endothelium

were precontracted with PE (3μM). When the contraction induced by PE reached a steady maximal response, the rings were treated with therapeutic levels (64mg/L) of water-soluble fractions of the drug.

Change in isometric force was recorded and the extent of vasrelaxation was expressed as percentage decrease in maximal tension obtained by PE-induced contraction.

b) Vasorelaxation induced by different concentrations of the drug:

Aortic rings precontracted with PE were treated with double and three times the therapeutic dosage (128mg/L and 192mg/L) and aortic relaxation was recorded.

c) Vasorelaxation induced by different components of the drug:

The concentration of the components selected was proportionate to that present in the formulation. Aortic rings were treated with different components of the formulation after precontraction with PE and vasorelaxation determined.

d) Mechanism of vasorelaxation:

Antihypertensives induce reduction of blood pressure by different mechanisms. Hence the possible mechanisms by which the drug can induce vasorelaxation were examined.

d i) Role of endothelium in mediating vasorelaxation by the drug:

Hypertension is associated with endothelial dysfunction (Bouloumie et al., 1997; Miligard and Lind, 1998). To examine whether vasorelaxation by the drug is mediated by endothelium, PE-precontracted endothelium- denuded aortic rings were

treated with the drug. The endothelium of the aorta was removed by gently rubbing the intimal surface with the tip of a small steel probe. The lack of a functional endothelium was confirmed by demonstrating the complete absence of relaxation by acetylcholine (1μM) in PE precontracted aortic rings. After confirmation of the absence of endothelium, the rings were washed and returned to base line tension. The extent of vasorelaxation induced by the drug in endothelium denuded aortae was calculated.

Nitric oxide is the primary endogenous vasodilator. For examining the involvement of endothelial-nitric oxide, rings with functional endothelium were exposed to the nitric oxide synthase inhibitor, L-NAME at a concentration of 100μM, 30 min prior to contraction with PE, and then exposed to the drug.

NO deficiency can potentiate excess production of constricting cyclooxygenases (Bratz and Kanagy, 2004). Constricting cyclo oxygenase products augment vascular reactivity to contribute to the development of hypertension. To examine whether the drug acts through the cyclooxygenase pathway, aortic rings with functional endothelium were incubated with 10μM indomethacin, an inhibitor of prostacyclin (a vasodilatory cyclooxygenase product), 30 min prior to contraction with PE. The response to the drug was recorded in the presence of the prostacyclin inhibitor.

dii) Role of the drug as an ATP-sensitive potassium channel (K_{ATP} channel) *agonist/ activator:*

Tissue factors such as K^+, adenosine, etc are released by the parenchymal cells surrounding the blood vessels and they significantly alter vessel diameter. Any change in the vessel tone can affect both organ blood flow and arterial pressure.

Pharmacologic activation of K_{ATP} channels increases potassium efflux, thereby causing membrane hyperpolarization and relaxation of vascular smooth muscle (Quast et al., 1994). Impaired function of K_{ATP} channels may contribute to impaired vasodilatation in hypertensive rats (Ghosh et al., 2004). K_{ATP} channel openers, exerting a potent vasodilatory action, are useful in the treatment of cardiovascular disorders including hypertension and angina pectoris. Also, endogenous vasodilators, such as nitric oxide and prostacyclin, act at least in part by opening K_{ATP} channels (Murphy and Brayden, 1995). Hyperpolarizing effect of K_{ATP} channel activation also reduces Ca^{2+} influx through voltage-dependent Ca^{2+} channels in vascular smooth muscle (Mironneau and Macrez-Lepretre, 1995).

To examine the ability of the drug to act as a K_{ATP} channel agonist, the aortic rings were incubated with 10μM glibenclamide, 30 min prior to contraction with PE. Glibenclamide is a K_{ATP} channel inhibitor. Percentage relaxation by the drug in the presence and absence of the inhibitor was recorded.

diii) Calcium antagonistic action by the drug:

Extracellular calcium induces vasoconstriction. To examine the role of the drug as a calcium antagonist, the rings were exposed to cumulative increase in extracellular calcium upto 10mM in the presence and absence of the drug. Verapamil, a known L-type calcium channel blocker, was used as positive control. The effect of treatment of aortic rings with verapamil (1μM), prior to addition of calcium was examined to confirm the results.

High K^+ concentration excites voltage-dependent Ca^{2+} channels (Karaki et al., 1997).

Hence aortic relaxation by drug on precontraction with 100mM KCl was determined. The extent of relaxation was expressed as percentage decrease in maximal tension obtained by KCl-induced contraction.

div) Role of the drug as an inhibitor of Ang II :

Renin angiotensin system plays an important role in the pathogenesis of hypertension (Laragh and Lewis, 1992). Hence it was decided to test whether the drug inhibits the action of Ang II.

Steady maximal contraction resulting in a plateau cannot be obtained on addition of 100nM of Ang II to the tissue bath as the increase in tension on addition of Ang II was sustained only for a few seconds (Fig.27). So the potency of relaxation of the drug could not be checked by precontracting the aorta using Ang II. Hence, the drug was added to the bath 30 minutes prior to addition of Ang II and the peak of isometric force was determined.

dv) Role of the drug as a β-adrenoreceptor antagonist:

Electrically driven rat papillary muscles were preferred for this particular experiment as the majority of β-adrenoreceptors are located in the heart rather than in the smooth muscle; and β-adrenoceptor blockers reduce blood pressure by decreasing cardiac output.

Isoproterenol (β-adrenoreceptor agonist, 1μM) treatment caused a positive inotropic response on the papillary muscle (Fig.29). To examine the role of the drug as a β-adrenoreceptor antagonist, the tissue bath was pre incubated with the drug for 30 minutes followed by treatment with isoproterenol. It was tested whether the drug affected the inotropic change induced by isoproterenol.

dvi) Delineation of the signaling pathways involved in vasorelaxation by the drug:

Vascular tone is maintained by a complex interaction among a variety of vasoactive agents that affect vascular smooth muscle cells. These vasoactive agents activate (or deactivate) an elaborate network of signal transduction pathways that modulate blood pressure (Walsh, 1994). Several signaling molecules, including ERK1/2, p38 MAPK, PKC and calcineurin are involved in systemic hemodynamics.

To examine the signaling pathways involved in the mediation of vasorelaxation by the drug, pathway specific inhibitors were added 30 min prior to addition of PE and the response to the drug was recorded. Decrease in relaxation in the presence of an inhibitor suggests the involvement of the pathway. ERK1/2, p38 MAPK, PKC and calcineurin were the pathways studied using the respective inhibitors: PD98059 (10μM), SB202190 (10μM), bisindolylmaleimide (1μM) and cyclosporin A (500ng/ml).

2.2 Determination of the effect of the drug on myocardial contractility

Antihypertensives can affect the mechanical function of the heart. Hence it was necessary to test the effect of the drug on myocardial contractility. The experiment was carried out in electrically stimulated papillary muscle. Contractile variation in response to different concentrations of water-soluble fractions of the antihypertensive drug was recorded. The response to therapeutic levels of the drug (64mg/L) and for increasing concentrations: 2 times, 4 times, 6 times, 8 times and 10 times the therapeutic dose were plotted. Steady state contraction was obtained within 30 minutes.

2.3. Testing the efficacy of the drug in the prevention of hypertrophy of cultured cardiac myocytes and prevention of proliferation of cultured cardiac fibroblasts

Left ventricular hypertrophy is characterized by the increase in cardiomyocyte size and excessive proliferation of cardiac fibroblasts. To examine the response of cardiomyocytes and fibroblasts to the drug independent of the systemic effects, cell cultures were used.

2.3a Testing the efficacy of the drug in the prevention of hypertrophy of cultured cardiac myocytes:

Stimulation of hypertrophy in cardiomyocytes: Cardiomyocyte hypertrophy was stimulated with Ang II (100nM) as well as the β agonist, isoproterenol (1μM). Ang II as well as isoproterenol induced myocyte hypertrophy. The ability of the drug to prevent the hypertrophic response was investigated. Cultured cells were pre treated with therapeutic levels of water-soluble fractions of the drug for 30 minutes, prior to addition of AngII or isoproterenol and the cell response was recorded after 96h of treatment.

Measurement of hypertrophic response: Myocyte hypertrophy was assessed by measuring the volume of differentially trypsinized cells after 96 h of treatment. Dishes with the cells were washed with buffer of the following composition (mM): NaCl, 117; KCl, 5.36; $MgSO_4$, 0.83; glucose, 5.5; KH_2PO_4, 0.44; Na_2HPO_4, 0.34; Hepes, 20. Trypsin (0.1%) prepared in the same buffer was added to the dishes and allowed to stand for 10 min at 37°C with gentle tapping in between. Medium- M199 with 10% FBS was added forcefully to detach the cells. The cell suspension was

centrifuged at 1200rpm for 10 minutes, resuspended in the buffer and viewed under phase contrast microscope for measurement of cell diameter using *Image-Pro Plus 5.1.* The cell volume was calculated as $4/3\ \pi r^3$.

2.3b. Testing the efficacy of the drug in the prevention of proliferation of cultured cardiac fibroblasts:

Fibroblast proliferation was stimulated by 100nM of Ang II in culture and the ability of the drug to prevent fibroblast proliferation was analyzed. The dishes with the cells were pretreated with the drug for 30 minutes prior to addition of Ang II and the cells were counted after 96 h of treatment. The cells were detached using trypsin-EDTA and cell density was determined using Neubauer's counting chamber.

2.4. Testing the antioxidant potential of the drug

Hypertension and hypertrophy are associated with excessive generation of reactive oxygen species (Nakagami et al., 2003; Redon et al., 2003). Hence the effectiveness of the drug as an antioxidant was examined. The antioxidant potential was assessed using three different parameters:

i) *Measurement of reduced glutathione levels (GSH) in human red blood cells (RBCs) exposed to drug and ROS*

ii) *Assessment of inotropic response of rat papillary muscle exposed to drug and oxidative stress*

iii) *Measurement of H_2 DCF-DA incorporation by cultured fibroblasts for assessment of intracellular ROS production*

i) Measurement of reduced glutathione levels (GSH) in human red blood cells:

Reduced glutathione level is used as an indicator of oxidative stress. Only about 1-3% of the total glutathione in the tissue and less than 1% in erythrocytes occurs in the oxidized form (GSSG). Glutathione reductase, a widely distributed enzyme, reduces GSSG to GSH in the presence of NADPH and thus maintains GSH as the predominant form of glutathione in tissues. This reaction provides an excellent *in vivo* reduction system that has been credited with a generalized function involving the maintenance of the sulfhydryl groups of enzymes and other essential thiols in reduced and thereby active states (Hull and Scott, 1976). The effects of ROS are generally believed to be mediated by covalent modification of critical protein sulphydryl residues.

To test the antioxidant effect of the drug on cellular membranes, red blood cells were chosen as an appropriate model. Reduced glutathione levels were determined by spectrophotometry in red blood cells exposed to ROS in the presence and absence of water-soluble fraction of the drug. Freshly prepared hypoxanthine (0.5mM) + xanthine oxidase (0.02U/ml) was used as the generator of ROS.

GSH levels were determined by titration with 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) according to a modified procedure by Beutler (Beutler, 1975).

RBCs were isolated from 25 healthy volunteers. 1% RBC suspension [500μL blood+ 1ml distilled water + 4 ml of 10% trichloroacetic acid (TCA)] was centrifuged (1600g, 10 min, 4°C). TCA was added to precipitate the proteins. The supernatant was separated from protein precipitate after 5 min by centrifugation and subsequently filtered using coarse filter paper. An aliquot of 0.5 ml of supernatant was combined

with 0.5 ml of 300mM Na_2HPO_4. To this, the drug was added and preincubated for 30 min followed by the ROS generator. This formed the first set of samples. A second set was obtained by adding the ROS generator in isolation. A third set of samples was kept as the control. The blank was obtained by combining 0.5 ml of supernatant with 0.5 ml of H_2O. 100 µL DTNB solution (20 mg DTNB in 100ml 1% sodium citrate) was added to the blank and to the samples and absorbance was read against the blank at 412 nm. The values are expressed as percentage of control.

ii)Assessment of inotropic response of papillary muscle exposed to drug and ROS::

Contractile variation on exposure to ROS was first established. To examine the role of the drug as an antioxidant, electrically stimulated papillary muscle was superfused with the therapeutic dose of the preparation for 15 minutes followed by the addition of the free radical generating system, and the contractile change recorded. The superoxide anion generator used for the study was freshly prepared hypoxanthine and xanthine oxidase (HX and XO). HX (0.5mM) was first mixed with the perifusate. Then contractile variation was recorded following addition of XO (0.04U/ml).

iii) Measurement of H_2 DCF-DA incorporation by cultured fibroblasts for assessment of intracellular ROS production

Intracellular oxidant stress was monitored by changes in fluorescence intensity resulting from intracellular probe oxidation (Privratsky, 2003). Cultured cardiac fibroblasts at a concentration of $1x10^5$cells/35mm dish were divided into 2 groups. One group was treated with HX and XO. The second group was treated with the drug

for 30 minutes prior to the addition of HX and XO. The concentrations of HX and XO were 1mM and $1x10^{-6}$U/ml respectively. After 24 hours of treatment, the cells were incubated in the dark with 2′-7′-dihydrodichlorofluorescin diacetate (H_2DCF-DA; 10μM) dissolved in fresh PBS at 37°C for 15 minutes. H_2DCF-DA is resistant to oxidation, but when taken up by cells, is de-acetylated by intracellular esterases to form the more hydrophilic nonfluorescent reduced dye dichlorofluorescin (DCFH), which then is rapidly oxidized to form a two-electron oxidation product, the highly fluorescent DCF in a reaction with the oxidizing species. The cells after incubation with DCFDA were rapidly scanned (to avoid photo-oxidation of the indicator-dye) for the presence of fluorescence under the fluorescent microscope. The cells were then trypsinized, inactivated with phenol-red free RPMI medium containing 10% FBS, pelleted at 1200 rpm for 10 minutes and resuspended in phenol red free-PBS for spectroflurometric analysis at excitation wavelength of 490nm and emission wavelength of 525nm.The whole procedure was carried out in the dark. The mean fluorescence intensities of hypoxanthine-xanthine oxidase and drug pre treated cells were determined.

DATA ANALYSIS

In vivo experiments were carried out in 6-8 animals/group and a minimum of 4 determinations for each *ex vivo* experiment using papillary muscles and aortic rings. Values are presented as mean ± standard deviation. Analysis of variance (ANOVA) was carried out where indicated and the significances of the differences between samples were determined by Student's t-test and probability value, $p \leq 0.05$ was considered significant.

CHAPTER 4

ANALYSIS OF DATA

RESULTS

1. IN VIVO EXPERIMENTS TO STUDY THE CARDIOVASCULAR RESPONSE TO CARDOGUARD

1.1. Assessment of antihypertensive potential of the drug:

The antihypertensive potential of the drug was determined by tail cuff method in male rats. The blood pressure of SHR was compared with that of normotensive Wistar rats. At 6 months of age, the mean systolic and diastolic pressure values of SHR (197mmHg and 169 mmHg) were significantly higher ($p<0.0001$) than that of age-matched Wistar rats (119mmHg and 82 mmHg). Systolic and diastolic blood pressures of SHR treated with *Cardoguard* at the therapeutic dose (5mg/animal/day) from 2months of age were found to be significantly less compared to that of untreated SHR at both 6 and 12 months of age (Table 2), thereby establishing the antihypertensive potential of the drug.

The effect of double the concentration of therapeutic dose (10mg/animal/day) was also examined in 12-month old rats. The systolic and diastolic blood pressures of SHR after 2 weeks of drug administration were 198± 9mm Hg and 163± 14 mm Hg against 216± 16 mmHg and 177± 19 mmHg in untreated SHR respectively. The differences were statistically significant ($p<0.05$). The blood pressure recorded in rats treated with double the concentration of therapeutic dose was comparable to that seen with the therapeutic dose.

1.2. Evaluation of efficacy of the drug in the prevention of left ventricular remodeling:

The efficacy of the drug in prevention of left ventricular remodeling was evaluated. It included assessment of morphological and histological (hypertrophy index, cardiomyocyte hypertrophy, ventricular fibrosis), electrical, functional and molecular changes in SHR treated with *Cardoguard* compared to untreated animals.

a. Assessment of hypertrophy by morphological and histological changes:

Hypertrophy was assessed by i) measurement of hypertrophy index [VW (mg) /BW (g)], ii) measurement of LV wall thickness, iii) measurement of left ventricular cardiomyocyte cross-sectional area and extent of fibrosis.

The mean body weight of 6 month old treated and untreated SHR was 311±20g. The mean ventricular weight of treated SHR (1123 ±57 mg) was significantly lower than that of untreated SHR (1230.5±116 mg; $p< 0.05$). The mean hypertrophy index of treated SHR was significantly lower ($p<0.01$) than that of SHR and comparable to that of Wistar rats (Fig.6). Treatment with *Cardoguard* reduced LV wall thickness ($p<0.01$) and the mean value of treated SHR was comparable to that of Wistar (Fig.7). The mean body weight of 12-month-old treated SHR (327±16g) was comparable to that of untreated animals (328±21g). The mean ventricular weight of treated SHR at 12-months was lower (1540±60 mg) than that of untreated animals (1737± 159 mg), though the difference was not statistically significant.The mean hypertrophy index of 12-month old treated SHR and untreated SHR was found to be 4.7± 0.39 and 5.3± 0.85 respectively. The mean LV wall thickness of 12-month old treated SHR and

untreated SHR was found to be 109 ±10 μm and 118 ±6 μm respectively. At 12-months, the mean hypertrophy index and LV wall thickness were found to be lower in treated animals compared to SHR, though the results were not as significant as that seen in younger rats.

Measurement of histologic myocyte size and cardiac fibrosis were carried out in 6-month old SHR. Cardiomyocyte cross sectional area was measured following immunohistochemical staining for dystrophin, which gives a clear outline of the cells. The mean cross sectional area of cardiomyocytes was found to be significantly lower in treated SHR ($p< 0.05$) (Fig.8). Fibrosis was measured by morphometric assessment of the interstitial collagen. Image analysis of Sirius red stained ventricular sections revealed that the mean percentage stained area in treated SHR was lower than that of SHR, but, the results were not statistically significant. The mean value for treated SHR was comparable to Wistar (Fig.9).

Both morphological and histological evaluations confirm that myocardial hypertrophy is attenuated by the drug.

Signaling pathways modified by the drug:

Immunostaining for the proteins of the major signaling pathways associated with hypertrophy showed that in 6 month old SHR, the drug acted possibly through ERK and PKC ε pathways as assessed by gray level intensity using *Imag –Pro Plus 5.1*. The mean gray level intensities in the ventricular sections of treated SHR were calculated as percentage of the mean value of untreated control (SHR). The signal intensity of pERK was found to be 10%±0.96 in ventricular sections of treated SHR

(Fig.10A)and that of p PKC ε was 6%±0.23 (Fig. 10B). The signal intensity was 88%± 2.14 for calcineurin (Fig.10C), 39%±17.04 for pJNK (Fig.10D) and 100%±6.05 for p -p38 (Fig.10E) in sections of treated SHR. The differences in signal intensities of pERK and pPKC ε in treated SHR were statistically significant ($p<0.001$) when compared to that in untreated animals. The signal intensity of pJNK was found to be lower in treated SHR, though the results were not statistically significant.

Negative controls were used by omitting primary antibodies. In the absence of primary antibodies, application of secondary antibodies failed to produce any staining.

b. Assessment of electrical remodeling:

ECG data were recorded in SHR using *BIOPAC Data Acquisition System.* No significant changes in ECG were observed in 6-month old rats. ECG variations were observed in 78% of untreated SHR and 44% of treated animals at 12 months. Presence of left axis deviation or LAD (mean QRS axis more negative than -30^{o}), which is suggestive of LVH, was found only in untreated SHR. A normal rat ECG is shown in Fig.11A. In left axis deviation, Lead I shows a tall R wave, Lead II shows rS complex with the S wave greater in amplitude than r wave and Lead III shows a deep S wave (Fig.11B). Animals with ST segment changes were slightly more among untreated SHR. ST segment changes mainly ST elevations (Fig.11C) are indicators of left ventricular overload and ischemia. In the normal state, ST segment is isoelectric in nature.

c. Measurement of cardiac output:

Cardiac output was determined as an index of the functional status. It was determined in anaesthetized SHR using *BIOPAC Data Acquisition System.* An age-dependent decrease in cardiac output was observed in SHR (Fig.12). The mean heart rates of both 6- month old and 12-month old treated and untreated SHR were comparable. At 6-months, a significantly higher cardiac output (Fig.12) ($p<0.05$) was observed in treated SHR consequent to an increase in stroke volume (757 ± 237μL vs. 520 ± 266μL). A similar beneficial effect was observed in treated rats even at 12 months of age. There was a significantly higher stroke volume (459 ± 145 μL vs. 319 ± 88 μL) ($p<0.05$) leading to an increase in cardiac output (Fig.12).

1.3. Evaluation of efficacy of the drug in the prevention of vascular remodeling:

Wall thickness and wall to lumen ratio of thoracic aortae and arteries in the kidney stroma of SHR and treated SHR at 6 months of age were measured. For aortic morphometry, H&E staining was carried out; and for arterial morphometry in the kidney stroma, Elastin Van Gieson's staining was carried out. The wall thickness and wall to lumen ratio of the aortic sections of treated SHR were comparable to that of Wistar rats and lower than that of untreated SHR; but the differences were not statistically significant (Fig.13). The wall thickness and wall to lumen ratio of arteries did not show a positive response to the treatment (Fig.14).

1.4. Determination of the toxicity of the drug:

Toxicity of the drug was determined by histopathological analysis of kidney and liver of SHR treated for 10 months. Six animals each from treated and untreated group were used for the study. On microscopic analysis of H&E stained kidney sections, it was found that the glomeruli and tubules in almost all sections appeared normal except for 1 glomerulus from an untreated SHR which was seen sclerosed with periglomerular fibrosis (Fig.15A). Mild interstitial inflammation was present in 2 sections of treated SHR. Focal tubular atrophy with hyaline casts were found in 2 foci in a section each from treated and untreated SHR (Fig.15B). On examination of liver sections, mononuclear cells were found in 2-3 loci each in the hepatic parenchyma of both treated and untreated SHR. Histological examination reveals no toxicity related features in drug–treated SHR.

1.5. Evaluation of neurodepressive action of the drug:

Reserpine, the isolated active principle of *Rauwolfia serpentina* is known to be depressogenic in patients on long term use for the management of hypertension. The activity of monoamine oxidase, a marker for depression was assayed in Wistar rats. The crude mitochondrial samples from the brain of control, reserpine- treated and *Cardoguard* treated -rats were used for the assay. Statistical significance of the fluorescence intensity values of the sample groups was determined by *ANOVA* and was found to be significant ($p<0.05$). The mean fluorescence intensity in reserpine-treated brain samples was higher than that of the control ($p<0.05$). Eventhough the mean fluorescence intensity of *Cardoguard*- treated brain samples was found to be

lower than that of reserpine treated samples; but slightly higher than that of controls;without statistical significance for either of the differences (Fig.16).

The brain MAO activity of *Cardoguard*- treated rats being lower than that of reserpine treated rats indicates that the drug is less depressogenic compared to the isolated active principle, reserpine.

2.1 DELINEATION OF THE MECHANISM OF ACTION OF CARDOGUARD USING RAT AORTA

Aortic rings were used to study the mechanism of action of the drug; as *ex vivo* models are more appropriate for this purpose.Aortic rings were isolated from normotensive rats.

a) Vasorelaxant capacity of the drug:

To establish the vasorelaxant property of the Ayurvedic drug, the aortic rings with endothelium were precontracted with PE (3μmol/L).

Addition of water soluble fraction of *Cardoguard* at the therapeutic dose (64 mg/L) induced relaxation of aortic rings precontracted with phenylephrine (Fig.17). It was a sustained and slowly developing relaxation and the percentage of vasorelaxation was found to be 82%. The extent of vasorelaxation by the drug was comparable to the vasorelaxation induced by verapamil, an L-type calcium channel blocker (82.9 %) in phenylephrine precontracted aortae.

b) Vasorelaxation induced by different concentrations of the drug:

Aortic rings precontracted with PE were treated with double and three times the therapeutic dosage (128mg/L and 192mg/L) and relaxation responses were recorded. The percentage vasorelaxation induced by double dosage of the drug was slightly lower than that induced by the therapeutic dose, but the difference was not statistically significant. The percentage vasorelaxation induced by triple dosage was significantly less compared to the therapeutic dose (Fig.18) Hence the therapeutic dose is found to be the most effective concentration and was used for further experimental studies.

c) Vasorelaxation induced by different components of the drug:

At the concentrations used in the drug, *Rauwolfia serpentina* induced the maximum vasorelaxation (57%) followed by *Terminalia chebula* (48%), *Emblica officinalis* (37%), *Terminalia arjuna* (22%), *Terminalia belerica* (15%) and *Boerhavia diffusa* (10%) (Fig.19). *ANOVA* showed significant difference between the groups ($p<0.0001$), with relaxation of each of the components being significantly lower than that of *Cardoguard.*

d) Mechanism of vasorelaxation: Aortic relaxation can be mediated by a number of mechanisms. The possible mechanisms of vasorelaxation by which reduction of blood pressure is attained were tested for *Cardoguard.*

d i) Role of endothelium in mediating vasorelaxation by the drug:

To determine the role of endothelium in inducing vasorelaxation by the drug, PE-precontracted endothelium- denuded aortic rings were treated with the drug. The

drug-induced vasorelaxation in endothelium-denuded rings was significantly less ($p<0.001$) when compared to that of endothelium intact rings (Fig.20 and Fig.21). Thus the vasorelaxation is found to be endothelium dependent.

It was observed that prazosin which is an α- adrenoreceptor antagonist and a commercially available antihypertensive, produced immediate relaxation in the same aortic ring in which *Cardoguard* failed to induce relaxation (Fig.20). This confirms that the vascular tissue was functional and also demonstrates the endothelium independent action of prazosin.

The nitric oxide synthase pathways and the cyclooxygenase pathways appear to interact in a complex way to maintain vascular tone under normal conditions (Bratz and Kanagy, 2004). For examining the specific involvement of endothelial-nitric oxide, rings with functional endothelium were exposed to the nitric oxide synthase inhibitor, L-NAME (100μM), 30 min prior to contraction with PE, and then exposed to the drug. To confirm the role of cyclooxygenase pathway in aortic relaxation, aortic rings with endothelium were incubated with indomethacin (10μM), an inhibitor of prostacyclin, prior to contraction with PE, and then exposed to the drug. The drug-induced vasorelaxation was significantly lowered both in L-NAME ($p<0.0001$) and indomethacin pretreated aortic rings ($p<0.001$), indicating the role of nitric oxide and cyclooxygenase pathways (Fig.22 and Fig.23). Prostacyclin may be an important factor that mediates endothelium-dependent vasorelaxation by the drug besides NO. These data confirm the endothelium dependent vasorelaxation by the drug.

dii) Role of the drug as an ATP-sensitive potassium channel(K_{ATP} channel) *agonist:*

To examine the ability of the drug to act as a K_{ATP} channel agonist, the aortic rings were incubated with glibenclamide (10μM), prior to contraction with PE, and then

exposed to the drug. It was observed that the vasorelaxation was significantly reduced ($p<0.001$) (Fig.24) in glibenclamide preincubated rings, signifying the role of the drug as a K_{ATP} channel activator.

diii) Calcium antagonistic action of the drug:

To examine the role of the drug as a calcium antagonist, aortic rings were exposed to calcium in the presence and absence of the drug. Verapamil, a known L-type calcium channel antagonist, was used as positive control. A second experiment was carried out in which the aortic rings were precontracted with high K^+ and vasorelaxant response of the drug examined.

Vasoconstriction of aortic rings was observed on addition of Ca^{2+} (Fig. 25A). Pretreatment with the drug significantly inhibited the contractile response induced by cumulative addition of calcium (6-10mM) (Fig.25B). Similar observations were noted when the drug was replaced with verapamil (1μM). This demonstrates the Ca^{2+} antagonistic action of *Cardoguard.*

Unlike *Cardoguard*, verapamil induced an endothelium independent relaxation. Vasorelaxation in response to verapamil was found to be 80% in endothelium denuded aortic rings which was significantly higher than that induced by *Cardoguard* ($p<0.001$); implicating different mechanisms in Ca^{2+} mediated vasorelaxation. *Cardoguard* induced vasorelaxation (36.5%) in 100mM KCl- precontracted aortic rings which was significantly less ($p <0.01$) than that in PE- precontracted rings (Fig.26). Phenylephrine as well as high K^+ induce vasoconstriction by calcium mobilization, albeit different mechanisms. Thus Ca^{2+} antagonistic action of the drug is also demonstrated by the occurrence of vasorelaxation in both PE- and high K^+ - precontracted aortae.

Based on the above results, it can be inferred that intracellular calcium mobilization is a target for the vasorelaxant effect of *Cardoguard.*

div) Role of the drug as an inhibitor of Ang II :

Unlike phenylephrine and KCl, Ang II does not induce sustained contraction (Fig.27).Hence aortic rings were preincubated with the drug for 30 minutes prior to addition of Ang II and the isometric force determined. The drug was not found to affect the vasoconstriction induced by Ang II (Fig.28). This suggests that the drug may not act as an inhibitor of Ang II in mediating the physiological change.

dv) Role of the drug as a β-adrenoreceptor antagonist:

Ventricular papillary muscle was used to examine whether the drug acts as a beta-blocker because the antihypertensive action of beta blockers is mediated by reducing the cardiac output. To examine the role of the drug as a β-adrenoreceptor antagonist, the electrically stimulated papillary muscle was preincubated with the drug followed by treatment with isoproterenol (1μM). Isoproterenol induces a positive inotropic effect (Fig.29).

Pre incubation with the drug was found to have no effect on the positive inotropic response of the papillary muscle to isoproterenol (Fig.30). This suggests that the drug does not act through β-adrenoreceptor pathway in mediating the functional change.

dvi) Delineation of the signaling pathways involved in vasorelaxation by the drug:

To examine the signaling pathways involved in the mediation of vasorelaxation by the drug, pathway specific inhibitors: PD98059 (10μM), SB202190 (10μM), bisindolylmaleimide (1μM) and cyclosporin A (500ng/ml) for the ERK, p38 MAPK, PKC and calcineurin pathways respectively were added one at a time 30 min prior to addition of PE and the response to the drug was recorded.

There was significant difference between the groups when *ANOVA* was carried out ($p<0.0001$).The vasorelaxation is found to be mediated by ERK, p38 MAPK and calcineurin pathways as the relaxation was affected in the presence of PD98059, SB 202190 and cyclosporin A respectively ($p<0.001$), but not in the presence of bisindolylmaleimide. Vasorelaxation in the presence of bisindolylmaleimide was comparable to vasorelaxation in the absence of any of the inhibitors suggesting that the PKC pathway was not involved in vasorelaxation by the drug (Fig.31).

2.2 EFFECT OF CARDOGUARD ON CARDIAC CONTRACTILITY ASSESSED IN VENTRICULAR PAPILLARY MUSCLE:

Some of the antihypertensive drugs are known to affect cardiac function. Hence contractile variation of the papillary muscle in response to different concentrations of water-soluble fractions of *Cardoguard* was recorded.

Exposure of papillary muscle to therapeutic levels of the drug did not induce any contractile variation. The contraction remained unaffected 1h after the addition of the drug. Decrease in force of contraction was seen at 4 times the normal dose and higher ($p<0.01$) (Fig.32).

2.3. STUDIES IN CULTURED CARDIAC CELLS TO TEST THE EFFICACY OF THE DRUG IN THE PREVENTION OF CARDIAC HYPERTROPHY:

Cardiomyocyte characteristics:

The cardiomyocytes could be seen attached to the culture surface under phase contrast optics 24 hours after their isolation. The cells remained viable and healthy for more than 2 weeks. The cardiomyocytes appeared dense with cross striations. They had coarse granular cytoplasm with dense inclusions. Nucleus was small and round

with single nucleolus. (Fig.33A) Beating myocytes could be observed when the culture medium was supplemented with serum. Immunohistochemical staining for antibody to desmin confirmed the identification of cardiomyocytes (Fig.33B).

Characteristics of fibroblasts:

Fibroblasts could be seen adhered to the culture surface 90 minutes after plating. The cells could be passaged about six times after which the cells appeared to go into senescence. They usually reach near confluence (Fig.33C) and become ready for the first subculture, by the second day after isolation. The fibroblasts appeared as irregularly shaped cells with thin, translucent cytoplasm with inclusions usually clustered near the nucleus. The nucleus was distinctively larger, less dense and more oval than that of myocytes. The fibroblasts immunostained positive for vimentin (Fig.33D) and negative for desmin and factorVIII.

Cardiomyocytes and fibroblasts were exposed to different concentrations of water-soluble fractions of the drug. At therapeutic levels of the drug, the cells remained healthy and viable for more than 6 days under controlled culture conditions. Thus the drug was found to be nontoxic to the cells in culture. The cell culture system was therefore found to be suitable for use as a model for assessing the antihypertrophic potential of the drug.

2.3a. Testing the efficacy of the drug in the prevention of hypertrophy of cultured cardiac myocytes:

Cardiomyocyte hypertrophy was stimulated with both: Ang II (100nM) as well as the β-adrenergic agonist, isoproterenol (1μM) and the ability of the drug to prevent the hypertrophic responses was investigated. The hypertrophic response was assessed by measurement of cell volume of myocytes isolated by differential trypsinization.

Differential trypsinization selectively dissociates the myocytes from the culture surface before non- myocytes.

Ang II induced hypertrophy of the cardiomyocytes. *ANOVA* showed significant difference between the control, Ang II and *Cardoguard* + Ang II treated cardiomyocyte groups ($p<0.001$). The mean volume of cardiomyocytes in Ang II treated dishes was significantly higher than that of control (40.9%, $p<0.0001$). On pretreatment with the drug, the mean volume of cells was significantly lower than that of Ang II treated dishes ($p<0.01$) (Fig.34 and Fig.35), and comparable to the control. Thus it can be assumed that Ang II induced hypertrophy was prevented by the drug.

Isoproterenol, a frequently used pharmacological inducer of cardiac hypertrophy, also produced hypertrophy of cardiomyocytes in culture. Significant difference between the groups was obtained by *ANOVA* ($p<0.0001$). The mean volume of cardiomyocytes was significantly higher ($p<0.0001$) in isoproterenol treated dishes compared to the control; and significantly lower ($p<0.0001$) in drug pretreated dishes compared to isoproterenol treated dishes (Fig.36), but higher than that of control ($p<0.05$). Thus, from the findings, it can be inferred that isoproterenol induced hypertrophy was prevented by the drug.

