The potential antihypertensive and antidiabetic activities of stevia in preventing chronic cardiovascular disease in rat models of hypertension and diabetes: Comparison to the calcium channel antagonist verapamil

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To my parents

To my husband

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Declaration by author

I hereby declare that I am the sole author of this thesis. I authorize CQUniversity to lend this thesis to other institutions or individuals for the purpose of scholarly research. The work contained in this thesis has not been previously submitted, either in whole or in part, for a degree at CQUniversity or any other tertiary institution. To the best of my knowledge and belief, the material presented in this thesis is original, except where due reference is made in text.

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Abstract

Diabetes and hypertension are major contributors to cardiovascular disease which is the leading causes of death worldwide. The impact of diabetes on the cardiovascular system is high, with almost 75% of diabetic deaths being directly attributed to some form of heart or blood vessel disorder. Diabetes and hypertension induce significant damage to the heart, blood vessels and kidney. Stevia, *Stevia rebaudiana* Bertoni, a naturally sweet herb, has been used for several hundred years as an anti-diabetic and cardiotonic in South America. In limited human and rat trials, stevia reduced hypertension, improved insulin-sensitivity and decreased blood lipids following hypertension and diabetes. The current study aimed to identify any cardioprotective benefits of stevia in reversing and/or preventing further cardiovascular damage in animal models of type 1 diabetes and hypertension compared to the calcium channel antagonist verapamil. A mechanistic study on normal healthy tissues was also performed to establish a putative mechanism of action of stevia.

The mechanistic study showed that stevia could reduce cardiac action potential duration (APD) at 20%, 50% and 90% of repolarisation, dilate vascular beds and decrease isolated ileum contraction induced by electrical field stimulation (EFS) and M_3 receptor agonist carbachol. In short, our results suggested that the mechanism of action of stevia is multimodal since stevia showed beneficial modulatory effects on cardiovascular and gastrointestinal tissues which can be related to calcium channel antagonism, activation of M_2 muscarinic receptor function, and via enhanced nitric oxide (NO) release.

The second study examined the chronic effects of hypertension in the spontaneously hypertensive rat (SHR). Chronic dosing (8 weeks) of stevia (200 mg/kg/day) or verapamil (4 mg/kg/day) were compared in the SHR to establish if either compound could reduce the severity of cardiovascular remodelling and peripheral neuropathy following hypertension. Stevia treatment prevented maladaptive left ventricular changes, restored cardiac contractility and end systolic pressure and reduced diastolic stiffness in the hypertensive rats. Stevia also normalised liver and kidney weights compared to body weight/tibial length ratio in the SHR rats. Stevia reduced oxidative stress and inflammation as observed by significant increase in TAC and serum NO levels comopared to the unrtreated SHR. Verapamil improved systolic and diastolic functions, except for the heart rate which was significantly reduced in the SHR.

The final study utilized the streptozotocin (STZ)-induced type 1 diabetic rat model with the effect of stevia and verapamil again investigated for an eight-week treatment period. The results showed that both stevia and verapamil treatment improved the maximal contractile and relaxation responses of thoracic aortic rings and mesenteric arteries, improved serum NO synthesis, total anti-oxidant capacity and malondialdehyde (MDA) levels following diabetes. Electrophysiological studies showed that stevia prevented prolongation of the action potential at 20%, 50% and 90% of repolarisation suggesting that stevia might restore sinus rhythm and reduce ventricular arrhythmogenesis following diabetes. Verapamil showed similar results in

these electrophysiological studies. In the isolated ileum, stevia prevented the gastrointestinal dysmotility following chronic diabetes.

In summary, this study established that stevia can prevent the cardiovascular remodelling following diabetes and hypertension with putative mechanisms thought to be through multiple pathways such as calcium channel blockade, reduction in insulin resistance, increased NO synthesis and reduction of oxidative damage. Verapamil was also shown to be effective in these animal models acting as an anti-inflammatory, antioxidant and NO promoting treatment in addition to its primary pharmacological role.

List of publications and presentations

Publications

1. **Yesmine, S**, Connolly, K, Hill, N, Coulson, FR & Fenning, AS. (2012), Electrophysiological, vasoactive and gastromodulatory effects of stevia in healthy Wistar rats: accepted in *Planta Medica* (PLAMED-2012-05-0486-OP)

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3. Islam, S, **Yesmine, S,** Khan, SA, Alam, NH & Islam, S. (2008), A comparative Study of Thyroid Hormone Levels in Diabetic and Non-Diabetic Patients, *South East Asian Journal of Tropical Medicine and Public Health*; Vol 39, No. 5.

Abstracts presented in refereed conferences

1. **Yesmine, S,** Connolly, K, Floyd, K, Coulson, FR & Fenning, AS. (2010), Vasoactive and gastrointestinal effects of Stevia, poster presentation at XXth World Congress of the international Society for Heart Research, Kyoto, Japan; May 13-16.
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List of Abbreviations

ABS	Australian Bureau of Statistics
ADMA	Asymmetric dimethyl arginine
ATR	Angiotensin II receptor
AIHW	Australian Institute of Health and Welfare
ANOVA	Analysis of variance
Ach	Acetylcholine
ACE	Angiotensin converting enzyme
AGE	Advanced glycation end products
APD	Action potential duration
ANP	Atrial natriuretic peptide
BP	Blood pressure
CCB	Calcium channel blocker
CHF	Chronic Heart Failure
CRP	C- reactive protein
CVD	Cardiovascular disease
DOCA	Deoxycorticosterone-acetate
DPPH	1-1-Diphenyl-2-picryl hydrazyl
ECM	Extra-cellular matrix
ERK ½	Extracellular signal-regulated kinase ½
ET_A	Endothelin type A receptor
eNOS	Endothelial nitric oxide synthase
GM-CSF	Granulocyte macrophage colony stimulating factor
HOPE	Heart Outcomes Prevention Evaluation (trial)
ICAM-1	Intercellular adhesion molecule 1
IDF	International diabetic federation
IL-6	Interlukin-6
I_{Kur}	Ultra-rapid potassium current
I_{to1}	Transient outward current
I_{Kr}	Rapid potassium current
I_{Ks}	Slow potassium current
I_{K1}	Inward rectifyer current
I_{Ca}	Calcium influx current
$I_{Ca\text{-}L}$	L-type calcium channel
I_{Na}	Inward sodium current
LDL	Low density lipoprotein
MCP	Monocyte chemotactic protein-1
MDA	Malondialdehyde
NO	Nitric oxide
NADPH	Nicotinamide adenine dinucleotide phosphate
NA	Noradrenaline
NaNO	Sodium nitroprusside
NOS	Nitric oxide synthase
PAR	Population attributable risk
plasminogen –Rs	plasminogen- receptors
PPAR	Peroxisome proliferator-activated receptor
PRA	Plasma renin activity

PVAT	Perivascular adipose tissue
RAAS	Renin-angiotensin aldosterone system
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
RV	Right ventricle
SMC	Smooth muscle cell
SHR	Spontaneously hypertensive rat
SHRSP	Stroke-prone spontaneously hypertensive rat
STZ	Streptozotocin
TGF-β	Transforming growth factor beta
TGR	Transgenic rat
TNF-α	Tissue necrosis factor α
VCAM-1	Vascular cell adhesion molecule 1
VSMC	Vascular smooth muscle cell
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation

Chapter 1 Introduction

1.1 Cardiovascular disease - diabetes and hypertension

Cardiovascular disease (CVD) induces damage to the heart, blood vessels and kidneys and includes conditions such as coronary heart disease, stroke, heart failure, peripheral vascular disease and rheumatic heart disease (Australian Bureau of Statistics [ABS] 2011). Although epidemiologists have made radical discoveries over the last 60 years to reduce its morbidity and mortality, cardiovascular disease remains one of the leading causes of death worldwide (ABS 2011). According to the World Health Organization (WHO), approximately 17.3 million people died of CVD in 2008, which is 30% of all deaths globally (Huang et al. 2009; WHO 2011). It was also predicted that by 2030, about 23.6 million deaths will attributable to CVD and it will remain the topmost cause of death worldwide, despite the extensive use of cholesterol lowering and antihypertensive agents (WHO 2011). CVD killed 811,940 people in the United States alone in 2008 with a further 79,400,000 diagnosed with one or more form of cardiovascular disease in that year (Roger et al. 2011). According to 2008 data, approximately 2200 people in America die of CVD every day (American Heart Association [AHA] 2009). Countries with a greater percentage of their population categorised in the lower socio-economic groupings are also adversely affected by CVD, as data showed that more than 80% of deaths due to CVD occur in these countries (WHO 2011).

In Australia, five of the top twenty primary causes of death were associated with cardiovascular disease representing 32.8% of all deaths in 2009 (ABS 2011). The prevalence of cardiovascular disease in Australia has increased by 18.2% over the last decade with approximately 3.67 million people affected (Australian Institute of Health and Welfare [AIHW] 2010). CVD has the highest level of expenditure in Australia which costed approximately $5.9 billion in 2004-05 (AIHW 2010). With the total economic burden of these diseases increasing over the coming decades, a better understanding of the underlying pathology and the development of novel pharmacological interventions is needed to reduce this trend.

Hypertension remains a primary cause in the initiation and development of cardiovascular disease particularly in relation to left ventricular remodelling and the development of heart failure (Bing et al. 2002; Boluyt & Bing 2000; Weber, K. 2002). Various studies have demonstrated a clear link to left ventricular remodelling and cardiovascular morbidity following diabetes and hypertension (Devereux & Roman 1999; Felicio et al. 2000). Poorly controlled hypertension leads to altered neuro-humoral activity and impaired vascular function leading end organ damage.

Worldwide, the diagnosis of diabetes and the metabolic syndrome is becoming a significant medical concern and public health burden. Diabetes is a rapidly growing disease which has been predicted to rise from 366 million in 2011 to 552 million by 2030 worldwide (The International Diabetic Federation [IDF] 2011). Currently 11,100,000 Americans have been diagnosed with diabetes and almost 800,000 new cases of type 2 diabetes are diagnosed every year (AHA 2004). The impact of the diabetic syndrome on the cardiovascular system is significant with almost 75% of diabetic deaths being directly attributed to some form of heart or blood vessel disorder (AHA 2004). According to AusDiab (2006), everyday, approximately 275 adult Australians develope diabetes and 3.0% of adult Australians are also developing hypertension annually. Moreover, the chance of developing high blood pressure was greater for people with diabetes or the metabolic syndrome (AusDiab 2006).

In Australia, the overall cost of type 2 diabetes was estimated to be 10.3 billion in 2005 with an average annual per person cost of $5360 (AusDiab 2006). These expenditures exclude productivity loss due to illness or premature death. According to the Access Economics Report (2006) for Diabetes Australia, the estimated annual cost of absenteeism for type 2 diabetes was $53.1 million and the loss for premature death was $64.7 million in 2005 (Access Economics Report 2006). Interestingly, there is increasing evidence showing that Asia is emerging as the epicentre of diabetes as India and China have large numbers of their population with the disease and it is predicted that by the year 2025 they could each have more than 20 million affected individuals (Yoon et al. 2006). It is clearly evident that chronic diseases like diabetes and hypertension have a negative effect on a country's population health as well as on the total socio-economic growth. The objective of our research was to have a better

understanding of the underlying pathophysiology of diabetes and hypertension and to establish the beneficial effects of novel pharmacological treatments such as stevia.

1.1.1 Modern treatment options versus natural medicine

The current treatment pattern for hypertension and cardiovascular disease encompasses many classes of well established agents such as combined alpha-/beta-adrenoceptor antagonists, peripheral alpha-adrenoceptor antagonists, diuretics, angiotensin converting enzyme (ACE) inhibitors, calcium channel blockers (CCBs) and also more recent renin-angiotensin-aldosterone system (RAAS) blockers. All these classes of drugs provide useful therapeutic options in lowering blood pressure. However, there has been increasing debate about whether the benefits of treatment are solely a function of the magnitude of blood pressure control or if the type of drug used might also have additional intrinsic therapeutic effects. ACE inhibitors or angiotensin receptor blockers with or without a diuretic are considered as important initial therapies for patients with hypertension and diabetes (Shammas, Sica & Toth 2009). A meta-analysis of trials for the treatment of hypertension with newer compounds showed that ACE inhibitors and calcium channel blockers are equally successful in reducing cardiovascular morbidity and mortality as beta-blockers or thiazides (Williams 2003). ACE inhibitors are found to reduce mortality and morbidity in patients with heart failure and also to slow the progression of cardiovascular disease and events. The Heart Outcomes Evaluation (HOPE) trial clearly showed beneficial effect of the ACE inhibitor, ramipril, on cardiovascular events and death (Arnold et al. 2003). Clinical studies have also indicated that CCBs may be effective in reducing organ injury associated with oxidative stress during the development of hypertension (Morimoto, Kureishi-Bando & Murohara 2010; Nishiyama, Nakano & Hitomi 2010). However it appears that compounds such as blockers of the RAAS or CCBs do induce some of their more desirable effects by inhibiting humoral as well as haemodynamic parameters.

Nutraceuticals have recently been proposed as potential treatments for chronic diseases such as diabetes, hypertension and obesity. In the field of cardiovascular medicine, plant extracts such as resveratrol and dietary supplements such as L-arginine and L-carnitine have been shown to have significant antioxidant and anti-

inflammatory effects which can prevent vascular and cardiac cell damage (Fenning et al. 2005; Miatello et al. 2005). Recently, stevia, a sweet herb native to South America, has shown notable results in the treatment of diabetes and hypertension. Studies showed that this naturally occurring replacement sweetener, stevia, could possess important cardiovascular and metabolic actions (Jeppesen et al. 2003). Initial studies showed stevia also has antioxidant and anti-inflammatory effects which help to reduce cardiovascular damage and metabolic disorders (Boonkaewwan, Toskulkao & Vongsakul 2006). Since cardiovascular disease and diabetes are now shown to be correlated with oxidative stress and inflammation, it is therefore of great interest to study the protective effect and influence of *Stevia rebaudiana* on these processes. There is some evidence that stevia has both directly acting vasorelaxant and antidiabetic properties which may act synergistically with its potential antioxidant effects (Boonkaewwan, Toskulkao & Vongsakul 2006; Wong et al. 2006). The importance of the natural antioxidant constituents of many plant sources to maintain health and to prevent cardiovascular disease, diabetes and other health conditions is increasing. If known antioxidant and anti-inflammatory agents such as resveratrol are able to improve end organ function and mediate weight loss in obese individuals it is mportant to further characterise these pathways and find new therapeutic targets. It is also of growing interest among researchers to develop new chemical entities with specific health benefits from plants. For this purpose, *Stevia rebaudiana* was selected in the present study to evaluate its potential vasculoprotective, cardioprotective and antioxidant properties in models of diabetes and high blood pressure compared to the known antihypertensive agent verapamil. This study also assessed and characterised the progression of cardiovascular and metabolic complications in these animal models. Verapamil, a calcium channel blocker was chosen to compare the effect of stevia in the animal models. Calcium channel blockers are an important group of drugs for the treatment of hypertension, heart failure and cardiac arrhythmias. CCBs relax arteriolar smooth muscles and decrease peripheral vascular resistance which ultimately reduce blood pressure (Ishimitsu 2010). Furthermore, it is the additional features of compounds like calcium channel blockers, other than simply their haemodynamic properties which confers significant cardiovascular protection and may provide a clue to the potential mechanism of action of novel compound such as stevia.

1.2 Aims and objectives

This thesis aimed to establish the degree of cardiovascular remodelling and end organ damage in animal models of type I diabetes (streptozotocin (STZ)-induced diabetic rat) and hypertension (spontaneously hypertensive rat – SHR) and to investigate the effects of stevia (both stevioside and rebaudioside A) and verapamil in reversing and/or preventing these maladaptive changes. The mechanistic study was designed to explore the vasoactive and gastro-modulatory effects of stevia on healthy rats and these effects were compared with verapamil to confirm stevia's mechanism of action in vascular, cardiac and mesenteric tissues.

The main objectives of this study were:

- to use rat models of hypertension (SHR) and diabetes (STZ rat) to establish chronic end-organ damage (cardiovascular, renal, neural and gastrointestinal)
- to add to the understanding of the pathophysiology of these conditions
- to use stevia as a proposed antihypertensive and anti-diabetic agent preventing end-organ damage in these models
- to establish the mechanisms of action of the proposed antioxidant and anti-inflammatory effect of stevia
- to compare the efficacy of stevia to a current best practise pharmaceutical agent of potentially similar mechanism of action - verapamil
- to compare stevia and verapamil by measuring different physiological parameters in diabetes and hypertension– body weight, water intake, blood glucose
- to determine the protective effects of stevia against the severity of vascular endothelial dysfunction and parasympathetic gastrointestinal function
- to expand upon the knowledge already established for the use of verapamil in these three conditions.

1.3 Conclusion

Both diabetes and hypertension accelerates cardiovascular complications where strict control of blood glucose and blood pressure are essential. Calcium channel blockers

show consistent hypotensive effects and are useful as combination drugs for the treatment of diabetes mellitus and chronic renal diseases (Rubio-Guerra et al. 2008). Clearly these protective roles of calcium channel blockade in the progression, severity and treatment of cardiovascular disease will provide us with a better option for the characterisation and assessment of a novel pharmacological therapy which is an important aim in current cardiovascular treatment options. Although, several studies have investigated the impact of stevia upon major cardiovascular and renal outcomes in diabetic and hypertensive patients, the precise pharmacological mechanism(s) of action are yet to be fully established. Our study explored the action of stevia on the cardiovascular system in chronic diseases such as diabetes and hypertension and also examined whether this natural therapy was comparable to modern pharmacological best practise.

1.4 Chapter overviews

Chapter 1- Introduction- describes a general overview of the thesis.

Chapter 2 - Literature review - presents a review on the effects of stevia and verapamil in preventing secondary complications of cardiovascular disease following diabetes and hypertension; reviews the mechanisms behind the progression of these diseases in both humans and also rat models of these diseases.

Chapter 3 – Methods- this chapter presents the complete experimental procedures for this project.

Chapter 4 – Study 1 ‘Electrophysiological, vasoactive and gastromodulatory effects of stevia in healthy Wistar rat “. This chapter presents the mechanistic studies of stevia.

Chapter 5 – Study 2 “Stevia and verapamil prevent cardiovascular remodelling following hypertension”. This chapter presents the findings of chronic dosing of stevia and verapamil in the spontaneously hypertensive rats to determine the involvement of hypertension in cardiac remodelling and vascular changes.

Chapter 6 - Study 3 “Effects of stevia and verapamil in diabetes-induced cardiovascular remodelling”. This chapter shows the activities of chronic

administration of stevia and verapamil in the STZ-induced diabetic rats and prevention of cardiovascular remodelling.

Chapter 7 – Conclusions- this chapter presents a summary of the results and discussions and also decribes the limitations of the study.

Chapter 2 Literature Review

2.1 Literature Review

This chapter briefly reviews the current literature relating to the pathophysiology of diabetes and hypertension leading to progressive loss of cardiovascular and renal function and the effects of stevia and verapamil in preventing this damage. The chapter also describes the rationale for use of STZ-induced diabetic and spontaneously hypertensive rats (SHR) models in this research.

2.2 Diabetes Mellitus

Diabetes mellitus is a metabolic syndrome characterised by hyperglycaemia resulting from absolute or relative insulin deficiency, insulin resistance or both (Kumar & Clark 2002). In the general population, glucose is metabolised to glucose-6-phosphate through glucokinase. The excess glucose is stored in the liver as glycogen. Glucokinase activity is controlled through changes in insulin levels which are secreted by the beta cells of the pancreas (Dean & McEntyre 2004).

Glucose regulates beta cell functioning directly by releasing insulin in the first phase response, followed by the secretion in pulses, which is the second phase response (Rorsman et al. 2000). Insufficient insulin release leads to ketoacidosis and hyperglycaemia (Arulmozhi, Veeranjaneyulu & Bodhankar 2004; Dean & McEntyre 2004). Symptoms of diabetes include polyphagia, polydipsia, hyperglycaemia and abnormal glucose tolerance (Takada et al. 2007). Among these, hyperglycemia has many detrimental effects on the body, including alterations of the immune system, cardiovascular damage, thrombosis and inflammation (Clement et al. 2004).

There are two types of diabetes mellitus- type 1 and type 2 and both share the features of hyperglycemia and various vascular pathologies. They differ in pathogenesis and in the ability of residual insulin secretion to suppress ketone formation from fatty acids in type 2 diabetes.

2.2.1 Pathophysiology of type 1 diabetes mellitus

Type 1 Diabetes Mellitus results from the autoimmune destruction of pancreatic beta islet cells. Type I diabetes mellitus is manifested by an inability to secrete even the modest amounts of insulin required to suppress ketone formation, resulting in recurrent episodes of diabetic ketoacidosis (Page et al. 2004). Usually, people with type 1 diabetes show acute symptoms of diabetes and markedly increased blood glucose levels (American Diabetes Association [ADA] 2007). It is considered as juvenile-onset diabetes since about half of the people with type 1 diabetes manifesting the disease before age 18 (AIHW 2008). Type 1 diabetes represents approximately 10% - 15% of all people with the condition.

It was suggested that type 1 diabetic patients are genetically predisposed to a trigger, such as a viral infection causing an autoimmune destruction of pancreatic β-cells (ABS 2006). These patients have a greater risk of developing other autoimmune disorders such as Graves' disease, Hashimoto's thyroiditis, Addison's disease, myasthenia gravis and pernicious anaemia (American Thyroid Association [ATA] 2010). However some forms of type 1 diabetes have no known aetiology and have no evidence of autoimmunity ('Diagnosis and classification of diabetes mellitus' 2009). This autoimmune role and potential inflammatory aetiology of the above disorders is interesting, considering most of them have a chronic inflammatory component which has similarities to the course of long-term cardiovascular disease.

Type 1 diabetes leads to severe endothelial dysfunction and increased cardiovascular risk because of reduced production of endothelially-derived NO which is a key mediator of vascular homeostasis (Baskaran 1990). Furthermore, patients with type 1 diabetes show a significant correlation between the degree of hyperglycaemia and the extent of coronary heart disease (Mazzone 2009).

2.2.2 Pathophysiology of type 2 diabetes mellitus

Type 2 diabetes or noninsulin-dependent diabetes is manifested by significantly reduced insulin release from the pancreas and/or cellular insulin resistance and increased levels glucose in blood. This disease is mostly prevalent in people aged 40

years and over (AIHW 2008). Type 2 diabetes comprises around 85%-90% of all people with diabetes. However, evidence showed that nowadays people at any age are being increasingly diagnosed with type 2 diabetes (Li et al, 2004).

Impaired function of pancreatic β-cells manifested by alteration in the first phase insulin secretion is a major contributing factor to glucose intolerance in type 2 diabetes (Fonseca 2009). The second phase of insulin synthesis and release is impaired with the progression of diabetes. In addition to these, insulin resistance at hepatocytes, skeletal muscle and adipose tissue reduced the ability of insulin to maintain normal glucose level in blood (Fonseca 2009). Most studies showed that β-cell mass is reduced in type 2 diabetic patients causing many distinct defects in insulin secretion including profound impairment in the first phase of insulin secretion in response to an intravenous glucose administration (Fonseca 2009). Increased hepatic glycogenolysis and gluconeogenesis following a decrease in glucose uptake by the tissue lead to hyperglycaemia in diabetes. Recent studies showed that combinations of genetic and environmental factors are involved in insulin resistance and β-cell dysfunction (Zhang, Y. et al. 2012). This study also demonstrated that a reduced expression of ZBTB20, a zinc finger protein present on the β-cell is observed in the diabetic mice compared to β cell of normal mice (Zhang, Y. et al. 2012). This zinc finger protein controls β cell function and maintains glucose haemostasis. Therefore, the ZBTB20 can act as a potential target for the treatment of type 2 diabetes.

Practically all forms of diabetes reduce the circulating insulin concentration and cause a decreased response of peripheral tissues to insulin. These abnormalities alter the metabolism of carbohydrates, lipids, ketones and amino acids leading to hyperglycaemia (Fonseca 2009). Overall chronic diabetes leads to many systemic problems including microvascular, macrovascular and cardiovascular complications, accelerated atherosclerosis and peripheral damage (Johansen et al. 2005). These severe complications result in significant pathological processes and system-wide dysfunction in the body however the gastrointestinal system, cardiovascular system, neural tissue, limbs and eyes are the most commonly affected.

Parameters such as sedentary lifestyle, changing dietary patterns and low birth weight contributed to increase the prevalence of diabetes. Liver maintains a balance between

uptake, storage and release of glucose by regulating glycogenesis, glycogenolysis and gluconeogenesis and thus plays a key role in blood glucose hemeostasis (Saravanan, Vengatash & Ramachandran 2012). Several regulatory enzymes and hormones such as insulin, glucagon, amylin, glucose-dependent insulinotropic peptide (GIP), epinephrine, cortisol, and growth hormone are involved in maintaining blood glucose hemeostasis. Diabetes causes an imbalance or disregulation of hepatic enzymes and glucoregulatory hormones that leads to overactivity of the endocrine system resulting in endocrine dysfunction (Saravanan, Vengatash & Ramachandran 2012). The main goal in diabetes treatment is tight control of blood glucose level and to maintain normoglycemia

2.3 Hypertension

Hypertension is defined conventionally as a sustained increase in blood pressure more than or equal to 140/90 mmHg, a criterion that characterizes a group of patients whose risk of hypertension-related cardiovascular disease is high. The risk of both fatal and nonfatal cardiovascular disease in adults is lowest if systolic and diastolic blood pressures remain less than 120 and 80 mmHg respectively (Rosendorff et al., 2007). Hypertension is the most common cardiovascular disease with its prevalence increasing with advancing age. A study in 2003 showed that about 50% of people between the ages of 60 to 69 years, had hypertension with a further increase in the over 70 age group (Rosendorff et al., 2007).

2.3.1 Pathophysiology of hypertension

Initially, enhanced sympathetic activity is observed in high blood pressure followed by increased peripheral resistance and greater cardiac output. Eventually sympathetic activity and cardiac output get back to normal levels except for the peripheral resistance. The increased peripheral resistance reflects the narrowing and hardening of the vessels and subsequent endothelial damage (Mulvany 2008). Recently, levels of atrial natriuretic peptides (ANP) were found to be raised in hypertension (Rubattu et al. 2010). Moreover, altered function of perivascular adipose tissue (PVAT) surrounding the systemic arteries contributed to the development of hypertension (Lu et al. 2011). An impaired PVAT function was observed in the mesenteric arteries of

the hypertensive rats (Gálvez-Prieto et al. 2008; Lu et al. 2011). Different animal studies showed that PVAT works through numerous mechanisms in the vascular bed such as, activation of protein kinase A and ATP-dependent K^+ channels were reported in rat aorta (Dubrovska et al. 2004), voltage dependent potassium channels in mesenteric arteries and opening of calcium activated potassium channels in human thoracic arteries (Lu et al. 2011)

It is now well documented that diabetes increases the chance of high blood pressure and other cardiovascular complications (Fiordaliso et al. 2006). In the United States, 73% of patients with diabetes are diagnosed with hypertension (ADA 2007). Diabetes potentiates atherosclerosis of the coronary arteries and autoimmune neuropathy which may play important roles in the high occurrence of cardiovascular disease in diabetes (Chiquette & Chilton 2002)

2.4 Impacts of diabetes and hypertension on CVD

The rapidly increasing incidence of diabetes mellitus diagnosis is becoming a serious threat to population health in all parts of the world. The control and treatment of diabetes and its complications mainly depend on chemical or biochemical agents but the fact remains that we have yet to completely cure this disease (Li, W. et al. 2004). Keeping this in mind, it is anticipated that this project will provide a better understanding of the underlying pathology of diabetes and hypertension and will open up potential new avenues of research into alternative pharmaceutical options for the treatment of these chronic diseases and related complications.

A recent study on 106 type 2 diabetic and 189 non diabetic multiethnic youths aged 10-22 years old showed that diabetes causes a greater occurrence of multiple CVD risk factors when compared with individuals without diabetes (West et al. 2009). Moreover, diabetes increases the likelihood of developing hypertension, due to angiotensin II stimulation in response to hyperglycaemia which increases vascular pressure and cardiac load (Fiordaliso et al. 2006).

Over the last few years, chronic subclinical inflammation has emerged as a causative factor associated with insulin resistance and progressive pancreatic beta cell failure (Pitsavos et al. 2007; Pradhan et al. 2003; Shoelson, Lee & Goldfine 2006). Several

studies demonstrated that chronic low-grade inflammation plays a major role in all steps of atherosclerosis including plaque initiation, progression and thrombosis (Libby, Ridker & Maseri 2002; Pitsavos et al. 2007). A strong and consistent relationship has been established between sensitive inflammatory markers and subsequent cardiovascular events by a number of epidemiological studies (Blake et al. 2003; Johnson et al. 2004; Pitsavos et al. 2007). Acute and chronic inflammation alters endothelial function causing a reduction in the bioavailability of NO, involvement of cytokines, such as TNF-∞, IL-6, raised concentration of C-reactive protein, fibrinogen, amyloid-A and increased expression of adhesion molecules which ultimately facilitate the formation of an atherosclerotic lesion in the vessel walls (Hermann & Ruschitzka 2006). However, initiation of low-grade inflammatory process expands from atherosclerosis towards the more complex metabolic syndrome, including pathologic glucose and lipid levels, hypertension, visceral obesity and end organ damage (Hermann & Ruschitzka 2006). Moreover, hypertension and other cardiovascular events increase angiotensin II synthesis stimulating the production of cytokines which is mediated via an activation of nuclear factor-κB dependent pathway (Hermann & Ruschitzka 2006).

Calcium channel blockers (CCBs) demonstrate antioxidant and anti-inflammatory effects in addition to their ample use for the treatment of hypertension and angina pectoris. In vitro experiments report that CCBs decrease oxidative stress via multiple pathways (Nishiyama, Nakano & Hitomi 2010). Various animal models have shown CCBs to reduce oxidative stress and protect organ injury by increasing endothelial NO release. The results indicate that organ-protective effects of CCBs are largely mediated through strong antioxidative effects and are not related to their antihypertensive action (Nishiyama, Nakano & Hitomi 2010; Shima et al. 2008). Amlodipine (10^{-6} mol/l) and manidipine (10^{-6} mol/l) reduced superoxide generation by the inhibition of the overexpression of NADPH oxidase in angiotensin II-stimulated endothelial cells (Toba et al. 2006). Data demonstrated that L-type Ca^{2+} channel (LTCC) blockers, represented by amlodipine and verapamil, show anti-inflammatory activities by the suppression of plasminogen -receptors (plasminogen -Rs) on macrophages (Das et al. 2009). Two weeks administration of amlodipine (20 mg/kg/day) and manidipine (10 mg/kg/day) to L-NAME-induced hypertensive rats

showed direct anti-inflammatory and antioxidative effects in the aorta which have been mediated by an enhanced eNOS expression and by the inhibition of the expression of ACE (Toba et al. 2005).

Studies showed that some calcium channel blockers (amlodipine, nicardipine, cilnidipine, benidipine) suppress O_2 release in human neutrophils stimulated by GM-CSF or TNF-α in atherosclerotic plaque at a relatively low concentration (1^{-10} μmol/l) (Nishiyama, Nakano & Hitomi 2010; Shima et al. 2008). While antihypertensive treatment can improve vascular functions, experimental studies demonstrated that anti-inflammatory treatment has beneficial effects. Calcium channel blockers, ACE-inhibitors and angiotensin receptor blockers show anti-inflammatory effects by blocking pro-inflammatory actions of angiotensin II (Hermann & Ruschitzka 2006).Therefore, assessment of anti-inflammatory and antioxidant activity of stevia in addition to anti-hypertensive and anti-diabetic effects would increase its clinical benefits.

Moreover, epidemiological studies have confirmed that hyperglycemia is the most important factor in the onset and progression of diabetic complications (Jakus & Rietbrock 2004; Méndez et al. 2010). Hyperglycemia leads to increased formation of advanced glycation end products (AGEs), enhanced production of reactive oxygen species (ROS), reduced nitric oxide (NO) synthesis, activation of protein kinase C (PKC), stimulation of the polyol pathway and the renin-angiotensin system (RAS) which are examples of metabolic and hemodynamic derangements that contribute to the characteristic histopathological changes in diabetes (Brownlee 2001; Méndez et al. 2010). It is postulated that AGEs and advanced lipoxidation end-products (ALEs) contribute to accelerated micro- and macrovascular damage observed in diabetes (Šebeková et al. 2002, Jakus & Rietbrock 2004).

AGEs, by interacting with their receptors (RAGE) activate transcription factor NF-κB, produce tumour necrosis factor (TNF) and interleukin-1 (IL-1), and induce interleukin-6 (IL-6) mRNA expression which all lead to an increase in oxidative stress and vascular wall cells inflammation (Lin, Park & Lakatta 2009; Méndez et al. 2010). Evidence show that RAGE plays vital pathogenic role in diabetic vascular disease

such as vascular wall remodelling, endothelial cell activation and also in atherosclerosis and arterial plaque formation in (Méndez et al. 2010).

There are a number of established therapies which are capable of reducing the formation and accumulation of AGEs in diabetes such as, angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor antagonists, calcium channel blockers (CCBs), metformin, peroxisome proliferators receptor agonists, and some antioxidants (Méndez et al. 2010; Thomas et al. 2005). Recent human clinical trials have confirmed the benefits of ACE inhibition beyond their hemodynamic effects in humans (Méndez et al. 2010; Ruiz-Ortega et al. 2000). In diabetic animals, ramipril reduces renal and serum AGEs demonstrated by a reduction in renal fluorescence, serum fluorescence, and immunostaining for AGEs. It has now been demonstrated that ACE inhibition decreases the renal and serum AGEs accumulation by the inhibition of endothelial NO synthase which is a mechanism of the oxidative pathways (HOPES 2000; Méndez et al. 2010). Recently, nifedipine, one of the most widely used Ca^{2+} channel blockers (CCBs), has been reported to show anti-oxidative and anti-AGE-RAGE axis properties. Thus the use of nifedipine in patients with diabetes prevents cardiorenal vascular damage in addition to ensuring effective blood pressure control (Yamagishi, Nakamura & Matsui 2009). Moreover, CCBs protect the vasculature by stimulating endothelial NO production and/or restoring normal adiponectin and HDL levels in the circulation. Furthermore, CCBs were found to increase circulating endothelial progenitor cell level which play major role in normal endothelial function (Morimoto, Kureishi-Bando & Murohara 2010; Sirmagül et al. 2007). Accordingly, these data suggest that CCB can be used as a vasculo-protective agent in patients with diabetes and hypertension.

Moreover, studies showed that natural compounds have a novel therapeutic potential in preventing diabetic complications such as l-arginine and spermine both shown to inhibit pyrraline formation because of the presence of a guanidine group and four amino groups (Méndez et al. 2004, 2010). There is now growing interest to see whether stevia has the potential to inhibit/reduce the formation of AGEs thereby reducing the complications of diabetes and hypertension.

2.4.1 Myocardial Infarction

Of all of the previously mentioned complications, a myocardial infarction (MI) poses the most significant and acute complication to this group of patients. Myocardial ischemia is characterized by chest pain reflecting insufficient oxygen supply to the myocardium. Arterial hypertension is an important risk factor for the development of myocardial ischemia which ultimately leads to myocardial infarction. The narrowed coronary artery, clogged by atherosclerosis, may be the underlying pathogenesis which impedes blood flow or causes myocardial infarction in the case of atherosclerotic plaque rupture (Stocker & Keaney 2004). Diabetes mellitus is another major risk factor for coronary artery disease and is associated with a higher incidence of myocardial infarction. Diabetic patients show a higher morbidity, mortality and re-infarction rate than the non-diabetic group with a 50% higher mortality in this group over a one-year time period (Tenerz et al. 2001; Williams & Zaman 2003). In general, mortality from acute MI in the diabetic population was 4 times higher among men and 7 times higher among women when compared to the non-diabetic population. In a human trial to find out the risk factor of MI in 1997, the population attributable risk (PAR), a gross estimate of all Acute MIs attributed to diabetes, was 11% in men and 17% in women (Lundberg et al. 1997).

Whilst a myocardial infarction involves both a vascular and ventricular component, the original insufficiency arises in the remodelled coronary vascular tree. Diabetes results in vascular endothelial damage affecting vascular contraction, adhesion of immune cells, platelet aggregation and smooth muscle cell growth (Gokce & Haznedaroglu 2008). NO is a potent vasodilator and diabetes reduces and impairs the availability of NO (Gokce & Haznedaroglu 2008). Hyperglycaemia also induces apoptosis of human microvascular endothelial cells (McClung et al. 2005). So, diabetes and hypertension cause vascular complications and induce changes that accelerate atherosclerosis. Tedesco et al. (2004) found significantly higher arterial stiffness in patients with both hypertension and diabetes compared to those with either diabetes or hypertension alone. Hypertension causes changes in vasculature resistance and high glucose levels encourage advanced glycation end (AGE) products formation promoting alterations in vessel walls. An increase in arterial stiffness leads to an

increase in pulse pressure which in turn increases the load on the ventricle and myocardial oxygen demand, causing either adaptive structural and functional changes or primary abnormalities of the vessel walls (Tedesco et al. 2004).

2.4.2 Endothelial dysfunction

Endothelial dysfunction is directly involved in the development of atherosclerosis leading to coronary and peripheral diseases. The endothelium functions to regulate vascular tone, platelet activity, thrombosis and leukocyte adhesion (Avogaro, Kreutzenberg & Fadini 2008). The process of atherosclerosis begins with adhesion of monocytes to the endothelium. Adhesion of monocytes is associated with plaque formation and it has been shown that hyperglycaemia stimulates monocyte adhesion to the vascular wall (Watada, Azuma & Kawamori 2007). Atherosclerosis is associated with substantial changes in the endothelium of blood vessels whilst increased oxidative stress accounts for a significant amount of damage to the endothelium as oxygen derived free radicals have been linked with changes in vasomotor function in experimental models of atherosclerosis (Shaikh & Suryakar 2008). Clinical data show that treatment with antioxidants improves the function of the endothelium in coronary artery disease (CAD) and with other risk factors (Heitzer et al. 2001). Quercetin, one of the potent bioflavonoid antioxidants reduces oxidative vascular damage by modulating gene expression thereby suppressing reactive oxygen and nitrogen species production (Luangaram et al. 2007). In phenylhydrazine-induced oxidant stressed rats, quercetin was shown to protect against vascular damage by inhibiting superoxide anion production (Luangaram et al. 2007). All these results are in support of our assumption that stevia could prevent vascular damage through its antioxidant activity in addition to its other cardioprotective mechanisms.

The function of the vascular endothelium includes synthesis of nitric oxide, endothelin and endothelium derived hyperpolarizing factor (EDHF). The endothelium is of paramount importance to correct immune reactions and regulates vascular tone. The endothelial cells regulate the inflammatory response by responding to chemokines that modifies the adhesion molecules such as intracellular adhesion molecule-1(ICAM), vascular adhesion molecule-1 (VCAM-1) and cytokines (Tesfamariam & DeFelice 2007).

2.4.3 Cardiac Remodelling

Left ventricular (LV) function is an important predictor of cardiac morbidity and mortality, both in the healthy population and in patients with heart disease. Diabetes causes an increase in the stiffness of the myocardium and enhances passive stretch properties produced by the accumulation of myocardial collagen (Loganathan et al. 2006). Left ventricular hypertrophy (LVH) can develop following endurance exercise, obesity, diabetes, hypertension and heart failure (Fenning et al. 2003). Exercise induced hypertrophy is physiological and adaptive in nature which improves cellular metabolism, coronary blood flow, left ventricular structure and overall cardiac function (Fenning et al. 2003). However, maladaptive pathological hypertrophy is induced by cardiovascular disease states which potentiate myocardial infarction, arrhythmias and heart failure. Patients with pathological LVH, present with a reduction in left ventricular longitudinal strain while there is an increase in circumferential deformation and torsion (Cappelli et al. 2009). From epidemiological studies, it is now evident that heart failure and arrhythmias which occur due to LVH and fibrosis are the major causes of cardiovascular diseases (Edwards et al. 2009; Fenning et al. 2003). Increased arterial stiffness due to chronic kidney disease, diabetes and systolic hypertension also contributes to the development of LVH and fibrosis (Edwards et al. 2009; Fenning et al. 2003; Tonelli et al. 2006). Thus pathological LV hypertrophy has a significant impact on the overall survival of the patients with cardiovascular disease and diabetes.

A study by Loganathan et al. (2006) in type 1 diabetic rat models has quantified a 95% increase of collagen deposits in diabetic LV sections compared to the controls. The stiff and fibrotic myocardium then tries to compensate for its poor contractility by increasing pressure through limiting LV filling during cardiac cycle (Loganathan et al. 2006). Recently, Meris et al. demonstrated that both type 1 and type 2 diabetes and hypertension contribute to increase LV filling pressure and LV dysfunction (Meris et al. 2009). In human trials using MRI, diabetic subjects demonstrated thicker LV walls compared to healthy groups (Loganathan et al. 2006; Remme et al. 2004). LV hypertrophy is an important cardiac phenotypic outcome of the streptozotocin rat model of diabetes (Loganathan et al. 2006). It is evident from the streptozotocin rat

model that diabetes causes an increase in LV myocardial mass which is not associated with hypertension (Grimm et al. 2002; Loganathan et al. 2006). The pathological effects of left ventricular hypertrophy include increased risk of stroke, ischemic heart disease, and finally congestive heart failure and death (Cohuet & Struijker-Boudier 2006). Modifications in left ventricular dimensions have been developed in the nitric oxide deficient (L-NAME), Dahl salt-sensitive and DOCA-salt hypertensive rat models (Sainz et al. 2005). Higher salt diets and/or prolonged treatment periods in Dahl salt-sensitive and DOCA-salt hypertensive rat models demonstrate more aggressive results with concentric left ventricular hypertrophy, left ventricular dilation and eventually sudden death taking place through induction of arrhythmias (Doggrell & Brown 1998). Spontaneously hypertensive rats (SHR) demonstrated significant concentric cardiac hypertrophy represented by increased ventricular wall thickness and decreased internal dimension by 6 months of age which leads to heart failure between 18 and 21 months of age (Heyen et al. 2002; Bing et al. 2002). Therefore, these models can be used as ideal representation for LVH and heart failure caused by diabetes or hypertension.

The direct changes in vessel wall structure and function lead to both increased humoral and haemodynamic outcomes which directly impact the heart muscle itself. The increased circulating components of inflammation and oxidative stress initiate cellular remodelling and growth in the ventricle in a direct manner (Kahan & Bergfeldt 2005; London et al. 2002). The decreased supply of nutrients through damaged arteries creates a metabolic debt situation which stresses the cardiac cells themselves (Kahan & Bergfeldt 2005; London et al. 2002). Compounding these more humoral actions, the loss of vascular compliance leads to increased haemodynamic loading and hence workload on the ventricle. The heart muscle is already in an adverse metabolic situation and is forced to complete more work which results in significant remodelling of the left ventricle.

Echocardiography is the tool of choice for the assessment of differentiation of physiologic and pathologic LVH in both human subjects and experimental animal models. This approach assesses the quantitative analysis of the left ventricle, in order to calculate left ventricular mass, left ventricular mass index and relative wall thickness for diagnosing concentric or eccentric LVH (Brown et al. 2002; D'Andrea et

al. 2009). The geometric pattern of concentric remodelling may arise in mildly hypertensive patients with increased relative wall thickness but without any discernible increased left ventricular mass. Eccentric LVH is characterized by an increased left ventricular mass without any relative wall thickness changes. Patients with both increased left ventricular mass and relative wall thickness are at a high risk of cardiovascular mortality. Also the cardiac action potential is coordinated by a number of ion channels and in the human heart early phase repolarisation is achieved by both the inactivation of voltage-gated Na^+ channels and the activation of ultrarapid and transient outward current (I_{Kur} & I_{to1}) (Tamargo et al. 2004). Potassium channels play a major role in cell repolarisation whereas the influx of calcium (Ica) also has an effect on repolarisation. The L-type Ca^{2+} channel (Ica-L) remains activated through repolarisation which is responsible for the characteristic plateau seen in cardiac action potentials (Brette et al. 2006). In cardiac remodelling the potassium channel Ito1 is significantly affected (Momtaz et al. 1996) resulting in prolonged action potentials as the rate of repolarisation is reduced. This shows that in most cases changes in cardiac dimensions are also correlated with electrophysiological changes which may potentiate arrhythmia formation.