2.3b. Testing the efficacy of the drug in the prevention of proliferation of cultured cardiac fibroblasts:

Fibroblast proliferation was stimulated with Ang II (100nM) and the ability of *Cardoguard* to prevent fibroblast proliferation was tested. The dishes with the cells were pre incubated with the drug for 30 minutes prior to addition of Ang II. The cells were detached by trypsinization after 96 hours of treatment, and the cell count was obtained. Statistical significance was observed by *ANOVA* ($p<0.01$). The mean cell

count in Ang II treated dishes was significantly higher ($p<0.01$) compared to control, whereas the mean cell count in drug pretreated dishes was significantly lower ($p<0.05$) compared to Ang II treated dishes (Fig.37). The mean cell count in drug pretreated dishes was comparable to that of control.Thus, from the findings, it can be inferred that Ang II- induced cell proliferation was prevented by the drug.

2.4. ASSESSMENT OF THE ANTIOXIDANT POTENTIAL OF CARDOGUARD

i) Measurement of reduced glutathione levels (GSH) in human red blood cells exposed to drug and ROS:

GSH levels were determined in red blood cells exposed to the free radical generator [HX (0.5mM) +XO (0.02U/ml)] in the presence and absence of water-soluble fraction of the drug.

GSH levels were found to be reduced on exposure of the erythrocytes to HX+XO. On pretreatment with the drug, the GSH levels were found to be significantly higher ($p<0.01$) than the levels without the drug and comparable to control (Fig.38). The finding establishes the antioxidant potential of the drug.

ii) Assessment of inotropic response of rat papillary muscle exposed to drug and ROS:

In another experiment designed to examine the antioxidant potential, papillary muscles were superfused with the therapeutic dose of the drug for 15 minutes followed by the free radical generating system, HX (0.5mM) +XO (0.04U/ml).

Addition of HX+XO to the bath induced a negative inotropic response. The contractile force significantly decreased by 30%. Pretreatment with the drug prior to

addition of HX+XO prevented the contractile variation. This was comparable to the response seen on pretreatment with superoxide dismutase (SOD) (120U/ml), the enzyme that catalyzes the reduction of superoxide (Fig.39).

iii) Measurement of H_2 DCF-DA incorporation by cultured cardiac fibroblasts as an assessment of intracellular ROS production:

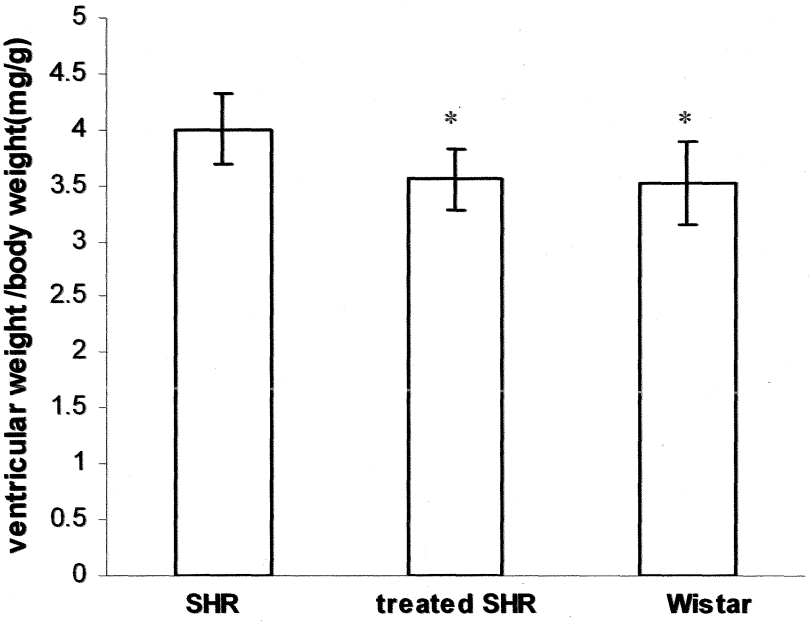
Quantitation of intracellular ROS production in cultured cardiac fibroblasts subject to oxidative stress gives an idea of the antioxidant potential of the drug. This was assessed by H_2DCF-DA incorporation. The values were determined by spectroflurometry. The DCF fluorescence intensity of drug pretreated fibroblasts was significantly less ($p<0.0001$) compared to that of HX (1mM) and XO ($1x10^{-6}$U/ml) treated cells without the drug (Fig.40).

Table 2: Systolic and diastolic blood pressures of SHR treated with *Cardoguard (5mg/rat daily)*

	6 month old SHR		12 month old SHR	
	Untreated	**Treated**	**Untreated**	**Treated**
SBP (mm/Hg)	**197 ± 1**	**188 ±5 ***	**216 ±16**	**194 ±21***
DBP (mm/Hg)	**169 ± 5**	**156 ± 8***	**177 ± 19**	**150 ±31***

Values presented as mean ±SD,*p< 0.05 compared to untreated rats

n =10/group



Values presented as mean ±SD, * p< 0.05 vs SHR, n=8 /group

Fig. 6 Hypertrophy index of 6-month old treated SHR compared to untreated SHR and Wistar rats

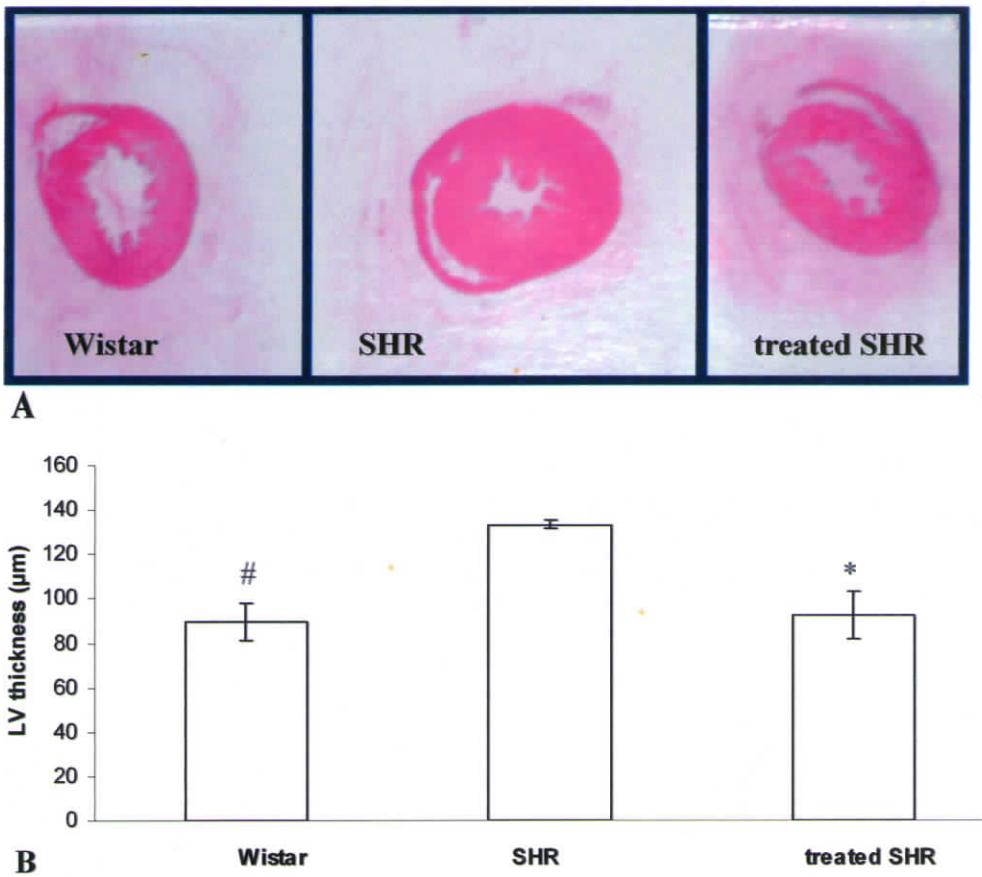
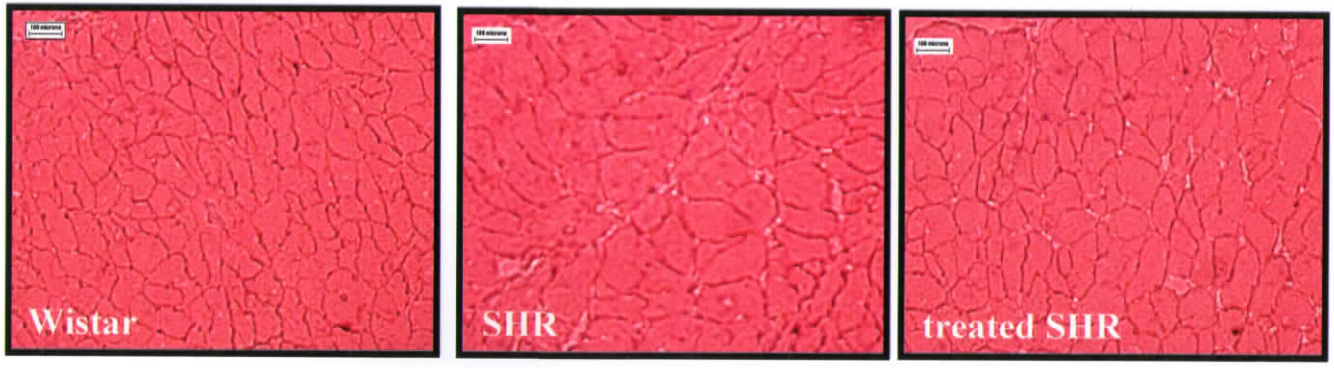


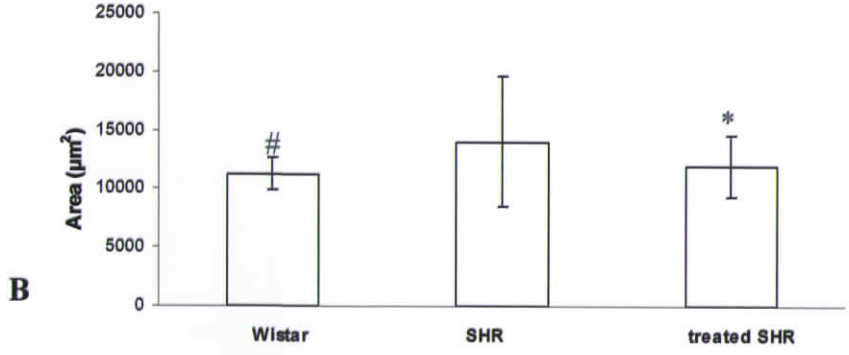
Fig.7 Left ventricular wall thickness of 6-month old treated SHR compared to untreated SHR and Wistar rats A. Macroscopic hematoxylin and eosin-stained histological section of hearts

B. Statistical graph -Values presented as mean ±SD,

*$p<0.01$ vs SHR, #$p<0.001$ vs SHR, n=8 /group



A

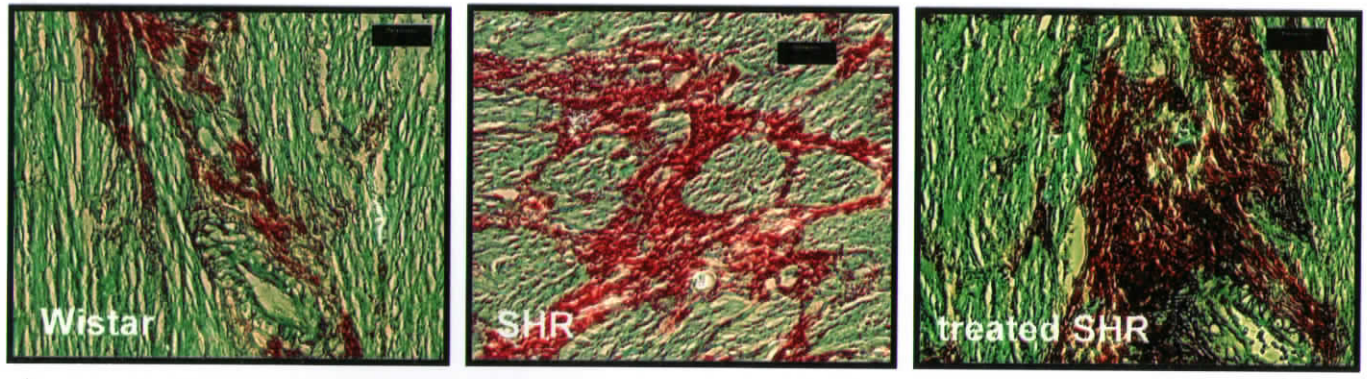


B

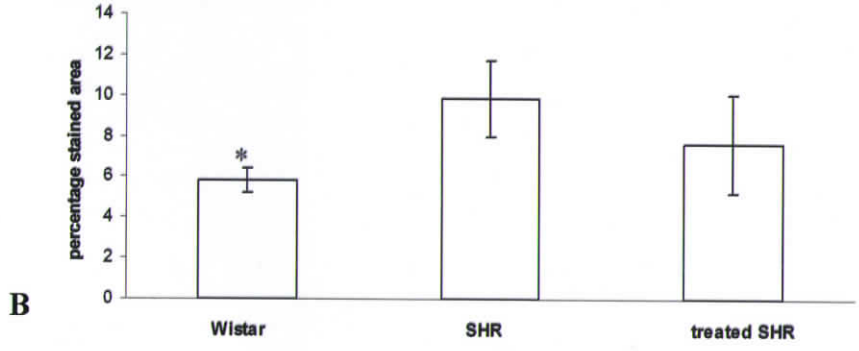
Fig. 8 Cross sectional area of LV cardiomyocytes of 6-month old treated SHR compared to untreated SHR and Wistar.

A. LV sections immunostained for dystrophin, scale bar-100µm B. Statistical graph- Values presented as mean ±SD,

* p <0.05 vs SHR, # p<0.01 vs SHR, n=4 /group



A



B

Fig. 9 Fibrosis in 6-month old treated SHR compared to untreated SHR and Wistar. A. LV myocardium stained for collagen (red), scale bar -100μm B. Statistical graph representing percentage area stained by Sirius red- Values presented as mean ±SD, *p<0.01 vs SHR, n=6/ group

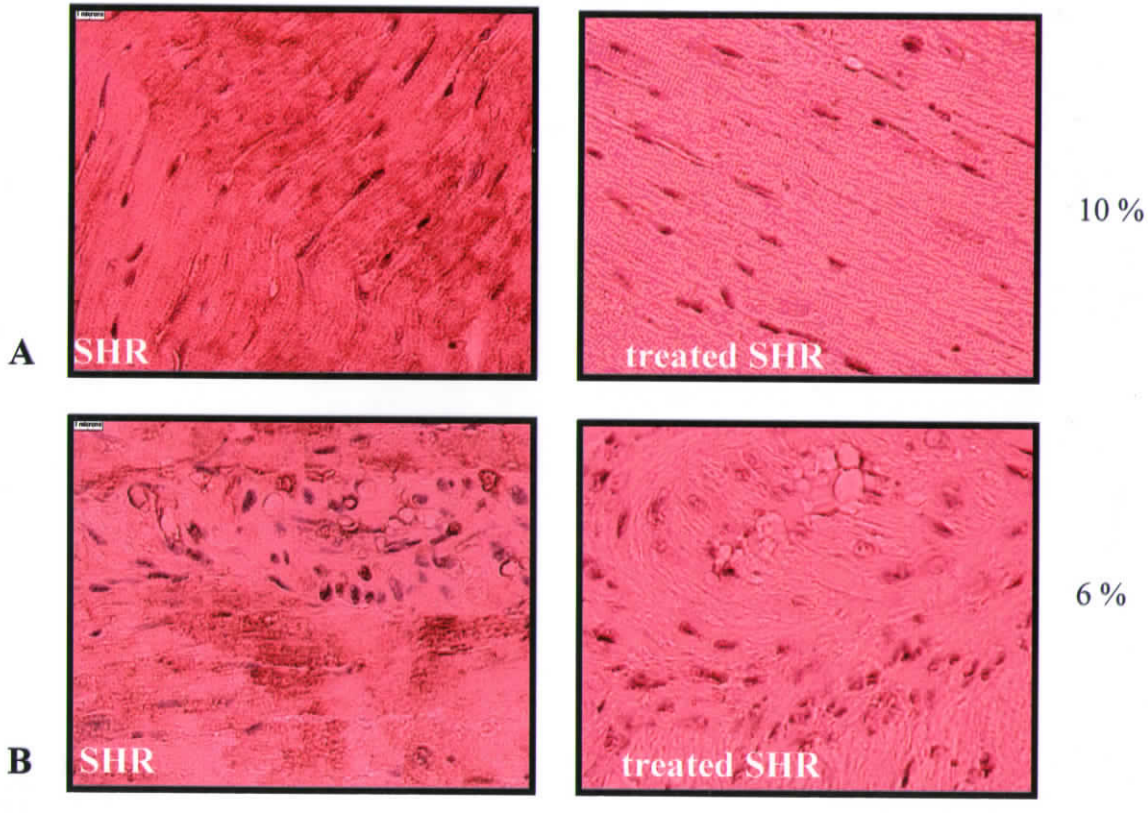


Fig. 10 LV myocardium of 6-month old SHR showing immunostaining for signaling pathways modified by *Cardoguard*
A. p ERK and B. pPKCε

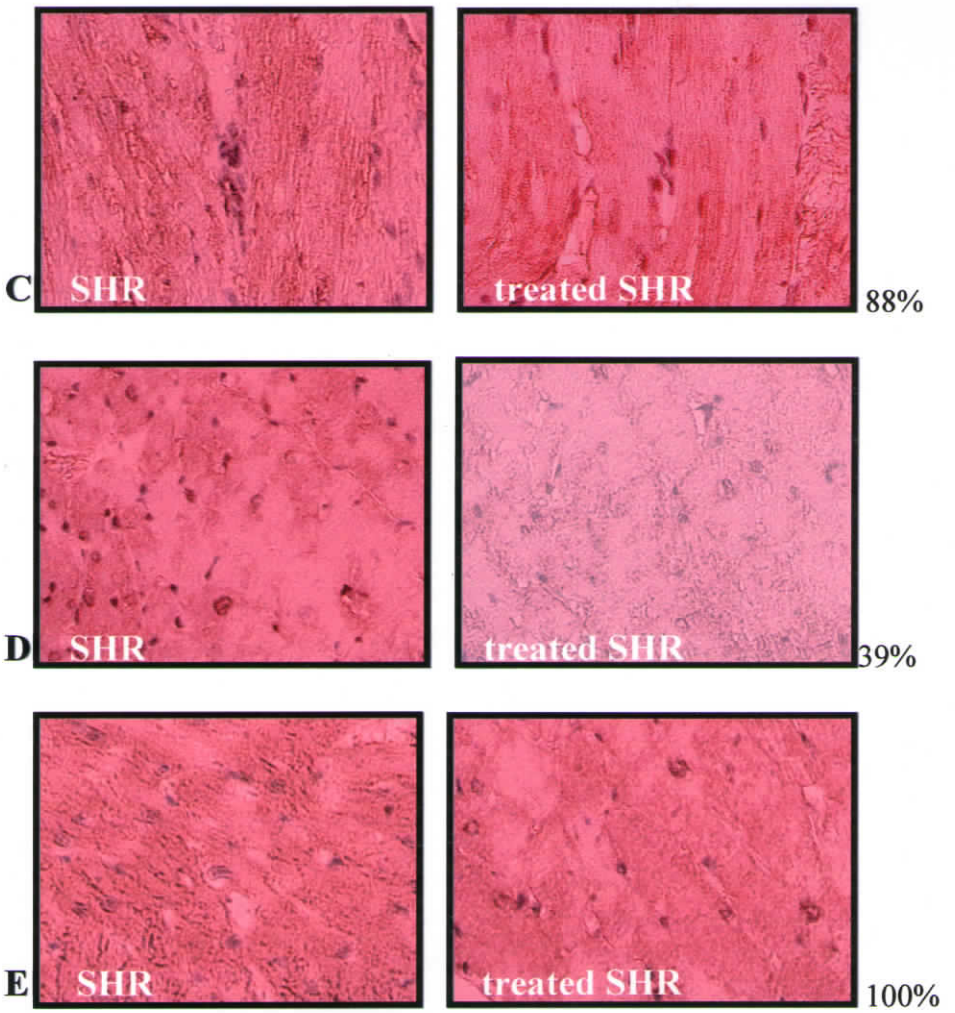


Fig. 10

C. calcineurin D. p JNK E. p-p38

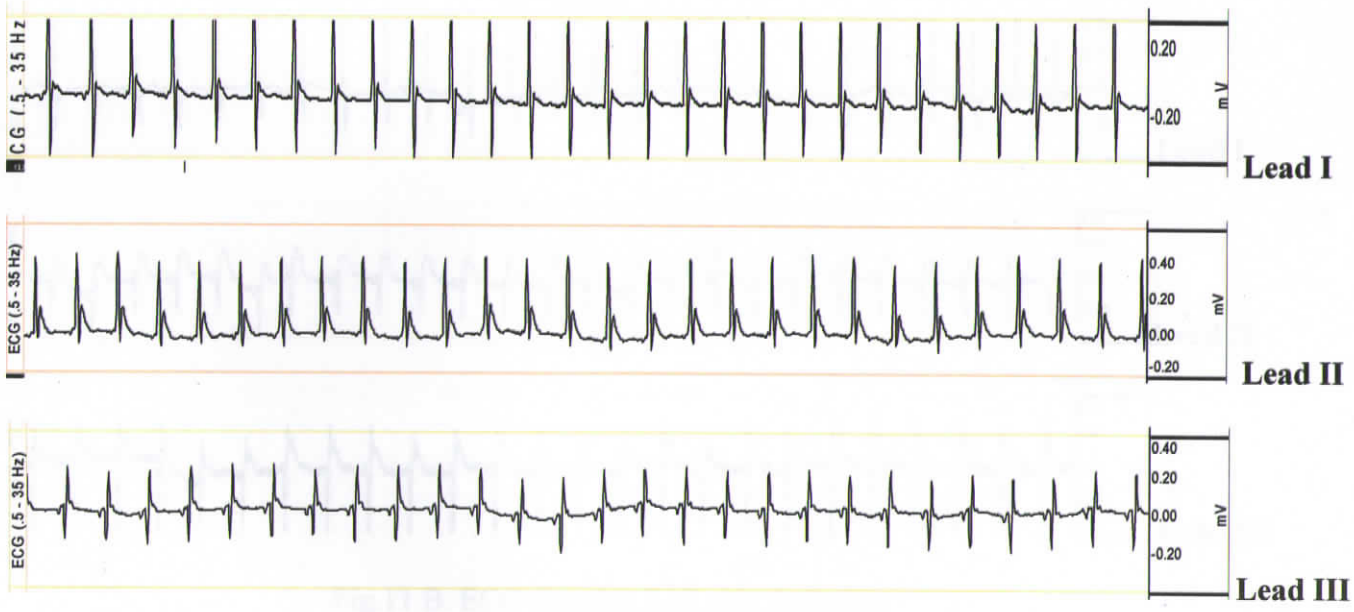


Fig.11A. Normal rat ECG

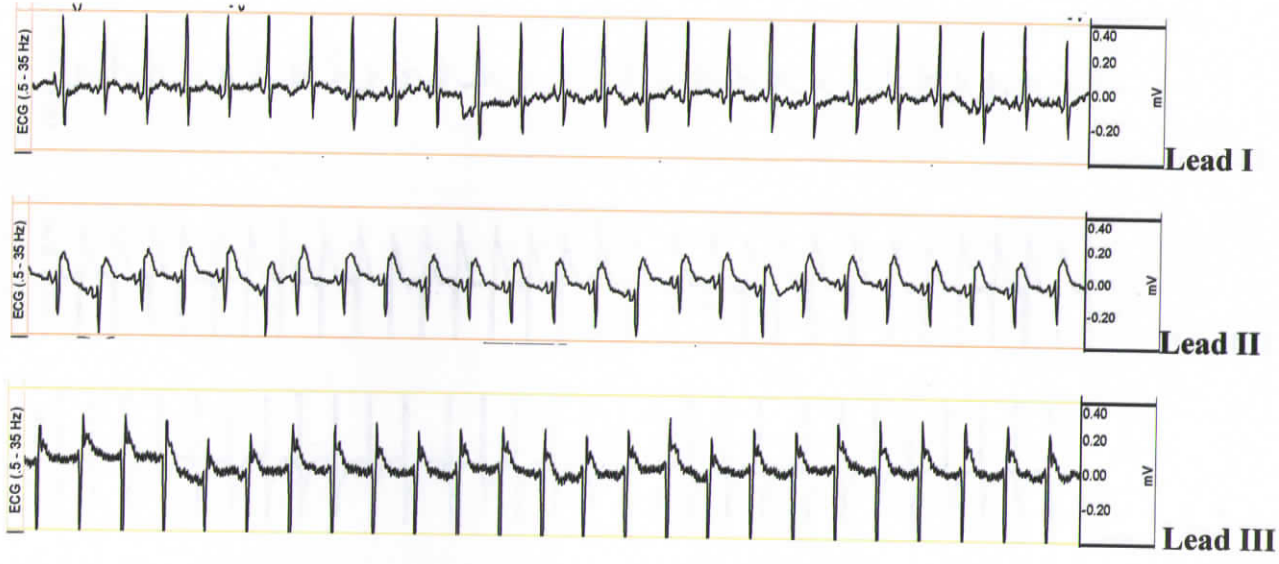


Fig.11 B. ECG showing Left axis deviation

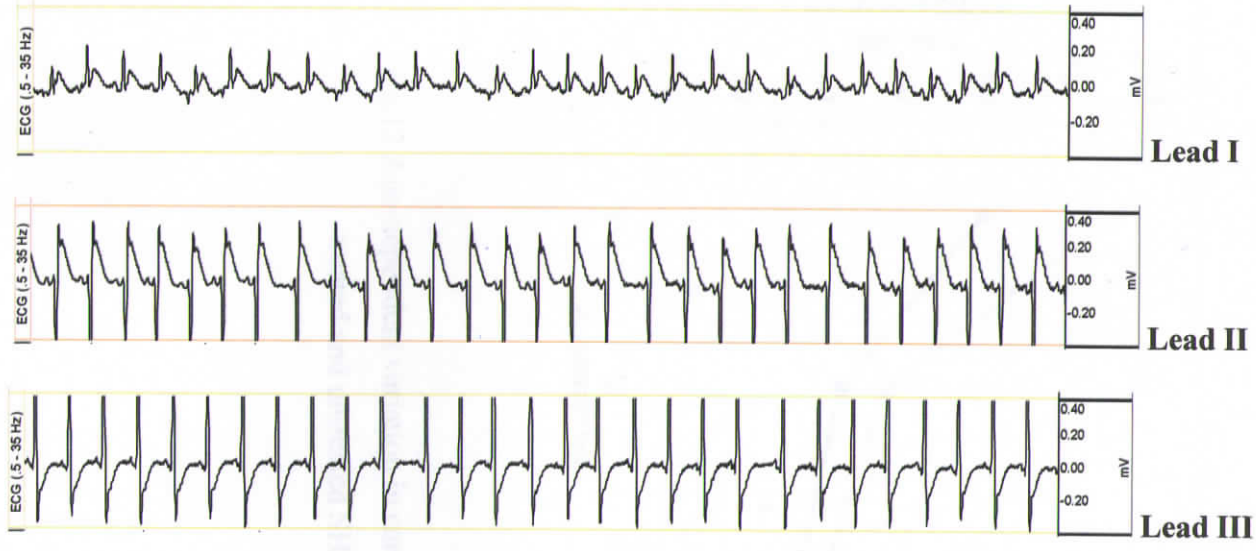


Fig.11C. ECG showing ST segment changes

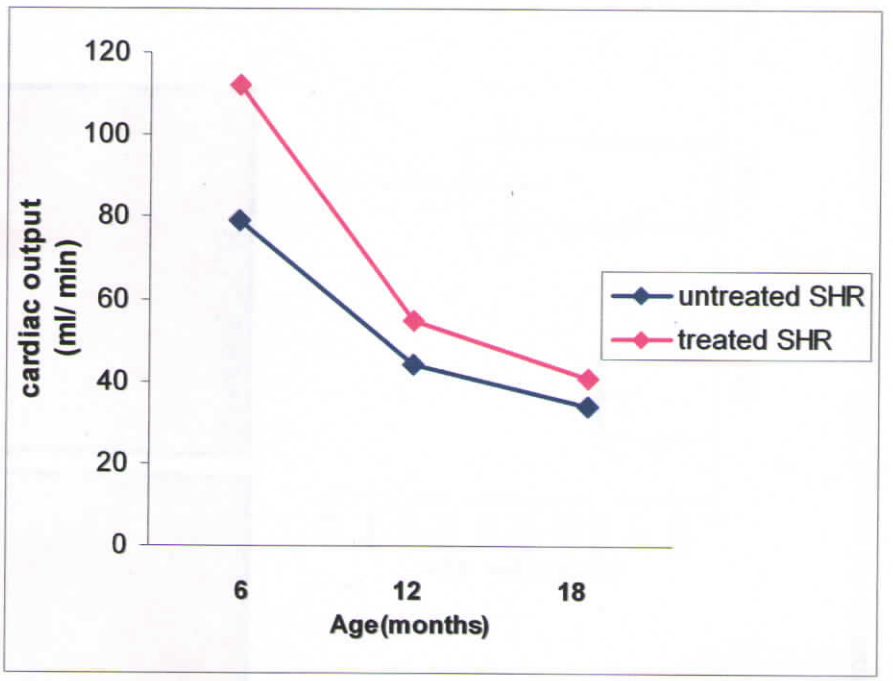


Fig.12 Age-dependent variation in cardiac output of treated and untreated SHR

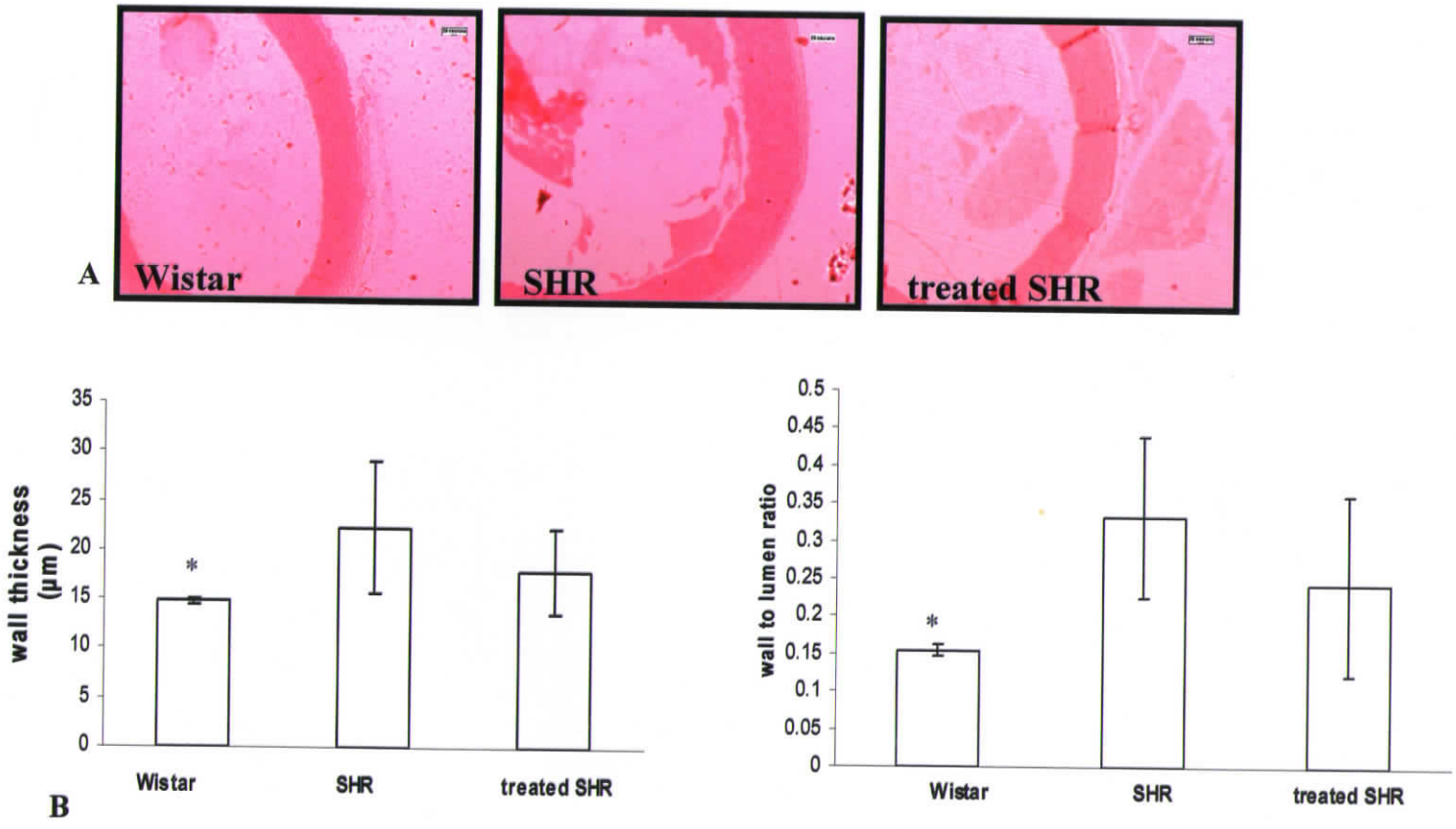


Fig. 13 Aortic wall thickness and wall to lumen ratio of 6-month old treated SHR compared to untreated SHR and Wistar. A. H&E stained aortic sections, scale bar 100µm B. Statistical graphs- Values presented as mean ±SD, *$p<0.05$ vs SHR, n=6/group

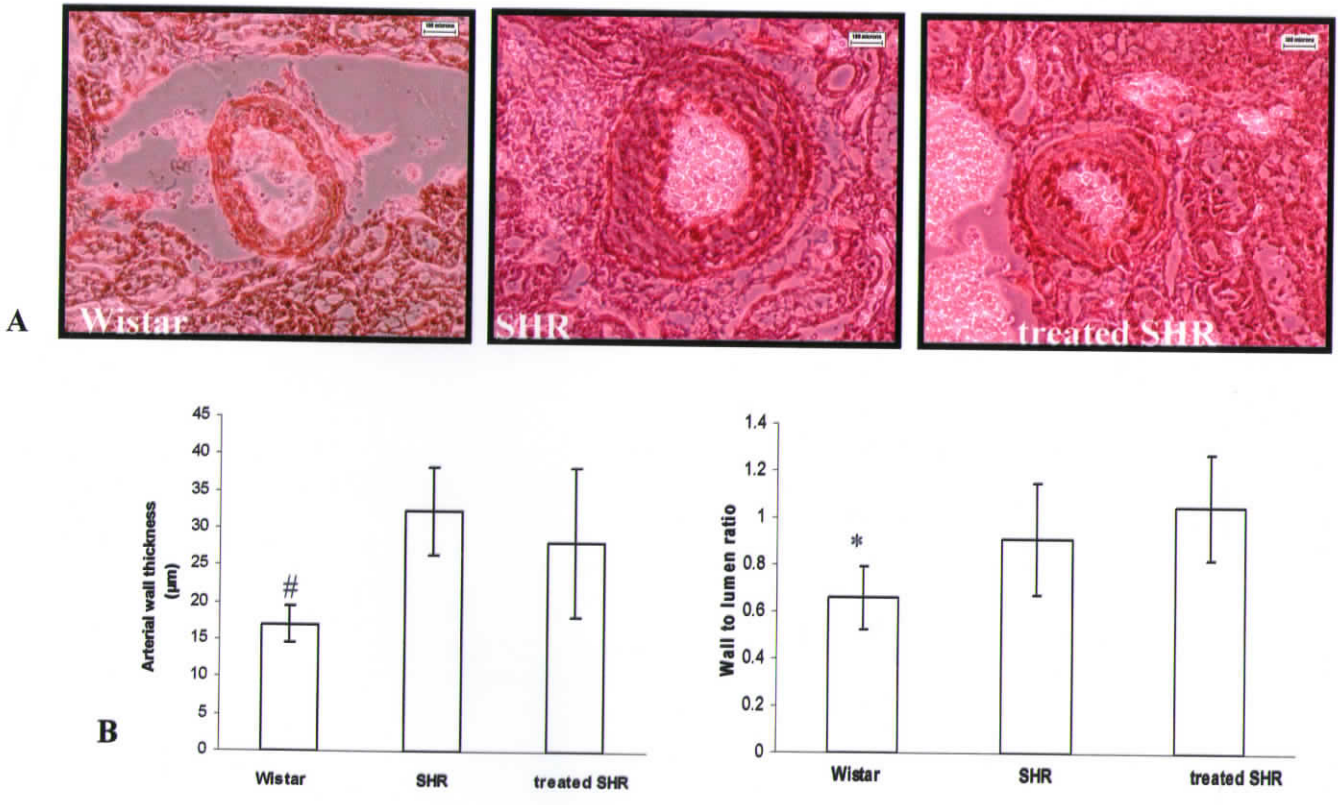


Fig. 14 Arterial wall thickness and wall to lumen ratio of 6-month old treated SHR compared to untreated SHR and Wistar. A. Elastin Van Gieson's stained arterial sections, scale bar 100µm B. Statistical graphs- Values presented as mean ±SD, #$p<0.01$ vs SHR, *$p\leq0.05$ vs SHR, n=6/group

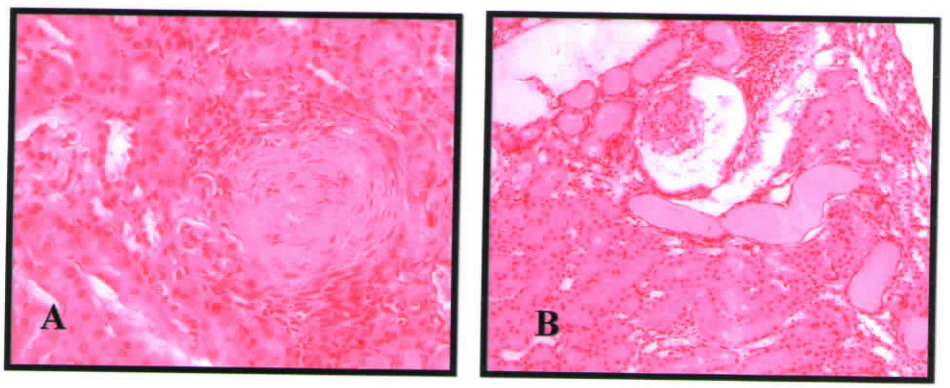
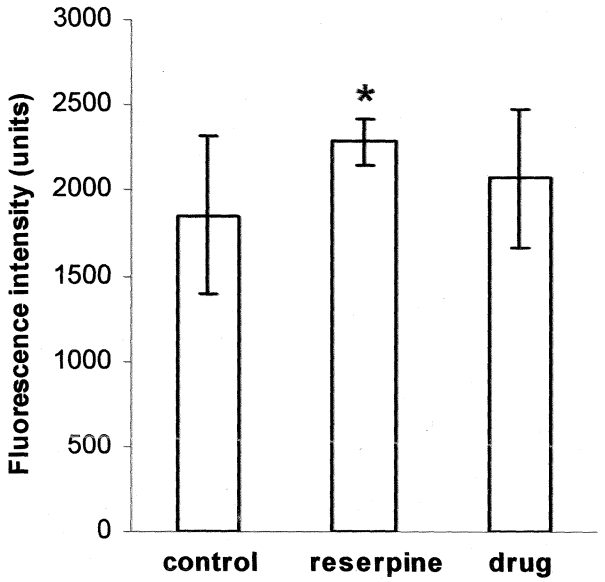


Fig. 15 Determination of long term drug toxicity: micrographs showing the histological changes observed in the kidneys of SHR A. Sclerosed glomerulus B. Focal tubular atrophy with hyaline casts



Values presented as mean ±SD, * $p<0.05$ vs control, n = 6 each

ANOVA- $p<0.05$

Fig.16 Brain monoamine oxidase activity of Wistar rats treated with *Cardoguard* (5mg/animal daily) and reserpine (10µg/animal daily) for 28 days

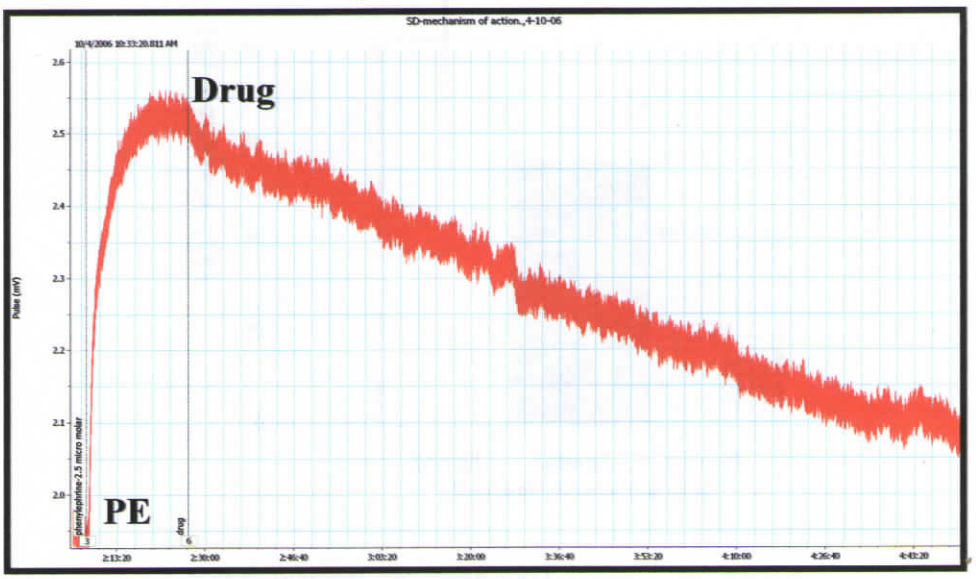
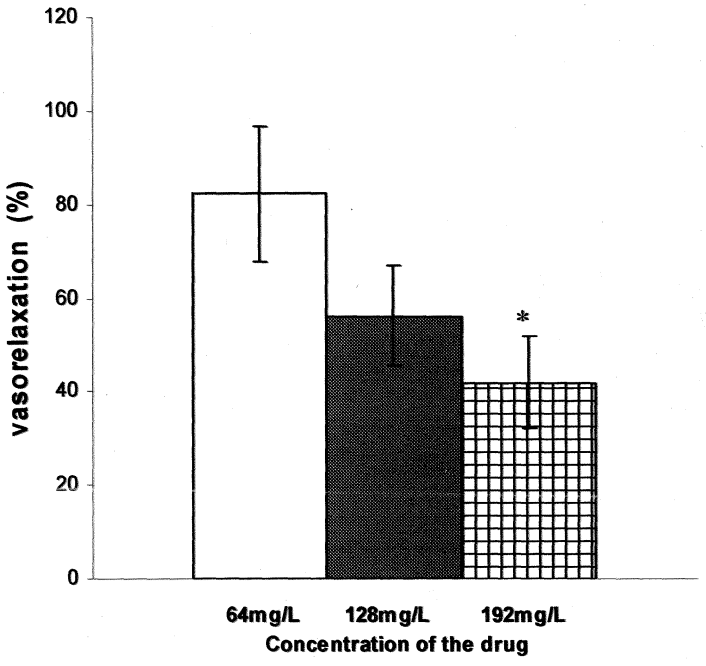
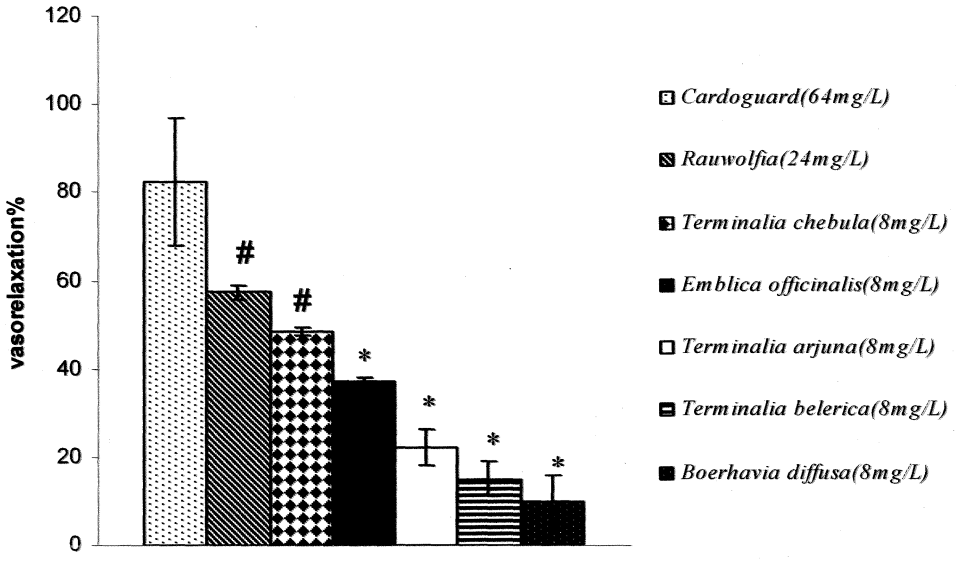


Fig. 17 Vasorelaxant capacity of *Cardoguard* (64mg/L): Recording showing the relaxant response in phenylephrine- precontracted aortic ring



Values presented as mean ±SD, * $p<0.01$ vs single dose,
n =4 determinations

Fig.18 Vasorelaxant effects of different concentrations of *Cardoguard*



Values presented as mean ±SD, # $p<0.05$, * $p<0.0001$ compared to *Cardoguard,* n=4 determinations

Fig. 19 Vasorelaxation induced by *Cardoguard* and each of its different components

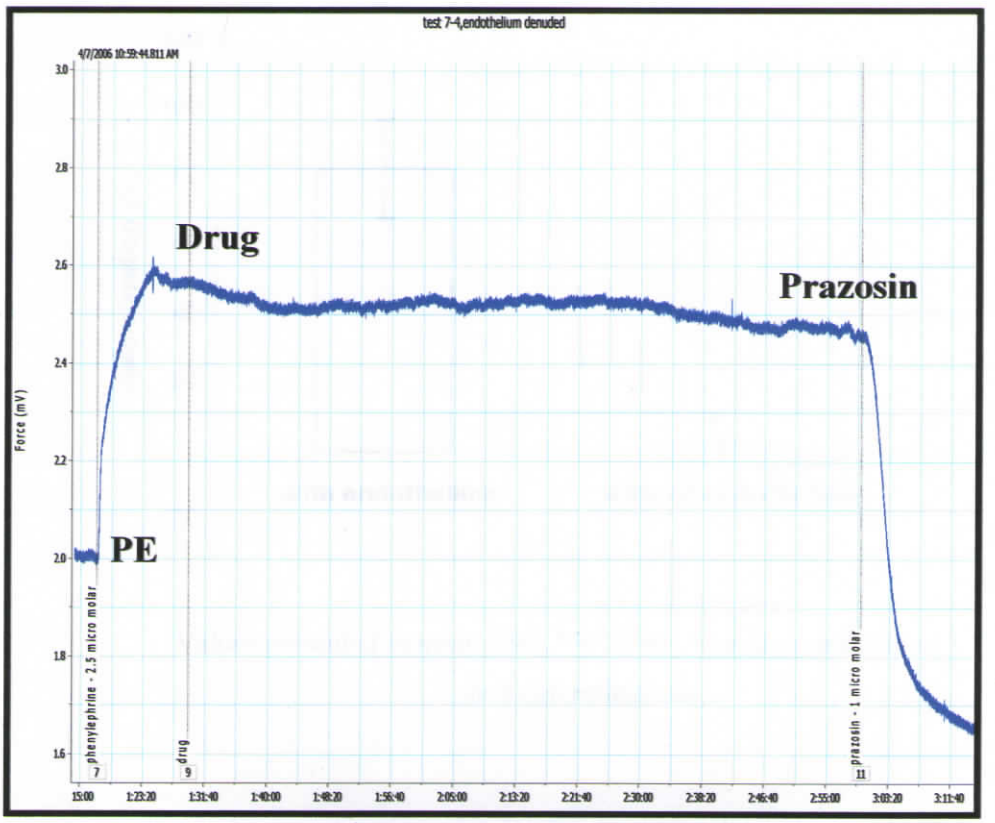
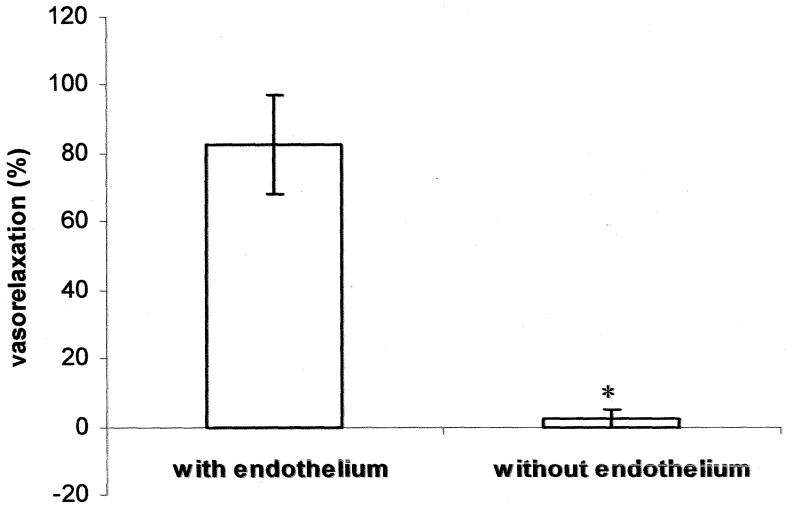


Fig.20 Recording showing inhibition of vasorelaxation by *Cardoguard* (64mg/L) in the absence of functional endothelium



Values presented as mean ±SD, * p< 0.001 vs with endothelium, n=6 determinations

Fig. 21 Percentage vasorelaxation induced by *Cardoguard* (64mg/L) in aortic rings with and without functional endothelium

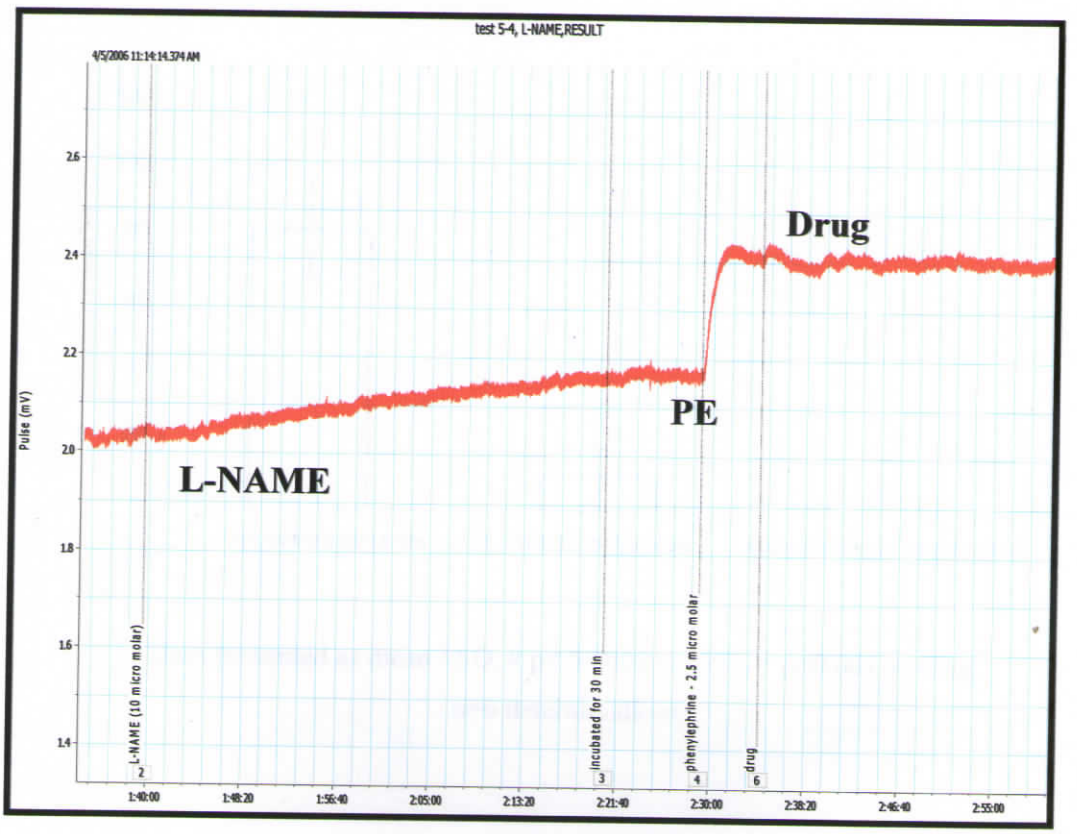
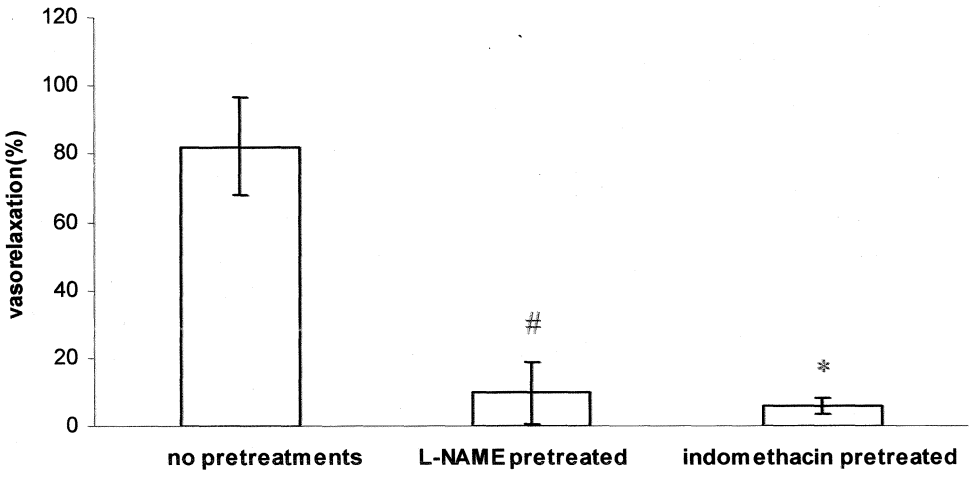
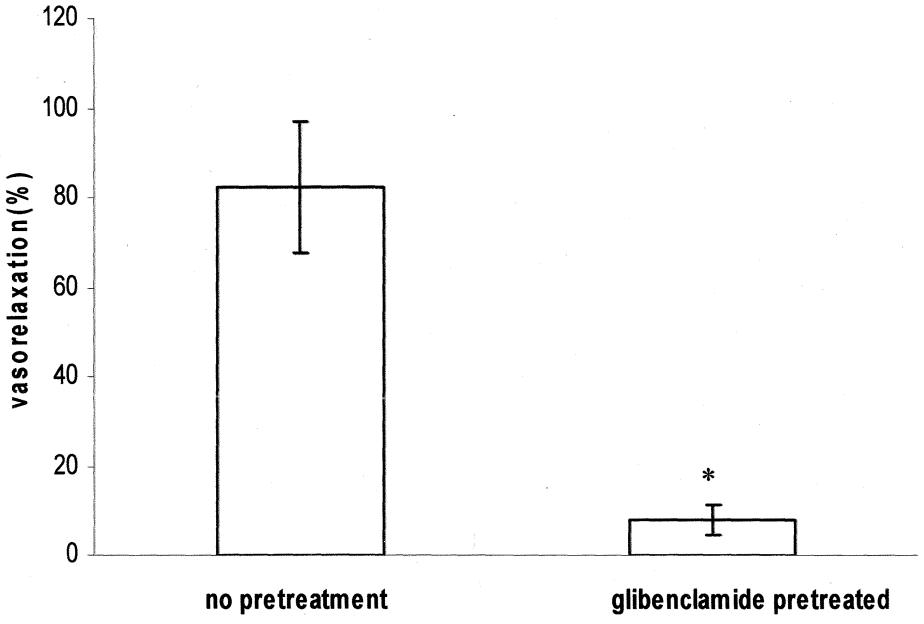


Fig. 22 Recording showing inhibition of vasorelaxation by *Cardoguard* (64mg/L) in L-NAME treated aortic ring



Values presented as mean ±SD, # p< 0.0001 vs drug, * p<0.001 vs drug, n=6 determinations

Fig. 23 Percentage vasorelaxation by *Cardoguard* (64mg/L) following L-NAME and indomethacin preincubation



Values presented as mean ±SD, *p<0.001, n=4 determinations

Fig.24 Percentage vasorelaxation by *Cardoguard* (64mg/L) following glibenclamide pretreatment

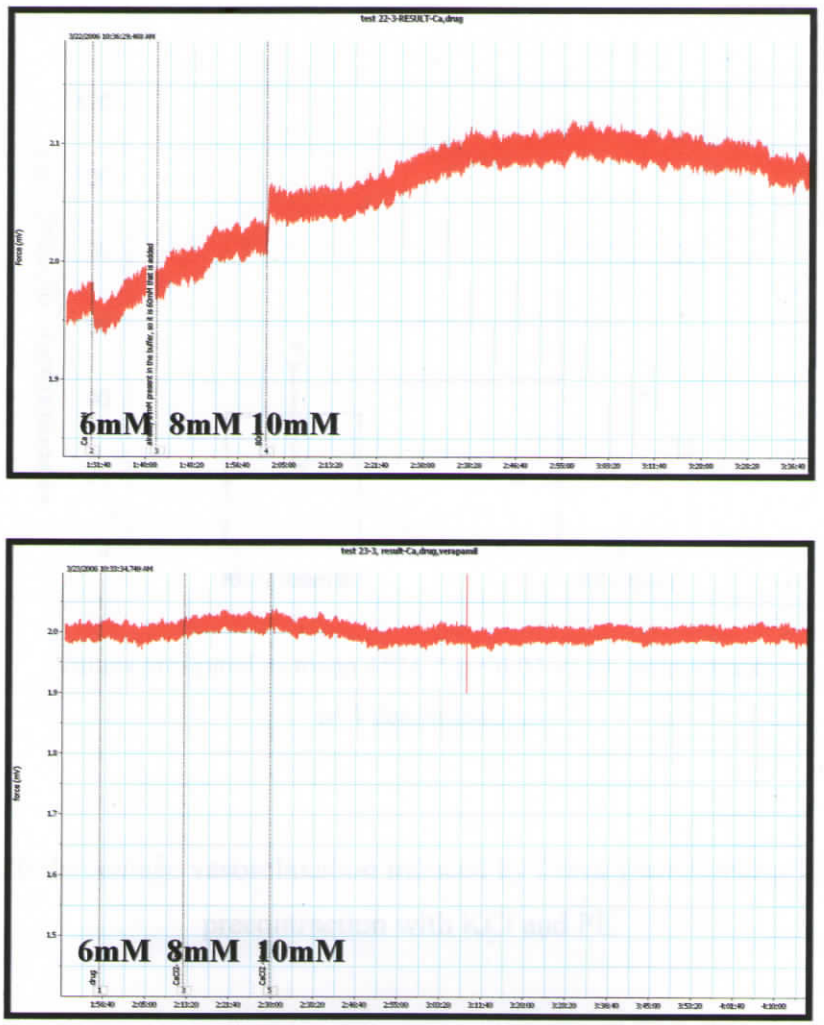
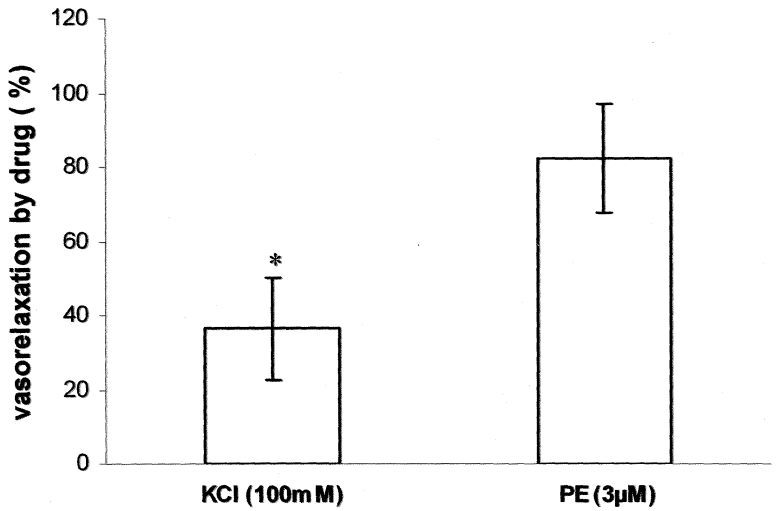


Fig.25 Recordings showing vasoconstriction by cumulative increase in Ca^{2+}concentration A. in the absence of pretreatment with *Cardoguard* B. on pretreatment with *Cardoguard* (64 mg/L)



Values presented as mean ±SD, * p <0.01 vs PE precontraction,
n=4 determinations

Fig.26 Percentage vasorelaxation induced by *Cardoguard* (64mg/L) on precontraction with KCl and PE

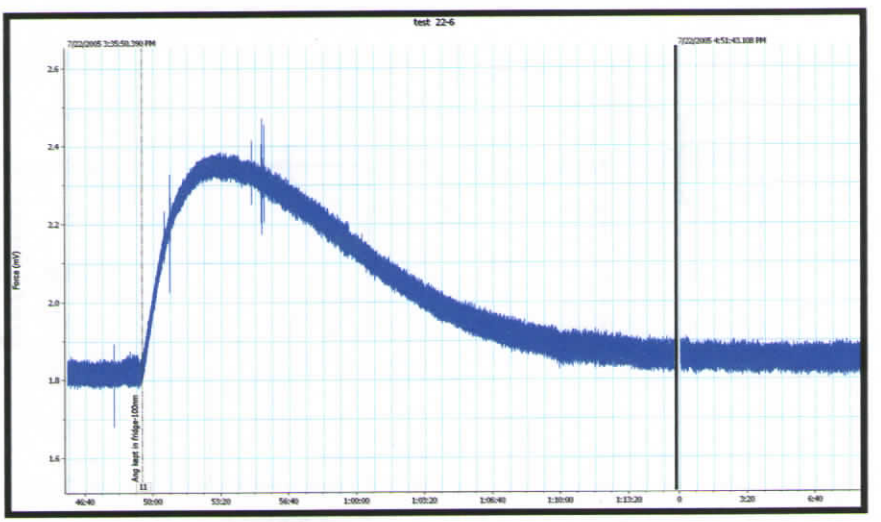
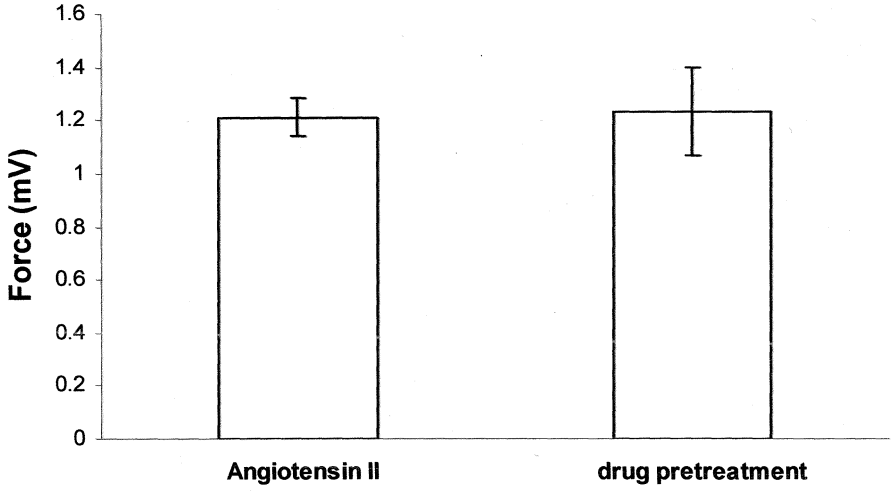


Fig. 27 Recording showing the contractile curve on addition of Ang II to the bath. Plateau formation is not observed unlike phenylephrine.