An estimated 20-30% of the population with mild to moderate hypertension develop LVH. A French study on a hypertensive population showed the prevalence of LVH was even higher than 50% for the patients studied (Cohuet & Struijker-Boudier 2006; Gosse et al. 1999). LHV causes remodelling of the myocardium, perivascular collagen matrix deposition, and coronary microcirculation alterations which play an important role in target organ damage (Cohuet & Struijker-Boudier 2006; Vogt et al. 1990). Recent studies in the aged spontaneously hypertensive (SHR) rats with heart failure have demonstrated changes in the expression of genes encoding for extracellular matrix (ECM) components (Cohuet & Struijker-Boudier 2006). Fibrosis and inflammatory mechanisms are considered important factors in the process of heart failure (Boluyt & Bing 1995; 2000; Cohuet & Struijker-Boudier 2006), However the mechanisms involved in the pathogenesis of cardiac hypertrophy are not yet fully established and need to be further investigated.

Diabetes causes maladaptive changes in the left ventricle such as hypertrophy and fibrosis by activating local cardiac renin-angiotensin system (RAS) components

(Weber, K. 2002). The generation of angiotensin II following activation of RAS also plays an important role in the progression of diabetic renal disease and end organ damage (Miller 1999). Furthermore, increasing evidence suggested that aldosterone induces progressive renal injury in chronic kidney disease and the use of aldosterone antagonists can slow down the progression of renal disease (Edwards et al. 2009). However, the use of aldosterone antagonists has been limited due to their adverse effect on serum potassium levels and renal function (Edwards et al. 2009). Cardiac arrhythmias due to decreased potassium currents causing action potential prolongation and is a major cause of diabetes associated death (Shimoni 2001). Toma et al. (2008) described that hyperglycemia increases RAS activity through a paracrine signalling pathway in the diabetic kidney which causes the citric acid cycle intermediate succinate to bind and activate the G-protein coupled GPR91 receptor thus causing renin release (Steckelings et al. 2009; Toma et al. 2008). Diabetes increases RAS activity causing an induction of AT1R by increased levels of angiotension II thus upregulating superoxide generation. Whereas, expression of the AT2R which is the 'protective arm' of RAS component, is decreased in diabetes because of hyperglycaemia-induced increase in transcription factor PARP in cells (Steckelings et al. 2009). Hyperglycemia- induced superoxide production and tissue damage are known to be stimulated by-(i) increased production and accumulation of advanced glycation end products (AGEs), (ii) stimulation of protein kinase C (PKC) by diacylglycerol production, (iii) increased polyol pathway flux, and (iv) increased hexosamine pathway flux (Steckelings et al. 2009). RAS is involved in almost all these events and eventually leads to cell damage. Angiotensin II is a strong inducer of oxidative stress and has been shown to enhance AGEs formation, thus establishing a vicious circle of cell injury (Steckelings et al. 2009).

2.4.4 Gastrointestinal dysfunction

The changes to the cardiovascular system are very significant following diabetes and hypertension however these are not the only major organ systems to be affected. Of particular interest are autonomic nervous system neuropathy, changes in gastrointestinal function and nerve signalling following diabetes. Gastrointestinal symptoms are frequent in diabetes mellitus and are thought to be due to autonomic

neuropathy. Evidence shows that acute changes in blood glucose concentrations reversibly influence upper-gut motor and sensory function in type 1 diabetes (Rayner et al. 2001; Samsom et al. 1997). Acute hyperglycemia disturbs normal functioning of every region of the gastrointestinal tract. Upper gastrointestinal motor function, especially the rate of gastric emptying is very much dependent on postprandial blood glucose concentrations. To optimize the glycaemic control in diabetes, scientists are now exploring the potential to utilize the modulation of gastric emptying (Rayner et al. 2001). These results show that glucose regulation is very important in overall gut organ function which can potentially provide a further medicinal outcome for stevia.

A non-selected population-based cohort of 110 young adults with both type 1 and type 2 diabetes showed increased occurrence of upper gastrointestinal symptoms such as anorexia and vomiting compared to age-and sex-matched control subjects (Bytzer et al. 2001; Schvarcz et al. 1996). Moreover, experiments in STZ- rat models demonstrated that diabetes induces the frequency depression of gastric motility and does not change contractility of GI tract (Cai et al. 2009). Hyperglycemia over a long period of time can lead to gastromucosal damage and diabetic gastroparesis by developing neuropathy and injury to the vagus nerve (Koch & Uwaifo 2008). In fasted STZ-induced diabetic rats, it has been demonstrated that spontaneous gastric damage is related to glutathione depletion because of limited availability of cysteine following diabetes and exogenous cysteine supplementation was found to attenuate the gastric damage. The study also showed that gastric mucosal blood flow was not influenced by diabetes (Goldin et al. 1997). The STZ-induced diabetic rats showed a potentiated C-type natriuretic peptide induced inhibitory effects on spontaneous contraction of gastric smooth muscle (Cai et al. 2009). Interestingly, stevia extract shows a protective effect against gastric mucosal damage in the rainbow trout against histamine (Shiozaki et al. 2006). However, the exact gastroprotective mechanism of stevia is not yet clear. The antioxidant activity of stevia is assumed to contribute to the attenuation of gastric damage (Goldin et al. 1997; Shiozaki et al. 2006). Thus, it is evident that stevioside holds promise to potentially be effective for the treatment of gastric disease following diabetes.

2.4.5 Neuropathy and Retinopathy

The condition of diabetes not only causes changes in parasympathetic nervous system functioning as demonstrated in the gastrointestinal tract but it also damages other nervous tissue types. A study in 2007 observed a substantial loss of both motor nerve eletrophysiology and motor endplates structure in a diabetic mouse model (Ramji et al. 2007). There is considerable evidence that diabetes leads to abnormal calcium signalling in dorsal root ganglion (DRG) neurons which develops sensory neuropathy (Hall et al. 2001). Therefore, abnormal calcium regulation in diabetes may lead to the exact mechanisms of both impaired neuronal conduction and neuronal injury. In animal and human models of diabetes, various neuronal dysfunctions and injury are observed, including segmental demyelination, atrophy, loss of myelinated and unmyelinated nerve fibres (Hall et al. 2001). Increased calcium currents in peripheral and spinal sensory nerves observed in rodent models of diabetes, leads to increased calcium release from internal stores followed by increased calcium concentration leading to abnormal neuronal conductance and neuronal injury (Hall et al. 2001). A study on STZ-induced diabetic rats also showed that elevated reactive oxygen species (ROS) in diabetes causes cumulative damage to neurons and Schwan cells, along with a injurious effect on nerve blood flow and an early nerve conduction velocity deficits and conduction failure (Skalska et al. 2008). Therefore, there is a growing body of evidence that appropriate antioxidants could prevent and decrease neural changes that occur in diabetes (Johansen et al. 2005). In recent years, it has been demonstrated that diacylglycerol-protein kinase C (DAG-PKC) pathway is associated with diabetic microvascular complications such as retinopathy, nephropathy and neuropathy (Das Evcimen & King 2007). Hyperglycemia-induced increase in oxidant production such as H_2O_2 can activate PKC either directly or by increasing DAG synthesis leading to changes in blood flow and vascular contractility (Das Evcimen & King 2007). Hyperglycemia-induced ischemia has been shown to play a major role in the progression of diabetic neuropathy and use of vasodilators appeared to improve nerve function in diabetic rodents (Cameron & Cotter 1197; Das Evcimen & King 2007). High blood glucose may decrease PKC activation in peripheral nerves which in turn may diminish the Na+-K+-ATPase phosphorylation causing a decrease in nerve conductance and nerve regeneration (Das Evcimen & King 2007).

Recent studies showed a direct association between β-adrenergic receptor function with diabetic retinopathy (Walker, R. et al. 2011). Walker et al. demonstrated that β-adrenergic receptors agonists improved retinal function in diabetes by preventing the failure of insulin receptor in retinal Muller cells (Walker, R. et al. 2011).

2.4.6 Renal damage

Perhaps one of the most significant complications observed in the diabetic patient is advanced chronic renal disease which further complicates the compromised cardiovascular system (Barış et al. 2008). Diabetic nephropathy is the most common cause of end stage renal disease in the diabetic population. It can be shown that 30-40% of all type 2 diabetic patients worldwide have diabetic nephropathy which results from an interaction between metabolic and hemodynamic factors (Barış et al. 2008; Trachtman et al. 2002). Hyperglycemia encourages multiple molecular and cellular events leading to the development of progressive kidney disease in diabetes (Kathryn et al. 2008). Increased production of endogenous renal endothelin in the presence of arterial hypertension or diabetes is one factor that promotes the development of glomerulosclerosis (Barton 2008, 2010; Barton & Luscher 1999). Endothelin directly stimulates glomerular production of collagen and fibronectin and contributes to proteinuria and glomerular injury leading to inflammatory responses in the diseased organ (Barton 2010; Barton 2008). The increased formation of pro-inflammatory and fibrogenic peptides such as angiotensin II plays an essential role in this process (Barton 2010; Suzuki et al. 2003). Renal endothelin also triggers pathways such as the reactive oxygen species production and inflammation that act independently of the physiopathology of hypertensive renal disease itself (Barton 2010; Huber & Benzing 2005). Endothelin is found to stimulate the formation of angiotensin II by increasing the activity of angiotensin converting enzyme (Barton 2010; Kawaguchi, Sawa & Yasuda 1991). It is now documented that angiotensin-mediated renal and podocyte injury is directly mediated via endothelin (Barton 2010; Barton et al. 1997) because angiotensin AT1 receptor blockade inhibits endothelin-1 production in vivo, and effects of AT1 receptor blockade are independent of podocyte AT1 receptor expression (Barton 2010; d'Uscio et al. 1998).

On the other hand, angiotensin II, the vasoconstrictor and pro-fibrotic peptide of renin–angiotensin system (RAS) stimulates renal endothelin production (Barton 2010; d'Uscio et al. 1998) resulting in renal inflammation (Barton 2010; Suzuki et al. 2003). Modifications within the RAS are pivotal for the development and progression of renal disease (Cooper & Johnston 2000; Velez 2009; Velkoska et al. 2009). Angiotensin-(1–7) produced from the breakdown of angiotensin II by Angiotensin-converting enzyme 2 (ACE2) have vasodilatory and anti-fibrotic actions in kidney (Ferrario 2006; Tipnis et al. 2000; Velkoska et al. 2009). ACE2 is highly expressed in the kidney in normal physiological conditions and remained localized to the proximal tubules and glomerulus, where it is expressed in podocytes and mesangial cells (Soler et al. 2009; Velkoska et al. 2009; Ye et al. 2006). Previous studies have recognized the therapeutic potential of increased ACE2 activity and suggested that in renal disease, ACE2 may have renoprotective effects. For example, glomerulosclerosis developed in ACE2-knockout mice can be prevented by angiotensin receptor blockade (Oudit et al. 2006; Velkoska et al. 2009).

In addition to angiotensin II, formation of AGEs on the matrix components exacerbates diabetic glomerulosclerosis by trapping and covalently cross-linking with the extravasated plasma proteins (Lin, Park & Lakatta 2009; Yamagishi, Nakamura & Matsui 2009). AGEs upregulate the production of type IV collagen formation, laminin, and fibronectin by stimulating insulin-like growth factor-I, -II, platelet-derived growth factor (PDGF), in mesangial cells (Lin, Park & Lakatta 2009). AGEs also directly stimulate reactive oxygen species (ROS) generation which subsequently prevents de novo protein synthesis in proximal tubular cells (Yamagishi, Nakamura & Matsui 2009). AGEs also increase production of transforming growth factor (TGF-b) which is an important factor in the pathogenesis of tubulointerstitial fibrosis in diabetic nephropathy (Lin, Park & Lakatta 2009; Ya magishi, Nakamura & Matsui 2009).

The rate of progression of diabetic nephropathy in both type 1 and type 2 diabetes is strongly related with the degree of hypertension. Moreover, renal vascular resistance must increase to permit the development of hypertension otherwise natriuresis would normalize blood pressure (Hall, J. et al. 1990). Trials show that lowering blood pressure decreases albuminuria and attenuates the rate of loss of GFR in both type 1 and type 2 diabetes (Fraser & Phillips 2007). Additionally, all the important classes of

antihypertensive agents reduce renal complications in diabetics but it is not solely linked to pressure alone. Nephropathy can also develop independently to hypertension but antihypertensive agents such as ACE inhibitors or other blockers of Ang II and RAAS show fantastic renoprotective properties. The ACE inhibitors were found more effective than other antihypertensive treatments to limit proteinuria, prevent GFR decline and risk of end-stage renal disease (Ruggenenti, Schieppati & Remuzzi 2001; The GISEN Group 1997; Zoja et al. 2002). A study on passive Heymann nephritis (PHN) rats with heavy proteinuria demonstrated that lisinopril given from 2 to 10 months after disease induction, improved urinary protein excretion at levels that were comparable to pre-treatment values (Zoja et al. 2002). The addition of angiotensin II receptor antagonist (AIIRA) L-158,809 to lisinopril was found to be more effective, as the combination significantly improved renal function and reduced proteinuria below pre-treatment values (Zoja et al. 2002).

There is increasing evidence that nitric oxide prevents diabetic nephropathy by altering intraglomerular hemodynamics and total accumulation of extracellular matrix within kidney (Trachtman et al. 2002). Nitric oxide is synthesized by the enzyme nitric oxide synthase (NOS) which has three distinct isoforms and all are expressed in kidney. Constitutive endothelial and neuronal NOS produce small (pM) transient burst of NO by following calcium dependent pathway. The inducible isoform iNOS is found mostly in the medullary thick ascending limb and inner medullary collecting duct and is stimulated by inflammatory cytokines. Following stimulation, iNOS facilitates large amounts (nM) of NO production for a longer period of time. However the excessive nitric oxide production is observed only within the first 1-2 weeks of the onset of STZ diabetes. The same study demonstrated that the net synthesis of nitric oxide was reduced in STZ diabetes which aggravated the chronic diabetic nephropathy. Moreover, the study manifested net production of all three isoforms and linked their changes to modulation in glomerulosclerosis and interstitial damage in chronic diabetic nephropathy (Trachtman et al. 2002). Angiotensin converting enzyme (ACE) inhibitors offer superior protection against renal damage by reducing protein trafficking and its long-term toxicity (Griffin et al. 2003). ACE inhibitors limit proteinuria and renal injury in virtually all experimental models of chronic proteinuric nephropathy when treatment starts soon after insult or early in the course of the

disease (Churchill et al. 2002; Griffin et al. 2003; Tanaka et al. 2001). Lisinopril is found to limit proteinuria and renal injury in the accelerated model of passive Heymann nephritis (PHN) if given 7 days after disease induction, whereas very high dose of lisinopril failed when given after 4 months (Griffin et al. 2003; Linz et al. 1998; Stier et al. 1989).

The stroke-prone spontaneously hypertensive rat (SHRSP) shows increased susceptibility to develop stroke and renal damage and thereby offers a suitable model to investigate hypertension induced target organ damage (Churchill et al. 2002; Griffin et al. 2003). It is believed that the pathogenesis of such target organ damage is blood pressure (BP)-independent and mediated by the renin-angiotensin-aldosterone system (RAAS) which directly promotes tissue damage (Griffin et al. 2003; Rubattu et al. 1996; Stier et al. 1989). This assumption is supported by the fact that ACE inhibitors or angiotensin receptor blockers and more recently, aldosterone receptor antagonists, demonstrated a significant reduction in the severity of renal damage and/or the incidence of stroke without markedly reducing BP (Griffin et al. 2003).

The renin-angiotensin-aldosterone system inhibitors along with antihypertensive regimens provide reductions in both BP-dependent and -independent renal and cardiovascular events (Griffin et al. 2003). Angiotensin II leads to the development of proteinuria and left ventricular hypertrophy (LVH) as it has direct detrimental effects on vasculature, end organs, including the kidney and the heart. Several large randomized clinical trials showed that RAAS inhibition prevents LVH and the blockade is also effective in preventing the cardiovascular and renal complications of diabetes (Cooper 2004).

Advanced glycation end products (AGEs) are pro-inflammatory mediators which play a major role in the pathogenesis of diabetic kidney disease (DKD). Patients with DKD treated with an angiotensin receptor blocker candesartan, showed slightly increased creatinine clearance and decreased urinary excretion of transforming growth factor-beta1 than in controls (Saha et al. 2009). It is assumed from the study that angiotensin receptor blockers show protective effect in DKD by reducing kidney exposure to AGEs (Saha et al. 2009). Therefore, antioxidant or anti-AGE treatments are of particular interest as an adjunct therapy in diabetic patients.

The renin-angiotensin-system (RAS) is known as a circulating hormonal system and is also a main cardiovascular regulator. Cardiovascular as well as non-cardiovascular tissues produce a 'local RAS' where they trigger the physiological and patho-physiological processes such as inflammation, fibrosis, proliferation or apoptosis (Steckelings et al. 2009). In both type I and type 2 diabetes, the angiotensin II level is increased by preferential stimulation of the local RASs in organs affected by hyperglycaemic injury such as the kidney or the retina. Thus increased angiotensin II concentrations contribute to diabetic tissue injury by stimulating the angiotensin AT1-receptor (Cooper et al. 2004). Angiotensin II also activates hyperglycaemia-induced pathobiochemical pathways such as oxidative stress, generation of advanced glycation end products, activation of protein kinase C and increased hexosamine pathway flux which in turn cause tissue damage (Steckelings et al. 2009). Furthermore in deoxycorticosterone-acetate hypertensive rats, Brown et al. (1999) confirmed humoral involvement in cardiac remodelling by demonstrating that blockade of the renin-angiotensin aldosterone system (RAAS) can reverse this process. Assessment by Langendorff heart preparations showed that left ventricular hypertrophy was developed along with changes to the functional parameters of the heart such as increased passive diastolic stiffness and increased contractility of the DOCA-salt hearts (Brown et al. 1999). Blockade of the various points of the RAAS were effective in reversing or attenuating these changes in ventricular stiffening and contractility, indicating that these pathological changes require the involvement of RAAS components (Brown et al. 1999).

It is also evident that pathological cardiac remodelling can be prevented by CCBs; however a reduction in heart size can be achieved by reducing blood pressure (Afzal et al. 1988). This statement has been supported by the evidence that verapamil (2, 4, or 8 mg.kg) was found to show a preventive effect on diabetes-induced myocardial changes. Verapamil also improved the diabetes-induced alterations in myocardial high-energy phosphate stores and ultra-structural damage without affecting their hyperglycemic status (Afzal et al. 1988). Moreover, in the salt-loaded stroke-prone spontaneously hypertensive rats (SHRSP) there is a paradoxical increase in plasma renin concentration (Kyselovic et al. 2001). Müller et al. (1998) demonstrated in Langendorff preparations that circulating renin can be taken up by the heart and

induce local generation of angiotensin II which could thus increase LV concentrations of renin (Dostal & Baker 1999; Kyselovic et al. 2001; Muller et al. 1998). A study in salt-loaded SHRSP showed that low and intermediate dosages of CCBs prevent increases in plasma renin activity (PRA) in the circulation (Kyselovic et al. 2001). The result signifies the protective effects of CCBs against kidney damage and excessive renin production and contributes to their beneficial cardiovascular effects. Thus the involvement of the RAS in the pathomechanisms underlying diabetic and hypertensive end organ damage gives us an insight that pharmacological RAS inhibition is an important therapeutic approach in these disorders.

In addition to the above information, verapamil, a calcium channel blocker was found to potentiate the renal vasodilating action of the stevioside in normotensive subjects (Melis 1991). Verapamil also increased hypotension, diuresis and per cent fractional sodium and potassium excretion promoted by stevioside. These results suggest that the vasculoprotective effect of stevioside is analogous to verapamil which is a specific inhibitor of calcium channels in cardiac and vascular muscle (Melis & Saitnati 1991; Melis 1992).

Our present research will encompass and utilize the emerging knowledge about cardiovascular and non-cardiovascular effects of the RAS, the mechanism of RAS involvement and calcium channel inhibition in diabetic end organ damage.

2.4.7 Inflammation

Over the last few years, chronic subclinical inflammation has emerged as a causative factor associated with insulin resistance and progressive pancreatic beta cells failure (Pitsavos et al. 2007; Pradhan et al. 2003; Shoelson, Lee & Goldfine 2006). Several studies demonstrated that chronic low-grade inflammation plays a major role in all steps of atherosclerosis including plaque initiation, progression and thrombosis (Libby, Ridker & Maseri 2002; Pitsavos et al. 2007). A strong and consistent relationship has been established between sensitive inflammatory markers and subsequent cardiovascular events by a number of epidemiological studies (Blake et al. 2003; Johnson et al. 2004; Pitsavos et al. 2007). Acute and chronic inflammation alters endothelial function causing a reduction in bioavailability of NO, involvement of

cytokines, such as TNF-∞, IL-6, raised concentration of C-reactive protein, fibrinogen, amyloid-A and increased expression of adhesion molecules which ultimately facilitate the formation of atherosclerotic lesion in the vessel walls (Hermann & Ruschitzka 2006). However, initiation of low-grade inflammatory process expands from atherosclerosis towards the more complex metabolic syndrome, including pathologic glucose and lipid levels, hypertension, visceral obesity and end organ damage (Hermann & Ruschitzka 2006). Moreover, hypertension and other cardiovascular events increase angiotensin II synthesis stimulating the production of cytokines which is mediated via an activation of nuclear factor-κB dependent pathway (Hermann & Ruschitzka 2006). The inflammatory responses within the vasculature provoke the release of pro-inflammatory cytokines which in turn causes the release of C-reactive protein (Hermann & Ruschitzka 2006). However, a single inflammatory marker may not be responsible for all aspects of the inflammatory processes contributing to cardiovascular disease risk. Indeed, there are other factors with a potential role as useful predictors of cardiovascular risk, such as cytokines and humoral factors interleukin (IL)-6, angiotensin II, serum amyloid- A, adhesion molecules and fibrinogen (Hermann & Ruschitzka 2006). As such, our focus is on the role of inflammation in hypertension and diabetes and emerging therapeutic approaches to inhibit these processes.