Values presented as mean ±SD, n = 3 determinations

Fig. 28 Vasoconstrictive response to Ang II after pretreatment with *Cardoguard* (64mg/L)

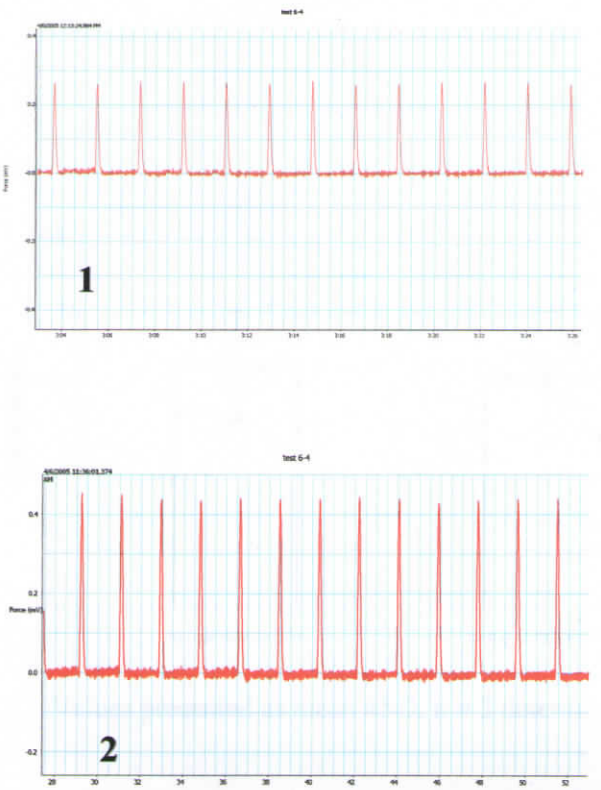
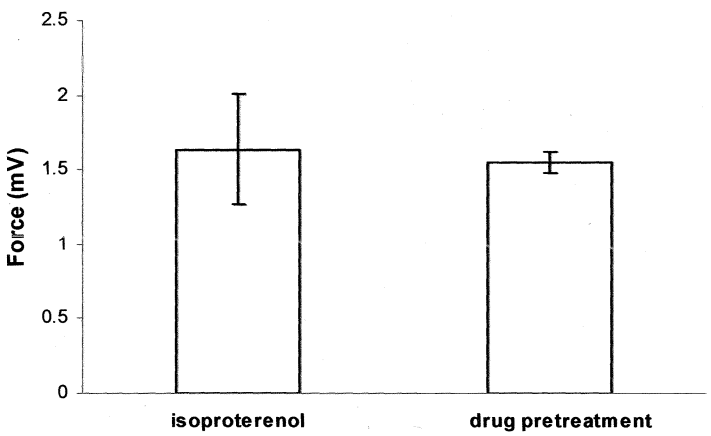
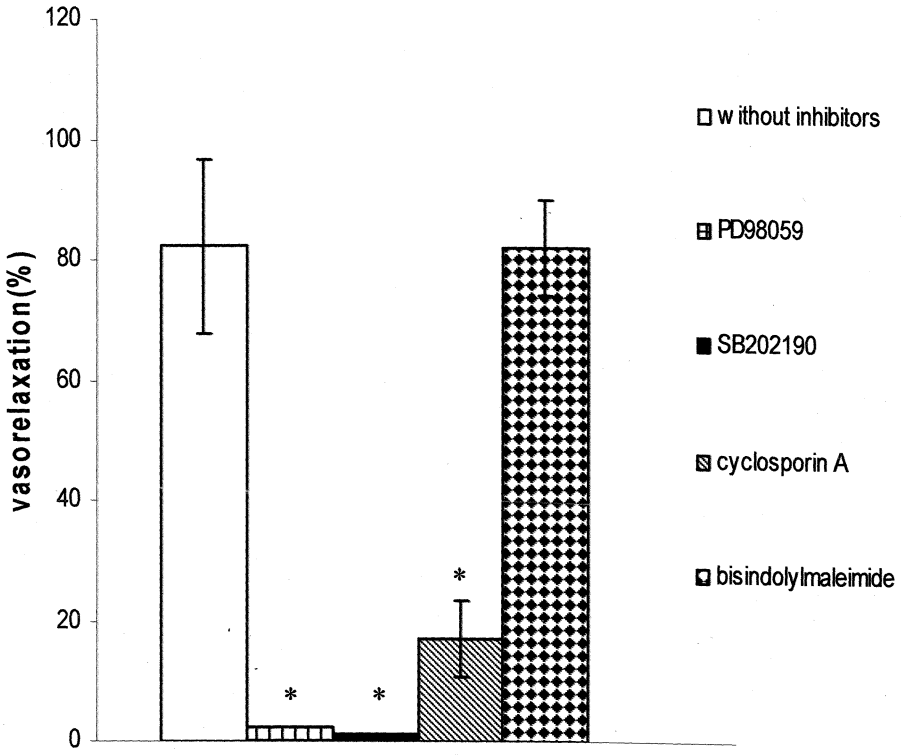


Fig.29 Recording showing papillary muscle contraction 1) control (baseline contraction) 2) on isoproterenol treatment



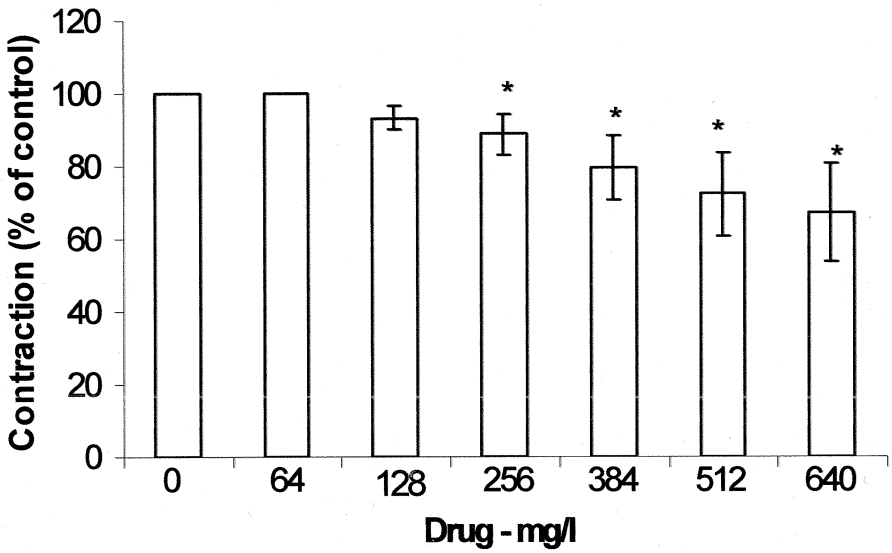
Values presented as mean ±SD, n = 3 determinations

Fig. 30 Contractile response of the papillary muscle to isoproterenol in the presence and absence of *Cardoguard* (64mg/L)



Values presented as mean ±SD, *p<0.001vs without inhibitor,
n=4 determinations

Fig.31 Percentage vasorelaxation by *Cardoguard* (64mg/L) on pretreatment with different signaling pathway inhibitors



Values presented as mean ±SD, * $p<0.01$ versus control,
n=6-8 determinations

Fig.32 Concentration dependent variations in inotropic response of papillary muscle to *Cardoguard*

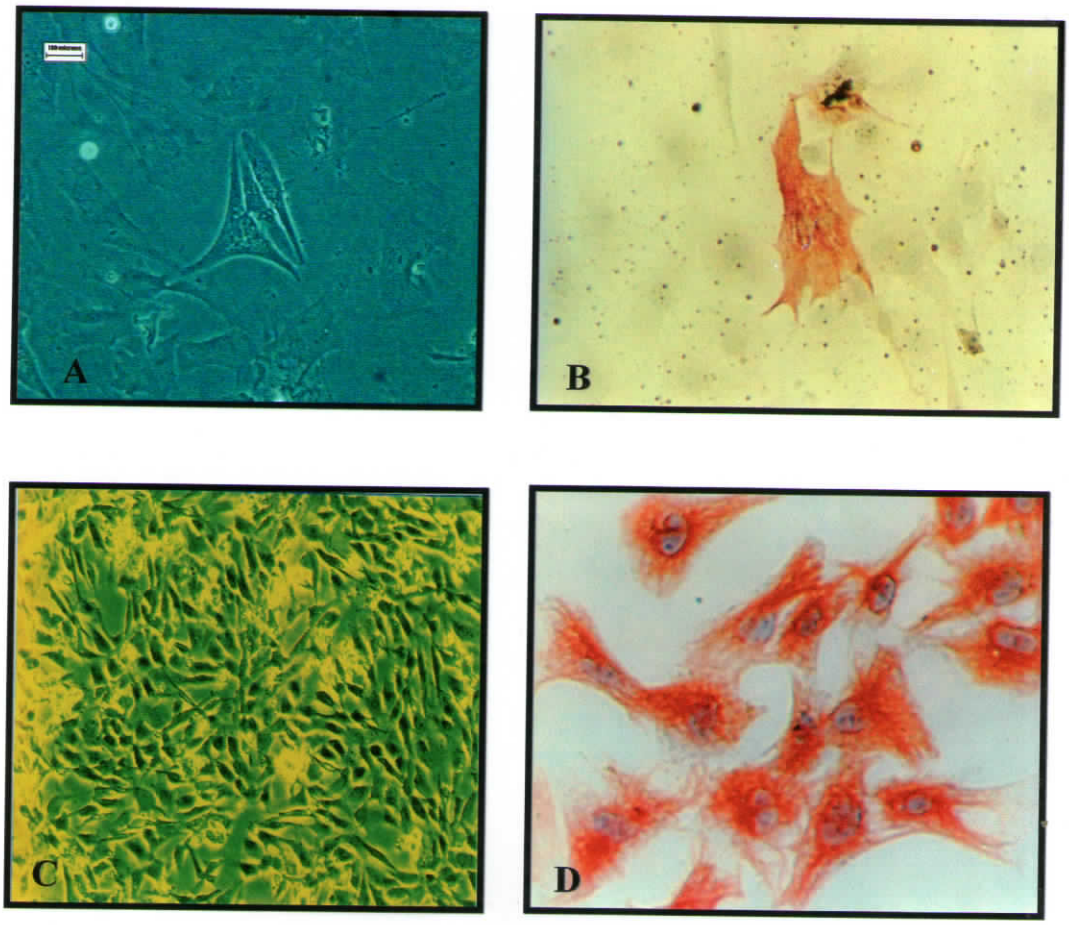


Fig.33 Photomicrographs of cardiomyocytes and fibroblasts A- Phase contrast image of cardiomyocytes (scale bar: 100μm) B- Cardiomyocyte stained with anti-desmin (magnification 200X) C- Phase contrast image of cardiac fibroblasts at near confluence (magnification 100X) D- Cardiac fibroblasts stained with anti- vimentin (magnification 200X)

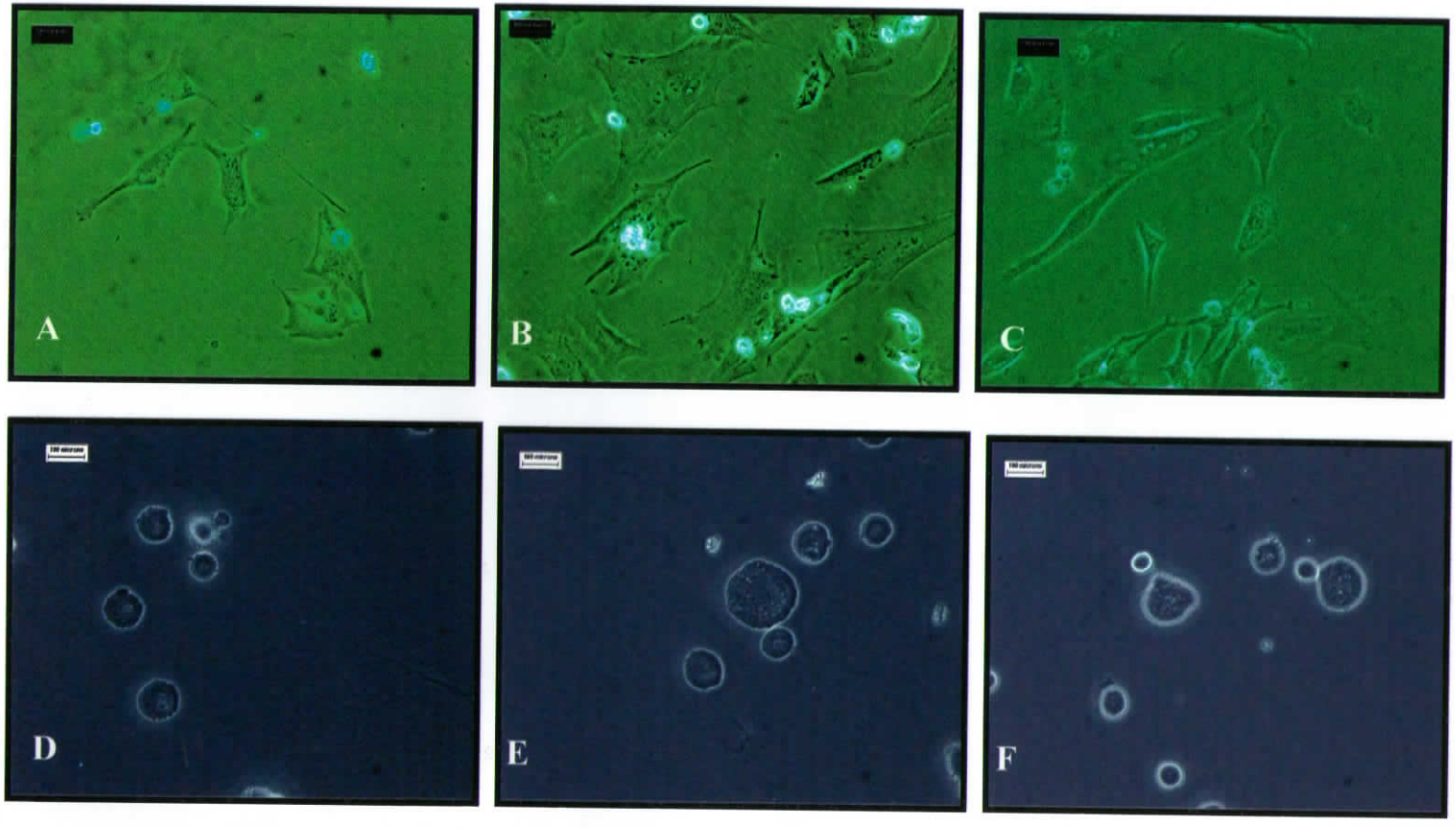
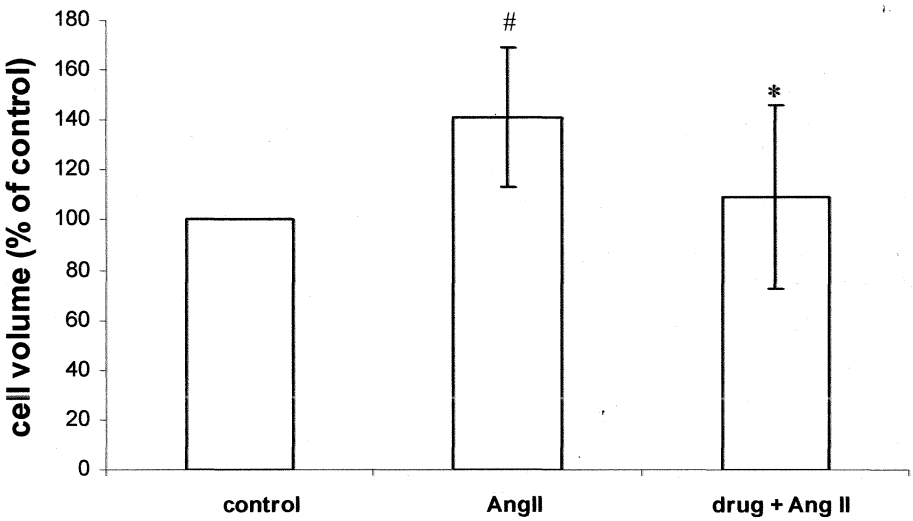
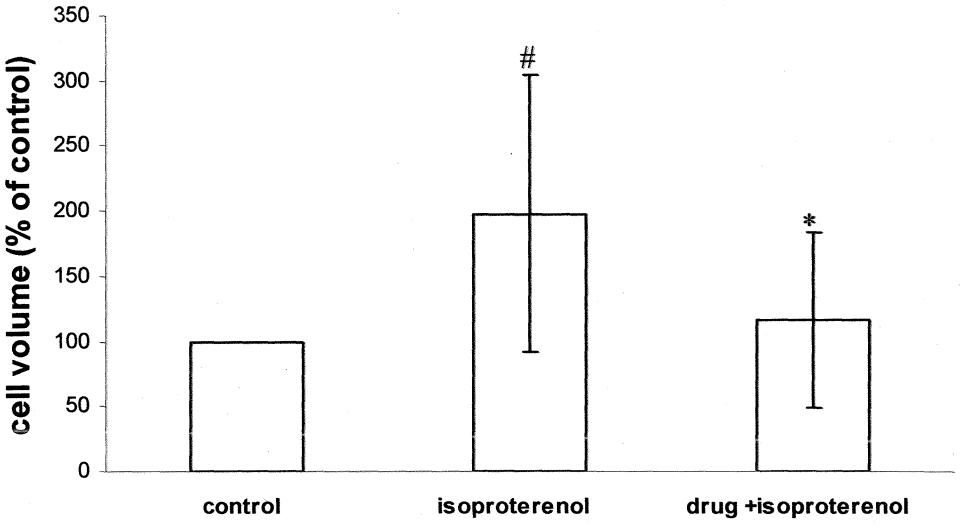


Fig.34 Cardiomyoctes attached to culture dish: A. control B. Ang II C. *Cardoguard* (64mg/L) +Ang II Differentially trypsinized cells: D. control E. Ang II F. *Cardoguard* (64mg/L) +Ang II; scale bar 100μm



Values presented as mean ±SD, # p <0.0001 vs control, * p <0.01 vs Ang II
No. of cells: control (n=100), Ang II (n=200), *Cardoguard* +Ang II (n=250)
n=3 determinations

Fig. 35 Volume of cardiomyocytes treated with Ang II with and without *Cardoguard* (64mg/L)



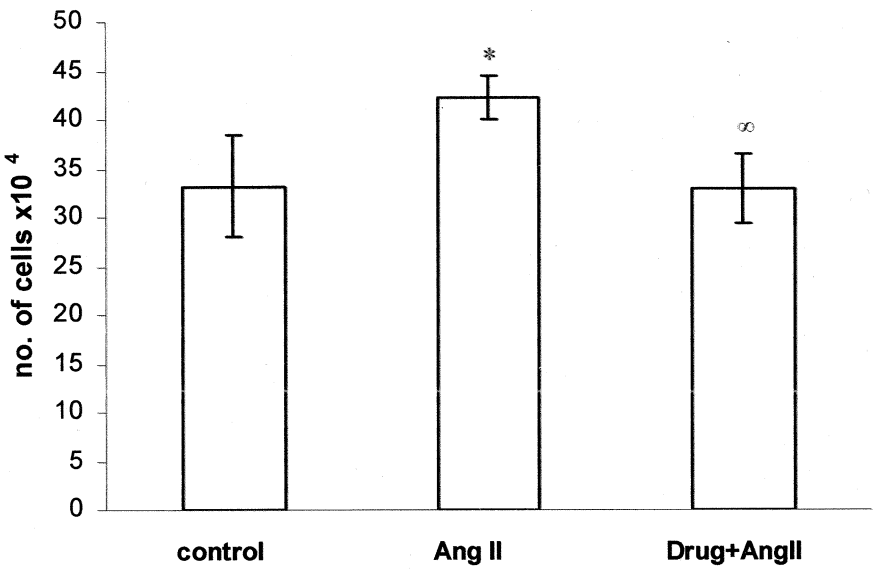
Values presented as mean ±SD, # $p<0.0001$ vs control, * $p<0.0001$ vs isoproterenol

No. of cells: control (n=100), isoproterenol (n=105),

Cardoguard +isoproterenol (n=100)

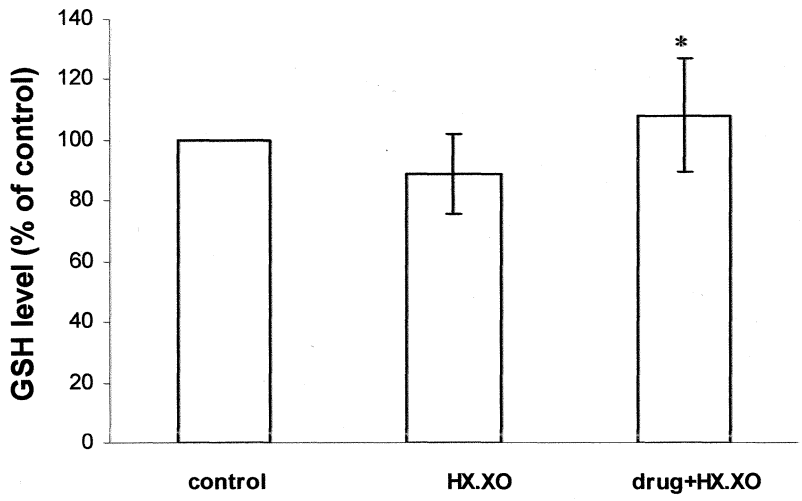
n =3 determinations

Fig. 36 Volume of cardiomyocytes treated with isoproterenol with and without *Cardoguard* (64mg/L)



Values presented as mean ±SD, * $p<0.01$ vs control,
∞ $p<0.05$ vs Ang II, n =4 determinations each

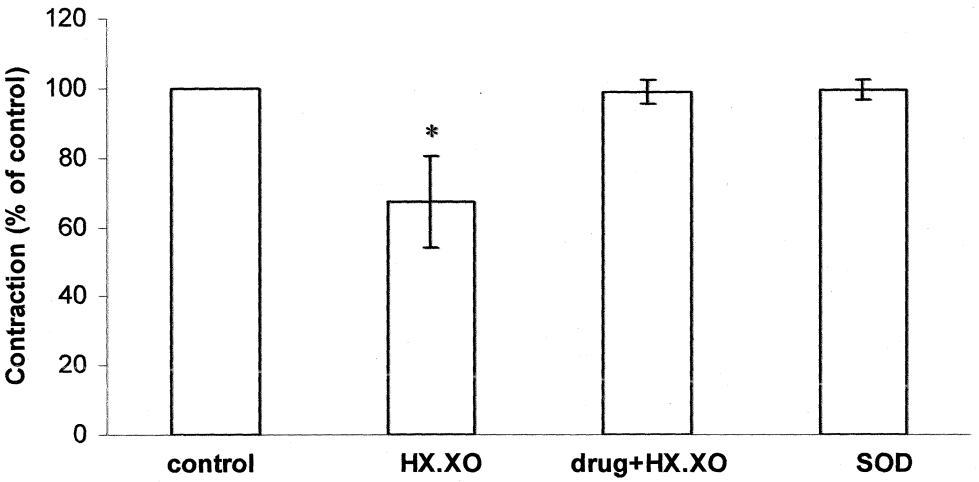
Fig. 37 Cardiac fibroblast cell count on treatment with Ang II in the presence and absence of *Cardoguard* (64mg/L)



Values presented as mean ±SD, *p<0.01 vs HX.XO, n=25

HX +XO (Hypoxanthine and xanthine oxidase)

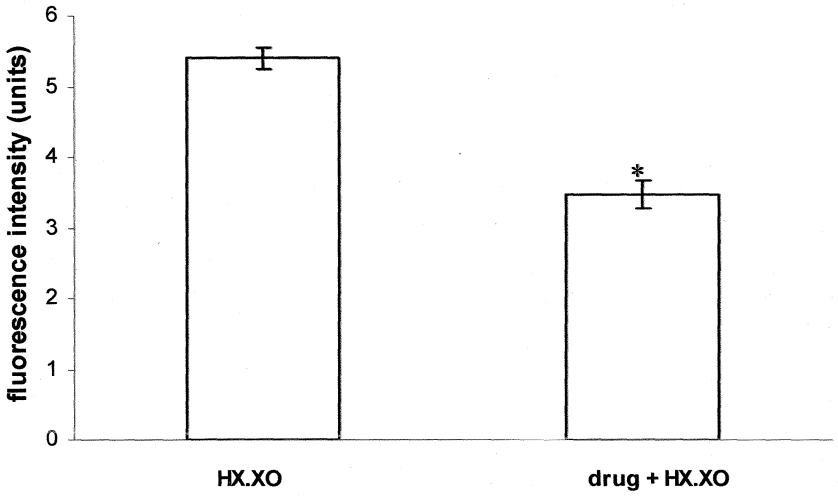
Fig. 38 Reduced glutathione levels in human RBC subjected to oxidative stress in the presence and absence of *Cardoguard* (64mg/L)



Values presented as mean ±SD, * $p<0.01$ vs control, n= 4 determinations

HX. XO (Hypoxanthine and xanthine oxidase), SOD (Superoxide dismutase)

Fig. 39 Inotropic response of papillary muscle to super oxide anion generator in the presence and absence of *Cardoguard* (64mg/L)



Values presented as mean ±SD, $p<0.0001$ vs HX.XO,
n= 4 determinations

Fig. 40 Assessment of ROS production by H_2DCF-DA incorporation in fibroblasts in the presence and absence of *Cardoguard* (64mg/L)

DISCUSSION

Ayurveda is a traditional Indian system of medicine. Widespread acceptance of Ayurvedic medicine is limited by the lack of authentic information on the mechanism of action, toxicity and the possible side effects of Ayurvedic formulations. Evaluation of the mechanism of action and efficacy of the Ayurvedic drugs using modern biological techniques will help to overcome the lacuna and will go a long way in better acceptance of these drugs.

Hypertension affects a large proportion of the middle-aged population worldwide. Sustained hemodynamic burden imposed by hypertension leads to cardiac remodeling that includes hypertrophy, fibrosis, electrical remodeling and functional changes. Cardiac remodeling is a serious risk factor for heart failure. Therefore, prevention of cardiac remodeling is a major therapeutic goal in the treatment of hypertension. *Cardoguard* is an Ayurvedic antihypertensive drug formulated by *Nagarjuna Herbal Concentrates Ltd.* This study was taken up with the aim of scientifically evaluating the efficacy of the drug in the reduction of blood pressure and prevention of cardiac remodeling. The study was also aimed at delineating the mechanism of action and toxicity testing.

The *in vivo* experimental procedures were carried out in spontaneously hypertensive rats. In accordance with Okamoto's original description, the elevation of arterial blood pressure in SHR could be divided into three stages: a *prehypertensive stage* when pressure is not different from that in normal controls, a stage of *labile hypertension* (140- 200 g body weight), and finally the stage of *established steady hypertension* which begins when the

rats mature to a weight of 200 g or more (usually when they are 8 weeks old) (Okamoto and Aoki, 1963). In contrast, arterial blood pressure in normal control (American Wistar or Kyoto-Wistar) rats remains more or less the same throughout their growth.

Many studies in literature have made use of Wistar Kyoto rat (WKY) as a control for SHR (Deschepper et al., 2002; Pfeffer et al., 1979; Slama et al., 2002). In a study by Aiello et al, a positive correlation between blood pressure and LV mass was found in 4-5 month old male SHR, whereas no such relationship was observed in age- matched WKY or Wistar rats. But in the same study, cardiomyocyte cross-sectional area was not significantly different in SHR and WKY rats; these values were significantly higher than those of Wistar rats. Papillary muscles isolated from the LV of WKY and SHR were stiffer than those isolated from Wistar rats. Consistently, a greater level of myocardial fibrosis was detected in WKY and SHR compared to Wistar rats. These findings demonstrate blood pressure-independent cardiac hypertrophy in normotensive WKY rats (Aiello et al., 2004). The findings by Aiello et al raise a concern about the use of WKY strain as an appropriate control for SHR when pharmacological interventions that may affect myocyte size and/or fibrosis are attempted. Hence in the present study, Wistar rat was used as the normontensive control wherever it was required.

In the study, the experiments carried out in SHR include validation of antihypertensive potential of the drug and its efficacy in prevention of cardiac and vascular remodeling.

Evaluation of antihypertensive potential of *Cardoguard*

The mean systolic blood pressure value of unanaesthetized 6-month old Wistar was found to be 119mmHg. This finding agrees with the mean systolic blood pressure value of 4-5 month old Wistar (118.5mm Hg) observed by Aiello et al in their study (Aiello et al., 2004). The mean systolic blood pressure value of 6-month old SHR was found to be

197mm Hg in the present study which was found to be higher than the mean systolic blood pressure value of 4-5 month old SHR (175 mm Hg) measured by Aiello et al using the tail cuff method similar to the present study (Aiello et al., 2004).

The antihypertensive potential of the drug is established by the capacity of the drug to reduce blood pressure in male SHR at 6 and 12 months of age (Table 2). Increasing the concentration of the drug did not have a beneficial effect. Studies using other antihypertensive drugs have also shown that the reduction of blood pressure in SHR is only marginal and does not attain the levels observed in normotensive rats. Doxazosin, an α-adrenoreceptor blocker, administered orally was found to decrease blood pressure in SHR by $\simeq$ 20 mmHg, and both atenolol, a β blocker and losartan, an AT1 blocker also lowered blood pressure to the same extent (Asai et al., 2005). This is comparable to the reduction of blood pressure in SHR at 12-months in the present study (Table 2). *In vitro* studies in isolated aortae have also established that the drug possesses vasorelaxant property with the maximum aortic relaxation being at the concentration assumed to be comparable to the therapeutic level (Fig.17 and 18).

There are reports on the antihypertensive properties of some of the constituent plants of *Cardoguard. Rauwolfia serpentina* is the main component of the drug and it is well documented as an antihypertensive (Fahim et al., 1995).Even in this study, maximum vasorelaxation was observed with *Rauwolfia serpentina* (57%) followed by *Terminalia chebula* (48%).The relaxation induced by the other components was less than 40% (Fig.19). *Terminalia arjuna and Emblica officinalis* has been reported to possess antioxidant and free radical scavenging functions (Bhattacharya et al., 2002; Gupta et al., 2001). The antihypertensive activity of *Terminalia arjuna* has also been described (Singh et

al., 1982). The antioxidant properties may also contribute to the hypotensive action (Aruoma et al., 1989). It is reported that production of oxygen free radicals such as super oxide anion is enhanced in vascular tissues from hypertensive animals including SHR and that oxidative stress is closely related to the development of hypertension (Kerr et al., 1999; Schnackenberg et al., 1998; Schnackenberg and Wilcox, 1999; Suzuki et al., 1995; Tschudi et al., 1996; Wu et al., 2001). Elevated levels of oxidative stress markers have also been detected in hypertensives (Carlos et al., 1998; Keith et al., 1998; Miller et al., 1998; Redon et al., 2003). According to Pechanova et al, mechanisms responsible for blood pressure reduction in SHR appeared to be related to both decrease in ROS level and increase in NO production indicated by the elevation of NO synthase activity and eNOS protein expression (Pechanova et al., 2006). *Ex vivo* studies have shown that *Cardoguard* possesses antioxidant property (Fig.38, 39, and 40). It has been reported that *Emblica officinalis* is a diuretic (Nadkarni and Nadkarni, 1999), but the diuretic property of the drug has not been tested due to lack of metabolic cages.One significant observation of this study is that none of the components in isolation can induce vasorelaxation comparable to that of *Cardoguard.*However, the effect is not purely additive suggesting an interaction between the components. Another interesting observation is the vasorelaxant capacity of *Terminalia chebula,* which at 1/3 the strength of *Rauwolfia serpentina* induces a relaxation close to 50%. This observation is significant because the vasorelaxant capacity of *T. chebula* has not been reported earlier. Moreover, this provides scope for modifying the formulation by reducing *R .serpentina* and substituting with *T.chebula.*

Role of *Cardoguard* in the prevention of cardiac remodeling

Cardiac remodeling is a complex pathological process that occurs in the clinical setting of heart failure (Cohn, 2004) and has been shown to be stimulated by the hemodynamic

effects of elevated blood pressure (Swynghedauw and Baillard, 2000). It is a complicated process that involves cardiomyocyte hypertrophy, proliferation of fibroblasts and interstitial fibrosis (Ritter and Neyses, 2003). ROS have been recognized as the molecular mediators of ventricular hypertrophy in pressure overload (Nakagami et al., 2003). Many of the deleterious cellular phenotypes presented in hypertrophied and failing myocardium might be attributed to oxidative stress (Sawyer et al., 2002). Oxidative stress can be a cause and/or consequence of hypertrophy development in SHR (Alvarez et al., 2008).

Cardiomyocyte hypertrophy and fibrosis in SHR have been studied by several investigators. Aiello et al, in their study, has observed that cross sectional area of cardiomyocytes from Wistar rats was 1.7 and 1.6 times smaller than that of WKY and SHR, respectively. Collagen, synthesized by the cardiac fibroblasts, is the major component of the interstitium that contributes to the structural integrity of the myocardium. In the hypertrophied heart, collagen accumulation resulting in fibrosis is one of the main determinants of myocardial stiffness and function (Sun and Weber, 2000; Weber, 1997; Weber et al., 1995). In SHR, left ventricular hypertrophy is known to be accompanied by increased collagen concentration (Brilla et al., 1996; Linz et al., 1995). Interstitial fibrosis is reported to be increased in SHR compared with Wistar rats as evidenced by the average collagen volume fraction values in the LV myocardium (Aiello et al., 2004). According to a study by Cingolani et al, total left ventricle collagen reduction *per se* does not necessarily benefit cardiac function. Hence, in hypertensive heart disease, other factors besides collagen quantity, such as myocyte hypertrophy, might be targeted to improve cardiac function (Cingolani et al., 2004).

In the present study, the mean ventricular weight to body weight ratio of 6- month old SHR was 14% greater than that of age-matched Wistar rats. The mean ventricular weight to body

weight ratio of treated SHR was found to be lower than that of age-matched SHR and comparable to control (Fig.6).

Morphological (hypertrophy index) and histological (LV wall thickness and cardiomyocyte CSA) evaluation established prevention of left ventricular hypertrophy in 6-month old treated SHR (Fig.6, 7, and 8.). The interstitial collagen deposition in treated SHR was lower than that in untreated animals though the difference was not statistically significant. However; the mean value was comparable to that of control (Fig.9).

At 12-months, the mean hypertrophy index and LV wall thickness was found to be lower in treated animals compared to SHR. The reduction in hypertrophy at 12 months was not as significant as that seen in younger rats. Increased oxidative stress in older rats may perhaps be responsible for the age dependent variation in response to treatment. A fall in the antioxidant levels in the 12-month old SHR compared to the 6-month old SHR (Vericel et al., 1994) and also age-related inhibition of vascular relaxation in SHR (Payne et al., 2003) has been reported. Pechanova et al. have documented that chronic effect of administration of an antioxidant, N-acetylcysteine (NAC) on cardiac hypertrophy in SHR is dependent on the age of SHR. NAC attenuated cardiac hypertrophy occurring in young spontaneously hypertensive rats and on the contrary, the effect of NAC was negligible in adult SHR (Pechanova et al., 2006). This may again serve as an explanation for the inability of the drug to effectively prevent LVH in SHR. Furthermore, the efficacy of the antihypertensive drug is dictated by age and the hypertensive stage of the animals (Demirci et al., 2005). It is well known that, antioxidants do not inhibit ROS production; they only ameliorate oxidative stress (Touyz, 2004).

Sen et al has demonstrated that antihypertensive therapy with either α-methyldopa, a centrally acting antihypertensive or hydralazine, a vasodilator, reduced blood pressure

especially in hypertensive rats; however, ventricular weight was reduced by methyldopa, but not by hydralazine. The dissociation between the arterial blood pressure response to hydralazine and the persistence of cardiac hypertrophy suggests that blood pressure might not be the sole factor contributing to cardiac hypertrophy in the spontaneously hypertensive rat (Sen et al., 1974). The effect of β-blockers in SHR is controversial; a few authors report no effect on blood pressures (Sen et al., 1977), whereas others describe that metoprolol decreased both blood pressure and cardiac weight (Lundin and Hallback-Nordlander., 1984).

Effect of *Cardoguard* on electrical and functional remodeling

At 6 months of age, there was significant increase in the left ventricular wall thickness of untreated SHR compared to treated SHR and Wistar rats (Fig.7).However, no apparent changes on ECG of treated and untreated SHR were observed at 6 months.Whereas in 12-month old, ECG changes were relatively more among untreated SHR. Left axis deviation (LAD), which is a distinguishing feature of left ventricular hypertrophy, was found only in untreated SHR. LAD is an abnormal extension of the QRS axis, which in the normal state, lies between -30^{o} and $+100^{o}$. LAD has been reported in SHR by Kleber et al (Kleber et al., 1982). ST segment abnormalities are associated with hypertrophy and transient myocardial ischemia. Animals with ST changes were slightly more among the untreated SHR. The changes in ST segment on ECG are considered to be disturbances of repolarization processes associated with LVH (Riabykina et al., 2008).

An age dependent decrease in cardiac output was observed in the experimental animals (Fig.12). But in normotensive rats, no change in cardiac output is observed at ages 6, 12 and 18 months (Chang et al., 2000). In SHR, cardiac output is maintained by the induction of compensatory hypertrophy secondary to the increased after load (Frohlich, 1983).

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However, pumping ability of the heart tends gradually to decline with age and finally results in a decompensated state (Pfeffer et al., 1979). This phase of cardiac decompensation is found after prolonged and severe hypertension. Recent studies cite evidence in support of the fact that at 10-12 months of age, the SHR showed delayed relaxation and increased diastolic stiffness whereas systolic performance is increased. Alterations in both active relaxation and passive compliance characterize the diastolic dysfunction observed in adult hypertensive animals. It is well known from clinical data, as well as from some animal models, that alterations in diastole precede the development of heart failure in hypertensive heart disease (Cingolani et al., 2003). The mean stroke volume in 12 month old SHR was found to be 319± 88μL, comparable to an early study by Cingolani et al, where the stroke volume was approximately 350μL (Cingolani et al., 2003). In the present study, at 6 and 12 months of age, the mean heart rates of treated and untreated SHR were comparable. At 6-months, a significantly higher cardiac output (Fig.12) ($p<0.05$) was observed in treated SHR consequent to an increase in stroke volume (757 ± 237μL vs. 520 ± 266μL).A similar beneficial effect was observed even at 12 months of age. There was a significantly higher stroke volume (459 ± 145 μL vs.319 ± 88 μL) ($p<0.05$) leading to an increase in cardiac output (Fig.12). The observation that the mean stroke volume and cardiac output of treated SHR was higher than that of untreated SHR in the absence of chronotropic variation suggests that cardiac function is relatively improved in treated SHR.

As a result of evaluation of electrical and functional parameters, it was found that the drug has a beneficial effect on the electrical and mechanical function in the experimental animals.

The effects of some of the components of the drug on the heart were studied by several investigators. *Terminalia arjuna* was reported to increase left ventricular stroke volume index (Bharani et al., 1995); and, *Terminalia chebula* was found to increase cardiac output, without increasing heart rate (Reddy et al., 1990). On the other hand, negative chronotropic effect of *Terminalia arjuna* has also been reported (Singh et al., 1982). Since there is absence of chronotropic variation on treatment in the present study, it is possible that in *Cardoguard*, some of the other components may be neutralizing the negative chronotropic effect of *Terminalia arjuna*.

An earlier study on treatment of SHR with felodipine, a calcium channel blocker has reported similar observations. A 12-week treatment of SHRs with felodipine lowered blood pressure and peripheral resistance; without change in heart rate, but cardiac output was significantly increased (Nordlander et al., 1985).

Signaling pathways modified by the drug

Pressure overload stimulates a medley of signaling pathways leading to hypertrophic remodeling. In an attempt to identify the signaling pathway modified by the drug, the major signaling proteins in cardiac hypertrophy were analyzed in 6-month old SHR by immunohistochemisty and quantified using Image analyzer. The quantitation was based on chromogen gray levels. It was observed that the ERK and PKC ε are modulated by the drug (Fig.10A, 10B). Hence the antihypertrophic changes are probably mediated by the down regulation of these pathways.

The role of extracellular signal - regulated kinases in cardiac hypertrophy has been investigated in quite a lot of studies. The ERK1/2 kinases have been thought to play important roles during hypertrophic responses of cardiomyocytes both *in vitro* (Kim et al., 2002) and *in vivo* (Takeishi et al., 2001) although the activation of ERKs does not always

lead to cardiomyocyte hypertrophy (Post et al., 1996). In a study by Zhai et al., it was observed that knockdown of glycogen synthase kinase-3alpha, a negative regulator of cardiac hypertrophy in mice increased ERK phosphorylation, an effect that was inhibited by an inhibitor of ERK, PD98059, and also protein kinase C ε inhibitor peptide (Zhai et al., 2007). An earlier study has demonstrated that oxidative stress evoked signal transduction pathways leading to activation of ERKs in cultured cardiomyocytes of neonatal rats (Aikawa et al., 1997). The activities of ERKs in the heart were elevated in young SHR and decreased with age (Aoyagi and Izumo, 2001). The p-ERK level of SHR was significantly higher than that of age-matched WKY rats from 8-weeks to 24-weeks of age indicating its role in the development of hypertension induced -myocardial hypertrophy (Huang et al., 2005).

There is cumulative *in vitro* and *in vivo* evidence suggesting an important role for PKC pathways as mediators of hypertrophic signals, but the precise pattern of the isozymes involved remains controversial. Many studies have proposed a role for PKC ε in pressure overload hypertrophy. Aortic banding in guinea pigs, which causes pressure overload hypertrophy, results in a temporally related increase in PKC ε (Paul et al., 1997). Cardiac-specific over expression of constitutive active PKCε or peptide activator molecules of either PKCε or PKCδ isozymes are all sufficient to produce cardiac hypertrophy with preserved contractile function in transgenic mice (Chen et al., 2001 a). It has been reported that PKCε was translocated by stretch in SHR (Ahn et al., 2007). Studies also delineate the involvement of ROS in the activation of PKC ε (Zhang et al., 2002). Of particular interest was the observation by Wu et al that PKC ε signaling is a compensatory event in myocardial hypertrophy, rather than a pathological event (Wu et al., 2000). Nonetheless, a role for PKC ε activity has been demonstrated during the progression to heart failure in

hypertensive Dahl rats (Inagaki et al., 2008).

Since increased oxidative stress has been implicated in SHR, it is suggested that the drug possessing antioxidant capacity may have suppressed the oxidative stress-evoked signaling pathways, ERK and PKCε. A schematic diagram is therefore presented showing the sites which are possibly modified by the drug, leading to the modulation of cardiac hypertrophy.

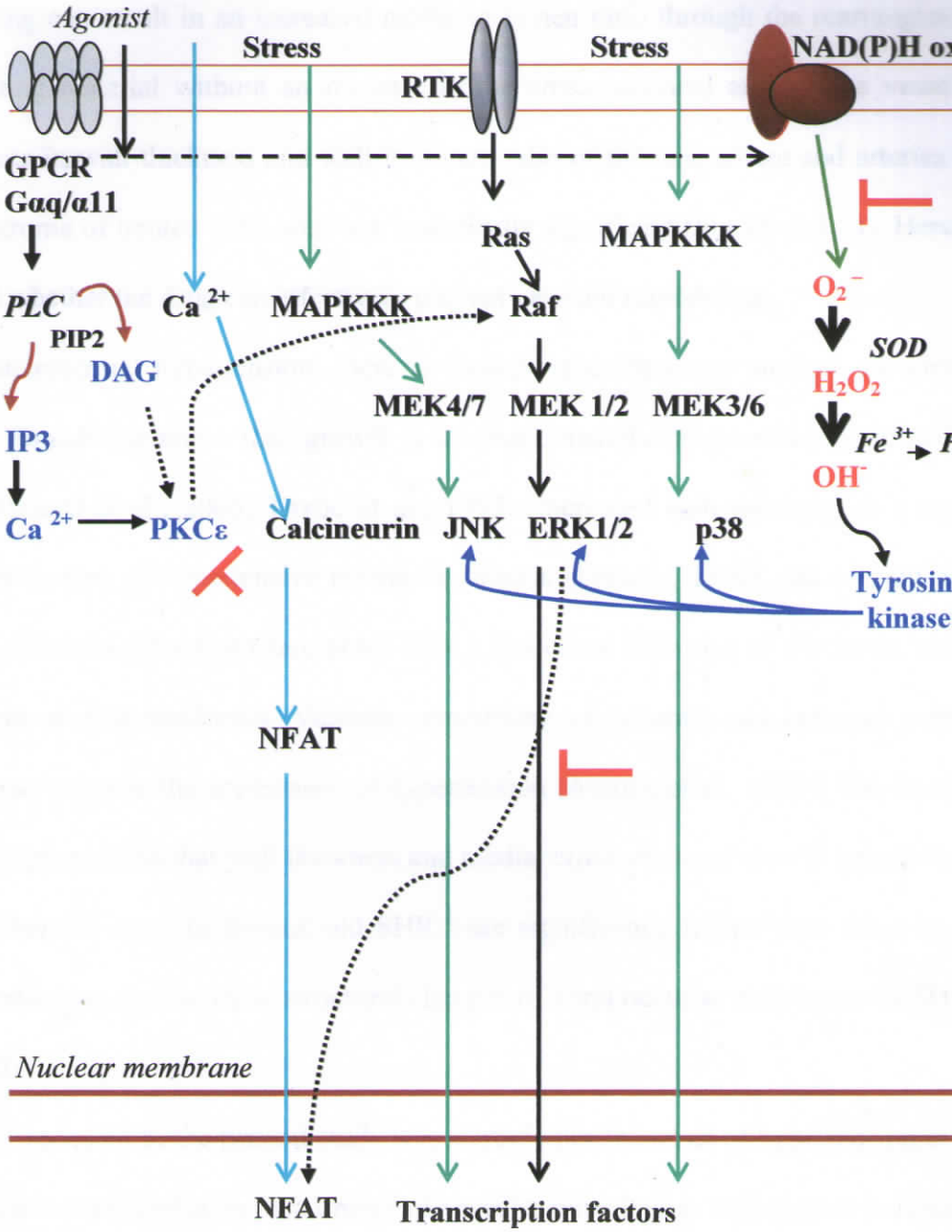

Fig. 41.Schematic representation of intracellular signal transduction pathways
coordinate the cardiac hypertrophic response and their modulation by *Cardogua*

⊣ denotes inhibition by *Cardoguard*

Role of *Cardoguard* in vascular remodeling

The drug was evaluated for its efficacy in prevention of vascular remodeling. Vascular remodeling can result in an increased media to lumen ratio through the rearrangement of the existing material without an increase in the cross sectional area of the vessel. The differences in wall thickness and wall to lumen ratio of thoracic aortae and arteries in the kidney stroma of treated SHR were not statistically significant (Fig.13 and 14). Hence it is doubtful whether the drug can effectively prevent vascular remodeling.

In human essential hypertension, there is increasing evidence to support the view that vascular remodeling rather than growth is the predominant change occurring in resistance vessels (Izzard et al., 2006; Thybo et al., 1995). Increased wall thickness is a common structural feature of hypertensive resistance vessels (Folkow, 1990) and conduit arteries such as, the aorta (Chamiot-Clerc et al., 2001). Structural alteration of the aortic wall may also affect arterial mechanics. Vascular remodeling of systemic and coronary resistance vessels may precede the appearance of hypertension (Antony et al., 1993). The findings of Ge et al demonstrated that wall thickness and medial cross-sectional area to lumen diameter ratio of thoracic aorta in 8-week old SHR were significantly higher than those in WKY group, indicating that vascular structural changes of aorta occur at early stage of SHR (Ge et al, 2003).

The drug treatment in the present study was started after the onset of hypertension in SHR, and almost certainly after the occurrence of vascular remodeling. This may also explain the lack of reduction of blood pressure in treated SHR to the level seen in normotensive rats.

Previous investigators have shown that pharmacologic treatment in SHR attenuates the

hypertensive structural changes in large arteries and this effect is associated with a reduction in blood pressure (Benetos et al., 1994; Benetos et al., 1997; Levy et al., 1994). Morphological analysis of aorta in DOCA-salt hypertensive rats showed significant increases in wall thickness and wall area. There was also thickening of small arteries in histological studies of the kidney (Matsumura et al., 2001). According to Matsumura et al, since hypertension itself is a major contributory factor for vascular hypertrophy, it is possible that the blunting of the rise in blood pressure of DOCA-salt rats fed with Brand's Essence of Chicken, a popular chicken extract is associated with the absence of vascular changes (Matsumura et al., 2001). On the other hand, an earlier study by Wang and Prewitt using experimental hypertensive rats demonstrated that the attenuation of vascular hypertrophy was observed after captopril treatment at a dose which did not lower blood pressure effectively (Wang and Prewitt, 1990). Moreover, vasorelaxing agents such as hydralazine failed to suppress vascular hypertrophy, even at a hypotensive dose (Owens, 1985). These observations suggest that factors other than blood pressure *per se* are involved in the vascular hypertrophy.

Evaluation of drug toxicity

Toxicity study was carried out in SHR to assess the safety of the drug. Organs such as kidney and liver, the functions of which can be transiently disrupted by adverse drug toxicity effects, were of investigative concern. Long-term toxicity was determined in 12-month old SHR by histopathological analysis of kidney and liver. No signs of toxicity were observed which shows that the drug does not produce any adverse reactions in the tissues of SHR.

Evaluation of neurodepressive action of the drug

The neurodepressive action in response to the drug was also evaluated. Reserpine, a component of *Rauwolfia serpentina*, though popular in the 1950's as an antihypertensive, went out of favor due to onset of psychotic depression on long-term use. According to Ayurveda, the use of whole plant reduces the risk of side effects that occur when isolated active components are used. An experiment was designed to examine whether the unfavorable side effect of reserpine will be neutralized in *Cardoguard*. The activity of brain mitochondrial monoamine oxidase, a marker for depression was assayed in Wistar rats treated with *Cardoguard* and compared with the values in rats treated with reserpine. MAO activity in the central nervous system can regulate levels of monoamine neurotransmitters and other biogenic amines; and alterations in MAO activity have been implicated in some nervous disorders (Pintar and Breakefield, 1982). Depression is caused by a functional deficiency of catecholamines, particularly norepinephrine (Heninger et al., 1996).

Rodriquez de Lores Arnaiz and De Robertis investigated the distribution of monoamine oxidase in rat brain homogenates and concluded that this enzyme is localized exclusively in the mitochondria and is not found in synaptic vesicles or intact nerve endings (Rodriguez De Lores Arnaiz and De Robertis, 1962). Hence brain MAO activity was assessed in crude mitochondrial samples. The mean fluorescence intensity of the brain samples of reserpine-treated rats was significantly higher than that of control; and the mean fluorescence intensity of the brain samples of *Cardoguard*- treated rats was found to be lower than that of reserpine- treated rats, and not significantly higher than that of control (Fig.16). The absence of a significant increase in brain MAO activity of *Cardoguard*- treated Wistar rats compared to control indicates the plausibility that the drug is less depressogenic compared

to the isolated active principle, reserpine. This may be due to synergistic effect of the various principles in the whole plant *(Rauwolfia)* or due to the combined effect of other components in the drug.

Contrary to the prevailing belief, there is a recent study that argues that reserpine is not depressogenic (Baumeister et al., 2003). This opens a new avenue for consideration.

Delineation of the mechanism of vasorelaxation by *Cardoguard*:

Vasorelaxation is the phenomenon that contributes to the lowering of blood pressure. *Ex vivo* studies using aortic rings from normotensive rats confirmed the vasorelaxant potential of the drug (Fig.17), thereby supporting the hypotensive effect of *Cardoguard* observed *in vivo* (Table 2). Maximum reduction of isometric force was seen at the therapeutic levels (Fig.18).

Hypertensinogenic factors are diverse; some of the important factors linked are endothelial dysfunction, impaired function of potassium channels, increased calcium entry into the cell, increased Ang II levels, increased β-adrenergic activity etc.

Since hypertension is associated with endothelial dysfunction (Bouloumie et al., 1997; Miligard and Lind, 1998), it was investigated whether vasorelaxation by the drug is endothelium dependent or not. From the observations, the mechanism of vasorelaxation was found to be endothelium dependent (Fig. 20, 21, 22 and 23).

Endothelial dysfunction in essential hypertension is characterized by an imbalance between the release of endothelium-dependent relaxing (EDRF) and contracting (EDCF) factors. In SHR, the production of EDCF is prominent in the arteries (Luscher and Vanhoutte, 1986; Vanhoutte et al., 2005). Decreased endothelium-derived relaxation was observed in the aorta (Luscher, 1990), mesenteric resistance vessels (Tesfamariam and Halpern, 1988; Watt and Thurston, 1989), cerebral arteries (Mayhan, 1990) and coronary arteries (Pourageaud

and Freslon, 1995) in SHR. It was recently demonstrated that cyclo oxygenase pathway may be crucial in the production of EDCF in SHR (Tang et al., 2007). Constricting cyclooxygenases augment vascular reactivity, contributing to the development of hypertension (Tomida et al., 2003). NO deficiency can potentiate excess production of constricting cyclooxygenases (Bratz and Kanagy, 2004). Also, endothelial cyclooxygenase-2 (COX-2) products induce vasoconstriction partly by inhibiting NO-induced dilatation (Taddei et al., 1997). In the study, the drug-induced vasorelaxation may be mediated by NO and/or prostacyclin as the relaxant responses were inhibited both in L-NAME and indomethacin- preincubated aortic rings respectively (Fig.22 and 23). Tang et al observed that oxygen-derived free radicals play a significant role in endothelium-mediated vasoconstriction (Tang et al., 2007). It is certain that oxidative stress plays an important role in hypertension. Although the mechanism by which antioxidant treatment improves vasorelaxation is unclear, it is generally accepted that antioxidants scavenge super oxides and thus increase the bioavailability of NO. *Cardoguard,* by virtue of its antioxidant capacity possibly induces vasorelaxation by enhancing endothelial NO/EDRF availability. Drugs with both antihypertensive and antioxidant properties offer promising results in the management of hypertension.

Additionally, the drug was found to act through activation of ATP-dependant K^+ channel which is apparent from the absence of vasorelaxation on preincubation with glibenclamide (Fig.24). In a study in DOCA- hypertensive rats, the antihypertensive effect of DU-1777, a long acting ACE-inhibitor was fully antagonized by glibenclamide (Nagata et al., 1997). However, *in vitro*, DU-1777 did not affect aortic ring contractions induced by high K^+. According to Nagata et al., DU-1777 itself does not open ATP-dependent K^+ channels; the antihypertensive effect observed *in vivo* was an indirect effect involving unknown mechanisms.

Endogenous vasodilators, such as NO and prostacyclin, act at least in part by opening K_{ATP} channels (Murphy and Brayden, 1995). Moreover; hyperpolarizing effect of K_{ATP} channel activation reduces Ca^{2+} influx through voltage-dependent Ca^{2+} channels in vascular smooth muscle (Mironneau and Macrez-Lepretre, 1995).

Cardoguard also antagonizes calcium influx as apparent from the absence of vasoconstriction on cumulative increase in extracellular calcium (Fig.25). This action of *Cardoguard* was similar to that of verapamil, a Ca^{2+} channel antagonist. The Ca^{2+} antagonistic action of the drug can also be explained by the data indicating the relaxant responses to both phenylephrine and high concentration of KCl (Fig.26) eventhough the extent of relaxation of high K^+ constricted rings was significantly less compared to phenylephrine- constricted rings. Both phenylephrine and KCl induce vasoconstriction by an increase in intracellular calcium concentration through calcium entry, even though by two different mechanisms (Karaki et al., 1997). Phenylephrine-induced vasoconstriction is mediated by the stimulation of G-protein coupled to α-adrenoceptors whereas KCl induces smooth muscle contraction by membrane depolarization, through the activation of voltage-dependent calcium channels and subsequent release of calcium from the sarcoplasmic reticulum. Moreover; activation of voltage operated calcium channels can occur directly by agonist-induced membrane depolarization and indirectly by agonist activated G proteins (McFadzean and Gibson, 2002). It has been reported that some of the Ca^{2+} channels regulated by agonists are voltage-dependent in as much as they are inhibited by blockers of these channels (Exton, 1988).

Thus it can be inferred that intracellular calcium mobilization is a target for the vasorelaxant effect of *Cardoguard.*

In a study by Dimo et al, *Bidens pilosa*, a plant widely used in Cameroon as an antihypertensive showed relaxant effects in rat aortic rings when contracted with KCl and norepinephrine and it was suggested that the relaxation effect was due to blockade of the influx of intracellular calcium (Dimo et al., 1998). The calcium channel antagonistic effect of *Carum copticum* seed extract possessing hypotensive action was established by the fact that the extract shifted the Ca^{2+} dose-response curves to right similar to verapamil (Gilani et al., 2005c). These observations agree with the findings of the present study. In an earlier study, Ress et al, reported that clonidine, a centrally acting antihypertensive, attenuated significantly the development of active tension by norepinephrine but did not inhibit significantly the development of active tension following membrane depolarization with KCl (Ress et al., 1979). Another study has reported that reserpine inhibits L-type Ca^{2+} channels thereby decreasing cytosolic calcium levels (Satoh et al., 1992).

Cardoguard did not inhibit Ang II induced vasoconstriction of the aortic ring and isoproterenol induced inotropic variation of the papillary muscle (Fig. 28 and 30), indicating that it does not have a direct action on these receptors.

The mechanisms of vasorelaxation induced by *Cardoguard* may be due to its different constituents operating in a synergistic manner. *Cardoguard* is found to induce endothelium dependent vasorelaxation, which appears to be mediated through NO / COX pathways, associated with activation of K_{ATP} channels (Fig.20, 21, 22, 23, and 24). Hyperpolarizing effect of K_{ATP} channel activation reduces Ca^{2+} influx through voltage-dependent Ca^{2+} channels in vascular smooth muscle. The drug was found to possess calcium antagonistic action as well (Fig.25 and 26).

Impairment of endothelium-dependent vascular relaxation / endothelial dysfunction is associated with oxidative stress that causes a reduction in NO availability (Moriguchi et al.,

2005). Nifedipine, a dihydropyridine calcium channel blocker, by its calcium antagonistic function increases endothelium-dependent vasodilation by restoring NO availability, an effect probably determined by antioxidant activity (Taddei et al., 2001). Polyphenolic antioxidants mimic the effects of dihydropyridines and show structural similarity to dihydropyridines (Carraway et al., 2004). Many of the constituent plants of *Cardoguard* such as *Terminalia chebula, Emblica officinalis and Terminalia arjuna* have tannins (water soluble polyphenols) as their active principles. The chemical structures of the dihydropyridines and polyphenols are similar; each possessing aromatic ring structures with redox capability (Carraway et al., 2004).The antioxidant capacity of the drug has also been established in the study (Fig. 38, 39, and 40.) Hence, it can be stated that the Ayurvedic antihypertensive formulation by virtue of its antioxidant capacity associated with its calcium antagonistic action enhances endothelium-dependent relaxing factor availability thereby inducing vasorelaxation.

The signaling pathways involved in vasorelaxation by the drug were studied and is found to be mediated by ERK, p38 MAPK and calcineurin pathways as the relaxation was affected in the presence of the respective inhibitors : PD98059, SB 202190 and cyclosporin A but not in the presence of bisindolylmaleimide, the PKC inhibitor (Fig.31).

Vasorelaxation by *Cardoguard* was found to be inhibited in the presence of ERK inhibitor (Fig.31). It was shown (Fig.23) that NO and prostacyclin are involved in vasorelaxation by the drug. According to Oliveira et al., NO and cGMP stimulate a signaling pathway involving p21Ras-Raf-1 kinase-MEK ERK1/2 (Oliveira et al., 2003). *Cardoguard* acts as a K_{ATP} activator as evident from the lack of relaxation on pretreatment with glibenclamide (Fig.24). The activation of K_{ATP} comes about through the release of vasodilators from endothelial cells and from surrounding tissue (Quayle et al., 1997). NO stimulation of K_{ATP}

is indirect and the Ras/ MAPK pathway underlies NO mediated enhancement of K_{ATP} channel function (Lin et al., 2004). Vasorelaxation was found to be inhibited in the presence of p38 MAPK inhibitor (Fig.31). According to a very recent study, p38 inhibition abrogated the upregulation of cyclooxygenase-2 products involved in vasoconstriction and PGI_2 (prostacyclin) release (Martinez-Gonzalez et al., 2008). Calcium activates the protein phosphatase, calcineurin. Protein phosphatases play an important role in the regulation of calcium sensitivity of the contractile apparatus of smooth muscle (Somlyo and Somlyo, 1994).Calcium dependent inactivation of L-type calcium channels is partly inhibited by cyclosporine A (Schuhmann et al, 1997).Vasorelaxation by *Cardoguard* was found to be inhibited in cyclosporine A- preincubated aortic rings (Fig.31). Kou et al has reported that cyclosporin A-induced hypertension involves, at least in part, the attenuation of endothelium-derived NO production through a calcineurin-sensitive pathway regulating eNOS dephosphorylation (Kou et al., 2002).

To summarize, vasorelaxation by *Cardoguard* appears to be mediated by the virtue of its antioxidant capacity associated with its Ca^{2+} antagonistic action, thereby enhancing NO/prostacyclin availability at the level of vascular smooth muscle. The release of these endothelium-dependent vasodilators is associated with activation of K_{ATP} channels whose activity appears to be modulated by *Cardoguard.* Vasorelaxation by the drug was found to involve MAPK (ERK and p38) and calcineurin pathways. Stimulation of NO by the drug may be associated with the signaling pathway involving Ras-Raf-MEK-ERK1/2. The involvement of p38 in drug-induced vasorelaxation was also demonstrated that indicates the participation of COX pathway. The Ras/ MAPK pathway may be involved in the NO-mediated enhancement of K_{ATP} channel function by the drug. Furthermore, the drug may

act via the calcineurin-sensitive pathway regulating eNOS dephosphorylation.

Figure 42 represents a schematic diagram showing the various hypertensinogenic factors and their modulation by the drug.

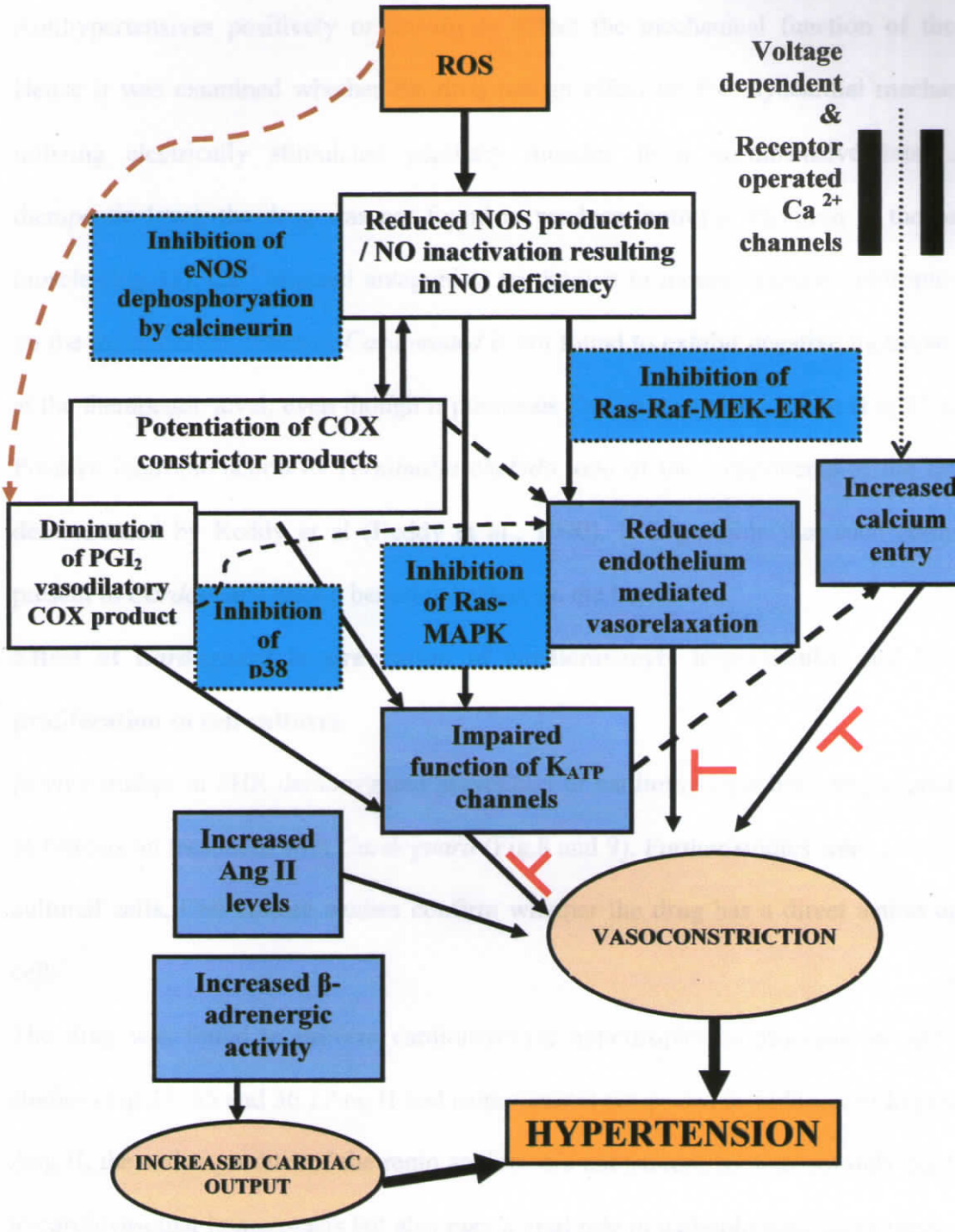

Fig.42. Schematic representation of hypertensinogenic factors and their modulation by *Cardoguard*. ⊢ denotes inhibition *by Cardoguard*. ▪ denotes the signaling pathways modulated by the drug.

Effect of *Cardoguard* on myocardial mechanics

Antihypertensives positively or negatively affect the mechanical function of the heart. Hence it was examined whether the drug has an effect on the myocardial mechanics by utilizing electrically stimulated papillary muscles from normotensive rats. At the therapeutic level, the drug was not found to produce inotropic variation in the papillary muscle (Fig.32). Ca^{2+} channel antagonists are known to induce negative inotropic effects on the myocardium, whereas *Cardoguard* is not found to exhibit negative inotropic effects at the therapeutic level, even though it possesses Ca^{2+} antagonistic action (Fig.25 and 26). Positive inotropic action of *Terminalia chebula*, one of the components of the drug was demonstrated by Reddy et al (Reddy et al., 1990). It is possible that such components present in *Cardoguard* have a beneficial effect on the heart.

Effect of *Cardoguard* in prevention of cardiomyocyte hypertrophy and fibroblast proliferation in cell cultures

In vivo studies in SHR demonstrated prevention of cardiomyocyte hypertrophy and extent of fibrosis on treatment with *Cardoguard* (Fig.8 and 9). Further studies were carried out in cultured cells. Cell culture studies confirm whether the drug has a direct action on these cells.

The drug was found to prevent cardiomyocyte hypertrophy as observed in cell culture studies (Fig 34, 35 and 36.).Ang II and isoproterenol were used as inducers of hypertrophy. Ang II, the active product of the renin angiotensin aldosterone system not only contributes to cardiovascular homeostasis but also play a vital role in pathophysiologic processes, such as myocardial hypertrophy and remodeling (Pagliaro and Penna, 2005; Paul et al., 2006). ROS plays an important role in cardiomyocyte hypertrophy mediated by AngII (Bendall et al., 2002; Nakamura et al., 1998; Sugden and Clerk, 1998). According to Li and Shah,

Ang II stimulates production of super oxide (Li and Shah, 2004). Suppression of ROS formation was found to inhibit Ang II–induced hypertrophy (Laskowski et al., 2006; Tanaka et al., 2001). Isoproterenol is a β-adrenoreceptor agonist and a known inducer of hypertrophy. β -adrenoreceptor stimulation also provokes cardiac oxidative stress similar to Ang II. An *in vivo* study by Zhang et al has demonstrated that cardiac oxidative stress increased in response to β-adrenoreceptor stimulation by acute isoproterenol infusion, and that cardiac ERK1/2, p38 and JNK MAP kinase activation triggered by the β-adrenoreceptor agonist was mediated through ROS generation. In the chronic phase of isoproterenol infusion, ROS appeared to participate in cardiac remodeling (Zhang et al., 2005). The efficacy of the drug in the prevention of cardiomyocyte hypertrophy as evidenced by a smaller mean cell volume in drug- preincubated dishes when compared to Ang II- and isoproterenol- treated dishes (Fig.35 and 36) is probably mediated by suppression of ROS formation.

Angiotensin II has been reported to stimulate proliferation of cardiac fibroblasts (Schorb et al., 1993). The effect of Ang II on cultured cardiac fibroblasts in the present study concurs with the above report by Schorb et al. In the study, the drug prevented fibroblast proliferation induced by Ang II as evidenced by a lesser mean cell count in drug preincubated dishes compared to Ang II- treated dishes (Fig.37). Thus the drug not only prevented cardiomyocyte hypertrophy, but also fibroblast proliferation in cell culture. The response of drug- retreated cells to Ang II and isoproterenol is paradoxical to the lack of inhibition of Ang II induced vasoconstriction of the aortic ring and isoproterenol induced inotropic variation of the papillary muscle (Fig.28 and 30).The drug does not seem to specifically inhibit Ang II / isoproterenol receptors; but it is probably

the suppression of ROS that might have led to prevention of cardiomyocyte hypertrophy and fibroblast proliferation.The lack of inhibition of changes in isometric contraction by the drug in the experiments involving aortic ring and papillary muscle may be because the production of ROS is time- dependent.

Antioxidant potential of *Cardoguard*

Oxidative stress is a consequence of the imbalance between ROS production and antioxidant capacity. Experimental studies with antioxidant supplements support the hypothesis that oxidative stress is pathogenic in myocardial remodeling and failure. In SHR, blood pressure is reduced and vascular remodeling is inhibited by a diet rich in vitamin C or E (Chen et al., 2001 b). Also, tempol (superoxide dismutase mimetic) decreases blood pressure, improves endothelium-dependent relaxation, media/lumen ratio, NO synthase activity, kidney damage and glomerular filtration (Chen et al., 2001b).

The antioxidant potential of *Cardoguard* was established in three different experimental models. One was the measurement of GSH levels in human RBC exposed to drug and ROS. The second was the assessment of inotropic response of rat papillary muscle exposed to drug and ROS. The third one was the measurement of H_2 DCF-DA incorporation by cultured cardiac fibroblasts exposed to drug and ROS for an assessment of intracellular ROS production. Hypoxanthine and xanthine oxidase was used as the free radical generator in all the three studies. Hypoxanthine plays a pivotal role in vascular injury as a substrate of xanthine oxidase overexpressed in hypertension (Taniyama and Griendling, 2003).

GSH is responsible for protecting cellular thiol against oxidation. As shown by the results, the GSH levels were significantly higher in RBC pretreated with the drug compared to that of cells subjected to oxidative stress in isolation (Fig.38). It can be established that the drug

maintains the cellular system in a more reduced state by neutralizing the ROS. GSH depletion can hamper cellular defenses against oxidative stress, resulting in cellular lipid peroxidation (Giugliano et al., 1996). Decreased GSH concentration and altered turnover rate of the GSH redox cycle with increased intracellular content of GSSG in different tissues has also been described in SHR (Wu and Juurlink, 2001).

The study on rat papillary muscle showed that ROS generated by hypoxanthine and xanthine oxidase induced a negative inotropic response. Negative inotropic response of the myocardium on exposure to ROS has been reported earlier (Ytrehus et al., 1986).The negative inotropic response to oxidative stress was observed to be mitigated in the muscle pretreated with the drug (Fig.39). It is widely accepted that an antihypertensive with antioxidant potential has beneficial effects on the heart. Antihypertensives such as beta blockers and salicylaldehyde isonicotinyl hydrozone are reported to prevent the free radical induced contractile changes in the heart (Horackova et al., 2000).

The mean fluorescent intensity obtained on H_2DCF-DA uptake of drug-pretreated cardiac fibroblasts exposed to ROS was significantly lower compared to that of ROS treated cells (Fig.40), which justifies the fact that ROS production was neutralized by the drug. The antioxidant effect of the drug is perhaps mediated by various substances like saponins, tannins, flavonoids including flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones. *Terminalia arjuna* bark powder has been proved to have significant antioxidant action that is comparable to vitamin E (Gupta et al., 2001). The tannoid principles of the fruits of *Emblica officinalis* have been reported to exhibit antioxidant activity *in vitro* and *in vivo* (Bhattacharya et al., 2002). *Terminalia chebula* and *Terminalia belerica* have also been reported to possess antioxidant activity (Naik et al., 2005).

Although the human body possesses defense mechanisms, as enzymes and antioxidant nutrients, which arrest the damaging properties of ROS, continuous exposure to chemicals and contaminants may lead to an increase in the amount of free radicals in the body beyond its capacity to control them, and cause irreversible oxidative damage. Therefore, antioxidants with free radical scavenging activities may have great relevance in the prevention and therapeutics of diseases in which oxidants are implicated.

To the best of my knowledge, such an extensive and systematic evaluation of an Ayurvedic antihypertensive has not been carried out earlier. Scientific evaluation of Ayurvedic drugs is an aspect that requires consideration. The study has also lent scope for modifying the formulation.

CHAPTER 5

SUMMARY & CONCLUSIONS

SUMMARY

The Ayurvedic system of medicine originated in India 3000 years ago and is still being practiced. One major lacuna in the widespread acceptance of Ayurvedic formulations is the lack of scientific validation. Systematic and scientific evaluation of the Ayurvedic drugs using modern biological techniques will make them more acceptable and popular.