Calcium channel blockers (CCB) demonstrate antioxidant and anti-inflammatory effects in addition to their use for the treatment of hypertension and angina pectoris. In vitro experiments exhibit that CCBs decrease oxidative stress via multiple pathways (Nishiyama, Nakano & Hitomi 2010). In various animal models, CCBs were found to reduce oxidative stress and protect organ injury by increasing endothelial NO release. The results indicate that organ-protective effects of CCBs are largely mediated through strong antioxidative effects and are not related to their antihypertensive action (Nishiyama, Nakano & Hitomi 2010; Shima et al. 2008). Amlodipine (10^{-6} mol/l) and manidipine (10^{-6} mol/l) reduced superoxide generation by the inhibition of the overexpression of NADPH oxidase in Ang II-stimulated endothelial cells (Toba et al. 2006). Data demonstrated that L-type Ca^{2+} channel (LTCC) blockers, represented by amlodipine and verapamil, show anti-inflammatory activities by the suppression of plasminogen -Rs on macrophages (Das et al. 2009).

Two weeks administration of amlodipine (20 mg/kg/day) and manidipine (10 mg/kg/day) to L-NAME-induced hypertensive rats showed a direct anti-inflammatory and antioxidative effects in the aorta which have been mediated by an enhanced eNOS expression and by the inhibition of the expression of ACE (Toba et al. 2005).

Studies showed that some calcium channel blockers (amlodipine, nicardipine, cilnidipine, benidipine) suppress O^{2-} release in human neutrophils stimulated by GM-CSF or TNF-α in atherosclerotic plaque at a relatively low concentrations (1–10 μmol/l) (Nishiyama, Nakano & Hitomi 2010; Shima et al. 2008). While antihypertensive treatment can improve vascular functions, experimental studies demonstrated that anti-inflammatory treatment has beneficial effects. Calcium channel blockers, ACE-inhibitors and angiotensin receptor blockers show anti-inflammatory effects by blocking pro-inflammatory actions of angiotensin II (Hermann & Ruschitzka 2006). Therefore, assessment of anti-inflammatory and antioxidant activity of stevia in addition to anti-hypertensive and antidiabetic effects would increase its clinical benefits.

Emerging evidence has suggested that diabetes, cardiovascular complications and low-grade chronic inflammation may be intimately linked. Inflammation, macrovascular and microvascular complications are closely related with diabetes. Inflammatory cytokine concentrations also increase following hyperglycemia. Uncontrolled diabetes is found to be positively associated with inflammatory markers linked with endothelial dysfunction including cytokines and adhesion molecules (Riad et al. 2007). A recent study of the assessment of early markers of inflammation and endothelial function in diabetes showed reduced availability of L- arginine, which is a NO precursor and plays an important role in endothelial dysfunction and microvascular and macrovascular complications (Barış et al. 2009). This demonstrates a significant link between inflammation, a decrease in NO, high blood pressure and blood vessel dysfunction (Ishimitsu 2010; Morimoto, Kureishi-Bando & Murohara 2010).

In the same way, cardiovascular disease also leads to endothelial dysfunction which is illustrated by a reduced capacity of endothelial cells to suppress the inflammatory process, oxidative stress and thrombosis. A healthy endothelium produces adequate

NO levels through the enzyme endothelial nitric oxide synthase (eNOS) (Schmitt & Dirsch 2009). Nowadays, NO is known as an antiatherogenic and vasoprotective agent which maintains vascular tone by direct relaxation of vascular smooth muscle (Schmitt & Dirsch 2009; Schulz et al. 2008). Several studies hypothesized that diabetes may alter the innate immune system responsible for cytokine-mediated acute phase response (Barış et al. 2009; Laakso 1999). In line with this evidence a prospective study in apparently healthy middle-aged women also showed that two systemic inflammatory markers, C-reactive protein and interleukin-6 (IL-6) were risk factors for the development of type 2 diabetes (Pradhan et al. 2001). Furthermore, high levels of IL-6 and tumor necrosis factor alpha (TNF-α) were observed in individuals both with insulin resistance syndrome and with clinically overt type 2 diabetes mellitus (Esposito et al. 2002). These cytokines production was more pronounced in hyperglycemic spikes than continuous hyperglycemia (Esposito et al. 2002). The other important outcome of this study was that hyperglycemia induced increase in plasma cytokines production was completely blocked by an antioxidant-glutathione. This suggests a fundamental role for hyperglycemia in the immune activation of diabetes (Esposito et al. 2002; Pickup et al. 2000; Shikano et al. 2000). Increased inflammatory markers and subsequent oxidative stress lead to the pathological conditions such as endothelial dysfunction reduced NO bioavailability, vascular smooth muscle cell (VSMC) proliferation and cell death (Rojas et al. 2006). Products of hyperglycaemia and pro-inflammatory cytokines increase the expression of adhesion molecules such as vascular adhesion molecule-1 (VCAM-1) and intracellular adhesion molecules (ICAM-1) (Libby, Ridker & Maseri 2002). VCAM-1 expression responds to endothelial cells and ICAM-1 responds to inflammatory cytokines and both regulate the attachment and migration of leukocytes. These cells secrete metalloproteinases (MMPs) that negatively impacts upon the function of vascular cells and causes vascular remodelling and destruction of arteries and the myocardium (Cipollone, Fazia & Mezzetti 2007).

A cross sectional study amongst hypertensive patients showed a significant relationship between BP variability index and inflammatory markers, especially IL- 6 which may be due to the hemodynamic stress on the vessel wall (Kim et al. 2008). However, the exact mechanism explaining the significance of BP variability is yet to

be established. Increased inflammation can be a promising answer to this question. An experimental study on animal models of BP variation on sinoaortic denervation (SAD) rats showed an increase in inflammation-related factors and treatment with antioxidant and anti-inflammatory agents showed a reduction in end organ damage in the SAD rats (Kim et al. 2008). So, it can be projected that inflammation is one of the mechanism underlying end organ damage in SAD rats (Kim et al. 2008). Some reports have shown a significant relationship between inflammatory markers and elevated BP in apparently healthy subjects suggesting that hypertension is, in part, an inflammatory disorder (Bautista et al. 2005; Chae et al. 2001; Kim et al. 2008; Sesso et al. 2003). Furthermore, C-reactive protein (CRP) was found to down regulate NO production which maintains cardiovascular homeostasis. CRP augments synthesis of the major inflammatory cytokines, endothelin- 1 (ET 1), and interleukin-6 in a synchronous fashion which is associated with a marked reduction in in vitro angiogenesis (Verma et al. 2002a; Verma et al. 2002b). Other studies also demonstrated the positive relationship of inflammation with endothelial dysfunction and suggested that CRP contributes to an increase in BP by reducing nitric oxide production both of which are related to renin-angiotensin system effects (Sesso et al. 2003; Venugopal et al. 2002; Verma et al. 2002b). While inflammation and its effects on nitric oxide production and the subsequent damage to the vessel wall have been identified as the positive and inevitable contributors to vascular remodelling and cardiac damage, the progression of oxidative stress is an important modulator of this damage as evidenced by positive responses observed following antioxidant therapy.

Changes in inflammation and a reduced release of nitric oxide often expose the cardiovascular system to increased oxidative stress. This is particularly important because it may be the case in some cardiovascular disease states that increasing oxidative stress generates inflammation and simultaneously reduces NO levels. Oxidative stress is characterized by imbalanced redox state in which pro-oxidants overpower the antioxidant power which results in enhanced production of reactive oxygen species (ROS). Increasing evidence showed that oxidative stress potentiates the pathogenesis of experimental and essential hypertension and ROS have been implicated in every stage of vascular damage (Fortuňo et al. 2005; Madamanchi, Hakim & Runge 2005; Mueller et al. 2005; Yesmine et al. 2009). ROS turns on

several intracellular signalling cascades which influence cellular processes in vascular remodelling by stimulating angiotensin type 1 (AT1) receptor mediated production of $.O_2$-generated radical formation by NADPH oxidase, leading to an increase in intracellular H_2O_2 which may act as a second messenger for the long term response and release of angiotensin II. This would then induce hypertrophy and hyperplasia of vascular smooth muscle cells (VSMCs) and other organs of the cardiovascular system like the heart (Fortuňo et al. 2005; Zafari et al. 1998). An enhanced NADPH oxidase-driven $.O_2$- production was observed in the aorta of the adult spontaneously hypertensive rat (SHR) model which leads to vascular wall hypertrophy (Fortuňo et al. 2005; Zalba et al. 2000). Another study using transgenic mice that over express the p22phox subunit in VSMCs showed that enhanced H_2O_2 production is linked with vascular hypertrophy (Fortuňo et al. 2005; Weber, D. et al. 2005). The importance of these studies in experimental animal models has been established in studies with human VSMCs showing increased NADPH oxidase-dependent oxidative stress. These changes are again associated with angiotensin II-induced vascular remodelling in essential hypertension following oxidative triggers. ROS can also transform vascular remodelling by increasing deposition of extracellular matrix proteins (Fortuňo et al. 2005; Touyz & Schiffrin 2001). A study in hypertensive patients suggested that essential hypertension enhances oxidative stress and augments growth-promoting actions of Ang II (Touyz & Schiffrin 2001). Many studies have demonstrated the effect of NADPH oxidase on the level of oxidative stress in endothelial cells (EC), VSMC and cardiac cells. NADPH oxidase levels were found to be elevated during hypertension, oxidative stress and heart failure in gene knockout studies (Heymes et al. 2003; Li, J. et al. 2002; Li, L. et al. 2003; Wu, F. et al. 2007b). Inhibition of NADPH oxidase by apocynin (1.5 mmol/L in drinking water) decreased both blood pressure and superoxide production in treated Sprague-Dawley DOCA-salt rats (Beswick et al. 2001). The attenuation of these parameters by simple NADPH inhibition suggests that NADPH oxidase mediated oxidative stress plays a role in the progression of DOCA-salt hypertension (Beswick et al. 2001). Human studies have also demonstrated the involvement of NADPH oxidase in oxidative stress and inhibition of NADPH in the left ventricle reduces the local elevation in ROS associated with heart failure (Heymes et al. 2003). This indicates that oxidative stress,

especially ROS production by NADPH oxidase, plays a major role on the dysfunctions of the cardiovascular system.

2.4.8 Oxidative Stress

There is increasing speculation that elevated oxidative stress may act as a likely mechanism connecting acute hyperglycaemia to cardiovascular diabetic complications by escalating cytokine secretion. Antioxidants have the capacity to reduce cytokine secretion and the subsequent inflammatory responses. In a study, the powerful antioxidant glutathione was observed to completely prevent cytokine increase by oscillatory hyperglycaemia in healthy humans (Esposito et al. 2002). It has been shown that a potent vasoconstrictor, endothelin-1 (ET-1) level is progressively elevated in diabetes, triggers the generation of reactive oxygen species. ET-1 causes increased superoxide production and impaired vasorelaxation to a higher degree in coronary artery bypass graft conduits obtained from diabetic patients as compared to non-diabetic patients (Elgebaly et al. 2007; Ergul et al. 2005; Touyz et al. 2004). Diabetic patients generates excess reactive oxygen species (ROS) may affect vascular function in several pathways. Immediately after production, augmented superoxide (O_2.) reacts with NO generating $ONOO^-$ and decreases NO bioavailability. This may directly impair vasorelaxation in patients with diabetes as NO is one of the major pathways that cause dilator responses. $ONOO^-$ can oxidize tetrahydrobiopterin, which is an important cofactor for eNOS causing a decrease in NO and increased superoxide production by eNOS (Ergul et al. 2005). Evidence show that ROS and the role of oxidative stress is directly related to hyperglycaemia experienced in diabetics (Choi, S. et al. 2008) and is implicated in complications of diabetes including nephropathy, retinopathy and atherosclerosis. There are many pathways in which diabetes produce ROS and include glucose auto-oxidation, altered mitochondrial metabolism, synthesis of AGEs and NADPH oxidase activation (Fiordaliso et al. 2006). Elevated ROS results in endothelial dysfunction, reduced NO bioavailability, vascular smooth muscle cell (VSMC) proliferation and cell death (Rojas et al. 2006; Jiang et al. 2006). All these changes induce systemic oxidative stress triggered by metabolic mitochondrial dysfunction that causes the oxidative radicals to spill out into the circulation (Sari-Sarraf, Pomposiello & Laurent 2008). The systemic oxidants migrate

and stimulate local free radical formation, which directly damages cells of the blood vessels, initiating cardiac remodelling. Reactive nitrogen species (RNS) such as peroxynitrite ($ONOO^-$) and nitric oxide (NO) are also important in the consideration of oxidative stress and vascular damage, as they can both influence cardiac remodelling. As nitric oxide is a potent vasodilator and a ROS scavenger, thus blockade of nitric oxide synthase has a direct effect on BP elevation (Sari-Sarraf, Pomposiello & Laurent 2008; Tarsitano et al. 2007). This effect is modulated to establish the L-NAME hypertensive rat model with NOS inhibition causing a rise in blood pressure. Studies in both human and animal models have clearly demonstrated an association between oxidative radical formation, local angiotensin II production and inhibition of endothelial NOS (eNOS) (Sydow & Münzel 2003). Meng et al (2003) showed that the DOCA-salt hypertensive and the Dahl-salt sensitive (SS) rat models also display increased production of reactive oxygen species. Production of O^{2-} is significantly increased in the kidneys when the rats of Dahl SS rats are fed on a high salt diet (Meng et al. 2003). DOCA-salt rats and STZ rats have displayed increased levels of reactive oxygen species in both the systemic circulation and in the blood vessels themselves (Fenning et al. 2005; Trinity 2005). STZ-rats also demonstrated increased superoxide and decreased cGMP levels in smooth muscle and endothelial cells (Trinity 2005). These increased levels of reactive oxygen molecules lead to oxidative stress causing damage to the vasculature and other tissues inducing further cardiovascular complications.

Published data also confirmed that hypertension in humans generates oxygen radicals like asymmetric dimethyl arginine (ADMA) which is an endogenous inhibitor of eNOS (Chen, S. et al. 2008; Sydow & Münzel 2003). In another study, the angiotensin II receptor antagonist telmisartan reduced plasma ADMA levels, improved blood glucose concentrations and normalised impaired vascular function in hypertensive patients (Ono et al. 2009).

Two weeks administration of the calcium channel antagonists amlodipine (20 mg/kg/day) and manidipine (10 mg/kg/day) to L-NAME-induced hypertensive rats showed a direct anti-inflammatory and antioxidative effects in the aorta which are assumed to have been mediated by an augmentation of eNOS expression and by the inhibition of the expression of ACE (Toba et al. 2005). Amlodipine (20 mg/kg/day)

and manidipine (10 mg/kg/day) given to L-NAME-induced hypertensive rats showed an anti-inflammatory and antioxidative effects in the aorta which may be mediated by an increased eNOS expression (Toba et al. 2005). ROS perpetuates the disease process, as glucose autoxidation and or non-enzymatic protein glycosylation leads to further damage to surviving pancreatic β-cells (Gokce & Haznedaroglu 2008). Diabetes also leads to endothelial cell dysfunction, generation of further ROS and is implicated in cardiovascular disease (CVD), neuropathy, nephropathy and retinopathy (Arulmozhi, Veeranjaneyulu & Bodhankar 2004). The most eminent factor is that vascular complications are central to diabetic fatalities. Therefore, antioxidants may be helpful in preventing/reducing complications that arise from hyperglycemia (Barbosa et al. 2008; Gokce & Haznedaroglu 2008).

2.5 Current Treatments of Type 2 Diabetes

Diabetes and cardiovascular disorders are the major causes of morbidity and mortality worldwide in spite of significant improvements in their diagnosis and treatment (Coccheri 2007). These complications are mainly due to increased serum glucose and greater generation of oxygen-derived free radicals which cause endothelial dysfunction. In addition, chronic inflammation is also regarded as an initiator of atherosclerosis and for other cardiovascular complications.

In an interesting twist, the modern treatments for the complications of diabetes are the same as those used to treat the complications arising from hypertension, heart failure and other cardiovascular diseases. The successful direct treatment of diabetes lies on improved glycaemic control which ultimately reduces microvascular risk (Bolen et al. 2007). The treatment of insulin resistance can prevent or delay the onset of diabetes as insulin resistance is a major factor in the development of diabetic macrovascular and microvascular complications. As mentioned previously, insulin resistance has a major inflammatory signalling component which links nicely to the hypothesis of low-grade chronic inflammation found in these conditions.

Among all the treatment options, sulfonylureas became the first clinically useful agent for the treatment of diabetes. These have been used since 1950s and they stimulate pancreatic beta cells to increase insulin secretion and are hence known as

secretagogues. Tolbutamide is the first widely used member of this group and clinical trials were carried out in patients with type 2 Diabetes in early 1950s. Since then more than 20 different agents of sulfonylureas have been in use worldwide.

In 1997, a new class of oral insulin secretagogues called meglitinides was accepted as a fast-acting pre-meal therapy to limit postprandial hyperglycemia. Repaglinide and nateglinide are the two members of this group that target early phase insulin release and reduce postprandial glucose excursion which is considered important in reducing long term cardiovascular complications of diabetes (Kikuchi 1996). They have a rapid onset and short duration of action which allows for a flexible meal schedule. Repaglinide also achieves good metabolic control as monotherapy or in combination with other antidiabetic agents, for example with metformin (Kikuchi 1996).

In 1957, the biguanides were introduced and of this group metformin has been used widely. Metformin lowers haemoglobin A_{1c} values by about 2% and can reduce plasma triglyceride by 15% to 20%. There is a strong consensus that reduction in haemoglobin A_{1c} by any therapy diminishes microvascular complications. Metformin has been shown to reduce macrovascular events in type 2 diabetes (UKPDS 1998). Diabetes is a progressive and complex disorder which is difficult to manage effectively in the long term. Sometimes only oral antidiabetic agents fail to achieve normoglycemia in obese or overweight patients and they eventually require insulin either as monotherapy or in combination with oral therapy.

There are several classes of antihypertensive drugs that lower blood pressure, improve maladaptive cardiovascular remodelling and improve quality of life. Some drugs only reduce blood pressure, such as diuretics, while others target more specific causes of the increased blood pressure. Effective antihypertensives are essential to lessen the risk of strokes, cardiac failure, and renal insufficiency due to hypertension. Pharmacological treatment of patients with hypertension decreases morbidity and mortality from cardiovascular diseases.

The beneficial effect of all major classes of antihypertensive drugs is due to blood pressure lowering, irrespective of their mechanism of action. Evidence from various clinical data has been used to establish specific lower BP targets with

pharmacological treatment in adults with different pathophysiological conditions (NHF 2008).

In 1940, it was demonstrated that bilateral excision of the thoracic sympathetic chain could lower blood pressure, and then a search started for effective sympatholytic agents. Since then, beta-blockers have been the first line of treatment for hypertension. Studies showed that beta blockers decrease the risk of hospitalization for heart failure in patients with diastolic dysfunction and stable coronary heart disease (Smith et al. 2010). This study also recommended β blockers for patients with acute coronary syndrome and demonstrated a reduction in mortality following their long term use in those with LV dysfunction on presentation (Smith et al. 2010). In addition, nebivolol, a 3rd generation β-antagonists, has demonstrated eNOS activation and NO production properties (Georgescu et al. 2005; Georgescu et al. 2007; Reidenbach et al. 2007). Nebivolol was found to reduce NG-nitro-l-arginine methylester (L-NAME) induced eNOS blockade in isolated renal arteries of mice and thus improved the ability of the arteries to relax (Georgescu et al. 2005). Georgescu et al (2005) also demonstrated that nebivolol mediated relaxation was impaired by blocking the Ca^{2+}ATP-ase pump in the sarcoplasmic reticulum (SR) leading to decreases in the Ca^{2+} levels within the endothelial cells (Georgescu et al. 2005). Therefore, these results indicated that the vasodilatation action of nebivolol was carried out through enhanced eNOS production and increased Ca^{2+} release from the SR. In 2007, Georgescu et al showed that nebivolol treatment improves endothelial dysfunction in diabetic mice and normalizes small vessel responses to ACh. So, it is evident that nebivolol shows vasculoprotective effect by NO dependent mechanism in addition to its beta-antagonist properties.

Type 2 diabetes mellitus and obesity are the most common endocrine-metabolic diseases characterized by insulin resistance and insulin secretion defects that can be demonstrated through various alterations in carbohydrate, lipid, and protein metabolism. Recently, the peroxisome proliferator-activated receptors (PPAR) have been identified as key regulators of glucose and lipid metabolism, because they act as transcription factors that stimulate the cascade of protein synthesis in a wide variety of processes including energy metabolism, proliferation, and cellular differentiation (Bermúdez et al. 2010). There are 3 types of PPAR, known as- alpha, beta/delta, and

gamma. Nowadays, the peroxisome proliferator-activated receptor-gamma (PPAR-gamma) agonists such as the thiazolidenediones are used in the treatment of type 2 diabetes and act by increasing the tissues sensitivity to the insulin. Telmisartan, an angiotensin II-receptor blocker (ARB), also acts as a partial agonist of the peroxisome proliferator-activated receptor-gamma (PPAR-gamma) which provides additional benefits to its RAS inhibiting effects (Goyal, S. et al. 2010). Telmisartan treatment increased down-regulated PPAR-gamma expression in myocardially infarcted diabetic hearts (Goyal, S. et al. 2010; Ono et al. 2009). In addition to its target effect as an ARB, telmisartan reduces oxidative stress and apoptosis and improves cardiac function via the PPAR-gamma pathway (Goyal, S. et al. 2010). Clearly this shows that the additional benefits of these compounds are based on more than just their primary activity by blocking oxidative stress and inflammatory signalling and enhancing NO production. However, the peroxisome proliferator-activated receptors (PPAR) agonists are reported to produce several adverse effects, such as increased weight increased, oedema, anaemia, pulmonary oedema, and congestive cardiac failure (Bermúdez et al. 2010).