Cardoguard is an Ayurvedic antihypertensive drug formulated by *Nagarjuna Herbal Concentrates Ltd.*, Kerala. It is prepared from the crude powders of 6 medicinal plants namely: *Rauwolfia serpentina, Terminalia arjuna, Boerhavia diffusa*, *Terminalia chebula*, *Terminalia belerica* and *Emblica officinalis*. The drug is being prescribed by the physicians at *Nagarjuna Herbal Concentrates Ltd.* and is found to be effective in the reduction of blood pressure in human subjects, but detailed scientific research has not been carried out. The aim of the study was to scientifically characterize the cardiovascular response to the Ayurvedic formulation.

Hypertension affects a significant percentage of middle -aged population worldwide. Persistent hemodynamic load imposed by hypertension leads to cardiac remodeling, a serious risk factor for heart failure and sudden death. For this reason, besides the antihypertensive action of the drug, the study was aimed to investigate its efficacy in prevention of cardiac remodeling. An antihypertensive with antioxidant properties is expected to be beneficial because hypertension and hypertrophy are associated with oxidative stress.

The major objectives of the study are:

i. Validate the antihypertensive potential and investigate its efficacy in the prevention of cardiac and vascular remodeling

ii. Determine the vasorelaxant potential of the drug and delineate the mechanism of action in vasorelaxation

iii. Study the effect of the drug on myocardial mechanics

iv. Examine the antioxidant potential of the drug

v. Evaluate the toxicity of the drug

vi. Evaluate the neurodepressive action of the drug

Spontaneously Hypertensive Rats (SHR) was used as the *in vivo* experimental model to determine the efficacy of the preparation in the reduction of blood pressure and prevention of cardiac and vascular remodeling. They provide a reliable model of a naturally developing pressure overload akin to essential hypertension. SHR develops hypertension at 6-12 weeks of age, hypertrophy at 6-9 months and cardiac failure at 18-24 months.

Treatment was initiated in two-month-old SHR. The drug was orally administered over a period of 10 months with the dosage based on body surface area. The dosage was comparable to that given to patients. Based on the body surface area, the dose was calculated to be 5mg/ animal/ day. Blood pressure was measured noninvasively using tail cuff method. Cardiac remodeling was assessed by determination of hypertrophy, fibrosis, electrical remodeling and functional changes. Vascular remodeling was assessed by morphometric analysis of thoracic aortae and arteries in the kidney stroma. Age-matched Wistar rats were used as control wherever required. Drug toxicity was evaluated by the histopathological examination of liver and kidney of SHR.

Rauwolfia serpentina is the main antihypertensive constituent plant of *Cardoguard.* Reserpine, the active principle of *Rauwolfia* is a known antihypertensive, but its use is

restricted owing to its side effect of psychotic depression. According to Ayurveda, the whole plant reduces the risk of side effects that occur when isolated active components are used. Hence it was hypothesized that the side effect of psychotic depression associated with reserpine will be mitigated in *Cardoguard*. The action of *Cardoguard* on brain monoamine oxidase activity was evaluated in normotensive Wistar rats.

Vasorelaxation contributes to the lowering of blood pressure. The vasorelaxant potential of the drug and the mechanism of vasorelaxation were assessed *ex vivo* using preparations of thoracic aortae isolated from adult Sprague Dawley rats. The effect of the drug on myocardial mechanics was also examined *ex vivo* using left ventricular papillary muscles isolated from Sprague Dawley rats. The efficacy of the drug in the prevention of cardiomyocyte hypertrophy and fibroblast proliferation, the two prominent features of cardiac hypertrophy, was tested in cardiac cells isolated from newborn Wistar rats. Therapeutic level of water-soluble fraction of the drug (64 mg/L) used for all *ex vivo* experiments was calculated based on average blood volume of adult.

Since oxidative stress is implicated in hypertension and cardiac hypertrophy, the antioxidant potential of the drug was examined in three different preparations subjected to oxidative stress: 1) reduced glutathione levels (GSH) in human RBC 2) inotropic response of rat papillary muscles 3) H_2DCF-DA incorporation by cultured cardiac fibroblasts.

Major findings of the study

1. The drug was found to reduce systolic and diastolic blood pressure in SHR.
2. Morphological and histological examination established prevention of left ventricular hypertrophy in treated SHR. The relative lowering in the

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Major findings of the study

1. The drug was found to reduce systolic and diastolic blood pressure in SHR.
2. Morphological and histological examination established prevention of left ventricular hypertrophy in treated SHR. The relative lowering in the

hypertrophy index and LV wall thickness was more significant in 6-month old rats compared to the 12-month old. Histological morphometry in 6-month old SHR revealed prevention of cardiomyocyte hypertrophy and interstitial fibrosis. Immunohistochemical studies indicated the involvement of ERK and PKC ε in the modulation of ventricular hypertrophy by the drug.

3. No apparent ECG changes were observed in 6-month old animals. At 12 months, animals with ECG changes were relatively more among untreated SHR. Left axis deviation which is a distinctive feature of LVH was found only in untreated SHR. ST segment abnormalities are associated with hypertrophy and transient myocardial ischemia. Animals with ST changes were slightly more among the untreated SHR.
4. At 6 and 12 months of age, the mean heart rates of treated and untreated SHR were comparable. A higher cardiac output was observed consequent to an increase in stroke volume in treated SHR compared to untreated SHR which suggests that cardiac function is improved in treated SHR.
5. Prevention of vascular remodeling by the drug is not indicated as observed from the lack of significant difference in wall thickness and wall to lumen ratio of thoracic aortae and arteries in the kidney stroma of treated SHR and untreated SHR.
6. Brain mitochondrial monoamine oxidase activities of reserpine and *Cardoguard* treated rats were determined by spectrofluorometry. The fluorescence intensity of the brain samples of *Cardoguard* treated rats was lower than that of reserpine treated rats, suggesting mitigation of neurodepression, but the difference was

not statistically significant.

7. No toxicity related features were observed on histopathological analysis of kidney and liver of treated SHR.
8. *Cardoguard* was found to possess vasorelaxant potential as evident from the *ex vivo* studies.The major components inducing vasorelaxation were *Rauwolfia serpentina* and *Terminalia chebula* with interaction between the components.
9. Vasorelaxation was found to be endothelium dependent. NO and cyclooxygenase pathways are implicated in the endothelium dependent relaxant effect by the drug. It was also found that the drug acts as an ATP-sensitive potassium channel activator. The drug also demonstrated Ca^{2+} antagonistic actions as evident from 1) the absence of vasoconstriction by cumulative increase in extracellular Ca^{2+} concentration on drug pretreatment 2) occurrence of vasorelaxation on preconstriction with phenylephrine and high concentration of K^{+} (both are responsible for intracellular calcium mobilization). The intracellular signaling mechanism associated with vasorelaxation when examined using pathway- specific inhibitors demonstrated that vasorelaxation by the drug involved ERK1/2, p38 and calcineurin pathways, but not PKC.
10. Negative inotropy was not found to be provoked by the drug at its therapeutic concentration as evident from the studies using rat papillary muscles. Thus the drug was not found to affect the mechanical function of the heart at the therapeutic level.
11. The drug prevented cardiomyocyte hypertrophy stimulated by Ang II and isoproterenol in cell culture. Fibroblast proliferation stimulated by Ang II was also found to be prevented by the drug.

12. GSH levels were significantly higher in red blood cells pretreated with the drug and subjected to oxidative stress. The negative inotropic response to oxidative stress was also attenuated in rat papillary muscles pre incubated with the drug. In addition, the DCF fluorescence intensity of drug-pretreated cardiac fibroblasts exposed to ROS was significantly lower compared to that of ROS treated cells in isolation.

CONCLUSIONS

Cardoguard is found to be effective as an antihypertensive as observed from its capacity to reduce blood pressure of spontaneously hypertensive rats supported by the finding of vasorelaxation by the drug in isolated aortae. Morphological examination showed that the drug attenuated hypertrophic changes in SHR. Histological morphometry and cell culture experiments revealed the effectiveness of the drug in the attenuation of cardiomyocyte hypertrophy and interstitial fibrosis. The attenuation of hypertrophy is possibly mediated by ERK and PKCε pathways. Cardiac function of treated SHR is found to be improved compared to SHR. As *Cardoguard* has been demonstrated to possess antioxidant properties, the hypotensive and antihypertrophic effects of the drug are speculated to be by the alleviation of oxidative stress. Vasorelaxation by *Cardoguard* appears to be mediated by the virtue of its antioxidant capacity associated with its Ca^{2+} antagonistic action, thereby enhancing EDRF availability at the level of vascular smooth muscle. The drug does not show any toxic effects. *Cardoguard* can therefore be safely used as an antihypertensive and is expected to have beneficial effects on the heart. As *Terminalia chebula* possesses significant vasorelaxation, it is suggested that a new formulation using the former as a major component may be designed.

RECOMMENDATIONS FOR FUTURE STUDIES

1. A major finding with impetus for future research is the observation of significant vasorelaxation by *Terminalia chebula.* Due to the general reluctance in the acceptance of *Rauwolfia serpentina* , it is suggested that the levels of *Rauwolfia serpentina* can be reduced or can be eliminated and supplemented with *Terminalia chebula.*
2. The diuretic capacity of the drug needs to be examined
3. Immunohistochemical studies carried out in the ventricular sections of SHR were pilot studies for determining the signaling pathways modulated by the drug. The efficacy in prevention of cardiac remodeling by the drug should be further characterized with gene expression changes.
4. The absence of changes in resistance arteries despite the reduction of blood pressure needs further investigation.

BIBLIOGRAPHY

BIBLIOGRAPHY

Abdul-Ghani AS, Amin R. Effect of aqueous extract of Commiphora opobalsamum on blood pressure and heart rate in rats. J Ethnopharmacol 1997; 57(3):219-22.

Adams MA, Bobik A, Korner PI. Differential development of vascular and cardiac hypertrophy in genetic hypertension. Relation to sympathetic function. Hypertension 1989; 14(2):191-202.

Adderley SR, Fitzgerald DJ. Oxidative damage of cardiomyocytes is limited by extracellular regulated kinases 1/2-mediated induction of cyclooxygenase-2. J Biol Chem 1999; 274(8):5038-46.

Adiga IK, Nair RR. Multiple signaling pathways coordinately mediate reactive oxygen species dependent cardiomyocyte hypertrophy. Cell Biochem Funct 2008; 26(3):346-51.

Ahlawat SK, Singh MM, Kumar R, Kumari S, Sharma BK. Time trends in the prevalence of hypertension and associated risk factors in Chandigarh. J Indian Med Assoc 2002; 100(9):547-52, 554-5, 572.

Ahn DS, Choi SK, Kim YH, Cho YE, Shin HM, Morgan KG, et al. Enhanced stretch-induced myogenic tone in the basilar artery of spontaneously hypertensive rats. J Vasc Res 2007; 44(3):182-91.

Aiello EA, Villa-Abrille MC, Escudero EM, Portiansky EL, Perez NG, de Hurtado MC, et al. Myocardial hypertrophy of normotensive Wistar-Kyoto rats. Am J Physiol Heart Circ Physiol 2004; 286(4):H1229-35.

Aikawa R, Komuro I, Yamazaki T, Zou Y, Kudoh S, Tanaka M, et al. Oxidative stress activates extracellular signal-regulated kinases through Src and Ras in cultured cardiac myocytes of neonatal rats. J Clin Invest 1997; 100(7):1813-21.

Aisaka K, Hattori Y, Kihara T, Ishihara T, Endo K, Hikino H. Hypotensive action of 3 alpha-dihydrocadambine, an indole alkaloid glycoside of Uncaria hooks. Planta Med 1985(5):424-7.

ALLHAT.Major outcomes in high-risk hypertensive patients randomized to angiotensin-converting enzyme inhibitor or calcium channel blocker vs diuretic: The Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT). Jama 2002; 288(23):2981-97.

ALLHAT.Diuretic versus alpha-blocker as first-step antihypertensive therapy: final results from the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT). Hypertension 2003; 42(3):239-46.

Alvarez MC, Caldiz C, Fantinelli JC, Garciarena CD, Console GM, Chiappe de Cingolani GE, et al. Is cardiac hypertrophy in spontaneously hypertensive rats the cause or the consequence of oxidative stress? Hypertens Res 2008; 31(7):1465-76.

Ancey C, Menet E, Corbi P, Fredj S, Garcia M, Rucker-Martin C, et al. Human cardiomyocyte hypertrophy induced in vitro by gp130 stimulation. Cardiovasc Res 2003; 59(1):78-85.

Anderson WP, Ramsey DE, Takata M. Development of hypertension from unilateral renal artery stenosis in conscious dogs. Hypertension 1990; 16(4):441-51.

Antony I, Nitenberg A, Foult JM, Aptecar E. Coronary vasodilator reserve in untreated and treated hypertensive patients with and without left ventricular hypertrophy. J Am Coll Cardiol 1993; 22(2):514-20.

Anversa P, Ricci R, Olivetti G. Quantitative structural analysis of the myocardium during physiologic growth and induced cardiac hypertrophy: a review. J Am Coll Cardiol 1986; 7(5):1140-9.

Aoyagi T, Izumo S. Hemodynamic Overload-Induced Activation of Myocardial Mitogen-Activated Protein Kinases In Vivo: Augmented Responses in Young Spontaneously Hypertensive Rats and Diminished Responses in Aged Fischer 344 Rats. Hypertension 2001; 37(1):52-57.

Arini PD, Valverde ER, Bertr´an GC, Laguna P. Quantification of ventricular repolarization dispersion on the electrocardiogram by means of T wave duration. XXXI International Conference in Computers in Cardiology IEEE Computer Society September 2004; 31:757–60.

Aruoma OI, Laughton MJ, Halliwell B. Carnosine, homocarnosine and anserine: could they act as antioxidants in vivo? Biochem J 1989; 264(3):863-9.

Asai T, Kushiro T, Fujita H, Kanmatsuse K. Different effects on inhibition of cardiac hypertrophy in spontaneously hypertensive rats by monotherapy and combination therapy of adrenergic receptor antagonists and/or the angiotensin II type 1 receptor blocker under comparable blood pressure reduction. Hypertens Res 2005; 28(1):79-87.

Ashraf MZ, Hussain ME, Fahim M.Endothelium mediated vasorelaxant response of garlic in isolated rat aorta: role of nitric oxide. J Ethnopharmacol 2004; 90(1):5-9.

Asokan N N. Abana in Cardiovascular Disorders. Probe 1986; 26 (1): 32-3.

Balaraman R, Hingorani N, Rathod SP. Studies on the antihypertensive effects of Abana in rats. Indian J Pharmacol 1993; 25:209-14.

Bauer JH, Reams GP. The angiotensin II type 1 receptor antagonists. A new class o antihypertensive drugs. Arch Intern Med 1995; 155(13):1361-8.

Baumeister AA, Hawkins MF, Uzelac SM. The myth of reserpine-induced depression: rol in the historical development of the monoamine hypothesis. J Hist Neurosci 2003 12(2):207-20.

Bendall JK, Cave AC, Heymes C, Gall N, Shah AM. Pivotal role of a gp91 (phox) containing NADPH oxidase in angiotensin II-induced cardiac hypertrophy in mice Circulation 2002; 105(3):293-6.

Benetos A, Levy BI, Lacolley P, Taillard F, Duriez M, Safar ME. Role of angiotensin I and bradykinin on aortic collagen following converting enzyme inhibition in spontaneousl hypertensive rats. Arterioscler Thromb Vasc Biol 1997; 17(11):3196-201.

Benetos A, Poitevin P, Prost PL, Safar ME, Levy BI. Life survival and cardiovascula structures following selective beta-blockade in spontaneously hypertensive rats. Am Hypertens 1994; 7(2):186-92.

Beutler E. Red cell metabolism. A manual of biochemical methods, 2nd ed. New York Grune and Stratton; 1975

Bharani A, Ganguly A, Bhargava KD. Salutary effect of Terminalia Arjuna in patients wit severe refractory heart failure. Int J Cardiol 1995; 49(3):191-9.

Bhatt JD, Panchakshari UD, Hemavati KG, Gulati OD. Effect of Abana, an Ayurvedi preparation of ethinyl estradiol induced hypertension in rats. Indian J Pharmacol 1998 30:399-403

Bhattacharya SK, Bhattacharya A, Sairam K, Ghosal S. Effect of bioactive tannoi principles of Emblica officinalis on ischemia-reperfusion-induced oxidative stress in ra heart. Phytomedicine 2002; 9(2):171-4.

Bing OH, Brooks WW, Robinson KG, Slawsky MT, Hayes JA, Litwin SE, et al. Th spontaneously hypertensive rat as a model of the transition from compensated le ventricular hypertrophy to failure. J Mol Cell Cardiol 1995; 27(1):383-96.

Bohlen HG, Lobach D. In vivo study of microvascular wall characteristics and restin control in young and mature spontaneously hypertensive rats. Blood Vessels 1978 15(5):322-30.

Bohm M, Moll M, Schmid B, Paul M, Ganten D, Castellano M, et al. Beta-adrenergi neuroeffector mechanisms in cardiac hypertrophy of renin transgenic rats. Hypertensio 1994; 24(6):653-62.

Bolton TB. Mechanisms of action of transmitters and other substances on smooth muscle Physiol Rev 1979; 59(3):606-718.

Bouloumie A, Bauersachs J, Linz W, Scholkens BA, Wiemer G, Fleming I, et al. Endothelial dysfunction coincides with an enhanced nitric oxide synthase expression and superoxide anion production. Hypertension 1997; 30(4):934-41.

Bratz IN, Kanagy NL. Nitric oxide synthase-inhibition hypertension is associated with altered endothelial cyclooxygenase function. Am J Physiol Heart Circ Physiol 2004; 287(6):H2394-401.

Braun M, Simonis G, Birkner K, Pauke B, Strasser RH .Regulation of protein kinase C isozyme and calcineurin expression in isoproterenol induced cardiac hypertrophy. J Cardiovasc Pharmacol 2003; 41: 946-54

Braunwald E. Mechanics and energetics of the normal and failing heart. Trans Assoc Am Physicians 1971; 84:63-94.

Braz JC, Bueno OF, Liang Q, Wilkins BJ, Dai YS, Parsons S, et al. Targeted inhibition of p38 MAPK promotes hypertrophic cardiomyopathy through upregulation of calcineurin-NFAT signaling. J Clin Invest 2003; 111(10):1475-86.

Brilla CG. Regression of myocardial fibrosis in hypertensive heart disease: diverse effects of various antihypertensive drugs. Cardiovasc Res 2000; 46(2):324-31.

Brilla CG, Matsubara L, Weber KT. Advanced hypertensive heart disease in spontaneously hypertensive rats. Lisinopril-mediated regression of myocardial fibrosis. Hypertension 1996; 28(2):269-75.

Brooksby P, Levi AJ, Jones JV. Contractile properties of ventricular myocytes isolated from spontaneously hypertensive rat. J Hypertens 1992; 10(6):521-7.

Brooksby P, Levi AJ, Jones JV. The electrophysiological characteristics of hypertrophied ventricular myocytes from the spontaneously hypertensive rat. J Hypertens 1993b; 11(6):611-22.

Brooksby P, Levi AJ, Jones JV. Investigation of the mechanisms underlying the increased contraction of hypertrophied ventricular myocytes isolated from the spontaneously hypertensive rat. Cardiovasc Res 1993 a; 27(7):1268-77

Bu PL, Zhao XQ, Wang LL, Zhao YX, Li CB, Zhang Y. Tong-xin-luo capsule inhibits left ventricular remodeling in spontaneously hypertensive rats by enhancing PPAR-gamma expression and suppressing NF-kappaB activity. Chin Med J (Engl) 2008; 121(2):147-54.

Calixto JB. Efficacy, safety, quality control, marketing and regulatory guidelines for herbal medicines (phytotherapeutic agents). Braz J Med Biol Res 2000; 33(2):179-89.

Capasso JM, Malhotra A, Scheuer J, Sonnenblick EH. Myocardial biochemical, contractile, and electrical performance after imposition of hypertension in young and old rats. Circ Res 1986; 58(4):445-60.

Capasso JM, Palackal T, Olivetti G, Anversa P. Left ventricular failure induced by long-term hypertension in rats. Circ Res 1990; 66(5):1400-12.

Capasso JM, Strobeck JE, Sonnenblick EH. Myocardial mechanical alterations during gradual onset long-term hypertension in rats. Am J Physiol 1981; 241(3):H435-41.

Cardillo C, Campia U, Iantorno M, Panza JA. Enhanced vascular activity of endogenous endothelin-1 in obese hypertensive patients. Hypertension 2004; 43(1):36-40.

Carlberg B, Samuelsson O, Lindholm LH. Atenolol in hypertension: is it a wise choice? Lancet 2004; 364(9446):1684-9.

Carlos DM, Goto S, Urata Y, Iida T, Cho S, Niwa M, et al. Nicardipine normalizes elevated levels of antioxidant activity in response to xanthine oxidase-induced oxidative stress in hypertensive rat heart. Free Radic Res 1998; 29(2):143-50.

Carraway RE, Hassan S, Cochrane DE. Polyphenolic antioxidants mimic the effects of 1, 4-dihydropyridines on neurotensin receptor function in PC3 cells. J Pharmacol Exp Ther 2004; 309(1):92-101.

Chabrashvili T, Tojo A, Onozato ML, Kitiyakara C, Quinn MT, Fujita T, et al. Expression and cellular localization of classic NADPH oxidase subunits in the spontaneously hypertensive rat kidney. Hypertension 2002; 39(2):269-74.

Chamiot-Clerc P, Renaud JF, Safar ME. Pulse pressure, aortic reactivity, and endothelium dysfunction in old hypertensive rats. Hypertension 2001; 37(2):313-21.

Chang KC, Peng YI, Dai SH, Tseng YZ. Age-related changes in pumping mechanical behavior of rat ventricle in terms of systolic elastance and resistance. J Gerontol A Biol Sci Med Sci 2000; 55(9):B440-7.

Chapleau MW, Cunningham JT, Sullivan MJ, Wachtel RE, Abboud FM. Structural versus functional modulation of the arterial baroreflex. Hypertension 1995; 26(2):341-7.

Chattopadhyay RR. Effect of Azadirachta indica hydroalcoholic leaf extract on the cardiovascular system. Gen Pharmacol 1997; 28(3):449-51.

Chen L, Hahn H, Wu G, Chen CH, Liron T, Schechtman D, et al. Opposing cardioprotective actions and parallel hypertrophic effects of delta PKC and epsilon PKC. Proc Natl Acad Sci U S A 2001a; 98(20):11114-9.

Chen X, Touyz RM, Park JB, Schiffrin EL. Antioxidant effects of vitamins C and E are associated with altered activation of vascular NADPH oxidase and superoxide dismutase in stroke-prone SHR. Hypertension 2001b;38(3 Pt 2):606-11.

Chen ZK, Hu SJ, Zhen X, Wang GB, Sun J, Xia Q, et al. [Effect of Astragalus membranaceus on baroreflex sensitivity in spontaneously hypertensive rats]. Zhongguo Zhong Yao Za Zhi 2003; 28(2):155-8.

Cherif S, Rahal N, Haouala M, Hizaoui B, Dargouth F, Gueddiche M, et al. [A clinical trial of a titrated Olea extract in the treatment of essential arterial hypertension]. J Pharm Belg 1996; 51(2):69-71.

Chien KR, Knowlton KU, Zhu H, Chien S. Regulation of cardiac gene expression during myocardial growth and hypertrophy: molecular studies of an adaptive physiologic response. Faseb J 1991; 5(15):3037-46.

Chiu KW, Fung AY. The cardiovascular effects of green beans (Phaseolus aureus), common rue (Ruta graveolens), and kelp (Laminaria japonica) in rats. Gen Pharmacol 1997; 29(5):859-62.

Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JL, Jr., et al. The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure: the JNC 7 report. Jama 2003; 289(19):2560-72.

Chubb J, Huxtable R. The effects of isoproterenol on taurine concentration in the rat heart. Eur J Pharmacol 1978; 48(4):357-67.

Cicogna AC, Matsubara BB, Matsubara LS, Okoshi K, Gut AL, Padovani CR, et al. Volume overload influence on hypertrophied myocardium function. Jpn Heart J 2002; 43(6):689-95.

Cingolani OH, Yang XP, Cavasin MA, Carretero OA. Increased systolic performance with diastolic dysfunction in adult spontaneously hypertensive rats. Hypertension 2003; 41(2):249-54.

Cingolani OH, Yang XP, Liu YH, Villanueva M, Rhaleb NE, Carretero OA. Reduction of cardiac fibrosis decreases systolic performance without affecting diastolic function in hypertensive rats. Hypertension 2004; 43(5):1067-73.

Cohen ME, Bing OH. Performance of papillary muscles from the aging spontaneously hypertensive rat: temporal changes in isometric contraction parameters. Proc Soc Exp Biol Med 1987; 185(3):318-24.

Cohn JN. New therapeutic strategies for heart failure: left ventricular remodeling as a target. J Card Fail 2004; 10(6 Suppl):S200-1.

Cohn JN, Ferrari R, Sharpe N. Cardiac remodeling--concepts and clinical implications: a consensus paper from an international forum on cardiac remodeling. Behalf of an International Forum on Cardiac Remodeling. J Am Coll Cardiol 2000; 35(3):569-82.

Conrad CH, Brooks WW, Robinson KG, Bing OH. Impaired myocardial function in spontaneously hypertensive rats with heart failure. Am J Physiol 1991; 260(1 Pt 2):H136-45.

Consolini AE, Baldini OA, Amat AG. Pharmacological basis for the empirical use o Eugenia uniflora L. (Myrtaceae) as antihypertensive. J Ethnopharmacol 1999; 66(1):33-9.

Contreras F, Rivera M, Vasquez J, De la Parte MA, Velasco M. Endothelial dysfunction i arterial hypertension. J Hum Hypertens 2000; 14 Suppl 1:S20-5.

Cory CR, Grange RW, Houston ME. Role of sarcoplasmic reticulum in loss of load sensitive relaxation in pressure overload cardiac hypertrophy. Am J Physiol 1994; 266(1 P 2):H68-78.

Cowley AW, Jr. Long-term control of arterial blood pressure. Physiol Rev 1992 72(1):231-300.

Cowley AW, Jr., Roman RJ. The role of the kidney in hypertension. Jama 1996 275(20):1581-9.

Crawford MH, Badke FR, Amon KW. Effect of the undisturbed pericardium on lef ventricular size and performance during acute volume loading. Am Heart J 1983 105(2):267-72.

Dahlof B, Hansson L. The influence of antihypertensive therapy on the structural arteriola changes in essential hypertension: different effects of enalapril and hydrochlorothiazide. Intern Med 1993; 234(3):271-9.

Dahlof B, Pennert K, Hansson L. Reversal of left ventricular hypertrophy in hypertensiv patients. A metaanalysis of 109 treatment studies. Am J Hypertens 1992; 5(2):95-110.

Dantas AP, Franco Mdo C, Silva-Antonialli MM, Tostes RC, Fortes ZB, Nigro D, et al Gender differences in superoxide generation in microvessels of hypertensive rats: role o NAD(P)H-oxidase. Cardiovasc Res 2004; 61(1):22-9.

Davy KP, Hall JE. Obesity and hypertension: two epidemics or one? Am J Physiol Regu Integr Comp Physiol 2004; 286(5):R803-13.

De Champlain J, Krakoff LR, Axelrod J. Catecholamine metabolism in experimenta hypertension in the rat. Circ Res 1967; 20(1):136-45.

De Champlain J, Mueller RA, Axelrod J. Turnover and synthesis of norepinephrine i experimental hypertension in rats. Circ Res 1969; 25(3):285-91.

De la Bastie D, Levitsky D, Rappaport L, Mercadier JJ, Marotte F, Wisnewsky C, et al Function of the sarcoplasmic reticulum and expression of its Ca 2(+)-ATPase gene i pressure overload-induced cardiac hypertrophy in the rat. Circ Res 1990; 66(2):554-64.

De Windt LJ, Lim HW, Bueno OF, Liang Q, Delling U, Braz JC, et al. Targeted inhibitio of calcineurin attenuates cardiac hypertrophy in vivo. Proc Natl Acad Sci U S A 2001 98(6):3322-7.

De Windt LJ, Lim HW, Haq S, Force T, Molkentin JD. Calcineurin promotes protei kinase C and c-Jun NH2-terminal kinase activation in the heart. Cross-talk between cardia hypertrophic signaling pathways. J Biol Chem 2000; 275(18):13571-9.

Demirci B, McKeown PP, Bayraktutan U. Blockade of angiotensin II provides additiona benefits in hypertension- and ageing-related cardiac and vascular dysfunctions beyond it blood pressure-lowering effects. J Hypertens 2005; 23(12):2219-27.

Deschepper CF, Picard S, Thibault G, Touyz R, Rouleau JL. Characterization o myocardium, isolated cardiomyocytes, and blood pressure in WKHA and WKY rats. Am Physiol Heart Circ Physiol 2002; 282(1):H149-55.

Devereux RB, Savage DD, Drayer JI, Laragh JH. Left ventricular hypertrophy and functio in high, normal, and low-renin forms of essential hypertension. Hypertension 1982 4(4):524-31.

Dimo T, Rakotonirina S, Kamgang R, Tan PV, Kamanyi A, Bopelet M. Effects of lea aqueous extract of Bidens pilosa (Asteraceae) on KCl- and norepinephrine-induce contractions of rat aorta. J Ethnopharmacol 1998; 60(2):179-82.

Ding B, Price RL, Goldsmith EC, Borg TK, Yan X, Douglas PS, et al. Left ventricula hypertrophy in ascending aortic stenosis mice: anoikis and the progression to early failure Circulation 2000; 101(24):2854-62.

Doggrell SA, Brown L. Rat models of hypertension, cardiac hypertrophy and failure Cardiovasc Res 1998; 39(1):89-105.

Dongmo AB, Kamanyi A, Franck U, Wagner H. Vasodilating properties of extracts fron the leaves of Musanga cecropioides (R. Brown). Phytother Res 2002; 16 Suppl 1:S6-9.

Donnelly R, Connell JM. Insulin resistance: possible role in the aetiology and clinica course of hypertension. Clin Sci (Lond) 1992; 83(3):265-75.

Dorn GW, 2nd, Brown JH. Gq signaling in cardiac adaptation and maladaptation. Trend Cardiovasc Med 1999; 9(1-2):26-34.

Drayer JI, Gardin JM, Weber MA, Aronow WS. Cardiac muscle mass during vasodilatio therapy of hypertension. Clin Pharmacol Ther 1983; 33(6):727-32.

Dubey VD. A study on blood pressure amongst industrial workers of Kanpur. J Indian Me Assoc 1954; 23(11):495-8.

Dubey GP, Agrawal A, Srivastava VK, Soni KL. Regulation of Cardiovascular Function with Abana (An Experimental Study). Probe 1985; 25(1): 27-30.

Duerr RL, Huang S, Miraliakbar HR, Clark R, Chien KR, Ross J, Jr. Insulin-like growth factor-1 enhances ventricular hypertrophy and function during the onset of experimenta cardiac failure. J Clin Invest 1995; 95(2):619-27.

Duncan AC, Jager AK, van Staden J. Screening of Zulu medicinal plants for angiotensin converting enzyme (ACE) inhibitors. J Ethnopharmacol 1999; 68(1-3):63-70.

Dwivedi S, Somani PN, Chansouria JP, Udupa KN. Cardioprotective effects of certain indigenous drugs in myocardial ischaemia in rabbits. Indian J Exp Biol 1988; 26:969-75.

Dzau VJ. Theodore Cooper Lecture: Tissue angiotensin and pathobiology of vascular disease: a unifying hypothesis. Hypertension 2001; 37(4):1047-52.

Ebadi MS. Pharmacodynamic Basis of Herbal Medicine. Florida: CRC Press; 2002.

Eble DM, Cadre BM, Qi M, Bers DM, Samarel AM. Contractile activity modulates atrial natriuretic factor gene expression in neonatal rat ventricular myocytes. J Mol Cell Cardiol 1998; 30(1):55-60.

Esler M, Rumantir M, Kaye D, Lambert G. The sympathetic neurobiology of essential hypertension: disparate influences of obesity, stress, and noradrenaline transporter dysfunction? Am J Hypertens 2001; 14(6 Pt 2):139S-146S.

Exton JH. Mechanisms of action of calcium-mobilizing agonists: some variations on a young theme. Faseb J 1988; 2(11):2670-6.

Fahim M, Khan MS, Hameed HA. Effect of Ajmaloon on the baroreceptor-heart rate reflex in anaesthetized rabbits and monkeys.Indian J Physiol Pharmacol 1995; 39(2):101-5.

Fauci AS, Braunwald E, Kasper DL, Hauser SL, Longo DL, Jameson JL, Loscalzo J. Harrison's Principles of Internal Medicine.17th ed. New York : McGraw Hill;2008.

Felder RA, Jose PA. Mechanisms of disease: the role of GRK4 in the etiology of essential hypertension and salt sensitivity. Nat Clin Pract Nephrol 2006; 2(11):637-50.

Feldman AM, Weinberg EO, Ray PE, Lorell BH. Selective changes in cardiac gene expression during compensated hypertrophy and the transition to cardiac decompensation in rats with chronic aortic banding. Circ Res 1993; 73(1):184-92.

Ferrannini E. Physiological and metabolic consequences of obesity. Metabolism 1995; 44(9 Suppl 3):15-7.

Ferrannini E, Buzzigoli G, Bonadonna R, Giorico MA, Oleggini M, Graziadei L, et al. Insulin resistance in essential hypertension. N Engl J Med 1987; 317(6):350-7.

Folbergrova J, Passonneau JV, Lowry OH, Schulz DW. Glycogen, ammonia and related metabolities in the brain during seizures evoked by methionine sulphoximine. J Neurochem 1969; 16(2):191-203.

Folkow B. "Structural factor" in primary and secondary hypertension. Hypertension 1990; 16(1):89-101.

Folkow B. Early structural changes in hypertension: pathophysiology and clinical consequences. J Cardiovasc Pharmacol 1993; 22 Suppl 1:S1-6.

Freireich EJ, Gehan EA, Rall DP, Schmidt LH, Skipper HE. Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey, and man. Cancer Chemother Rep 1966; 50(4):219-44.

Freis FD. How diuretics lower blood pressure. Am Heart J 1983; 106:185-87

Freund C, Schmidt-Ullrich R, Baurand A, Dunger S, Schneider W, Loser P, et al. Requirement of nuclear factor-kappaB in angiotensin II- and isoproterenol-induced cardiac hypertrophy in vivo. Circulation 2005; 111(18):2319-25.