2.5.1 Calcium channel blockers in the treatment of cardiovascular disease

In addition to beta- blockers and PPAR agonists, calcium channel blockers are an important group of drugs for the treatment of hypertension, heart failure and cardiac arrhythmias and diabetes. Hypertension is generally the result of increased peripheral vascular resistance. Since an increase in free intracellular calcium concentration causes the contraction of vascular smooth muscle, inhibition of transmembrane movement of Ca^{2+} through voltage sensitive Ca^{2+} channels can decrease the total amount of calcium that reaches intracellular sites. All calcium channel blockers relax arteriolar smooth muscles and decrease peripheral vascular resistance which ultimately reduce blood pressure (Weber, K. 2002).

In patients with coronary artery disease, long-acting CCBs -either dihydropyridines or nondihydropyridines, were associated with a reduction in the risk of stroke, angina pectoris, and heart failure, with similar outcomes for other cardiovascular events as the comparison group (Bangalore, Parkar & Messerli 2009). Several studies have

compared the effects of calcium channel blockers with placebo and other antihypertensive therapies in the primary and secondary prevention of stroke. Two placebo control trials presented showed evidence of a 39% reduction in stroke risk with calcium channel blockers vs. placebo (Kassiri et al. 2009; Lawes et al. 2004). Furthermore, in patients with hypertension and stable angina, calcium channel blockers reduce the risk of any stroke and transient ischemic attack (TIA) by 30% and heart failure by 28% when compared with placebo. The results were similar for both dihydropyridines and nondihydropyridine calcium channel blockers (Bangalore, Parkar & Messerli 2009; Kassiri et al. 2009).

In diabetes, calcium channel antagonists (amlodipine and nifedipine) were found to produce an increase in insulin sensitivity in rat models of insulin resistance e.g- SH, neonatal STZ-induced NIDDM, and fructose fed hypertensive rats (Goyal, R. 1999). Chronic treatment of STZ-induced diabetic rats (Sprague-Dawley) with verapamil (8 mg/kg daily for 4-8 wk) resulted in an improvement of the altered myofibrillar ATPase activity, myosin ATPase, myosin isoenzyme distribution, and sarcoplasmic reticular Ca^{2+}-pump activities in ventricular tissue (Afzal et al. 1989). Verapamil (2, 4, or 8 mg.kg-1.day-1 for 4 weeks) shows a preventive effect on diabetes-induced myocardial changes. Verapamil improved diabetes-induced alterations in myocardial high-energy phosphate stores and ultrastructural damage without affecting their hyperglycemic status (Afzal et al. 1988). Further data suggested that calcium channel blockade by verapamil also improve glucose metabolism in Cohen-Rosenthal Diabetic Hypertensive Rats (Rosenthal et al. 1997).

Proteinuria is highly prevalent in diabetic patients and the onset and levels of proteinuria are directly associated with end-stage renal dysfunction in type 2 diabetes. Verapamil in combination with trandolapril was found to be effective in reducing proteinuria in both normotensive and type 2 diabetic patients (Rubio-Guerra et al. 2004).

Generally, high blood pressure or uncontrolled blood pressure remains the largest contributor to stroke events in humans. In the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT), amlodipine-based treatment achieved better blood pressure lowering effects compared to atenolol-based treatment with an average difference of 2.7/1.9

mmHg (Poulter et al. 2005). Moreover, in a small scale study, amlodipine reduced the risk of stroke by 24% compared with enalapril (Wang, J. 2009). In a recent seven-day study on SHR rats, Ca^{2+}-channel blockers (nifidipine 1mg/kg/day, diltiazem 5mg/kg/day and verapamil 4mg/kg/day) showed either a significant reduction or complete reversal of hypertensive changes of the morphometric parameters in the thoracic aorta such as- wall thickness, cross-sectional area, media-to-lumen ratio compared to age-matched WKY controls (Vaja et al. 2009). The same study also showed that diltiazem and verapamil also decreased internal diameter of aorta in SHRs (Vaja et al. 2009). The calcium channel blockers show a rapid vasculoprotective effects on large arteries which are independent of their blood pressure lowering effects (Ferrante et al. 1994; Vaja et al. 2009). A recent study demonstrated that a single-dose application of three calcium channel blockers- verapamil, diltiazem and nicardipine induced the production of NO in rabbits (Sirmagül et al. 2007). As such, it can be hypothesized that the cardioprotective effects of calcium channel blockers relate to their NO supporting properties which would reduce oxidative stress, inflammation, remodelling and improve vascular function To support this postulation, data demonstrated that L-type Ca^{2+} channel (LTCC) blockers, represented by amlodipine and verapamil, show anti-inflammatory activity by the suppression of plasminogen -Rs on macrophages (Das et al. 2009). Two weeks administration of amlodipine (20 mg/kg/day) and manidipine (10 mg/kg/day) to L-NAME-induced hypertensive rats showed a direct anti-inflammatory and antioxidative effect in the aorta which appear to have been mediated by an augmentation of eNOS expression and by the inhibition of the expression of ACE (Toba et al. 2005). These results clearly show the potential of calcium channel inhibition in CVD states and that their effects can also positively modulate RAS activity and reduce reactive oxygen species production and inflammatory cytokine signalling.

All of the clinical data in CVD and diabetes show protective benefits of calcium channel blockers by reducting of oxidative stress, and subsequent inflammatory signalling in addition to their calcium channel blocking activity. Therefore, stevia, which has been shown to act in part by blockade of calcium channels, has the potential to reduce or prevent cardiovascular damage following oxidative stress,

diabetes, hypertension and CVD. A study with stevia showed that verapamil (infused systemically at 0.015 mg/min) potentiates the renal vasodilating action of stevioside (infusion rate at 16 mg/kg per h) in normal Wistar rats (Melis 1991). Verapamil also increased hypotension, diuresis and percent fractional sodium and potassium excretion promoted by stevioside. These results suggest that stevioside's vasodilating effect is analogous to verapamil which is a specific inhibitor of calcium channels in cardiac and vascular muscle (Melis 1991, 1992, 1995).

In isolated aortic rings from normal rats, stevioside could dose-dependently relax the vasopressin-induced vasoconstriction in both the presence and absence of endothelium. In addition, stevioside lost its influence on vasopressin-induced vasoconstriction in Ca^{2+} free medium. The results indicate that stevioside caused vasorelaxation via an inhibition of Ca^{2+} influx into the blood vessel (Lee et al. 2001). Stevioside produced a significant increase in myocardial sensitivity to Verapamil without any toxicity (Vasović et al. 2006). This shows an important link between calcium channel antagonists and the potential therapeutic potential of stevia in CVD states.

2.5.2 Renin-angiotensin-aldosterone system (RAAS) blockers

The renin-angiotensin-aldosterone system has been shown to be an important player in the progression of diabetes-induced cardiovascular disease. The first-in-class direct renin inhibitor, aliskiren, blocks the RAAS at its point-of-activation, suppressing plasma renin activity and thus attenuating blood pressure similar to other antihypertensives. Aliskiren with or without other agents provides significant and prolonged blood pressure reductions in a broad range of hypertensive patients and is well tolerated (Ram 2009). Angiotensin II and aldosterone lead to several adverse cardiovascular effects including left ventricular hypertrophy and increased arterial stiffness (Edwards et al. 2009; Ram 2009). Studies showed that ACE inhibitors and angiotensin receptor blockers (ARBs) alone do not offer complete suppression of aldosterone production which is a powerful stimulus to the development of left ventricular hypertrophy, fibrosis and vascular inflammation (Edwards et al. 2009). Therefore, therapy with renin-angiotensin-aldosterone system blockers is expected to demonstrate additive beneficial effects on endothelial dysfunction and insulin

resistance in patients with cardiovascular risk factors. Recent studies have also showed that many tissues have a local RAAS such as pancreatic islets and adipose tissue. In the diabetic rat model (Zucker diabetic fatty rats), pancreatic islets exhibit an increased intra-islet expression of ACE and AT1 as well as increased intra-islet fibrosis, apoptosis, and oxidative stress (Cooper 2004, Edwards et al. 2009). Thus, studies demonstrated that inhibition of Ang II action in these tissues may be responsible for many of the clinical benefits observed following RAAS inhibition (Cooper 2004).

The ACE inhibitors are an important advancement in the treatment of hypertension particularly for patients with diabetes, hypertension and heart failure. They slow down the development and progression of diabetic glomerulopathy and other forms of chronic renal complications such as glomerulosclerosis and an ACE inhibitor is recognized as the first-line treatment in these patients (Coppey et al. 2006). The Heart Foundation guide to management of hypertension 2008 has ranked ACE inhibition as the initial therapy for newly diagnosed hypertension as these drugs have been shown to be effective in preventing ventricular dysfunction and other cardiovascular end points and also in lowering blood pressure.

Chronic hypertension and diabetes produce both macrovascular and microvascular pathophysiological changes. The renin-angiotensin system is implicated in the progression of diabetic complications including microvascular and end-organ damage (Ichihara 2006). Arterial stiffening enhances resistance artery remodelling and capillary rarefaction and leads to increased peripheral resistance, thereby contributing to hypertension and amplifying the detrimental haemodynamic effects (Feihl, Liaudet & Waeber 2009). The result is target organ destruction such as left ventricular hypertrophy, decreased coronary perfusion pressure, reduced coronary reserve and further vascular remodelling, terminating in coronary artery disease and stroke. Angiotensin-converting enzyme inhibitors (ACEI) such as perindopril with indapamide combination, have been shown to modify both arterial and arteriolar remodelling, leading to reduced central systolic blood pressure and enhancing vascular bed perfusion (Agabiti-Rosei 2008). Pharmacological studies have found that blockage of the renin-angiotensin-system (RAS), increases life expectancy in diabetic patients. The main driving force of this process is angiotensin II as hyperglycaemia

increases the production of this hormone in cardiac cells (Fiordaliso et al. 2006). Controlled diabetes and blood pressure reduce heart disease, stroke, retinopathy and nephropathy.

Therapies that modify the RAS, such as ACE inhibitors and angiotensin receptor blockers (ARBs) increase insulin sensitivity, meanwhile reducing diabetic development in hypertensive patients (Östergren 2007). These effects may also extend to reducing increased markers of oxidative stress and inflammation seen with hypertension and diabetes. Angiotension II is also known to stimulate NAD(P)H oxidase leading to subsequent increases in oxidative stress in the kidney. In diabetic neuropathy, NAD(P)H oxidase acts as a primary source of reactive oxygen species generation in vascular tissues (Coppey et al. 2006; Cotter & Cameron 2003; Inoguchi et al. 2000; Mollnau et al. 2002; Seshiah et al. 2002; Wang, H. et al. 2001; Wautier et al. 2001). Moreover, hyperglycemia and advanced glycation end products, two common complications in diabetes also stimulate reactive oxygen species generation in vascular tissues via activation of NAD(P)H oxidase (Coppey et al. 2006 Cotter & Cameron 2003; Wang, H. et al. 2001). Different experimental trials confirmed that inhibition of angiotensin II production either using angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers reverses remodelling and also increases tissue insulin sensitivity (Coppey et al. 2006). Moreover, controlling the renin-angiotensin system clinically delays the onset of type 2 diabetes, but the mechanisms involved are not clearly understood. (Fleming, Kohlstedt & Busse 2006)

A recent study showed that ACE inhibitor, perindopril is effective in reducing tubular apoptosis which was increased in experimental diabetic rats thus reflecting its capacity to attenuate endoplasmic reticulum stress (Sun et al. 2009). ACE inhibitors confer benefits at both incipient and overt stages of nephropathy in trials with type 1 and type 2 diabetes (Fraser & Phillips 2007). Renoprotective effects are observed by angiotensin receptor blocking drugs and ACE inhibitors were shown to reduce the risk of microalbuminuria, the initial step in renal disease in diabetes. Animal studies on the combination of the ACE-inhibitor perindopril and the diuretic indapamide showed a trend towards reducing albumin excretion and improving cardiovascular events (Mogensen 2005). Large clinical trials showed that reduction in the activity of renin-angiotensin system improved cardiovascular outcomes (Sleight 2002). Myocardial

infarction is one of the leading causes of heart failure. ACE inhibitors prevent the development of clinically significant ventricular dysfunction and mortality after acute myocardial infarction (Sleight 2002). The ACE inhibitors appear to confer a special advantage in the treatment of patients with diabetes, slowing the development and progression of diabetic glomerulopathy. Among the ACE inhibitors, enalapril improves vascular function and possess antioxidant activity (Chen, S. et al. 2008).

The first study with Enalapril was the Cooperative North Scandinavian enalapril Survival Study (CONSENSUS-I) and it evaluated the influence of enalapril (2.5 to 40 mg/day) on the outcomes of severe heart failure. Significant improvement was observed in the enalapril group with a 40% reduction in mortality compared to the placebo group in conjunction with reduced heart size and less need for further heart failure medication. After treatment with enalapril, the beneficial effect is maintained for at least 4 years and overall survival time is prolonged by 50% (Sleight 2002; Swedberg et al. 1999; The CONSENSUS Trial Study Group 1987). Enalapril also restores the reduced endothelium dependent vasodilation in experimental streptozotocin-induced diabetic rats (Baluchnejadmojarad, Roghani & Imani 2004a). Endothelial dysfunction is ca used by altered vascular contraction and dilatation, inflammation, and thrombosis in the vascular wall. Several studies also have demonstrated that advanced oxidation protein products are associated with impaired endothelium in uraemia (Chen, S. et al. 2008; Descamps-Latscha et al. 2005), diabetes mellitus (Kalousová, Skrha & Zima 2002), and coronary artery disease (Kaneda et al. 2002).

Captopril and enalapril are shown to possess potent antioxidant activity, which can scavenge oxygen free radicals or oxidant and inhibit lipid peroxidation (Chen, S. et al. 2008). In male Sprague-Dawley rats, chronic treatment of captopril or enalapril demonstrated a protective effect to endothelial dysfunction elicited by advanced oxidation protein products-BSA and reduced the elevated serum levels of malondialdehyde (MDA) derived from lipid peroxidation as well as increased NO production in endothelial cells (Chen, S. et al. 2008). Moreover, It was reported that ACE inhibitors stimulate basal NO production by suppressing bradykinin breakdown (Chen, S. et al. 2008; Grafe et al. 1993). These results provide important evidence that beneficial effects of captopril and enalapril on advanced oxidation protein products-

induced endothelial dysfunction are related to its antioxidant activity and enhancing NO production. Furthermore, non sulfhydryl ACE inhibitor, enalapril also reversed the advanced oxidation protein products-BSA induced endothelial dysfunction in this study. Therefore, captopril and enalapril are effective pharmacological approach to preventing the oxidation stress (Chen, S. et al. 2008). Similarly, in STZ-induced diabetic rats, long-term administration of enalapril results in partial restoration of endothelium-dependent contractile vascular reactions and endothelium-dependent dilatation of aorta and coronary vessels (Prysiazhna et al. 2007). Enalapril also reduces oxygen cost of smooth muscles and myocardial work. Thus enalapril improves vascular function by increasing nitric oxide synthesis and reducting oxidative stress in tissues of animals with diabetes mellitus (Prysiazhna et al. 2007). Thus both CCBs and ACE inhibitors are current and effective antihypertensive drugs. Moreover, CCBs show protective effect against cardiac and renal damage in salt-loaded stroke-prone spontaneously hypertensive rats (SHRSP) by reducing renin level and increasing endothelial NO production (Kyselovic et al. 2001).

2.6 Alternative therapy

Despite the major advancements in the pharmacological treatment of hypertension, cardiovascular diseases and diabetes, they remain major health problems in both developing and developed countries. Moreover some antihypertensive drugs such as, beta-blockers and diuretics have a negative effect on quality of life including altered sexual function (Paul et al. 2000; Wong et al. 2004a). Nowadays, nutraceuticals and different plant derived glycosides, nonsteroids and other components have been investigated for their therapeutic advantages in diabetes and cardiovascular diseases and in disease prevention therapy. Naturally occurring isoflavones and phytoestrogens like genistein are found to be cardioprotective and vasorelaxant in recent studies which confer beneficial effects on the incidence of coronary heart diseases in high soya-based food consuming populations (Baluchnejadmojarad & Roghani 2008).

2.7 Plant based therapies

Natural medicines have played major roles in preventing and treating different diseases of humans and animals for thousands of years. Among all natural products,

plant extracts offer vast sources for new medical entities with the improvement of modern scientific techniques for separation, structure elucidation, screening and synthesis (Huang et al. 2009).

Since antiquity several plants extract have been used for the treatment of diabetes and hypertension. In vitro studies showed that common spices like cinnamon, cloves, turmeric, bay leaves and tea improve insulin receptor function and enhance insulin sensitivity (Anderson & Polansky 2002; Broadhurst, Polansky & Anderson 2000; Cao, Polansky & Anderson 2007). Recent scientific investigation has confirmed the efficacy of many of these preparations, some of which are remarkably effective. Among them Momordica charantia (Bitter Melon), Aloe greatheadii var. davyana (Asphodelaceae), Ginkgo Biloba, Cinnamon, and Blueberry leaves are most commonly used in diabetes. Studies have demonstrated that Aloe greatheadii var. davyana extracts contain compounds with antioxidant capacity beneficial to prevention and treatment of diabetes, hypertension and cardiovascular diseases (Botes, van der Westhuizen & Loots 2008). A recent study showed that green tea recovers the normal redox state and reduces indicators of nephropathy but does not show any effect on glycemia and blood pressure levels in diabetic SHR rats (Ribaldo et al. 2009). Resveratrol (an extract from red wine) and Δ^9-THC (a plant based cannabinoid) have been shown to be cardioprotective via antioxidant and anti-inflammatory mechanisms suggesting a potential benefit for treatment of oxidative stress and inflammation-induced complications in hypertension and diabetes (Baur & Sinclair 2006; Steffens et al. 2005). Quercetin, another plant-derived polyphenol present in red wine, is drawing much research interest as an antioxidant agent offering cardioprotective effects. Clinical data showed that quercetin can reduce both malondialdehyde (MDA) levels produced by lipid peroxidization and left ventricular hypertrophy with oral doses of 5 and 10 mg/day (Han et al. 2009). Quercetin given by oral gavage at 10 mg/day was able to produce a hypotensive effect, reduce MDA concentrations and increase and normalise vascular responses to ACh in the SHR model (Duarte, J. et al. 1993).

In recent years, resveratrol has attracted scientists' attention by demonstrating improved endothelial functioning and decreased superoxide production in animal models for metabolic diseases (Schmitt & Dirsch 2009). Resveratrol is a nonflavonoid

polyphenol present in many plants and fruits and, at especially high concentration in grape skin (*Vitis vinifera*) and therefore present in red wine. Emerging evidence suggests that resveratrol plays an important role in the prevention of many human diseases (Kanti & Syed 2009). This compound has strong cytoprotective action and has ability to reduce oxidative stress in cells (Berardi et al. 2009). It has been hypothesized that resveratrol protects against cardiovascular diseases by its antioxidant effects which forms the basis for the so-called French paradox (the lower incidence of cardiovascular diseases in France than other Western countries) despite a saturated fat-rich diet (Iannelli et al. 2007). An increasing amount of data confirms that resveratrol has cardiovascular protective actions due to interference with several functions of human polymorphonuclear leukocytes, the regulation of several kinases such as protein kinase C and D, the phytoestrogenic modulation of oestrogen receptors, the influence on cell survival and apoptotic pathways (Iannelli et al. 2007; Rotondo et al. 1998; Slater et al. 2003; Stewart, Christman & O'Brian 2000). Very recently resveratrol demonstrated activity as sirtuins activator that enables this compound to mimic caloric restriction thus delaying ageing in yeast and metazoans (Iannelli et al. 2007; Rotondo et al. 1998; Slater et al. 2003; Stewart, Christman & O'Brian 2000; Wood et al. 2004).

Another study in 2009 showed that resveratrol confers significant protection against the t-BHP-induced oxidative stress of erythrocytes/membranes as evidenced by the increase in GSH level and membrane -SH content (Kanti & Syed 2009). Evidence suggests that resveratrol prevents or delays the onset of chronic diseases such as diabetes, inflammation, Alzheimer’s disease and cardiovascular disease and inhibits proliferation of human cancer cell lines (Brown et al. 2009; Das Evcimen & King 2007). In a recent study, resveratrol was shown to be beneficial for treating obesity and age-related diseases as it extended the lifespan and improved motor function in mice fed a high-calorie diet (Brown et al. 2009; Baur et al. 2006).

Overall, there is moderate evidence emerging for natural medicines to be explored further for their potential use as novel therapeutic agents or in the design of future pharmaceutical products used in the treatment of diabetes and hypertension.

2.8 Stevia offers new hope for the treatment of diabetes and hypertension

Whilst scientists are working on different natural products, they have identified a plant, *Stevia rebaudiana* Bertoni which is native to Brazil and Paraguay for its medicinal properties. The first written article about stevia dates back to 1900. However, it was not until 1931 when it was found that stevia contained two major glycosides, stevioside and rebaudioside. These chemicals are the active constituents and are responsible for the sweetening properties of stevia leaves. Stevioside is extremely sweet and is approximately 250-300 times sweeter than sucrose (Bridel & Lavielle 1931; Sehar et al. 2008). Since antiquity, extracts of stevia have been used in the treatment of diabetes among Indians in South America, specially Brazil and Paraguay. Evidence showed that stevioside and steviol possess a blood glucose-lowering effect and possibly have potential in the treatment of diabetes (Jeppesen et al. 2000). Stevioside lowered blood pressure in spontaneously hypertensive rats (SHR) when administered intravenously (Chan et al. 1998; Sehar et al. 2008). Indigenous Guarani people of Brazil and Praguay have used its leaves to sweeten their tea and also used as a heart tonic. Besides hypertension and diabetes, it is used against obesity, stomach burn and to lower uric acid levels (Lewis 1992; Sehar et al. 2008).

So, it would be of significant benefit for patients if the antihypertensive, antioxidant and anti-inflammatory effects of stevia could be established with good efficacy and tolerability. Lessening oxidative stress is an effective pharmacological approach to attenuate advanced oxidation protein products-BSA induced endothelial dysfunction and is potentially one of the actions of stevia.