Frize ED. Mental depression in hypertensive patients treated for long periods with high doses of reserpine. N Engl J Med 1954; 251:1006-1008.

Frohlich ED. Hemodynamics and other determinants in development of left ventricular hypertrophy. Fed Proc 1983; 42(10):2709-15.

Fukunaga T, Ide T, Yamashiro M, Takeya K, Itokawa H. [Studies on pharmacological activity of the Japanese and European mistletoe]. Yakugaku Zasshi 1989; 109(8):600-5.

Ge CJ, Hu SJ, Wu YS, Chen NY. Effects of atorvastatin on vascular remodeling in spontaneously hypertensive rats. J Zhejiang Univ Sci 2003; 4(5):612-5.

Ghayur MN, Gilani AH, Afridi MB, Houghton PJ. Cardiovascular effects of ginger aqueous extract and its phenolic constituents are mediated through multiple pathways. Vascul Pharmacol 2005; 43(4):234-41.

Ghosh M, Hanna ST, Wang R, McNeill JR. Altered vascular reactivity and K_{ATP} channel currents in vascular smooth muscle cells from deoxycorticosterone acetate (DOCA)-salt hypertensive rats. J Cardiovasc Pharmacol 2004; 44(5):525-31.

Gilani AH, Jabeen Q, Ghayur MN, Janbaz KH, Akhtar MS. Studies on the antihypertensive, antispasmodic, bronchodilator and hepatoprotective activities of the Carum copticum seed extract. J Ethnopharmacol 2005 c; 98(1-2):127-35.

Gilani AH, Khan AU, Jabeen Q, Subhan F, Ghafar R. Antispasmodic and blood pressure lowering effects of Valeriana wallichii are mediated through K^+ channel activation. J Ethnopharmacol 2005 b; 100(3):347-52.

Gilani AH, Khan AU, Shah AJ, Connor J, Jabeen Q. Blood pressure lowering effect of olive is mediated through calcium channel blockade. Int J Food Sci Nutr 2005 a; 56(8):613-20.

Gilani AH, Rahman AU. Trends in ethnopharmocology. J Ethnopharmacol 2005; 100(1-2):43-49.

Giugliano D, Ceriello A, Paolisso G. Oxidative stress and diabetic vascular complications Diabetes Care 1996; 19(3):257-67.

Gopalakrishnan C, Dhananjayan R, Kameswaran L. Studies on the pharmacological action of Cardiospermum helicacabum. Indian J Physiol Pharmacol 1976; 20(4):203-8.

Gopinath N, Chadha SL, Sood AK, Shekhawat S, Bindra SP, Tandon R. Epidemiologica study of hypertension in young (15-24 yr) Delhi urban population. Indian J Med Res 199 99:32-7.

Gore A. The management of hypertension with Abana. Probe 1985; 25(1):64-8.

Goto H, Sakakibara I, Shimada Y, Kasahara Y, Terasawa K. Vasodilator effect of extra prepared from Uncariae ramulus on isolated rat aorta. Am J Chin Med 2000; 28(2):19 203.

Goto H, Shimada Y, Tanigawa K, Sekiya N, Shintani T, Terasawa K. Effect of Uncaria ramulus et Uncus on endothelium in spontaneously hypertensive rats. Am J Chin Me 1999; 27(3-4):339-45.

Griendling KK, Sorescu D, Ushio-Fukai M. NAD (P) H oxidase: role in cardiovascul biology and disease. Circ Res 2000; 86(5):494-501.

Grisk O, Rettig R. Interactions between the sympathetic nervous system and the kidneys arterial hypertension. Cardiovasc Res 2004; 61(2):238-46.

Grossman E, Oren S, Messerli FH. Left ventricular filling and stress response pattern essential hypertension. Am J Med 1991; 91(5):502-6.

Grossman W, Jones D, McLaurin LP. Wall stress and patterns of hypertrophy in the huma left ventricle. J Clin Invest 1975; 56(1):56-64.

Gu X, Bishop SP. Increased protein kinase C and isozyme redistribution in pressur overload cardiac hypertrophy in the rat. Circ Res 1994; 75(5):926-31.

Gupta R, Singhal S, Goyle A, Sharma VN. Antioxidant and hypocholesterolaemic effec of Terminalia arjuna tree-bark powder: a randomised placebo-controlled trial. J Asso Physicians India 2001; 49:231-5.

Gupta S, Young D, Sen S. Inhibition of NF-kappaB induces regression of cardia hypertrophy, independent of blood pressure control, in spontaneously hypertensive rat Am J Physiol Heart Circ Physiol 2005; 289(1):H20-9.

Haider AW, Larson MG, Benjamin EJ, Levy D. Increased left ventricular mass ar hypertrophy are associated with increased risk for sudden death. J Am Coll Cardiol 199 32(5):1454-9.

Hall JE. The kidney, hypertension, and obesity. Hypertension 2003; 41(3 Pt 2):625-33.

Haller H, Cosentino F, Luscher TF.Endothelial dysfunction, hypertension and atherosclerosis. A review of the effects of lacidipine. Drugs R D 2002; 3(5):311-23.

Hanes DS, Weir MR, Sowers JR. Gender considerations in hypertension pathophysiology and treatment. Am J Med 1996; 101(3A):10S-21S.

Hansen K, Nyman U, Smitt UW, Adsersen A, Gudiksen L, Rajasekharan S, et al. In vitro screening of traditional medicines for anti-hypertensive effect based on inhibition of the angiotensin converting enzyme (ACE). J Ethnopharmacol 1995; 48(1):43-51.

Hansson L, Hedner T, Lund-Johansen P, Kjeldsen SE, Lindholm LH, Syvertsen JO, et al. Randomised trial of effects of calcium antagonists compared with diuretics and beta-blockers on cardiovascular morbidity and mortality in hypertension: the Nordic Diltiazem (NORDIL) study. Lancet 2000; 356(9227):359-65.

Hansson L, Zanchetti A, Carruthers SG, Dahlof B, Elmfeldt D, Julius S, et al. Effects of intensive blood-pressure lowering and low-dose aspirin in patients with hypertension: principal results of the Hypertension Optimal Treatment (HOT) randomised trial. HOT Study Group. Lancet 1998; 351(9118):1755-62.

Harauma A, Moriguchi T. Aged garlic extract improves blood pressure in spontaneously hypertensive rats more safely than raw garlic. J Nutr 2006; 136(3 Suppl):769S-773S.

Hasebe N, Kikuchi K. Controlled-release nifedipine and candesartan low-dose combination therapy in patients with essential hypertension: the NICE Combi (Nifedipine and Candesartan Combination) Study. J Hypertens 2005; 23(2):445-53.

Haynes WG, Noon JP, Walker BR, Webb DJ. Inhibition of nitric oxide synthesis increases blood pressure in healthy humans. J Hypertens 1993; 11(12):1375-80.

Hazarika NC, Biswas D, Narain K, Kalita HC, Mahanta J. Hypertension and its risk factors in tea garden workers of Assam. Natl Med J India 2002; 15(2):63-8.

He H, Giordano FJ, Hilal-Dandan R, Choi DJ, Rockman HA, McDonough PM, et al. Overexpression of the rat sarcoplasmic reticulum Ca $^{2+}$ATPase gene in the heart of transgenic mice accelerates calcium transients and cardiac relaxation. J Clin Invest 1997; 100(2):380-9.

Hearse DJ, Sutherland FJ. Experimental models for the study of cardiovascular function and disease. Pharmacol Res 2000; 41(6):597-603.

Hein S, Arnon E, Kostin S, Schonburg M, Elsasser A, Polyakova V, et al. Progression from compensated hypertrophy to failure in the pressure-overloaded human heart: structural deterioration and compensatory mechanisms. Circulation 2003; 107(7):984-91.

Heninger GR, Delgado PL, Charney DS. The revised monoamine theory of depression: a modulatory role for monoamines based on new findings from monoamine depletion experiments in humans. Pharmacopsychiatry 1996; 29(1):2-11.

Higuchi Y, Ono K, Sekita S, Onodera H, Mitsumori K, Nara Y, et al. Preventive effects of Shichimotsu-koka-to on renal lesions in stroke-prone spontaneously hypertensive rats. Biol Pharm Bull 1998; 21(9):914-8.

Hingtgen SD, Tian X, Yang J, Dunlay SM, Peek AS, Wu Y, et al. Nox2-containing NADPH oxidase and Akt activation play a key role in angiotensin II-induced cardiomyocyte hypertrophy. Physiol Genomics 2006; 26(3):180-91.

Horackova M, Ponka P, Byczko Z. The antioxidant effects of a novel iron chelator salicylaldehyde isonicotinoyl hydrazone in the prevention of H_2O_2 injury in adult cardiomyocytes. Cardiovasc Res 2000; 47(3):529-36.

Hosokawa N, Hohenadl C, Satoh M, Kuhn K, Nagata K. HSP47, a collagen-specific molecular chaperone, delays the secretion of type III procollagen transfected in human embryonic kidney cell line 293: a possible role for HSP47 in collagen modification. J Biochem 1998; 124(3):654-62.

Hropot M, Grotsch H, Klaus E, Langer KH, Linz W, Wiemer G, et al. Ramipril prevents the detrimental sequels of chronic NO synthase inhibition in rats: hypertension, cardiac hypertrophy and renal insufficiency. Naunyn Schmiedebergs Arch Pharmacol 1994 350(6):646-52.

Huang ZY, Liu Z, Zhu JH, Li S. [ERK expression and activation in myocardial hypertrophy of spontaneously hypertensive rats with different ages]. Zhejiang Da Xue Xue Bao Yi Xue Ban 2005; 34(6):542-6.

Hughes AD. How do thiazide and thiazide-like diuretics lower blood pressure? J Renin Angiotensin Aldosterone Syst 2004; 5(4):155-60.

Hull SJ, Scott ML. Studies on the changes in reduced glutathione of chick tissues during onset and regression of nutritional muscular dystrophy. J Nutr 1976; 106(2):181-90.

Hunter JJ, Chien KR. Signaling pathways for cardiac hypertrophy and failure. N Engl J Med 1999; 341(17):1276-83.

Hwang HS, Bleske BE, Ghannam MM, Converso K, Russell MW, Hunter JC, et al. Effects of Hawthorn on Cardiac Remodeling and Left Ventricular Dysfunction after 1 Month of Pressure Overload-induced Cardiac Hypertrophy in Rats. Cardiovasc Drugs Ther 2008; 22(1):19-28.

Hypertension study Group. Prevalence, Awareness, treatment and control of hypertension among elderly in Bangladesh and India: a multicentric study. Bulletin of the World Health Organization 2001; 79(6): 490-500. Hypertension study group: Md.Iftekhar Quasem, Mrunal S Shetye, Shiney C Alex, Anjan Kumar Nag, P S Sarma, Thankappan KR, R S Vasan

Iams SG, Wexler BC. Inhibition of the development of spontaneous hypertension in SH rats by gonadectomy or estradiol. J Lab Clin Med 1979; 94(4):608-16.

Ibarrola DA, Ibarrola MH, Vera C, Montalbetti Y, Ferro EA. Hypotensive effect of crude root extract of Solanum sisymbriifolium (Solanaceae) in normo- and hypertensive rats. J Ethnopharmacol 1996; 54(1):7-12.

Idikio H, Fernandez PG, Triggle CR, Kim BK. Regression of left ventricular hypertrophy and control of hypertension in the spontaneously hypertensive rat (SHR): oxprenolol versus hydrochlorothiazide. Clin Invest Med 1983; 6(1):43-8.

Ikeda H, Shino A, Matsuo T, Iwatsuka H, Suzuoki Z. A new genetically obese-hyperglycemic rat (Wistar fatty). Diabetes 1981; 30(12):1045-50.

Iliescu R, Yanes LL, Bell W, Dwyer T, Baltatu OC, Reckelhoff JF. Role of the renal nerves in blood pressure in male and female SHR. Am J Physiol Regul Integr Comp Physiol 2006; 290(2):R341-4.

Inagaki K, Koyanagi T, Berry NC, Sun L, Mochly-Rosen D. Pharmacological inhibition of epsilon-protein kinase C attenuates cardiac fibrosis and dysfunction in hypertension-induced heart failure. Hypertension 2008; 51(6):1565-9.

Ingwall JS, Roeske WR, Wildenthal K. The fetal mouse heart in organ culture: maintenance of the differentiated state. Methods Cell Biol 1980; 21A:167-86.

Izumo S, Nadal-Ginard B, Mahdavi V. Protooncogene induction and reprogramming of cardiac gene expression produced by pressure overload. Proc Natl Acad Sci U S A 1988; 85(2):339-43.

Izzard AS, Horton S, Heerkens EH, Shaw L, Heagerty AM. Middle cerebral artery structure and distensibility during developing and established phases of hypertension in the spontaneously hypertensive rat. J Hypertens 2006; 24(5):875-80.

Jalil JE, Doering CW, Janicki JS, Pick R, Shroff SG, Weber KT. Fibrillar collagen and myocardial stiffness in the intact hypertrophied rat left ventricle. Circ Res 1989a; 64(6):1041-50.

Jalil JE, Janicki JS, Pick R, Abrahams C, Weber KT. Fibrosis-induced reduction of endomyocardium in the rat after isoproterenol treatment. Circ Res 1989b; 65(2):258-64.

Julius S, Alderman MH, Beevers G, Dahlof B, Devereux RB, Douglas JG, et al. Cardiovascular risk reduction in hypertensive black patients with left ventricular hypertrophy: the LIFE study. J Am Coll Cardiol 2004; 43(6):1047-55.

Kahonen M, Tolvanen JP, Sallinen K, Wu X, Porsti I. Influence of gender on control of arterial tone in experimental hypertension. Am J Physiol 1998; 275(1 Pt 2):H15-22.

Karaki H, Ozaki H, Hori M, Mitsui-Saito M, Amano K, Harada K, et al. Calcium movements, distribution, and functions in smooth muscle. Pharmacol Rev 1997; 49(2):157-230.

Katholi RE. Renal nerves in the pathogenesis of hypertension in experimental animals and humans. Am J Physiol 1983; 245(1):F1-14.

Kearney PM, Whelton M, Reynolds K, Muntner P, Whelton PK, He J. Global burden of hypertension: analysis of worldwide data. Lancet 2005; 365(9455):217-23.

Kearney PM, Whelton M, Reynolds K, Whelton PK, He J. Worldwide prevalence of hypertension: a systematic review. J Hypertens 2004; 22(1):11-9.

Keith M, Geranmayegan A, Sole MJ, Kurian R, Robinson A, Omran AS, et al. Increased oxidative stress in patients with congestive heart failure. J Am Coll Cardiol 1998; 31(6):1352-6.

Kerr S, Brosnan MJ, McIntyre M, Reid JL, Dominiczak AF, Hamilton CA. Superoxide anion production is increased in a model of genetic hypertension: role of the endothelium. Hypertension 1999; 33(6):1353-8.

Khan AU, Gilani AH. Cardiovascular inhibitory effects of Hyoscyamus niger. Methods Find Exp Clin Pharmacol 2008; 30(4):295-300.

Kim J, Eckhart AD, Eguchi S, Koch WJ. Beta-adrenergic receptor-mediated DNA synthesis in cardiac fibroblasts is dependent on transactivation of the epidermal growth factor receptor and subsequent activation of extracellular signal-regulated kinases. J Biol Chem 2002; 277(35):32116-23.

Kishi T, Hirooka Y, Kimura Y, Ito K, Shimokawa H, Takeshita A. Increased reactive oxygen species in rostral ventrolateral medulla contribute to neural mechanisms of hypertension in stroke-prone spontaneously hypertensive rats. Circulation 2004; 109(19):2357-62.

Kiss E, Ball NA, Kranias EG, Walsh RA. Differential changes in cardiac phospholamban and sarcoplasmic reticular Ca (2+)-ATPase protein levels. Effects on Ca^{2+} transport and mechanics in compensated pressure-overload hypertrophy and congestive heart failure. Circ Res 1995; 77(4):759-64.

Kleber FX, Pfeffer MA, Pfeffer JM. Alterations in the electrocardiogram of spontaneously hypertensive rats by chronic antihypertensive therapy with captopril. Clin Exp Hypertens A 1982; 4(6):977-87.

Kline RL, Patel KP, Ciriello J, Mercer PF. Effect of renal denervation on arterial pressure in rats with aortic nerve transection. Hypertension 1983; 5(4):468-75.

Kobayashi N, Kobayashi K, Hara K, Higashi T, Yanaka H, Yagi S, et al. Benidipine stimulates nitric oxide synthase and improves coronary circulation in hypertensive rats. Am J Hypertens 1999; 12(5):483-91.

Kou R, Greif D, Michel T. Dephosphorylation of endothelial nitric-oxide synthase vascular endothelial growth factor. Implications for the vascular responses to cyclospo A. J Biol Chem 2002; 277(33):29669-73.

Kutty VR, Balakrishnan KG, Jayasree AK, Thomas J. Prevalence of coronary heart dise in the rural population of Thiruvananthapuram district, Kerala, India. Int J Cardiol 19 39(1):59-70.

Kutty VR, Soman CR, Joseph A, Kumar KV, Pisharody R. Random capillary blood su and coronary risk factors in a south Kerala population. J Cardiovasc Risk 2002; 9(6):361

Ladva S. NICE and BHS launch updated hypertension guideline.2006. National Instit for Health and Clinical Excellence

Langer SZ, Cavero I, Massingham R. Recent developments in noradrener neurotransmission and its relevance to the mechanism of action of certain antihypertens agents. Hypertension 1980; 2(4):372-82.

Laragh JH. Lewis K. Dahl Memorial Lecture. The renin system and four lines hypertension research. Nephron heterogeneity, the calcium connection, the prore vasodilator limb, and plasma renin and heart attack. Hypertension 1992; 20(3):267-79.

Laskowski A, Woodman OL, Cao AH, Drummond GR, Marshall T, Kaye DM, et Antioxidant actions contribute to the antihypertrophic effects of atrial natriuretic peptide neonatal rat cardiomyocytes. Cardiovasc Res 2006; 72(1):112-23.

Lecarpentier Y, Bugaisky LB, Chemla D, Mercadier JJ, Schwartz K, Whalen RG, et Coordinated changes in contractility, energetics, and isomyosins after aortic stenosis. Ar Physiol 1987; 252(2 Pt 2):H275-82.

Ledingham JM, Laverty R. Renal afferent arteriolar structure in the genetica hypertensive (GH) rat and the ability of losartan and enalapril to cause structu remodelling. J Hypertens 1998; 16(12 Pt 2):1945-52.

Lee MA, Bohm M, Paul M, Bader M, Ganten U, Ganten D. Physiological characterizati of the hypertensive transgenic rat TGR(mREN2)27. Am J Physiol 1996; 270(6 Pt 1):E9 29.

Leuchtgens H. [Crataegus Special Extract WS 1442 in NYHA II heart failure. A place controlled randomized double-blind study]. Fortschr Med 1993; 111(20-21):352-4.

Levy BI, Ambrosio G, Pries AR, Struijker-Boudier HA. Microcirculation in hypertensi a new target for treatment? Circulation 2001; 104(6):735-40.

Levy BI, Duriez M, Phillipe M, Poitevin P, Michel JB. Effect of chronic dihydropyridir (isradipine) on the large arterial walls of spontaneously hypertensive rats. Circulation 199 90(6):3024-33.

Lewis JF, Maron BJ. Elderly patients with hypertrophic cardiomyopathy: a subset wi distinctive left ventricular morphology and progressive clinical course late in life. J A Coll Cardiol 1989; 13(1):36-45.

Li JM, Shah AM. Endothelial cell superoxide generation: regulation and relevance f cardiovascular pathophysiology. Am J Physiol Regul Integr Comp Physiol 200 287(5):R1014-30.

Liang Q, Bueno OF, Wilkins BJ, Kuan CY, Xia Y, Molkentin JD. c-Jun N-terminal kinas (JNK) antagonize cardiac growth through cross-talk with calcineurin-NFAT signalin Embo J 2003; 22(19):5079-89.

Liddle GW, Bledsoe T, Coppage WS Jr. A familial renal disorder simulating prima aldosteronism but with negligible aldosterone secretion. Trans Assoc Am Physicians 196 76:199–213

Lifton RP. Molecular genetics of human blood pressure variation. Science 199 272(5262):676-80.

Lima-Landman MT, Borges AC, Cysneiros RM, De Lima TC, Souccar C, Lapa A Antihypertensive effect of a standardized aqueous extract of Cecropia glaziovii Sneth rats: an in vivo approach to the hypotensive mechanism. Phytomedicine 2007; 14(5):31 20.

Lin YF, Raab-Graham K, Jan YN, Jan LY. NO stimulation of ATP-sensitive potassiu channels: Involvement of Ras/mitogen-activated protein kinase pathway and contributi to neuroprotection. Proc Natl Acad Sci U S A 2004; 101(20):7799-804.

Lindholm LH, Carlberg B, Samuelsson O. Should beta blockers remain first choice in t treatment of primary hypertension? A meta-analysis. Lancet 2005; 366(9496):1545-53.

Lindpaintner K, Kreutz R, Ganten D. Genetic variation in hypertensive and 'control' strain What are we controlling for anyway? Hypertension 1992; 19(5):428-30.

Linz W, Gohlke P, Unger T, Scholkens BA. Experimental evidence for effects of ramipr on cardiac and vascular hypertrophy beyond blood pressure reduction. Arch Mal Coe Vaiss 1995; 88 Spec No 2:31-4.

Linz W, Schaper J, Wiemer G, Albus U, Scholkens BA. Ramipril prevents left ventricul hypertrophy with myocardial fibrosis without blood pressure reduction: a one year study rats. Br J Pharmacol 1992; 107(4):970-5.

Liu JC, Chan P, Chen JJ, Lee HM, Lee WS, Shih NL, et al. The inhibitory effect trilinolein on norepinephrine-induced beta-myosin heavy chain promoter activity, react oxygen species generation, and extracellular signal-regulated kinase phosphorylation neonatal rat cardiomyocytes. J Biomed Sci 2004; 11(1):11-8.

Lohmeier TE. The sympathetic nervous system and long-term blood pressure regulati Am J Hypertens 2001; 14(6 Pt 2):147S-154S.

Lohmeier TE. Interactions between angiotensin II and baroreflexes in long-term regulat of renal sympathetic nerve activity. Circ Res 2003; 92(12):1282-4.

Lohmeier TE, Irwin ED, Rossing MA, Serdar DJ, Kieval RS. Prolonged activation of baroreflex produces sustained hypotension. Hypertension 2004; 43(2):306-11.

Lombard JH, Eskinder H, Kauser K, Osborn JL, Harder DR. Enhanced norepinephr sensitivity in renal arteries at elevated transmural pressure. Am J Physiol 1990; 259(1 2):H29-33.

Lombard JH, Hess ME, Stekiel WJ. Neural and local control of arterioles in SH Hypertension 1984; 6(4):530-5.

Lombard JH, Hess ME, Stekiel WJ. Enhanced response of arterioles to oxygen dur development of hypertension in SHR. Am J Physiol 1986; 250(5 Pt 2):H761-4.

Lorell BH, Carabello BA. Left ventricular hypertrophy: pathogenesis, detection, prognosis. Circulation 2000; 102(4):470-9.

Lorenz JN, Kranias EG. Regulatory effects of phospholamban on cardiac function in in mice. Am J Physiol 1997; 273(6 Pt 2):H2826-31.

Loscalzo J, Welch G. Nitric oxide and its role in the cardiovascular system. P Cardiovasc Dis 1995; 38(2):87-104.

Loukotova J, Kunes J, Zicha J. Gender-dependent difference in cell calcium handling VSMC isolated from SHR: the effect of angiotensin II. J Hypertens 2002; 20(11):2213-9

Lowenstein CJ, Dinerman JL, Snyder SH. Nitric oxide: a physiologic messenger. A Intern Med 1994; 120(3):227-37.

Lundin SA, Hallback-Nordlander MI. Regression of structural cardiovascular changes antihypertensive therapy in spontaneously hypertensive rats. J Hypertens 1984; 2(1):11-8

Luscher TF. The endothelium. Target and promoter of hypertension? Hypertension 19 15(5):482-5.

Luscher TF, Vanhoutte PM. Endothelium-dependent contractions to acetylcholine in aorta of the spontaneously hypertensive rat. Hypertension 1986; 8(4):344-8.

Magos GA, Vidrio H, Enriquez R. Pharmacology of Casimiroa edulis; III. Relaxant a contractile effects in rat aortic rings. J Ethnopharmacol 1995; 47(1):1-8.

Malhotra P, Kumari S, Kumar R, Jain S, Sharma BK. Prevalence and determinants hypertension in an un-industrialised rural population of North India. J Hum Hyperte 1999; 13(7):467-72.

Mann DL. Mechanisms and models in heart failure: A combinatorial approach. Circulati 1999; 100(9):999-1008.

Mansour P, Bostrom PA, Mattiasson I, Lilja B, Berglund G. Low blood pressure levels a signs of myocardial ischaemia: importance of left ventricular hypertrophy. J Hu Hypertens 1993; 7(1):13-8.

Marteau JB, Zaiou M, Siest G, Visvikis-Siest S. Genetic determinants of blood pressu regulation. J Hypertens 2005; 23(12):2127-43.

Martinez-Gonzalez J, Rodriguez-Rodriguez R, Gonzalez-Diez M, Rodriguez C, Herre MD, Ruiz-Gutierrez V, et al. Oleanolic acid induces prostacyclin release in human vascu smooth muscle cells through a cyclooxygenase-2-dependent mechanism. J Nutr 20(138(3):443-8.

Matsubara LS, Matsubara BB, Okoshi MP, Franco M, Cicogna AC. Myocardial fibro rather than hypertrophy induces diastolic dysfunction in renovascular hypertensive ra Can J Physiol Pharmacol 1997; 75(12):1328-34.

Matsumura Y, Okui T, Ono H, Kiso Y, Tanaka T. Antihypertensive effects of chick extract against deoxycorticosterone acetate-salt-induced hypertension in rats. Biol Pha Bull 2001; 24(10):1181-4.

Mayhan WG. Impairment of endothelium-dependent dilatation of basilar artery duri chronic hypertension. Am J Physiol 1990; 259(5 Pt 2):H1455-62.

McCall E, Ginsburg KS, Bassani RA, Shannon TR, Qi M, Samarel AM, et al. Ca flu contractility, and excitation-contraction coupling in hypertrophic rat ventricular myocyt Am J Physiol 1998; 274(4 Pt 2):H1348-60.

McFadzean I, Gibson A. The developing relationship between receptor-operated and sto operated calcium channels in smooth muscle. Br J Pharmacol 2002; 135(1):1-13.

McGill G, Shimamura A, Bates RC, Savage RE, Fisher DE. Loss of matrix adhesi triggers rapid transformation-selective apoptosis in fibroblasts. J Cell Biol 19 138(4):901-11.

McGuire PG, Twietmeyer TA. Aortic endothelial junctions in developing hypertensic Hypertension 1985; 7(4):483-90.

McMullen JR, Sherwood MC, Tarnavski O, Zhang L, Dorfman AL, Shioi T, et al Inhibition of mTOR signaling with rapamycin regresses established cardiac hypertrophy induced by pressure overload. Circulation 2004; 109(24):3050-5.

Michel JB, Salzmann JL, Ossondo Nlom M, Bruneval P, Barres D, Camilleri JP Morphometric analysis of collagen network and plasma perfused capillary bed in the myocardium of rats during evolution of cardiac hypertrophy. Basic Res Cardiol 1986 81(2):142-54.

Miller FJ, Jr., Gutterman DD, Rios CD, Heistad DD, Davidson BL. Superoxide production in vascular smooth muscle contributes to oxidative stress and impaired relaxation in atherosclerosis. Circ Res 1998; 82(12):1298-305.

Millgard J, Lind L. Acute hypertension impairs endothelium-dependent vasodilation. Clin Sci (Lond) 1998; 94(6):601-7.

Mironneau J, Macrez-Lepretre N. Modulation of Ca^{2+} channels by alpha 1A- and alpha 2A-adrenoceptors in vascular myocytes: involvement of different transduction pathways Cell Signal 1995; 7(5):471-9.

Misra A, Pandey RM, Devi JR, Sharma R, Vikram NK, Khanna N. High prevalence of diabetes, obesity and dyslipidaemia in urban slum population in northern India. Int J Obes Relat Metab Disord 2001; 25(11):1722-9.

Mohan V, Shanthirani S, Deepa R, Premalatha G, Sastry NG, Saroja R. Intra-urban differences in the prevalence of the metabolic syndrome in southern India -- the Chennai Urban Population Study (CUPS No. 4). Diabet Med 2001; 18(4):280-7.

Molkentin JD, Dorn GW, 2nd. Cytoplasmic signaling pathways that regulate cardiac hypertrophy. Annu Rev Physiol 2001; 63:391-426.

Montfort I, Perez-Tamayo R. The muscle-collagen ratio in normal and hypertrophic human hearts. Lab Invest 1962; 11:463-70.

Moore JH, Williams SM. New strategies for identifying gene-gene interactions in hypertension. Ann Med 2002; 34(2):88-95.

Moriguchi J, Itoh H, Harada S, Takeda K, Hatta T, Nakata T, et al. Low frequency regular exercise improves flow-mediated dilatation of subjects with mild hypertension. Hypertens Res 2005; 28(4):315-21.

Mossa JS, Al-Yahya MA, Al Mehal IA, Tariq M. Phytochemical and biological screening of Saudi medicinal plants. Fitoterapia 1983; 54(4): 147-52.

Muller DN, Hilgers KF, Bohlender J, Lippoldt A, Wagner J, Fischli W, et al. Effects of human renin in the vasculature of rats transgenic for human angiotensinogen. Hypertension 1995; 26(2):272-8.

Murphy ME, Brayden JE. Nitric oxide hyperpolarizes rabbit mesenteric arteries via ATP sensitive potassium channels. J Physiol 1995; 486 (Pt 1):47-58.

Nabauer M, Kaab S. Potassium channel down-regulation in heart failure. Cardiovasc Re 1998; 37(2):324-34.

Nadkarni KM, Nadkarni AK. In: Indian Materia Medica - with Ayurvedic, Unani-Tibb Siddha, Allopathic, Homeopathic, Naturopathic and Home remedies. Popular Prakasha Private Ltd., India; 1999.

Nagata S, Takeyama K, Hosoki K, Karasawa T. Possible involvement of ATP-dependen K-channel related mechanisms in the antihypertensive and cough suppressant effects of th novel ACE inhibitor (2S, 3aS, 7aS)-1-(N2-nicotinoyl-L-lysyl-gamma-D glutamyl)octahydro-1H- indole-2-carboxylic acid. Arzneimittelforschung 1997; 47(6):726 30.

Naik GH, Priyadarsini KI, Bhagirathi RG, Mishra B, Mishra KP, Banavalikar MM, et al. I vitro antioxidant studies and free radical reactions of triphala, an ayurvedic formulation an its constituents. Phytother Res 2005; 19(7):582-6.

Naik GH, Priyadarsini KI, Mohan H. Free radical scavenging reactions and phytochemica analysis of *triphala*, an ayurvedic formulation. Current Sci 2006; 90:1100-51.

Nair R R, Gupta P N.Isolation and culture of beating cells from human fetal heart. J Tissu Culture Meth 1989 ; 11:211-16.

Nakagami H, Takemoto M, Liao JK. NADPH oxidase-derived superoxide anion mediate angiotensin II-induced cardiac hypertrophy. J Mol Cell Cardiol 2003; 35(7):851-9.

Nakamura K, Fushimi K, Kouchi H, Mihara K, Miyazaki M, Ohe T, et al. Inhibitory effect of antioxidants on neonatal rat cardiac myocyte hypertrophy induced by tumor necrosi factor-alpha and angiotensin II. Circulation 1998; 98(8):794-9.

Nakaoka Y, Nishida K, Fujio Y, Izumi M, Terai K, Oshima Y, et al. Activation of gp13 transduces hypertrophic signal through interaction of scaffolding/docking protein Gab with tyrosine phosphatase SHP2 in cardiomyocytes. Circ Res 2003; 93(3):221-9.

Nand V, Doggrell SA, Barnett CW. Effects of veratridine on the action potentials an contractility of right and left ventricles from normo- and hypertensive rats. Clin Ex Pharmacol Physiol 1997; 24(8):570-6.

New MI, Levine LS, Biglieri EG, Pareira J, Ulick S. Evidence for an unidentified steroid i a child with apparent mineralocorticoid hypertension. J Clin Endocrinol Metab 1977 44(5):924-33.

Nishizuka Y. Studies and perspectives of protein kinase C. Science 1986; 233(4761):305 12.

Nissen SE, Tuzcu EM, Libby P, Thompson PD, Ghali M, Garza D, et al. Effect o antihypertensive agents on cardiovascular events in patients with coronary disease and normal blood pressure: the CAMELOT study: a randomized controlled trial. Jama 2004 292(18):2217-25.

Nordlander M, Di Bona GF, Ljung B, Yao T, Thoren P. Renal and cardiovascular effects o acute and chronic administration of felodipine to SHR. Eur J Pharmacol 1985;113(1):25 36.

Okamatsu S, Lefer AM. The protective effects of nifedipine in the isolated cat heart. J Surg Res 1983; 35(1):35-40.

Okamoto K. Spontaneous hypertension in rats. Int Rev Exp Pathol 1969; 7:227-70.

Okamoto K, Aoki K. Development of a strain of spontaneously hypertensive rats. Jpn Circ J 1963; 27:282-93.

Okoshi MP, Matsubara LS, Franco M, Cicogna AC, Matsubara BB. Myocyte necrosis i the basis for fibrosis in renovascular hypertensive rats. Braz J Med Biol Res 1997 30(9):1135-44.

Oliveira CJ, Schindler F, Ventura AM, Morais MS, Arai RJ, Debbas V, et al. Nitric oxid and cGMP activate the Ras-MAP kinase pathway-stimulating protein tyrosin phosphorylation in rabbit aortic endothelial cells. Free Radic Biol Med 2003; 35(4):381-96

Onodera T, Tamura T, Said S, McCune SA, Gerdes AM. Maladaptive remodeling o cardiac myocyte shape begins long before failure in hypertension. Hypertension 1998 32(4):753-7.

Oren S, Grossman E, Frohlich ED. Arterial and venous compliance in obese and nonobes subjects. Am J Cardiol 1996; 77(8):665-7.