Polar extract of stevia administered orally normalized blood glucose levels, prevented loss of body weight, restored normal organ weights in alloxan treated diabetic rats (Misra et al. 2011). Chronic treatment with glyburide reduced glucose stimulated insulin secretion and increased basal insulin secretion in the diabetic rats causing desensitization and secondary beta-cells failure (Chen, J. et al. 2006; Misra et al. 2011). Stevioside cancelled out this desensitizing action of glyburide reflecting that stevioside is highly effective in the treatment of diabetes.

2.8.1 Stevia: Composition

Stevia has been used for several hundred years as a natural sweetener and traditional medicine exclusively in South America, Japan, China and throughout the rest of Asia for decades as a replacement sweetener. Stevioside and rebaudioside A are the two most abundant glycosides present in Stevia leaves accounting for 5-10% and 2-4% of the dry matter, respectively. These two glycosides are responsible for the intense sweet taste of Stevia leaves (Figure 2.1) (Abudula et al. 2004). In Asia, Japan first marketed stevioside as a sweetener in food and the pharmaceutical industry. Nowadays, the use of stevioside has increased dramatically, substituting sugar and other artificial sweeteners due to health concerns, dental caries, obesity and diabetes (Chatsudthipong & Muanprasat 2009). These compounds are estimated to be up to 300 times sweeter than sucrose and comparable in sweetness to artificial sweeteners with the compound containing minimal calories. Stevia is useful in many food preparations as it is stable to 120-140^{o}C, is non-fermentable and is stable at a wide pH range (3–9) (Kroyer 1999; Sapna et al. 2008). Stevia has been safely used as a sweetener in Asia for decades and is used in many foods including yogurt, soft drinks and soy sauce (Tadhani, Patel & Subhash 2007).

Stevioside and rebaudioside A are diterpenoid glycosides which differ by one additional glucose moiety on rebaudioside A (Wheeler et al. 2008). Our study incorporates both rebaudioside A (2-4%) and stevioside (5-10%) as active compounds from stevia dry leaf. Stevia leaf also contains Rebaudioside C, Dulcoside A and C as well as other glycoside compounds and antioxidants (Midmore & Rank 2002). Many studies have looked individually at the medicinal effects of either rebaudioside A or stevioside however many medicinal benefits may be observed when these compounds are used in conjunction. Structurally, stevioside and rebaudioside A differ by a glucose moiety on the rebaudioside A molecule (Figure 2.1) and both are metabolised to steviol (Maki et al. 2008). Both rebaudisiode A and stevioside are by far the most concentrated of the glycosides in the plant.

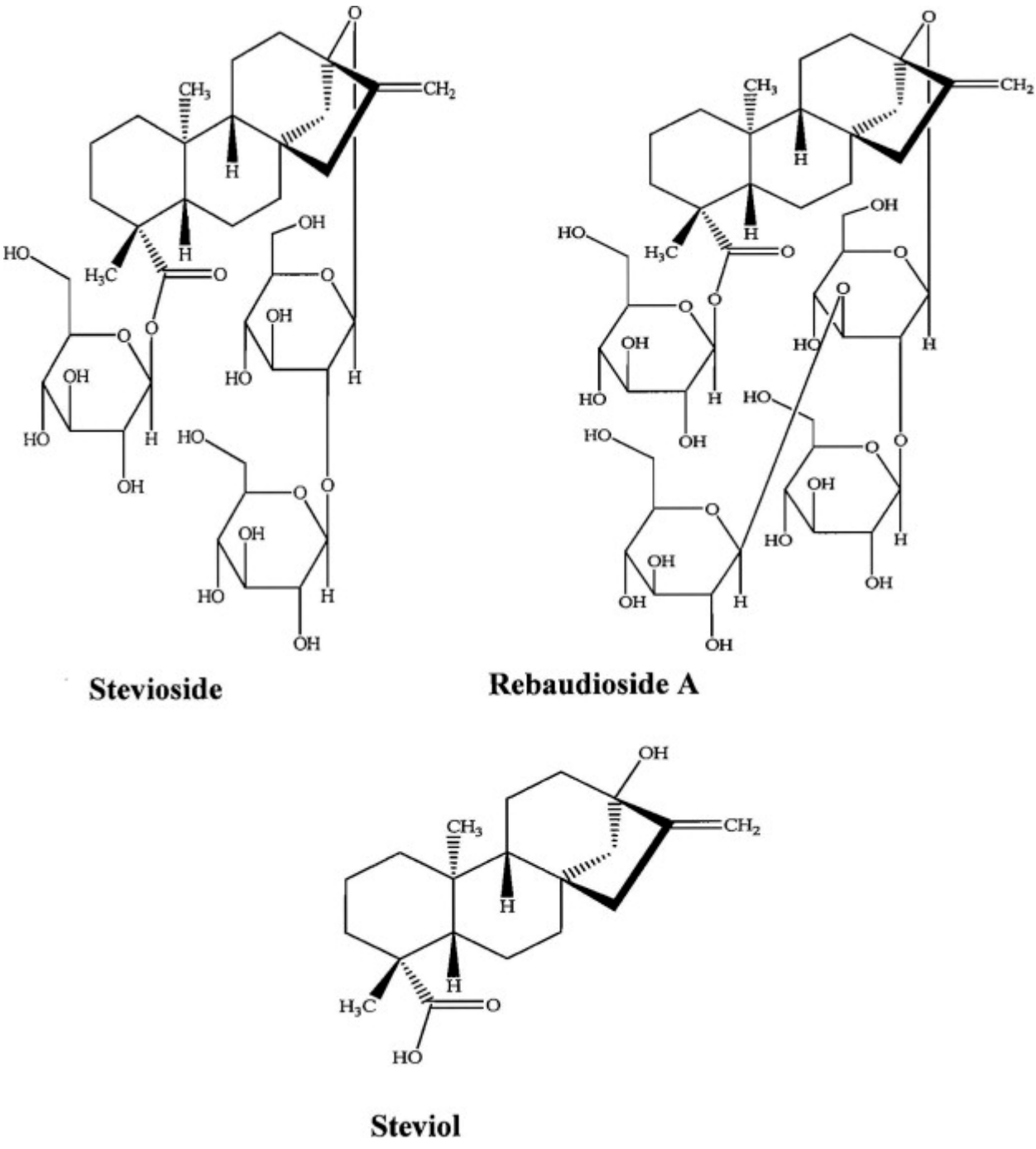


Figure 2.1: The chemical structure of rebaudioside A and stevioside ($C_{38}H_{60}O_{18}$) and steviol. (Carakostas et al. 2008)

2.8.2 Uptake and metabolism of stevioside

Both these glycosides are metabolized to steviol but they are not hydrolysed by digestive enzymes of the mouth, stomach and small intestine (Koyama et al. 2003).

Several in vitro and in vivo studies have shown that both rebaudioside A and stevioside are hydrolysed to the aglycone steviol by the microflora in the colon (Gardana et al. 2003; Geuns et al. 2003, 2007; Wheeler et al. 2008) and that this hydrolysis is required before absorption can occur. Steviol was found in the maximum concentration in the blood of the animals after 8 hours of intake (Geuns 2003; Koyama et al. 2003).

Steviol glucuronide is the common major metabolite for stevioside and rebaudioside and in human urinary excretion plays a major role to eliminate this metabolite from body, whereas biliary excretion is predominant in rats. This difference in excretion of steviol glucuronide in rats is because they have a lower molecular weight threshold for biliary excretion compared to humans (Chatsudthipong & Muanprasat 2009; Renwick 2008).

2.8.3 Mechanism of action of stevioside

Stevioside, the main diterpene glycoside of Stevia rebaudiana, possesses insulinotropic effect and glucagonostatic, antihyperglycemic and blood pressure-lowering effects (Chan et al. 1998; Gregersen et al. 2004; Hong, J. et al. 2006; Jeppesen et al. 2000, 2003; Jianguo et al. 2006; Paul et al. 2000). Stevioside and steviol stimulate insulin secretion via a direct action on beta cells. Both stevioside and steviol were observed to potentiate insulin secretion from INS-1 cells without any influence on the plasma membrane K^+ adenosine triphosphate (K+)ATP)-sensitive channel activity, or alteration of cyclic adenosine monophosphate (cAMP) levels in islets. The insulinotropic effects of both stevioside and steviol were conserved in the absence of extracellular Ca^{2+} (Jeppesen et al. 2000). The results indicate that stevia has a potential as an antihyperglycemic agent in the treatment of type 2 diabetes mellitus. It also has been reported that isosteviol- a derivative of stevioside, reduces the activity of liver glucose-6-phosphate which in turn decreases glucose production in the liver. Moreover, isosteviol inhibits monosaccharide transport in the intact rat liver (Wong et al. 2004a). Other studies have shown that isosteviol induces reduced glucose production and inhibits oxygen uptake in rat renal tubules (Wong et al. 2004a; Yamamoto, Kelmer Bracht & Ishii 1985). These findings have increased interest into establishing and evaluating the blood pressure-lowering effects of stevia which could

provide potential benefits for patients with hypertension and other cardiovascular complications with lesser side effects.

Effects on blood pressure

A study on healthy mongrel anesthetized dogs confirmed the antihypertensive effects of stevioside and suggested that its hypotensive mechanism may be due to inhibition of the Ca^{2+} influx (Liu et al. 2003). Another animal study suggested that isosteviol induces vasorelaxation by the opening of small conductance calcium-activated potassium channels (SK_{Ca}) and ATP-sensitive (K_{ATP}) channels (Wong et al. 2004a). Results obtained from this study demonstrated that selective opening of potassium channel with subsequent reduction of Ca^{2+} concentration to produce vasorelaxation could be one of the mechanisms of isosteviol (Wong et al. 2004a). Moreover, isosteviol showed a dose-dependent vasorelaxation of the vasopressin induced vasoconstriction in isolated aortic rings with or without endothelium which was also consistent with the report of opening of potassium channels (Topouzis, Schott & Stoclet 1991; Wong et al. 2004a). However, mediation of voltage dependent potassium channel in the vasodilation needs further investigation. Various data suggest that stevioside's vasodilating effect is analogous to verapamil which is a specific inhibitor of calcium channels in cardiac and vascular muscle (Melis 1991; 1992a; 1994).

In isolated aortic rings from normal rats, stevioside could dose-dependently relax the vasopressin-induced vasoconstriction in both the presence and absence of endothelium. In addition, stevioside lost its influence on vasopressin-induced vasoconstriction in Ca^{2+}-free medium. The results indicate that stevioside caused vasorelaxation via an inhibition of Ca^{2+} influx into the blood vessel (Lee et al. 2001)

Anti-inflammatory effects

Stevioside attenuates synthesis of inflammatory mediators in LPS-stimulated THP-1 cells by interfering with the IKKβ and NF-κB signaling pathway and induces TNF-α secretion which is partially mediated through TLR4 (Boonkaewwan, Toskulkao & Vongsakul 2006). Isosteviol (1-100 µmol/l) acts by inhibiting angiotensin-II-induced DNA synthesis and endothelin-1 secretion as observed in cultured rat aortic smooth

muscle (Wong et al. 2006). Evidence showed that increased angiotensin II levels leads to an increased production of reactive oxygen species in Angiotensin-converting enzyme 2 (ACE2)-deficient hearts by inhibiting metabolism of angiotensin II into angiotensin 1-7 (Kassiri et al. 2009). Deficiency of ACE2 and subsequent potentiation of angiotensin II levels also lead to increased neutrophilic infiltration in the infarct and peri-infarct regions, resulting in upregulation of inflammatory cytokines, interferon-gamma, interleukin-6, and the chemokine, monocyte chemo-attractant protein-1 (Kassiri et al. 2009). Moreover, aggravated end-organ damage has been observed in hypertensive patients with large blood pressure (BP) variability and the cardiac angiotensin II system could play a role in the pathogenesis of cardiac remodelling and dysfunction induced by a combination of hypertension and exaggerated BP variability (Kudo et al. 2009) Recent studies also suggested that circulating inflammatory markers are associated with BP variability in hypertensive patients (Kai et al. 2009). A study by Wong et al. (2006) showed an isosteviol-mediated inhibition of angiotensin II induced intracellular reactive oxygen species generation measured by a redox-sensitive fluorescent dye - 2'7'-dichlorofluorescin diacetate. Thus, isosteviol inhibits angiotensin-II-induced cell proliferation and endothelin-1 secretion via a reduction of reactive oxygen species generation (Wong et al. 2006). Therefore stevia has the potential to be used as a natural antioxidant and potential anti-inflammatory agent for cardiovascular and metabolic disorders.

2.8.4 Acute and chronic toxicity of stevioside

Stevia (stevioside and rebaudioside A) has a very low toxicity profile in different rodents models (Table 1.1) with a typical acceptable daily intake (ADI) of 7.9 mg stevioside/kg body weight (Geuns 2003). However, this ADI is considered as a minimum value because the test was not done with a concentration of stevioside higher than 793mg/kg BW (Geuns 2003). From a three-month chronic subacute toxicity study with rats (Aze et al. 1991; Geuns 2003) and hamsters over several generations (Geuns 2003; Yodyingyuad & Bunyawong 1991), an ADI of 25mg stevioside/kg BW has now been deduced (safety factor 100).

Long term use of stevioside (108 weeks) has not shown any evidence of carcinogenic potential (Brusick 2008). Stevia can safely be used as a sugar substitute for diabetics

and for other metabolic conditions, such as phenylketonuria. Different studies showed that oral stevioside is a well-tolerated and effective compound that may be considered as an alternative or supplementary therapy for patients with hypertension and diabetes (Chan et al. 1998; Chatsudthipong & Muanprasat 2009; Jeppesen et al. 2000, 2003; Gregersen et al. 2004; Paul et al. 2000). So, stevia has the potential to offer a new avenue to reduce the amount of internal damage experienced in the diabetic patient, particularly by using antioxidant and anti-inflammatory mechanisms.

Table 2.1 Acute toxicity of steviol glycosides and related substances

Species	**Sex**	**Test Substance**	**Route**	**LD_{50} (mg/kg bw)**	**Reference**
Mouse	Male and Female	93–95% Stevioside	Gavage	>15 000	Akashi & Yokoyama (1975)
Rat	Male	Isosteviol (purity not supplied)	Oral	>500	Bazotte et al. (1986)
Rat	Male	Isosteviol (purity not supplied)	Intraperitoneal	273.36	Bazotte et al. (1986)
Rat	Male	Isosteviol (purity not supplied)	Intravenous	55.27	Bazotte et al. (1986)

LD_{50}: median lethal dose (World Health Organisation 1999).

2.8.5 Stevia as a prevention and treatment for diabetes and hypertension

2.8.5.1 Effects of stevia in diabetes

The long term prognosis of type 2 diabetes leads to microvascular and macrovascular complications (Jeppesen et al. 2003). Diabetes causes oxidative stress by both an increased production of plasma free radical concentrations and a reduction of antioxidant defences (Ceriello 2000; Ceriello et al. 2000). This is also typically manifested inside the cell with altered mitochondrial metabolism generating increased oxidative radicals. Release of NO is inhibited in diabetes causing the attenuation of

adenosine receptor responses and reduced endothelial functioning. The vascular endothelium is impaired following diabetes due to a decrease in EDRF release and a decrease in the sensitivity of diabetic vascular smooth muscle to EDRF (Fahim, Hussain & Mustafa 2001). In another study, Sicrad et al. (2006) showed that hypertensive rats had elevated markers of oxidative stress demonstrated by increased vascular NAD(P)H oxidase activity and enhanced dihydroethidium (DHE) oxidation caused by superoxide anion in the aorta due to the impairment of plasma antioxidant capacity. It has now been well documented that inflammatory processes are a major cause of vascular injury following reperfusion injury. Steviosides have been shown to reduce rat heart ischemia-reperfusion injury damage to some extent (Xu et al. 2006). Thus with inflammatory signalling and ROS playing a significant role in ischemia-induced damage, stevia appears to confer some beneficial effect through ROS inhibition and possibly calcium channel blockade.

Stevia has also been used for a long time in the treatment of diabetes in South America and some clinical studies have been completed to prove the anti-hyperglycemic effect of stevia in patients suffering from diabetes. A recent trial showed that stevia can cause up to a 35% reduction in blood glucose in diabetic subjects after a single oral intake of Stevia extracts (Gregersen et al. 2004). However this finding needs to be fully examined, with our initial research indicating a much more conservative reduction following oral dosing in STZ diabetic rats.

As such our research aims to examine the protective effects of stevia 200 mg/kg/day (orally) against the severity of cardiovascular and parasympathetic gastrointestinal dysfunction in streptozotocin-induced diabetic rats (STZ).

2.8.5.2 Effects of stevia in hypertension

Stevioside has been reported to have blood-pressure-lowering effects and studies have demonstrated that stevioside causes bradycardia and hypotension both in humans and rats (Melis 1992; Melis, Rocha & Augusto 2009). Interestingly, the most pronounced antihypertensive effects appear to be exclusively reported in only the hypertensive animals or humans, not in the normotensive controls. This gives stevia a novel therapeutic profile by only acting on those individuals suffering from the disease.

Chan et al. (2000) demonstrated that 250 mg of stevioside three times a day, administered over a three month period, was able to produce a mean systolic blood pressure reduction of 12 mmHg and a mean diastolic blood pressure reduction of 8 mmHg in humans. Stevia has also been shown to induce a discrete shortening of the duration of the cardiac electrical systole, signifying a positive inotropic effect (Melis, Rocha & Augusto 2009). This finding has additional implications in potentially reducing the incidence of dysrhythmias which occurs during hypertensive heart disease. A recent study showed that the antihypertensive effect of crude stevia extract requires long term administration (Chatsudthipong & Muanprasat 2009). Melis (1996) has observed no significant change in blood pressure following oral administration of stevia extract to normal and hypertensive rats for the first 20 days. It required 40 to 60 days of stevia extract administration to observe a clear hypotensive effect (Melis 1996) which corroborates with the findings of Jeppesen et al. (2003) where a significant reduction in blood pressure was observed in rats only after repeated oral administration of stevioside at 25 mg/kg/day for 6 weeks (Jeppesen et al. 2003). These changes observed at the lower doses seem to take time to manifest and may show a more systemic or signalling effect via decreasing oxidative stress or inflammation or improving NO rather than a direct channel blocking action of stevia. Evidence showed that hypertensive rats had elevated markers of oxidative stress due to the impairment of plasma antioxidant capacity and an increase in the vascular production of reactive oxygen species (Sicard et al. 2006). It has now been well documented that inflammatory processes are a major cause of vascular injury following reperfusion. Steviosides have been shown to reduce rat heart ischemia-reperfusion injury damage to some extent (Xu et al. 2006). Stevioside induces hypotension, diuresis, natriuresis and kaliuresis and reduces the tubular reabsorption of glucose in rats (Melis 1992; Melis, Rocha & Augusto 2009). Stevia has been shown to possess antiglycemic, antihypertensive and antioxidant properties, all which promote its ability to provide an element of cardioprotection. Such protective attributes may be a result of its antioxidant properties. By reducing oxidative stress, NO availability is improved and promotes improved vascular and cardiac function.

Stevia also effectively reduces hypertension in human subjects. A double-blind placebo-controlled study examining the effectiveness and tolerability of oral

stevioside on 106 randomized Chinese subjects showed that stevioside is not only an effective drug for lowering blood pressure, but that it has no adverse effects on sexual function and other factors of daily living. This finding is particularly important from a patient compliance perspective, especially for male subjects, and shows that stevia is effective and well tolerated as a treatment for hypertension. Overall, stevioside is a safe and effective compound for the treatment of hypertension but its blood pressure lowering amplitude was found to be slightly less than that of other antihypertensive therapy (Paul et al. 2000). However this finding needs to be taken into perspective given that stevioside is a naturally derived product with many potential mechanisms of action compared to an empirically designed pharmaceutical agent.

Studies suggest that hypertension and diabetes induces systemic organ damage by cellular changes initiated by oxidative stress and inflammation that lead primarily to endothelial dysfunction (Dandona et al. 2006; Sciarretta et al. 2007). Moreover, there are several studies showing that oxidative stress leads to diabetic nephropathy. As scientists widely accept that both inflammation and oxidative stress play an integral role in the pathogenesis of hypertension and diabetes, the link between them is yet to be fully established (Prabhakar et al. 2007). So, we have planned to investigate the antioxidant and anti-inflammatory effect of stevia and determine to what extent stevia can prevent diabetes and hypertension-related oxidative stress and lessen cardiovascular and gastrointestinal injury.

It is now well known that oxygen radicals are closely associated with several disease processes including diabetes, CVD and aging. Therefore, an increasing interest in natural antioxidants present in medicinal and dietary plants products is occurring in the scientific community which might underpin a natural remedy in preventing this oxidative damage. A recent in vitro study demonstrated that the ethanolic extract of stevia leaves has strong antioxidant activity which can inhibit DPPH, hydroxyl radical, inducible nitric oxide, superoxide anion scavenging and hydrogen peroxide scavenging activity compared to a standard antioxidant like ascorbic acid (Shukla et al. 2009). Stevia extracts were found to contain substantial amounts of total phenols which are the major components acting as typical antioxidants (Shukla et al. 2009). However not much is known about the effect of stevia on vascular dysfunction and in prevention of end organ damage. Currently there is growing interest into whether

stevioside provides protection to the cardiovascular system, particularly the heart and blood vessels in hypertension- and diabetes-induced damage by putative antioxidant and anti-inflammatory mechanisms.

2.9 The Streptozotocin-induced diabetic rat models

Untreated diabetes leads to the development of characteristic complications including heart disease, strokes and macrovascular diseases like atherosclerosis, retinopathy which leads to blindness; impaired kidney function, end-stage renal disease and neuropathy which may lead to tactile allodynia, ulcers and amputations (Dresslerová & Vojácek 2010; Felicio et al. 2000; Fraser & Phillips 2007). To investigate these complications and cardiovascular changes, scientists typically use the streptozotocin-induced diabetic rat model which was first introduced in 1963. Since then this model has been used in more than 7600 published journal articles (Wei et al. 2003). A single, rapid intravenous injection of streptozotocin destroys the pancreatic β-cells inducing type 1 diabetes. The STZ model also demonstrates symptomatic complications of diabetes including hyperglycaemia, polydipsia, polyphagia and polyuria. Tactile allodynia occurs due to a decrease in nerve conductivity and occurs concurrently with diabetic neuropathy. Thus, the STZ model can be effectively used in studying diabetes-induced nerve damage (Davidson et al. 2007; Oltman et al. 2005). Moreover, benefits of the STZ rat model is that the induction of diabetes is fast, relatively easy, reproducible and reasonably cheap to reproduce (Wei et al. 2003).

The STZ-induced diabetic rat models present with vascular and neural dysfunction earlier than Zucker diabetic rats due to the more direct influence of hyperglycaemia (Oltman et al. 2005). This means that the animal model is easier to manipulate with more well defined diabetic and cardiovascular disease induced end-points. STZ-diabetic rats gradually develop diastolic and systolic dysfunction, diastolic stiffness with increased collagen deposition and a significant increase in the duration of the cardiac action potential in all phases of repolarisation (Jarrin et al. 2002). The current research will attempt to further characterise the cardiovascular changes and end organ damage following diabetes and requires an animal model that closely imitates the changes observed in humans. Results from different studies proved that the STZ-diabetic rat model is a dependable and suitable option for producing many chronic

complications observed in human diabetic patients and also for direct pathophysiological and biochemical investigation of diabetes itself. Previous studies with STZ-induced diabetic rats show a significant generation of superoxide radicals causing increased oxidative stress. It has been implied from this study that hyperglycaemia-induced oxidative stress may be a major factor for diabetes-induced vascular and neural dysfunction (Davidson et al. 2007). A recent study showed an increase in free radical concentrations and production of cytotoxic cytokines TNF-α in STZ-diabetic rats' myocardium (Drimal et al. 2008). This study also demonstrated that high blood glucose, increased free radicals and inflammatory TNF-α production in the myocardium was a leading feature in the development of STZ diabetes and also in the initiation of cardiac pathology. The STZ diabetic myocardium also showed that an increased release of free radicals and pro-inflammatory TNF-α contributed predominantly to the induction of inflammatory responses resulting in a graded left ventricular dysfunction. A significant glutathione deficiency and TNF-alpha induced myocardial inflammation were observed in the rats following five weeks of STZ diabetes (Drimal et al. 2008). All this information leads to an important assumption that there may be the loss of hemodynamic control in this model. It is now evident that myocardial and vascular damage caused by chronic diabetes result either from the persistence of chronic inflammatory signalling directly in the heart or from the dysfunction of anti-inflammatory systems. Furthermore, STZ rat models have also been shown to develop positive correlations between oxidative stress, free radicals, inflammatory cytokines and chemoattractants in the development of diabetes, chronic heart failure and vascular dysfunction and end organ damage similar to that seen in the diabetic human patient (Drimal et al. 2008; Kiss et al. 2009).

2.10 The spontaneously hypertensive rat models

Hypertension and its relevant effects on different organs are replicated in many different rat models such as the DOCA-salt, two kidney one clip and the L-NAME hypertensive rat. However, one of the most important rat models for studying the effects of hypertension and heart failure is the spontaneously hypertensive rat (SHR). The SHR is a genetic model of essential hypertension in humans and if left untreated leads to heart failure and death. The SHR rat model together with the Wistar-Kyoto

normotensive (WKY) rats as control is the most commonly used experimental model of human essential hypertension with over 13000 literature citations, (Bing, O. et al. 1995 ; Kodavanti & Costa 2001). This model shares many of the cardiovascular abnormalities observed in essential hypertension in humans (Hughes & Bund 2002). SHR rats attain a steady value of increased blood pressure and early hypertension at approximately 8-12 weeks of age. At the age of 3 months, SHR rat models show an extremely high blood pressure with increased vascular resistance (Guinamard et al. 2006). Following this initial hypertensive insult, SHR hearts develop pathological cardiac remodelling known as left ventricular hypertrophy with progressive increases in perivascular and interstitial fibrosis, thus the SHR represents a realistic model of human cardiac hypertrophy (Boluyt & Bing 2000; Guinamard et al. 2006). During the compensated stage, the SHR shows an increase in myocyte contractility, amplitude of the intracellular Ca^{2+} transient and action potential duration (APD) with no changes in resting membrane potential (Guinamard et al. 2006; Weisser-Thomas et al. 2007). A prolonged QT interval leads to APD prolongation on the ECG and is mainly due to a reduction of the transient outward current (Ito) density (Guinamard et al. 2006; Shipsey, Bryant & Hart 1997; Weisser-Thomas et al. 2007). In addition to this, SHR ventricular myocytes were found to show a higher incidence of delayed-after-depolarization (DAD) and associated-after-contractions compared to WKY rats. As DADs are generated by intracellular Ca^{2+} waves released from intracellular stores, SHR cardiomyocytes have greater incidence of presenting calcium waves (Guinamard et al. 2006). In SHR, vascular structural alterations represented by a larger medial volume and a higher number of smooth muscle cell layers are observed at the age of 3-4 weeks before a significant increase of the blood pressure takes place (Gao et al. 2008). This is an important finding and shows that it is not just the haemodynamic changes in this model but many humoral factors which play a significant role in SHR-induced hypertension. A greater contractile response in SHR arteries and an elevated peripheral vascular resistance are developed from these changes in blood vessel structure. Therefore, the SHR vessel walls are subjected to greater shear stress compared with those from the normotensive WKY which causes the functional impairment of the endothelium (Gao et al. 2008). These changes then compound the systemic increases in oxidative stress and inflammation with a reduced ability to release and manufacture endothelial derived nitric oxide.

Furthermore, in order to achieve a better understanding of the genetic basis behind the pathology of endothelial dysfunction, genetic hypertensive animal strain has been extensively studied which has led to some advancement in this area. A study by McBride et al. (2003) identified a candidate gene, Gstm2, in the stroke-prone spontaneously hypertensive rat (SHRSP) and showed that Gstm2 was significantly down regulated in the SHRSP compared with both the congenic strain SP.WKYGla2c* (produced by introgressing regions of rat chromosome 2 from Wistar Kyoto rats (WKY) into the recipient SHRSP strain) and WKY strains. The stroke-prone spontaneously hypertensive rat (SHRSP) offers an effective experimental model for human essential hypertension because of the complex genetic determination of blood pressure variation due to multiple gene-gene and gene-environment interactions which are similar to the human disease (McBride et al. 2003). The SHRSP manifests several vascular complications as observed in human, including cardiac hypertrophy, cardiac failure, and stroke (Jeffs et al. 1997; McBride et al. 2003). Gstm2 is a member of large gene family encoding glutathione S-transferases and plays a major role in increasing cellular resistance against oxidative stress (Cnubben et al. 2001; McBride et al. 2003). Thus, endothelial dysfunction in the SHRSP model establishes a possible link between increased oxidative stress and hypertension.

SHR rats are used to produce STZ-induced diabetes and serve as an important model of both hypertension-diabetes as well as the singular hypertension model. SHR-STZ induced diabetic rats are reproducible, easy to handle and offer a suitable means for pharmacological and biochemical investigation of diabetes and hypertension together (Van Zwieten et al. 1996). SHR rats also demonstrate elevated oxidative stress markers as a result of the impaired plasma antioxidant capacity and an increase in the production of vascular reactive oxygen species comparable to the human condition (Hughes & Bund 2002; Sicard et al. 2006). This shows humoral factors, such as endothelin, pro-fibrotic and pro-inflammatory cytokines, along with oxidative stress play important roles in the pathogenesis and pathology of hypertension. SHR have few glomeruli resulting in a reduced glomerular volume. This is consistent with the hypothesis that the kidney plays an important role in hypertension and makes the

animal itself more susceptible to cardiovascular complications (Nakanishi et al. 2005; Rettig et al. 1990).

Chapter 3 Methods

This chapter gives a detailed description of the animal models and materials and methods used in this research project. The specific animal models used in each of the individual studies are described in the annotated method sections outlined in the individual chapter.

3.1 Animals

Eight-week old male Wistar (300-320 g) rats were used in study 1; Spontaneously Hypertensive Rats (SHR) were used as the hypertensive model in study 2 (210-250 g) along with Wistar Kyoto (WKY) control animals (200-230 g). Male Wistar rats were used to develop the diabetic (STZ) model in study 3 by a single, rapid intravenous dose of streptozotocin (65 mg/kg) dissolved in 0.1 M citrate buffer via the femoral vein. All rats were obtained from the Animal Resource Centre in Western Australia and housed in the animal housing facility at CQUniversity, Australia. Animals were acclimatized and housed at a constant temperature (22 ± 2 ^{o}C) on a twelve-hour light/twelve-hour darkness cycle to simulate a normal living environment. They had unlimited access to water and chow pellets.

Ethical clearance was sought prior to use of the laboratory animals. Ethics approvals for use of animals in research were obtained from CQUniversity Australia's Animal Research Ethics Committee (ethics number# A06-190).

3.2 Study 1: Mechanistic study

Healthy male Wistar rats (8 weeks of age; weighing ≈300g) were used for the mechanistic study to establish a comparative putative mechanism of action of stevia. The animals were euthanized via an intra-peritoneal injection of 375 mg/kg of pentobarbitone sodium (Lethabarb®).

3.2.1 Measurement of cardiac electrophysiological changes

Following the methods of Fenning et al. (2005), the left ventricular papillary muscles were rapidly dissected out from the left ventricle and submerged in cold gassed (95% O_2/ 5% CO_2) Tyrode's physiological salt solution (in mM: NaCl 136.9. KCl 5.4, $MgCl_2.H_2O$ 1.0, $NaH_2PO_4.2H_2O$ 0.4, $NaHCO_3$ 22.6, $CaCl_2.2H_2O$ 1.8, glucose 5.5, ascorbic acid 0.3, Na_2EDTA 0.05) (Fenning et al. 2005). A stainless steel hook was then inserted into one end of the papillary muscle and the muscle placed in a 1.0 ml experimental chamber between two platinum electrodes (used for EFS). The experimental chamber was continually perfused with warm (37º ± 0.5 °C) Tyrode's solution at a rate of 3 ml/min. The muscle was then impaled with a potassium chloride filled glass electrode (World Precision Instruments, filamentated borosilicate glass, outer diameter 1.5 mm, tip resistance of 5-15 mΩ when filled with 3M KCl). A silver/silver chloride electrode was used as a reference. Control recordings were taken for a minimum of 20 minutes after impalement with the microelectrode.

The microelectrode measurements of the LVPM muscles were recorded for the following treatments: (i) normal Tyrode's solution (control) (ii) effect of stevia addition (3 x 10^{-4} M) (iii) effect of Ca^{2+}-enhanced solution (3 x 10^{-7} M) (iv) effect of stevia (3 x 10^{-4} M) in Ca^{2+}-enhanced solution (3 x 10^{-7} M) (v) effect of Ca^{2+}-channel blocker verapamil (3 x 10^{-5} M) in Ca^{2+}-enhanced solution (3 x 10^{-7} M) (vi) effect of the Ca^{2+}-channel blocker verapamil (3 x 10^{-5} M). Parameters analysed included action potential duration (APD) at 20%, 50% and 90% of repolarisation (APD_{20}, APD_{50}, APD_{90} respectively), action potential amplitude (APA), resting membrane potential (RMP) as well as the force of contraction and time to 90% relaxation of force (TR90). All data were acquired, derived and analysed using PowerLab Chart 5.5 software (AD Instruments).

3.2.2 Isolation of thoracic aortic tissues

Following heart incision after euthanasia, the thoracic aorta was promptly removed and placed in gassed Tyrode's physiological salt solution (Brown et al. 2001). The aortic vessels were then cleaned of adipose tissue, cut into 4 mm segments and

suspended in 25 mL Tyrode's filled organ baths with a resting tension of 10 mN to equilibrate for 30 minutes.

3.2.3 Isolation of mesenteric arteries

According to the procedures described by Thuraisingham and Raine (1999), second or third order branches of mesenteric arteries were identified using a dissecting microscope (Thuraisingham & Raine 1999). Then the surrounding fat and connective tissues were removed and the artery was dissected free from the surrounding mesentery and vein. Approximately 2 mm segments of the free arteries were cut and mounted on a DMT 610 M multi wire Mulvany myograph system (DMT, Denmark) connected to a PowerLab 8/30 recording system (AD Instruments) using Chart Pro software on an iMac computer. Each blood vessel mounting process involved threading the vessels with a 40μm stainless steel wire and then attaching each wire to one of the two jaws. The vessels were then left to equilibrate for 30min in Tyrode's solution bubbled with carbogen (95% O_2; 5% CO_2) at 37^0 C.

3.2.4 Assessment of vasodilator activity in isolated thoracic aorta and mesenteric arteries

After an equilibration period all vessels were subjected to a submaximal contractile dose of noradrenaline ($3x10^{-6}$ M) followed by acetylcholine ($1x10^{-8}$ to $3x10^{-4}$ M) in order to ensure each vessel possessed a functional endothelium. The submaximal contractile dose of noadrenaline was determined as the dose which achieved a 70% of maximal response to noadrenaline in the vessel. Stevia concentrations from $1x10^{-9}$ M to $3x10^{-4}$ M were then administered to the baths and a relaxation concentration response curve was calculated on noradrenaline precontracted tissues. A verapamil concentration curve ($1x10^{-9}$ to $3x10^{-4}$) was also calculated for comparative purposes in noradrenaline precontracted tissues. A number of aortic rings and mesenteric strips were then incubated with a calcium channel inhibitor (verapamil), nitric oxide synthase inhibitor (L-NAME) or a potassium channel inhibitor (4-AP) for 15 minutes followed by concentration curves of stevia ($1x10^{-9}$ M to $3x10^{-4}$ M) and verapamil ($1x10^{-9}$ M to $3x10^{-4}$ M) in noradrenaline precontracted blood vessels.

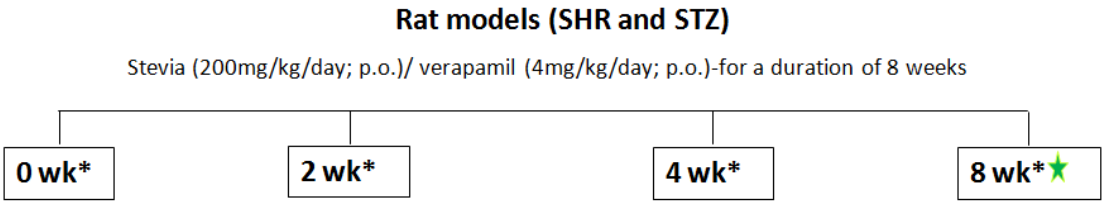
3.2.5 Assessment of contractile function of rat isolated ileum

The ileum was prepared according to the protocol of Coulson et al. (2002). Briefly, the ileum was removed from each animal and placed in Krebs-Henseleit solution of the following composition (mM): NaCl 117.5, KCl 5.6, $MgSO_4$ 1.18, $CaCl_2.2H_20$ 2.5, NaH_2PO_4 1.47, $NaHCO_3$ 25.0 and dextrose 5.54 containing propranolol (10^{-6} M). The ileum was rinsed with Krebs-Henseleit solution and cut into 1 to 2-cm segments after discarding the most distal 10 cm. Four sections of ileum were mounted longitudinally between two platinum electrodes in a 30 ml water jacketed organ bath containing Krebs-Henseleit solution bubbled with 5% CO_2 and 95% O_2, and kept at 37°C and were placed under 1.0 g isometric tension.

After a 60 minutes equilibration, a cumulative concentration-response curve (CRC) to carbachol (10^{-8} to 10^{-2} M) followed by a frequency-response curve to electrical field stimulation (EFS, 1–20 Hz, 100 V, 0.2-ms pulse duration for 5s every minute) were obtained on two ileum sections. The remaining two ileum sections were incubated with stevia (3×10^{-4} M) for 15 minutes followed by a concentration-response curve to carbachol (10^{-8} to 10^{-2} M) and then a frequency-response curve to electrical field stimulation (EFS, 1–20 Hz, 100 V, 0.2-ms pulse duration for 5 s every minute). Each ileum preparation was washed every 15 minutes for 60 minutes with Krebs-Henseleit solution to remove carbachol or endogenous acetylcholine from the bath and to regain baseline tension of the tissues. All tissues were then stimulated with 15 Hz of EFS and stevia was added to two organ baths followed by an M_2 muscarinic receptor agonist - pilocarpine CRC in all four organ baths. Pilocarpine was added cumulatively every 5 minutes to all tissues to investigate the effects of stevia on pilocarpine CRC and to test the M_2 muscarinic receptor functions by measuring the ability of pilocarpine (10^{-11} – 10^{-3} M) to inhibit an EFS-induced contraction. M_3 muscarinic receptor function was tested by measuring the ability of carbachol (M_3 muscarinic receptor agonist) to contract ileum. The muscarinic antagonist atropine (10^{-4} M) was added to the organ baths at the end of each experiment to confirm that EFS-induced contractions were mediated by muscarinic receptors.

3.3 Study 2 and Study 3: Chronic studies in animal models of hypertension and diabetes

In the chronic studies, we used the spontaneously hypertensive rats (SHR) for "study model 2" and the STZ-induced diabetic (STZ) rats as "study model 3". These chronic studies were designed to establish the organ specific damage caused by hypertension and diabetes and to investigate the effects of stevia and verapamil to reverse or prevent cardiovascular damage following diabetes and hypertension.



*Body weight, Water intake, Systolic blood pressure, Heart rate, Vonfrey measurements

Terminal experiments: Biochemical measurements, assessment of *ex vivo* cardiac function via Langendorff isolated hearts, electrophysiology of cardiomyocytes and organ bath experiments (thoracic aorta, ileums and mesenteric blood vessels).

3.4 Study model 2: The effects of stevia and verapamil therapy on chronic hypertension

The spontaneously hypertensive rat (SHR) and the Wistar-Kyoto control (WKY), were used as a genetic model of hypertension.

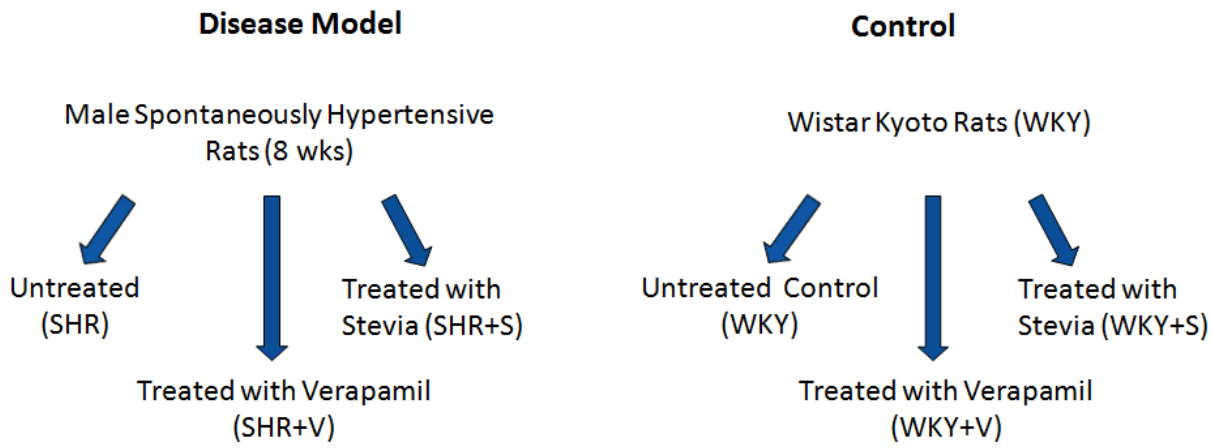


Figure 3.1: Flow chart of study design for chronic hypertensive rat model

3.5 Study model 3: The effects of stevia and verapamil therapy on chronic diabetes

STZ rats were used as an animal model of type I diabetes. Diabetes was induced using a single, rapid intravenous dose of streptozotocin (65 mg/kg) dissolved in 0.1 M citrate buffer via the femoral vein as described by Wei et al. (2003). Age-matched male Wistar rats were used as the control. Diabetes mellitus was confirmed by blood glucose monitoring and by an increase in water consumption (>100 ml/day) one week post STZ administration. The treated STZ-rats either received stevia (200 mg/ kg/day) from 0 to 8 weeks or the calcium channel antagonist verapamil (4 mg/kg/day) from 0 to 8 weeks. All rats were monitored by measurement of body weight, water and food intake.

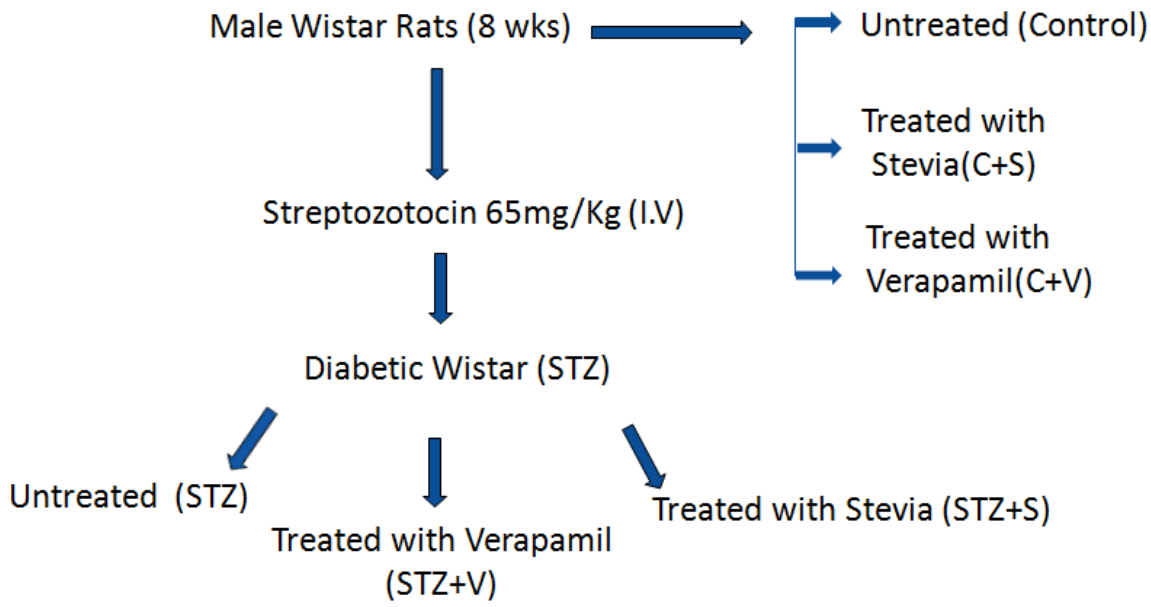


Figure 3.2: Flow chart of study design for chronic diabetic rat model

3.6 Treatment Organization

Animals of each species were randomly assigned to either treatment (stevia or verapamil) or non-treatment groups.