Ormezzano O, Cracowski JL, Quesada JL, Pierre H, Mallion JM, Baguet JP. EVAluatio of the prognostic value of BARoreflex sensitivity in hypertensive patients: the EVABA study. J Hypertens 2008; 26(7):1373-8.

Otterstad JE. Ischaemia and left ventricular hypertrophy. Eur Heart J 1993; 14 Suppl F: 2 6.

Ouvina SM, La Greca RD, Zanaro NL, Palmer L, Sassetti B. Endothelial dysfunction, nitri oxide and platelet activation in hypertensive and diabetic type II patients. Thromb Re 2001; 102(2):107-14.

Owens GK. Differential effects of antihypertensive drug therapy on vascular smoot muscle cell hypertrophy, hyperploidy, and hyperplasia in the spontaneously hypertensiv rat. Circ Res 1985; 56(4):525-36.

Packer M, Fowler MB, Roecker EB, Coats AJ, Katus HA, Krum H, et al. Effect o carvedilol on the morbidity of patients with severe chronic heart failure: results of th carvedilol prospective randomized cumulative survival (COPERNICUS) study. Circulatio 2002; 106(17):2194-9.

Pagliaro P, Penna C. Rethinking the renin-angiotensin system and its role in cardiovascula regulation. Cardiovasc Drugs Ther 2005; 19(1):77-87.

Panidis IP, Kotler MN, Ren JF, Mintz GS, Ross J, Kalman P. Development and regressio of left ventricular hypertrophy. J Am Coll Cardiol 1984; 3(5):1309-20.

Pasnani JS, Hemavathi KG, Gulati OD, Rajani AP. Effects of Abana, an Ayurvedi preparation, on rabbit atrium and intestine. J Ethnopharmacol 1988; 24(2-3):287-302.

Paul K, Ball NA, Dorn GW, 2nd, Walsh RA. Left ventricular stretch stimulates angiotensi II--mediated phosphatidylinositol hydrolysis and protein kinase C epsilon isofor translocation in adult guinea pig hearts. Circ Res 1997; 81(5):643-50.

Paul M, Poyan Mehr A, Kreutz R. Physiology of local renin-angiotensin systems. Physic Rev 2006; 86(3):747-803.

Payne JA, Reckelhoff JF, Khalil RA. Role of oxidative stress in age-related reduction o NO-cGMP-mediated vascular relaxation in SHR. Am J Physiol Regul Integr Comp Physic 2003; 285(3):R542-51.

Pechanova O, Zicha J, Kojsova S, Dobesova Z, Jendekova L, Kunes J. Effect of chronic N acetylcysteine treatment on the development of spontaneous hypertension. Clin Sci (Lond 2006; 110(2):235-42.

Pegram BL, Frohlich ED. Cardiovascular adjustment to antiadrenergic agents. Am J Me 1983; 75(3A):94-9.

Pepine CJ, Handberg EM, Cooper-DeHoff RM, Marks RG, Kowey P, Messerli FH, et al. A calcium antagonist vs a non-calcium antagonist hypertension treatment strategy for patient with coronary artery disease. The International Verapamil-Trandolapril Study (INVEST): randomized controlled trial. Jama 2003; 290(21):2805-16.

Perticone F, Ceravolo R, Pujia A, Ventura G, Iacopino S, Scozzafava A, et al. Prognosti significance of endothelial dysfunction in hypertensive patients. Circulation 2001 104(2):191-6.

Peterson JC, Adler S, Burkart JM, Greene T, Hebert LA, Hunsicker LG, et al. Bloo pressure control, proteinuria, and the progression of renal disease. The Modification of Die in Renal Disease Study. Ann Intern Med 1995; 123(10):754-62.

Petkov V. Plants and hypotensive, antiatheromatous and coronarodilatating action. Am Chin Med 1979; 7(3):197-236.

Pewsner D, Juni P, Egger M, Battaglia M, Sundstrom J, Bachmann LM. Accuracy of electrocardiography in diagnosis of left ventricular hypertrophy in arterial hypertension: systematic review. Bmj 2007; 335(7622):711.

Pfeffer JM, Pfeffer MA, Fishbein MC, Frohlich ED. Cardiac function and morphology with aging in the spontaneously hypertensive rat. Am J Physiol 1979; 237(4):H461-8.

Pfeffer JM, Pfeffer MA, Mirsky I, Braunwald E. Prevention of the development of heart failure and the regression of cardiac hypertrophy by captopril in the spontaneously hypertensive rat. Eur Heart J 1983; 4 Suppl A: 143-8.

Pfeffer MA, Pfeffer JM, Frohlich ED. Pumping ability of the hypertrophying left ventricle of the spontaneously hypertensive rat. Circ Res 1976; 38(5):423-9.

Piano MR, Bondmass M, Schwertz DW. The molecular and cellular pathophysiology of heart failure. Heart Lung 1998; 27(1):3-19.

Pimentel DR, Amin JK, Xiao L, Miller T, Viereck J, Oliver-Krasinski J, et al. Reactive oxygen species mediate amplitude-dependent hypertrophic and apoptotic responses to mechanical stretch in cardiac myocytes. Circ Res 2001; 89(5):453-60.

Pintar JE, Breakefield XO. Monoamine oxidase (MAO) activity as a determinant in human neurophysiology. Behav Genet 1982; 12(1):53-68.

Pinto YM, Paul M, Ganten D. Lessons from rat models of hypertension: from Goldblatt to genetic engineering. Cardiovasc Res 1998; 39(1):77-88.

Popping S, Rose H, Ionescu I, Fischer Y, Kammermeier H. Effect of a hawthorn extract on contraction and energy turnover of isolated rat cardiomyocytes. Arzneimittelforschung 1995; 45(11):1157-61.

Post GR, Goldstein D, Thuerauf DJ, Glembotski CC, Brown JH. Dissociation of p44 and p42 mitogen-activated protein kinase activation from receptor-induced hypertrophy in neonatal rat ventricular myocytes. J Biol Chem 1996; 271(14):8452-7.

Potenza MA, Marasciulo FL, Chieppa DM, Brigiani GS, Formoso G, Quon MJ, et al. Insulin resistance in spontaneously hypertensive rats is associated with endothelial dysfunction characterized by imbalance between NO and ET-1 production. Am J Physiol Heart Circ Physiol 2005; 289(2):H813-22.

Pourageaud F, Freslon JL. Impaired endothelial relaxations induced by agonists and flow in spontaneously hypertensive rat compared to Wistar-Kyoto rat perfused coronary arteries. J Vasc Res 1995; 32(3):190-9.

Prabhakaran D, Shah P, Chaturvedi V, Ramakrishnan L, Manhapra A, Reddy KS. Cardiovascular risk factor prevalence among men in a large industry of northern India. Natl Med J India 2005; 18(2):59-65.

Privratsky JR, Wold LE, Sowers JR, Quinn MT, Ren J. AT1 blockade prevents glucose-induced cardiac dysfunction in ventricular myocytes: role of the AT1 receptor and NADPH oxidase. Hypertension 2003; 42(2):206-12.

Purcell NH, Tang G, Yu C, Mercurio F, DiDonato JA, Lin A. Activation of NF-kappa B is required for hypertrophic growth of primary rat neonatal ventricular cardiomyocytes. Proc Natl Acad Sci U S A 2001; 98(12):6668-73.

Quast U, Guillon JM, Cavero I. Cellular pharmacology of potassium channel openers in vascular smooth muscle. Cardiovasc Res 1994; 28(6):805-10.

Quayle JM, Nelson MT, Standen NB. ATP-sensitive and inwardly rectifying potassium channels in smooth muscle. Physiol Rev 1997; 77(4):1165-232.

Rakotoarison DA, Gressier B, Trotin F, Brunet C, Dine T, Luyckx M, et al. Antioxidant activities of polyphenolic extracts from flowers, in vitro callus and cell suspension cultures of Crataegus monogyna. Pharmazie 1997; 52(1):60-4.

Rakusan K, Cicutti N, Kazda S, Turek Z. Effect of nifedipine on coronary capillary geometry in normotensive and hypertensive rats. Hypertension 1994; 24(2):205-11.

Ramachandran A, Snehalatha C, Vijay V, King H. Impact of poverty on the prevalence of diabetes and its complications in urban southern India. Diabet Med 2002; 19(2):130-5.

Raskoff WJ, Goldman S, Cohn K. The "athletic heart". Prevalence and physiological significance of left ventricular enlargement in distance runners. Jama 1976; 236(2):158-62.

Ray M, Srivastava S, Maitra SC, Dubey MP. The hamster heart is resistant to calcium paradox. Pharmacol Res 2000; 41(4):475-81.

Reckelhoff JF, Zhang H, Srivastava K. Gender differences in development of hypertension in spontaneously hypertensive rats: role of the renin-angiotensin system. Hypertension 2000; 35(1 Pt 2):480-3.

Reckelhoff JF, Zhang H, Srivastava K, Granger JP. Gender differences in hypertension in spontaneously hypertensive rats: role of androgens and androgen receptor. Hypertension 1999; 34(4 Pt 2):920-3.

Redon J, Oliva MR, Tormos C, Giner V, Chaves J, Iradi A, et al. Antioxidant activities and oxidative stress byproducts in human hypertension. Hypertension 2003; 41(5):1096-101.

Reddy KS, Prabhakaran D, Chaturvedi V, Jeemon P, Thankappan KR, Ramakrishnan L, et al. [Methods for establishing a surveillance system for cardiovascular diseases in Indian industrial populations]. Bull World Health Organ 2006; 84(6):461-9.

Reddy VRC, Kumari SVR, Reddy BM, Azeem MA, Prabhakar MC, Appa Rao AVN. Cardiotonic activity of the fruits of Terminalia chebula. Fitoterapia 1990; 61: 517-25.

Ress RJ, Field FP, Lockley OE, Fregly MJ. Effect of clonidine on the contractile responsiveness of aortic smooth muscle to norepinephrine. Pharmacology 1979; 18(3):149-54.

Riabykina GV, Liutikova LN, Saidova MA, Botvina Iu V, Kozhemiakina E, Shchedrina EV, et al. [Changes in ST segment on ECG of hypertensive patients]. Ter Arkh 2008; 80(5):67-73.

Ritter O, Neyses L. The molecular basis of myocardial hypertrophy and heart failure. Trends Mol Med 2003; 9(7):313-21.

Robert V, Van Thiem N, Cheav SL, Mouas C, Swynghedauw B, Delcayre C. Increased cardiac types I and III collagen mRNAs in aldosterone-salt hypertension. Hypertension 1994; 24(1):30-6.

Rockman HA, Koch WJ, Lefkowitz RJ. Seven-transmembrane-spanning receptors and heart function. Nature 2002; 415(6868):206-12.

Rodriguez De Lores Arnaiz G, De Robertis ED. Cholinergic and non-cholinergic nerve endings in the rat brain. II. Subcellular localization of monoamine oxidase and succinate dehydrogenase. J Neurochem 1962; 9:503-8.

Rouet-Benzineb P, Gontero B, Dreyfus P, Lafuma C. Angiotensin II induces nuclear factor- kappa B activation in cultured neonatal rat cardiomyocytes through protein kinase C signaling pathway. J Mol Cell Cardiol 2000; 32(10):1767-78.

Rowlands DB, Glover DR, Ireland MA, McLeay RA, Stallard TJ, Watson RD, et al. Assessment of left-ventricular mass and its response to antihypertensive treatment. Lancet 1982; 1(8270):467-70.

Ruggenenti P, Fassi A, Ilieva AP, Bruno S, Iliev IP, Brusegan V, et al. Preventing microalbuminuria in type 2 diabetes. N Engl J Med 2004; 351(19):1941-51.

Sachse FB, Seemann G, Taccardi B. Insights into electrophysiological studies with papillary muscle by computational models. In: Franji AF et al. ed. Lecture Notes in Computer Science. Springer; 2005: 216-25.

Satoh T, Moriyama T, Kuriki H, Karaki H. Calcium channel blocker-like action of reserpine in smooth muscle. Jpn J Pharmacol 1992; 60(3):291-3.

Sawyer DB, Siwik DA, Xiao L, Pimentel DR, Singh K, Colucci WS. Role of oxidative stress in myocardial hypertrophy and failure. J Mol Cell Cardiol 2002; 34(4):379-88.

Scheler S, Motz W, Strauer BE. Transient myocardial ischaemia in hypertensives: missing link with left ventricular hypertrophy. Eur Heart J 1992; 13 Suppl D: 62-5.

Schenk J, McNeill JH. The pathogenesis of DOCA-salt hypertension. J Pharmacol Toxicol Methods 1992; 27(3):161-70.

Schlant RC, Felner JM, Blumenstein BA, Wollam GL, Hall WD, Shulman NB, et al. Echocardiographic documentation of regression of left ventricular hypertrophy in patients treated for essential hypertension. Eur Heart J 1982; 3 Suppl A: 171-5.

Schmidt MI, Watson RL, Duncan BB, Metcalf P, Brancati FL, Sharrett AR, et al. Clustering of dyslipidemia, hyperuricemia, diabetes, and hypertension and its association with fasting insulin and central and overall obesity in a general population. Atherosclerosis Risk in Communities Study Investigators. Metabolism 1996; 45(6):699-706.

Schnackenberg CG, Welch WJ, Wilcox CS. Normalization of blood pressure and renal vascular resistance in SHR with a membrane-permeable superoxide dismutase mimetic: role of nitric oxide. Hypertension 1998; 32(1):59-64.

Schnackenberg CG, Wilcox CS. Two-week administration of tempol attenuates both hypertension and renal excretion of 8-Iso prostaglandin F_2alpha. Hypertension 1999; 33(1 Pt 2):424-8.

Schorb W, Booz GW, Dostal DE, Conrad KM, Chang KC, Baker KM. Angiotensin II is mitogenic in neonatal rat cardiac fibroblasts. Circ Res 1993; 72(6):1245-54.

Schuhmann K, Romanin C, Baumgartner W, Groschner K. Intracellular Ca2+ inhibits smooth muscle L-type Ca2+ channels by activation of protein phosphatase type 2B and by direct interaction with the channel. J Gen Physiol 1997; 110(5):503-13.

Schunkert H, Sadoshima J, Cornelius T, Kagaya Y, Weinberg EO, Izumo S, et al. Angiotensin II-induced growth responses in isolated adult rat hearts. Evidence for load-independent induction of cardiac protein synthesis by angiotensin II. Circ Res 1995; 76(3):489-97.

Schussler M, Holzl J, Fricke U. Myocardial effects of flavonoids from Crataegus species. Arzneimittelforschung 1995; 45(8):842-5.

Schwartzkopff B, Motz W, Vogt M, Strauer BE. Heart failure on the basis of hypertension. Circulation 1993; 87(5 Suppl):IV66-72.

Seidl PR. Pharmaceuticals from natural products: current trends. An Acad Bras Cienc 2002; 74(1):145-50.

Sen S. Regression of cardiac hypertrophy. Experimental animal model. Am J Med 1983; 75(3A):87-93.

Sen S, Tarazi RC. Regression of myocardial hypertrophy and influence of adrenergic system. Am J Physiol 1983; 244(1):H97-101.

Sen S, Tarazi RC, Bumpus FM. Cardiac hypertrophy and antihypertensive therapy. Cardiovasc Res 1977; 11(5):427-33.

Sen S, Tarazi RC, Khairallah PA, Bumpus FM. Cardiac hypertrophy in spontaneously hypertensive rats. Circ Res 1974; 35(5):775-81.

Serur JR, Galyean JR, Urschel CW, Sonnenblick EH. Experimental myocardial ischemia: dynamic alterations in ventricular contractility and relaxation with dissociation of speed and force in the isovolumic dog heart. Circ Res 1976; 39(4):602-7.

Shah J, Jandhyala BS. Age-dependent alterations in Na+, K (+)-ATPase activity in the central nervous system of spontaneously hypertensive rats: relationship to the development of high blood pressure. Clin Exp Hypertens 1995; 17(5):751-67.

Shannon TR, Bers DM. Assessment of intra-SR free [Ca] and buffering in rat heart. Biophys J 1997; 73(3):1524-31.

Shapiro LM, McKenna WJ. Left ventricular hypertrophy. Relation of structure to diastolic function in hypertension. Br Heart J 1984; 51(6):637-42.

Share L, Crofton JT, Lee-Kwon WJ, Shade RE. One-clip, one-kidney hypertension in rats with hereditary hypothalamic diabetes insipidus. Clin Exp Hypertens A 1982;4(8):1261-70.

Sharifi AM, Darabi R, Akbarloo N. Study of antihypertensive mechanism of Tribulus terrestris in 2K1C hypertensive rats: role of tissue ACE activity. Life Sci 2003; 73(23):2963-71.

Sharma AM. Is there a rationale for angiotensin blockade in the management of obesity hypertension? Hypertension 2004; 44(1):12-9.

Shepherd JT. Franz Volhard lecture. Increased systemic vascular resistance and primary hypertension: the expanding complexity. J Hypertens Suppl 1990; 8(7):S15-27.

Shih NL, Cheng TH, Loh SH, Cheng PY, Wang DL, Chen YS, et al. Reactive oxygen species modulate angiotensin II-induced beta-myosin heavy chain gene expression via Ras/Raf/extracellular signal-regulated kinase pathway in neonatal rat cardiomyocytes. Biochem Biophys Res Commun 2001; 283(1):143-8.

Shon YH, Kim JH, Nam KS. Effect of Astragali radix extract on lipopolysaccharide-induced inflammation in human amnion. Biol Pharm Bull 2002; 25(1):77-80.

Shorofsky SR, Aggarwal R, Corretti M, Baffa JM, Strum JM, Al-Seikhan BA, et al. Cellular mechanisms of altered contractility in the hypertrophied heart: big hearts, big sparks. Circ Res 1999; 84(4):424-34.

Sica DA. Centrally acting antihypertensive agents: an update. J Clin Hypertens (Greenwich) 2007; 9(5):399-405.

Silver MA, Pick R, Brilla CG, Jalil JE, Janicki JS, Weber KT. Reactive and reparative fibrillar collagen remodelling in the hypertrophied rat left ventricle: two experimental models of myocardial fibrosis. Cardiovasc Res 1990; 24(9):741-7.

Simpson P, McGrath A, Savion S. Myocyte hypertrophy in neonatal rat heart cultures and its regulation by serum and by catecholamines. Circ Res 1982; 51(6):787-801.

Singh N, Kapur KK, Singh SP, Shanker K, Sinha JN, Kohli RP. Mechanism of Cardiovascular Action of Terminalia arjuna. Planta Med 1982; 45(6):102-4.

Slama M, Susic D, Varagic J, Frohlich ED. High rate of ventricular septal defects in WKY rats. Hypertension 2002; 40(2):175-8.

Soares De Moura R, Costa Viana FS, Souza MA, Kovary K, Guedes DC, Oliveira EP, et al. Antihypertensive, vasodilator and antioxidant effects of a vinifera grape skin extract. J Pharm Pharmacol 2002; 54(11):1515-20.

Somanadhan B, Varughese G, Palpu P, Sreedharan R, Gudiksen L, Smitt UW, et al. An ethnopharmacological survey for potential angiotensin converting enzyme inhibitors from Indian medicinal plants. J Ethnopharmacol 1999; 65(2):103-12.

Somlyo AP, Somlyo AV. Signal transduction and regulation in smooth muscle. Nature 1994; 372(6503):231-6

Spelman K, Burns J, Nichols D, Winters N, Ottersberg S, Tenborg M. Modulation of cytokine expression by traditional medicines: a review of herbal immunomodulators. Altern Med Rev 2006; 11(2):128-50.

Staessen JA, Fagard R, Thijs L, Celis H, Arabidze GG, Birkenhager WH, et al. Randomised double-blind comparison of placebo and active treatment for older patients with isolated systolic hypertension. The Systolic Hypertension in Europe (Syst-Eur) Trial Investigators. Lancet 1997; 350(9080):757-64.

Struijker Boudier HA, le Noble JL, Messing MW, Huijberts MS, le Noble FA, van Essen H. The microcirculation and hypertension. J Hypertens Suppl 1992; 10(7):S147-56.

Sugden PH, Clerk A. Cellular mechanisms of cardiac hypertrophy. J Mol Med 1998; 76(11):725-46.

Sullivan JC, Sasser JM, Pollock JS. Sexual dimorphism in oxidant status in spontaneously hypertensive rats. Am J Physiol Regul Integr Comp Physiol 2007; 292(2):R764-8.

Sun Y, Weber KT. Infarct scar: a dynamic tissue. Cardiovasc Res 2000; 46(2):250-6.

Sutherland DJ, Ruse JL, Laidlaw JC. Hypertension, increased aldosterone secretion and low plasma renin activity relieved by dexamethasone. Can Med Assoc J 1966; 95(22):1109-19.

Sutherland FJ, Hearse DJ. The isolated blood and perfusion fluid perfused heart. Pharmacol Res 2000; 41(6):613-27.

Suzuki H, Swei A, Zweifach BW, Schmid-Schonbein GW. In vivo evidence f microvascular oxidative stress in spontaneously hypertensive rats. Hydroethidi microfluorography. Hypertension 1995; 25(5):1083-9.

Swynghedauw B. Molecular mechanisms of myocardial remodeling. Physiol Rev 199 79(1):215-62.

Swynghedauw B, Baillard C. Biology of hypertensive cardiopathy. Curr Opin Card 2000; 15(4):247-53.

Taddei S, Virdis A, Ghiadoni L, Magagna A, Favilla S, Pompella A, et al. Restoration nitric oxide availability after calcium antagonist treatment in essential hypertensio Hypertension 2001; 37(3):943-8.

Taddei S, Virdis A, Ghiadoni L, Magagna A, Salvetti A. Cyclooxygenase inhibiti restores nitric oxide activity in essential hypertension. Hypertension 1997; 29(1 Pt 2):27 9.

Tain YL, Baylis C. Dissecting the causes of oxidative stress in an in vivo model hypertension. Hypertension 2006; 48(5):828-9.

Takeishi Y, Huang Q, Abe J, Glassman M, Che W, Lee JD, et al. Src and multiple MA kinase activation in cardiac hypertrophy and congestive heart failure under chron pressure-overload: comparison with acute mechanical stretch. J Mol Cell Cardiol 200 33(9):1637-48.

Takimoto Y, Aoyama T, Iwanaga Y, Izumi T, Kihara Y, Pennica D, et al. Increas expression of cardiotrophin-1 during ventricular remodeling in hypertensive rats. Am Physiol Heart Circ Physiol 2002; 282(3):H896-901.

Tanaka K, Honda M, Takabatake T. Redox regulation of MAPK pathways and cardi hypertrophy in adult rat cardiac myocyte. J Am Coll Cardiol 2001; 37(2):676-85.

Tang EH, Leung FP, Huang Y, Feletou M, So KF, Man RY, et al. Calcium and reacti oxygen species increase in endothelial cells in response to releasers of endothelium-deriv contracting factor. Br J Pharmacol 2007; 151(1):15-23.

Taniyama Y, Griendling KK. Reactive oxygen species in the vasculature: molecular a cellular mechanisms. Hypertension 2003; 42(6):1075-81.

Tarazi RC, Fouad FM, Ferrario CM. Can the heart initiate some forms of hypertensio Fed Proc 1983; 42(10):2691-7.

Terracio L, Rubin K, Gullberg D, Balog E, Carver W, Jyring R, et al. Expression collagen binding integrins during cardiac development and hypertrophy. Circ Res 199 68(3):734-44.

Tesfamariam B, Halpern W. Endothelium-dependent and endothelium-independent vasodilation in resistance arteries from hypertensive rats. Hypertension 1988; 11(5):440-4.

Thybo NK, Stephens N, Cooper A, Aalkjaer C, Heagerty AM, Mulvany MJ. Effect of antihypertensive treatment on small arteries of patients with previously untreated essential hypertension. Hypertension 1995; 25(4 Pt 1):474-81.

Tomaselli GF, Beuckelmann DJ, Calkins HG, Berger RD, Kessler PD, Lawrence JH, et al. Sudden cardiac death in heart failure. The role of abnormal repolarization. Circulation 1994; 90(5):2534-9.

Tomaselli GF, Marban E. Electrophysiological remodeling in hypertrophy and heart failure. Cardiovasc Res 1999; 42(2):270-83.

Tomida T, Numaguchi Y, Nishimoto Y, Tsuzuki M, Hayashi Y, Imai H, et al. Inhibition of COX-2 prevents hypertension and proteinuria associated with a decrease of 8-iso-PGF2alpha formation in L-NAME-treated rats. J Hypertens 2003; 21(3):601-9.

Touyz RM. Reactive oxygen species, vascular oxidative stress, and redox signaling in hypertension: what is the clinical significance? Hypertension 2004; 44(3):248-52.

Touyz RM, Schiffrin EL. Signal transduction mechanisms mediating the physiological and pathophysiological actions of angiotensin II in vascular smooth muscle cells. Pharmacol Rev 2000; 52(4):639-72.

Tschudi MR, Mesaros S, Luscher TF, Malinski T. Direct in situ measurement of nitric oxide in mesenteric resistance arteries. Increased decomposition by superoxide in hypertension. Hypertension 1996; 27(1):32-5.

Uozumi H, Hiroi Y, Zou Y, Takimoto E, Toko H, Niu P, et al. gp130 plays a critical role in pressure overload-induced cardiac hypertrophy. J Biol Chem 2001; 276(25):23115-9.

Vaidya AD, Devasagayam TP. Current Status of Herbal Drugs in India: An Overview. J Clin Biochem Nutr 2007; 41(1):1-11.

van Zwieten PA. Centrally acting antihypertensive drugs. Present and future. Clin Exp Hypertens 1999; 21(5-6):859-73.

Vanhoutte PM, Feletou M, Taddei S. Endothelium-dependent contractions in hypertension. Br J Pharmacol 2005; 144(4):449-58.

Vericel E, Narce M, Ulmann L, Poisson JP, Lagarde M. Age-related changes in antioxidant defence mechanisms and peroxidation in isolated hepatocytes from spontaneously hypertensive and normotensive rats. Mol Cell Biochem 1994; 132(1):25-9.

Villari B, Vassalli G, Monrad ES, Chiariello M, Turina M, Hess OM. Normalization of diastolic dysfunction in aortic stenosis late after valve replacement. Circulation 1995; 91(9):2353-8.

Vohora SB, Parasar GC. Phytochemical and pharmacological studies on Ficus bengalensis, Linn. (Preliminary report). Indian J Physiol Pharmacol 1969; 13(3):143-5.

Wagner DR, Combes A, McTiernan C, Sanders VJ, Lemster B, Feldman AM. Adenosine inhibits lipopolysaccharide-induced cardiac expression of tumor necrosis factor-alpha. Circ Res 1998; 82(1):47-56.

Walsh, MP.Regulation of vascular smooth muscle tone.Can J Physiol Pharmacol 1994; 72(8): 919-36.

Wang DH, Prewitt RL. Captopril reduces aortic and microvascular growth in hypertensive and normotensive rats. Hypertension 1990; 15(1):68-77.

Wang Z, Kutschke W, Richardson KE, Karimi M, Hill JA. Electrical remodeling in pressure-overload cardiac hypertrophy: role of calcineurin. Circulation 2001; 104(14):1657-63.

Wasir HS, Ramachandran P, Nath LM. Prevalence of hypertension in a closed urban community. Indian Heart J 1984; 36(4):250-3.

Watt PA, Thurston H. Endothelium-dependent relaxation in resistance vessels from the spontaneously hypertensive rats. J Hypertens 1989; 7(8):661-6.

Weber KT. Extracellular matrix remodeling in heart failure: a role for de novo angiotensin II generation. Circulation 1997; 96(11):4065-82.

Weber KT. Targeting pathological remodeling: concepts of cardioprotection and reparation. Circulation 2000; 102(12):1342-5.

Weber KT, Brilla CG, Janicki JS. Myocardial remodeling and pathologic hypertrophy. Hosp Pract (Off Ed) 1991; 26(4):73-80.

Weber KT, Sun Y, Guarda E, Katwa LC, Ratajska A, Cleutjens JP, et al. Myocardial fibrosis in hypertensive heart disease: an overview of potential regulatory mechanisms. Eur Heart J 1995; 16 Suppl C: 24-8.

Wen C, Wu L, Ling H, Li L. Salutary effects of Corydalis yanhusuo extract on cardiac hypertrophy due to pressure overload in rats. J Pharm Pharmacol 2007; 59(8):1159-65.

Wettschureck N, Rutten H, Zywietz A, Gehring D, Wilkie TM, Chen J, et al. Absence of pressure overload induced myocardial hypertrophy after conditional inactivation of Galphaq/Galpha11 in cardiomyocytes.Nat Med 2001;7(11):1236-40.

WHO 2002.WHO Traditional Medicine Strategy 2002-2005. World Health Organization, Geneva.

Wickenden AD, Kaprielian R, Kassiri Z, Tsoporis JN, Tsushima R, Fishman GI, et al. The role of action potential prolongation and altered intracellular calcium handling in the pathogenesis of heart failure. Cardiovasc Res 1998; 37(2):312-23.

Williams RR, Hunt SC, Hopkins PN, Hasstedt SJ, Wu LL, Lalouel JM. Tabulations and expectations regarding the genetics of human hypertension. Kidney Int Suppl 1994; 44:S57-64.

Wing LM, Reid CM, Ryan P, Beilin LJ, Brown MA, Jennings GL, et al. A comparison of outcomes with angiotensin-converting--enzyme inhibitors and diuretics for hypertension in the elderly. N Engl J Med 2003; 348(7):583-92.

Wollam GL, Hall WD, Porter VD, Douglas MB, Unger DJ, Blumenstein BA, et al. Time course of regression of left ventricular hypertrophy in treated hypertensive patients. Am J Med 1983; 75(3A):100-10.

Wu G, Toyokawa T, Hahn H, Dorn GW, 2nd. Epsilon protein kinase C in pathological myocardial hypertrophy. Analysis by combined transgenic expression of translocation modifiers and Galphaq. J Biol Chem 2000; 275(39):29927-30.

Wu L, Juurlink BH. The impaired glutathione system and its up-regulation by sulforaphane in vascular smooth muscle cells from spontaneously hypertensive rats. J Hypertens 2001; 19(10):1819-25.

Wu R, Millette E, Wu L, de Champlain J. Enhanced superoxide anion formation in vascular tissues from spontaneously hypertensive and desoxycorticosterone acetate-salt hypertensive rats. J Hypertens 2001; 19(4):741-8.

Yamakawa T, Tanaka S, Tamura K, Isoda F, Ukawa K, Yamakura Y, et al. Wistar fatty rat is obese and spontaneously hypertensive. Hypertension 1995; 25(1):146-50.

Yamori Y. Importance of genetic factors in stroke: an evidence obtained by selective breeding of stroke-prone and -resistant SHR. Jpn Circ J 1974; 38(12):1095-100.

Yamori Y, Horie R, Handa H, Sato M, Fukase M. Pathogenetic similarity of strokes in stroke-prone spontaneously hypertensive rats and humans. Stroke 1976; 7(1):46-53.

Yan GX, Rials SJ, Wu Y, Liu T, Xu X, Marinchak RA, et al. Ventricular hypertrophy amplifies transmural repolarization dispersion and induces early afterdepolarization. Am J Physiol Heart Circ Physiol 2001; 281(5):H1968-75.

Yoshida M, Yoshida E, Satoh S. Effect of renal nerve denervation on tissue catecholamine content in spontaneously hypertensive rats. Clin Exp Pharmacol Physiol 1995; 22(8):512-7.

Ytrehus K, Myklebust R, Mjos OD. Influence of oxygen radicals generated by xanthine oxidase in the isolated perfused rat heart. Cardiovasc Res 1986; 20(8):597-603.

Yu XC, Wu S, Chen CF, Pang KT, Wong TM. Antihypertensive and anti-arrhythmic effects of an extract of Radix Stephaniae Tetrandrae in the rat. J Pharm Pharmacol 2004; 56(1):115-22.

Zachariah MG, Thankappan KR, Alex SC, Sarma PS, Vasan RS. Prevalence, correlates, awareness, treatment, and control of hypertension in a middle-aged urban population in Kerala. Indian Heart J 2003; 55(3):245-51.

Zaoui A, Cherrah Y, Lacaille-Dubois MA, Settaf A, Amarouch H, Hassar M. [Diuretic and hypotensive effects of Nigella sativa in the spontaneously hypertensive rat]. Therapie 2000; 55(3):379-82.

Zarzuelo A, Duarte J, Jimenez J, Gonzalez M, Utrilla MP. Vasodilator effect of olive leaf. Planta Med 1991; 57(5):417-9.

Zhai P, Gao S, Holle E, Yu X, Yatani A, Wagner T, et al. Glycogen synthase kinase-3alpha reduces cardiac growth and pressure overload-induced cardiac hypertrophy by inhibition of extracellular signal-regulated kinases. J Biol Chem 2007; 282(45):33181-91.

Zhang GX, Kimura S, Nishiyama A, Shokoji T, Rahman M, Yao L, et al. Cardiac oxidative stress in acute and chronic isoproterenol-infused rats. Cardiovasc Res 2005; 65(1):230-8.

Zhang HY, McPherson BC, Liu H, Baman TS, Rock P, Yao Z. H(2)O(2) opens mitochondrial K(ATP) channels and inhibits GABA receptors via protein kinase C-epsilon in cardiomyocytes. Am J Physiol Heart Circ Physiol 2002; 282(4):H1395-403.

Zhou Q, Rowlands DK, Gou YL, Tsang LL, Chung YW, Chan HC. Cardiovascular protective effects of traditional Chinese medicine bak foong pills in spontaneously hypertensive rats. Biol Pharm Bull 2003; 26(8):1095-9.

Zhu Z, Zhu S, Liu D, Cao T, Wang L, Tepel M. Thiazide-like diuretics attenuate agonist-induced vasoconstriction by calcium desensitization linked to Rho kinase. Hypertension 2005; 45(2):233-9.

Zierhut W, Zimmer HG. Significance of myocardial alpha- and beta-adrenoceptors in catecholamine-induced cardiac hypertrophy. Circ Res 1989; 65(5):1417-25.

Zierhut W, Zimmer HG, Gerdes AM. Effect of angiotensin converting enzyme inhibition on pressure-induced left ventricular hypertrophy in rats. Circ Res 1991; 69(3):609-17.

Zile MR, Gaasch WH. Mechanical loads and the isovolumic and filling indices of left ventricular relaxation. Prog Cardiovasc Dis 1990; 32(5):333-46.

Zou Y, Hiroi Y, Uozumi H, Takimoto E, Toko H, Zhu W, et al. Calcineurin plays a critical role in the development of pressure overload-induced cardiac hypertrophy. Circulation 2001; 104(1):97-101.

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