Stevia

Stevia extract was sourced from the Plant Science Centre of CQUniviersity Australia, Rockhampton. The compound was water-extracted from a dry-leaf parent product and contains a purified mixture of both stevioside and rebaudioside A. Stevia was dissolved in milli-Q water, producing a final concentration of 200 mg/ml. All stevia treated rats were dosed with stevia (200 mg/kg/ day) via oral gavage.

Verapamil

Verapamil was administered via oral gavage to the appropriate groups at a dose of 4 mg/kg/day. All treatment regimes were maintained for an eight-week period beginning when the animals were eight weeks of age (treatment week- zero) and continued until sixteen weeks of age (treatment week- eight).

3.7 Common experiments used in the chronic studies of hypertensive (Study 2) and diabetic (Study 3) rat models

This section describes all of the experimental procedures performed on the diabetic and hypertensive rats models.

Non-terminal assessments

3.7.1 Biometric parameter assessments

Every week, all rats were weighed and their water intakes recorded. For the diabetic groups, daily measurements were taken over the first week of treatment to ensure a diabetic state was present. Non-fasting blood glucose levels were monitored at the beginning and at the end of the study.

3.7.2 Systolic blood pressure, heart rate and heart rate variability assessments

Heart rate, heart rate variability and systolic blood pressure were measured at treatment weeks 0, 2, 4 and 8. Assessments of heart rate and systolic blood pressure were accomplished using the tail cuff plethysmography method as outlined by Fenning et al. (2005) with each rat lightly immobilized with a 0.1 ml intraperitoneal injection of Zoletil (tiletamine 15mg/kg with zolazepam 15mg/kg) (Fenning et al. 2005). The assessment of heart rate variability was carried out using a method based upon a study by Pereira-Junior et al. (2010). Electrodes connected to a PowerLab Bio amplifier were placed subcutaneously in the left and right hind leg and left front leg allowing for the collection of controlled electrocardiograms over a two-minute period.

3.7.3 Assessment of neuropathic pain

The development of tactile allodynia was measured by von Frey testing on each rat at treatment weeks 0, 2, 4 and 8. Rats were exposed to calibrated filaments (3-20 g) against the plantar surface of the hind paw in an ascending order until a brisk hind paw withdrawal was observed (Wei et al. 2003). The strength of the filament that induced hind paw withdrawal was then recorded.

Terminal experiments

For all terminal experiments rats were anesthetized and euthanased with an intraperitononeal injection of 375 mg/kg sodium pentobarbitone (*Lethabarb*®).

3.7.4 Organ weights and blood extraction

Immediately following euthanasia, blood samples were obtained directly from the abdominal vena cava. Blood was allowed to clot, then centrifuged and the serum was separated and stored at -80º C for later analysis. Blood glucose was determined using a MediSense® glucometer (Optium Xceed™, Abott Diabetes Care Inc., Almeda, USA). The left and right ventricles, kidneys, liver and spleen were removed, blotted dry and wet weights were taken. Organs weights were normalised according to final body weight for each rat, so as to allow for comparison between each animal.

3.7.5 Biochemical assessment

A number of biochemical parameters were assessed including total nitrate/nitric oxide, total antioxidant capacity (TAC), malondialdehyde (MDA), tumor necrosis factor-alpha, interleukin-6 using serum prepared from whole blood stored at -80º C to prevent sample degradation.

A. Serum MDA determination

A 96 well test kit for MDA detection, OxiSelect™ MDA Adduct ELISA Kit (Cell Biolabs, Inc.), was used to quantify the levels of MDA in the rat serum. Briefly, this kit was based on the principle that MDA bound to proteins in the blood to produce advanced lipid peroxidation end products, and by using an ELISA method their concentration was measured. The ELISA, in brief, required serum proteins that were incubated in a 96 plate to allow binding of the protein/MDA adducts to the wells. All standards and samples were assayed in duplicate. Anti-MDA antibody was added so as to bind to the bound protein/MDA adducts. Horse radish peroxidase was subsequently added to sandwich the anti-MDA and allowed colour development when the substrate was added. Once the colour developed the plate was put in a plate reader and absorbance was read at 450 nm. Duplicate readings were averaged and the

standards used to produce a four parameter logic curve, using Graphpad Prism 4.0 software, from this the unknown concentrations were determined.

B. Serum nitric oxide (NO) determination

NO was measured using a Total Nitric Oxide kit on rat serum (R&D Systems, Minneapolis, United States of America). Each sample and standard was assayed in duplicate and intra-subject means were than calculated. Both endogenous nitrate and total nitrite were measured.

The assay established a concentration of nitric oxide via an enzymatic conversion of nitrate to nitrite using nitrate reductase, the Griess reaction was used for the colorimetric detection of the nitrite (R&D Systems, Minneapolis, United States of America). The optical density for each sample was then measured using a Labsystems Multiskan Ascent microplate reader, which was set at 540 nm/ 690 nm. A four parameter logic line of best fit was generated using Graphpad Prism 4 software and the unknown concentrations were determined.

C. Serum total antioxidant capacity (TAC) determination

TAC was measured using Cayman's total Antioxidant Assay kit (Antioxidant Assay kit; item no. 709001; Australia). This method does not separate aqueous and lipid soluble antioxidants; therefore it determines combined total antioxidant activities of all its constituents. The assay principal relies on the capacity of antioxidant in the sample to inhibit the oxidation of ABTS (2, 2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) by metmyoglobin and measured by reading the absorbance at 405 nm. The total antioxidant capacity of the sample was compared a water-soluble tocopherol analogue called Trolox and was expressed as mM trolox equivalent.

$$\text{Antioxidant (mM)} = \frac{\text{Sample average absorbance} - \text{(y-intercept)}}{\text{Slope}} \text{ x Dilution}$$

D. Serum interleukin-6 (IL-6) determination

IL-6 was measured using the rat IL-6 Immunoassay kit (R&D Systems, Minneapolis, United States of America). This assay was based on the ability of the sandwich enzyme immunoassay technique to determine concentrations of proteins in serum samples. Briefly a monoclonal antibody specific for IL-6 (anti-IL-6) was pre-coated onto a 96 well microplate. Samples and standards were then pipetted into the wells of the microplate, in duplicate which allowed the anti-IL-6 to bind to any available IL-6 within the samples. Wells were washed thoroughly to ensure that only bound IL-6 is left in the wells before an enzyme-linked polyclonal antibody, specific for IL-6 was added to each well. After incubation a colour developed in direct proportion to the concentration of IL-6. The optical density for each sample is then measured using a Labsystems Multiskan Ascent microplate reader, which was set at 490 nm with λ correction at 650nm. Concentrations were then determined using the standard curve using Graphpad Prism 4.0 performing the analysis.

3.7.6 Vascular reactivity studies in isolated thoracic aorta and mesenteric arteries

A. Isolation of thoracic aortic tissues

Following heart excision after euthanasia, the thoracic aorta was promptly removed as per the methods discussed in section 3.2.2, page 66.

B. Isolation of mesenteric arteries

The process of isolation of mesenteric arteries was discussed in section 3.2.3, page 66.

C. Assessment of vascular reactivity in isolated thoracic aorta and mesenteric arteries

After an equilibration period, all large and small vessels were subjected to a sub maximal contractile dose of noradrenaline followed by acetylcholine in order to ensure each vessel possessed a functional endothelium.

Then the vessels were tensioned to (10 mN and 2 mN for aorta and mesenreric vessel respectively) before cumulative concentration-response curves were performed for noreadrenaline ($1x10^{-9}$ M to $3x10^{-4}$ M) and either acetylcholine ($1x10^{-8}$ M to $3x10^{-4}$ M) or sodium nitroprusside ($1x10^{-8}$ M to $3x10^{-4}$ M) in the presence of a submaximal contraction to noradrenaline.

3.7.7 Measurement of cardiac electrophysiological changes

The left ventricular papillary muscles were dissected out from the left ventricle as per the method discussed in section 3.2.1, page 66. Parameters analysed included action potential duration (APD) at 20%, 50% and 90% of repolarisation (APD_{20}, APD_{50}, APD_{90} respectively), action potential amplitude, resting membrane potential and action potential voltage over time (dV/dTmax) and the force of contraction over time (dF/dt).

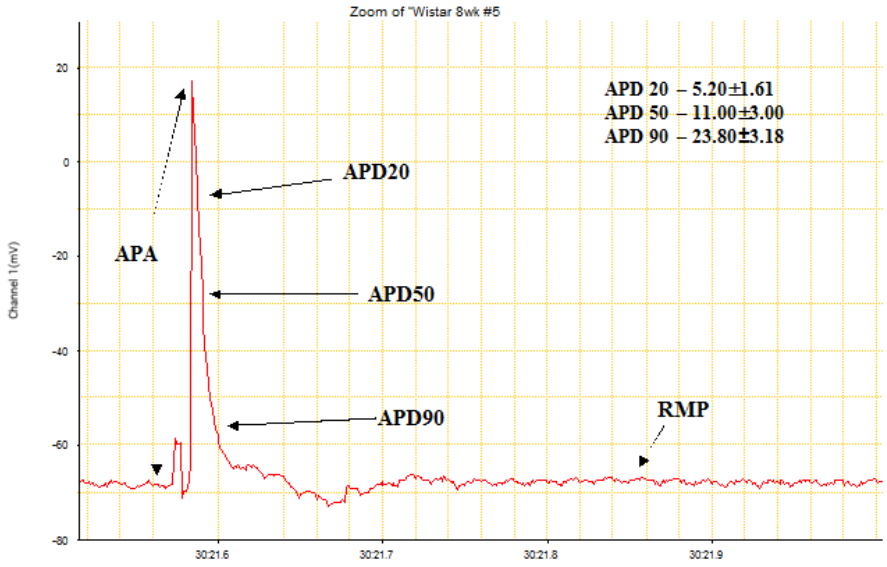


Figure 3.3: Electrophysiological recordings from left ventricular papillary muscles from a young Wistar rat demonstrating the key features.
APA = action potential amplitude, APD 20, 50 and 90 = action potential duration at 20%, 50% and 90% of repolarisation, RMP = resting membrane potential.

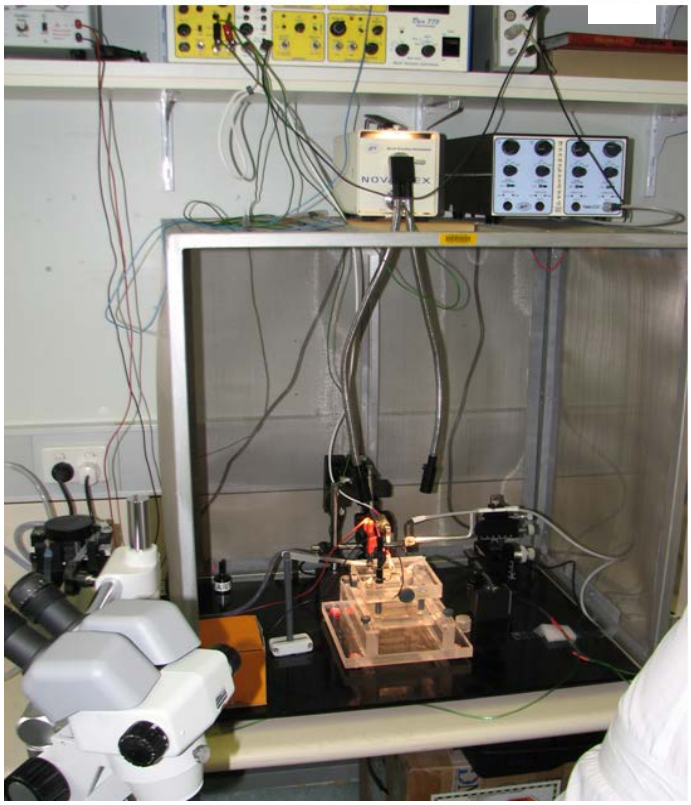


Figure 3.4: Cardiac electrophysiological chamber with single-cell microelectrode

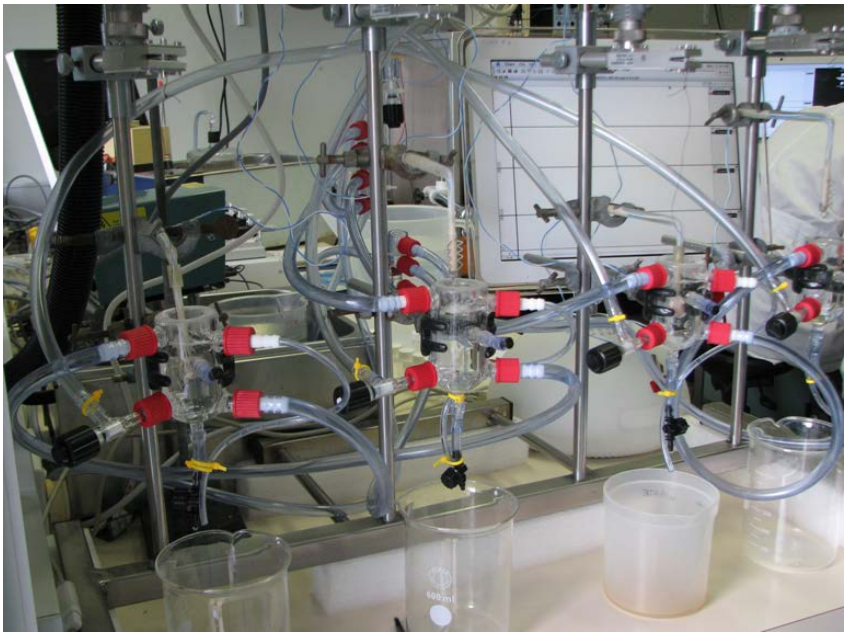


Figure 3.5: Ileum organ bath apparatus

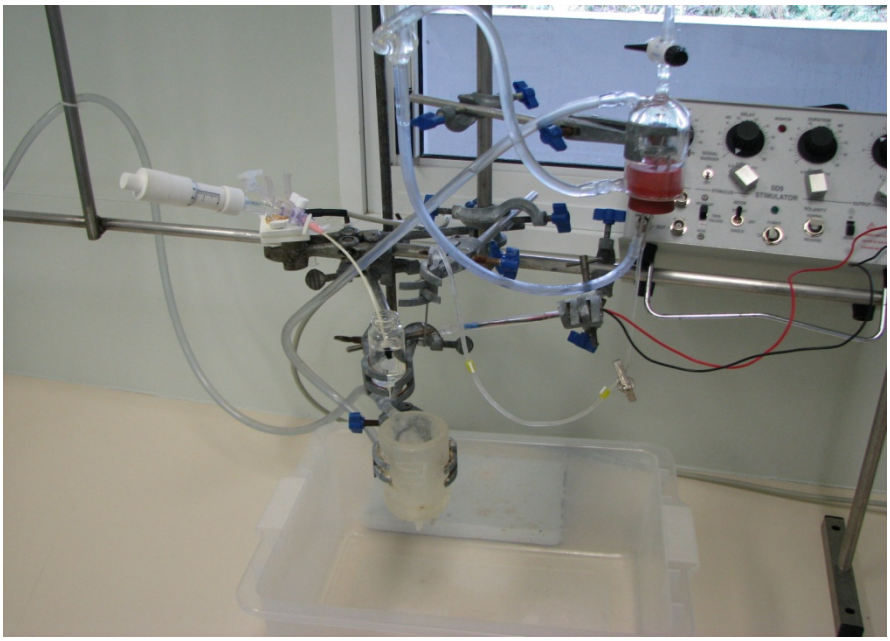


Figure 3.6: Isolated Langendorff's heart preparation machine

3.7.8 Determination of cardiac function in the isolated heart

To assess isolated myocardial function, the non-recirculating Langendorff heart preparation was used (Brown et al. 2001). Following euthanasia, the hearts were rapidly excised and placed in ice-cold modified Krebs-Henseleit solution. Then aorta was isolated and cannulised via the dorsal root. Retrograde perfusion was maintained

at a constant pressure (100 mmHg) with modified Krebs-Henseleit buffer containing (in mM): NaCl 119.1, KCl 4.75, $MgSO_4$ 1.19, KH_2PO_4 1.19, $NaHCO_3$ 25.0, and glucose 11.0, $CaCl_2$ 2.16 kept at 37˚C and bubbled with a mixture of 95% O_2 / 5% CO_2. A latex balloon was placed in the left ventricle to measure the myocardial diastolic stiffness and contractile functions through a connection to Capto SP844 pressure transducer (MLT844/D) which was then attached to a PowerLab program and recording system. Approximately 25 minutes of equilibration period was allocated for all hearts. At the end of the experiment, the atrium was removed and the weight of the ventricles plus septum recorded.

A. Diastolic stiffness

To evaluate end-diastolic pressure, measurements were taken for 3 minutes at every 5mmHg increase starting at 0mmHg up to a maximum of 30mmHg. Diastolic stiffness and left ventricular developed pressure were calculated by measuring diastolic pressure and systolic pressure at the last 1 minute of each 3 minutes recording. Myocardial diastolic stiffness was expressed by the stiffness constant k, which was determined by the slope of the linear relation between the tangent elastic modulus (E, dyne/cm^2) and stress (ծ dyne/cm^2) (Brown et al. 1999). Contractile function was assessed by measuring maximal +dP/dt values at a diastolic pressure of 10 mmHg.

3.7.9 Gastrointestinal function in isolated ileum

The ileum is removed from each animal following the methods discussed in section 3.2.5, page 68.

After 60 minutes equilibration of the isolated ileum, a cumulative concentration-response curve to carbachol (10^{-8} – 10^{-2} M) or a frequency-response curve to electrical field stimulation (EFS, 1–20 Hz, 100 V, 0.2-ms pulse duration for 5 s every minute) were created on each preparation. Then the ileum-preparation was washed every 15 min for 60 minutes with Krebs-Henseleit solution to remove carbachol from the bath and to regain baseline tension of the tissues. An M_2 receptor agonist - pilocarpine was added cumulatively every 5 minutes to the bath after a 15 minutes stimulation with 15Hz EFS to test the M_2 receptor functions by measuring the ability

of pilocarpine (10^{-11} – 10^{-3} M) to inhibit and methoctramine (10^{-9} – 10^{-4} M) to potentiate EFS-induced contraction. M_3 muscarinic receptor function is tested by measuring the ability of carbachol to contract ileum. The muscarinic antagonist atropine (10^{-4} M) was added to the organ baths at the end of each experiment. Addition of atropine to the baths blocked EFS-induced contractions, confirming that these contractions were mediated via muscarinic receptors. The 'gastrointestinal function' study signifies the some important parameters of diabetes which also has strong relevance with hypertension, such as: change in parasympathetic nerve functions in the gut and heart (reduced heart rate) and increase or decrease in the neuronal muscarinic receptor (M_2, M_3) functions.

Chemicals

Verapamil, carbachol, pilocarpine, propranolol, L-NAME and 4-Aminopyridine were purchased from Sigma-Aldrich (Australia). All drugs were dissolved in Milli-Q water (Millipore; USA). Stevia extract (mixture of stevioside 90% and rebaudioside A 10%) was obtained from the Centre for Plant Sciences, CQUniversity, Rockhampton, Australia.

3.8 Data Analysis

All data are presented as mean ± SEM with 'n' values representing the number of animals that contributed to the mean. The results were analysed using two-way ANOVA. When ANOVA showed a significant treatment effect, Bonferroni's multiple comparison tests was used to compare individual. A Student's t-test was used to compare and evaluate two group means via paired/ unpaired where appropriate. All standard curves were formulated using Graphpad Prism 4.0 to create a four parameter logic curve from which unknown values were determined. A p value of less than 0.05 was considered significant. *$p<0.05$ vs control and **$p<0.05$ vs STZ were considered statistically significant**.**

Chapter 4 Electrophysiological, vasoactive and gastromodulatory effects of stevia in healthy Wistar rats

4.1 Introduction

The extracts of stevia, *Stevia rebaudiana* Bertoni (Family-Asteraceae) have been widely used throughout the world for traditional medicinal purposes to treat diabetes, hypertension, heartburn and infection (Jeppesen et al. 2000; 2002). The first written account of stevia usage was published in 1900. However, it was not until 1931 when scientists found that stevia leaves contain two main glycosides: stevioside (4-13% dry weight) and rebaudioside A (2-4% of dry weight), of which stevioside is the principal sweetening component (Bridel & Lavielle 1931; Midmore & Rank 2002). Stevioside is approximately 250-300 times sweeter than sucrose.

With the alarming rise in rates of obesity, diabetes and cardiovascular disease (Meigs 2010), stevia attracts special attention as a preferred replacement sweetener in food and beverages. Data showed that intake of fructose based sweeteners and artificial non-caloric sweeteners can lead to significant alterations of lipid profiles and liver damage in healthy humans and animals (Figlewicz et al. 2009; Kelley, Allan & Azhar 2004). In contrast, stevia is well tolerated as a sugar substitute and does not induce any rebound weight gain due to changes in satiety potential (Bloomgarden 2011; Figlewicz et al. 2009; Geuns et al. 2003; Paul et al. 2000; Yodyingyuad & Bunyawong 1991).With other non-caloric sweeteners such as aspartame showing potential adverse effects whilst being devoid of any therapeutic actions (Kinghorn & Soejarto 1985; Nabors & Gelardi 1991) stevia shows some promising beneficial pharmacological activity (Chatsudthipong & Muanprasat 2009; Geeraert et al. 2010; Jeppesen et al. 2000, 2002). Stevia has been shown to have insulinotropic, antihyperglycemic, glucagonostatic and anti-atherosclerotic effects in animals and human subjects (Chatsudthipong & Muanprasat 2009; Geeraert et al. 2010; Jeppesen et al. 2000, 2002). Both human and animal studies demonstrated blood pressure lowering effects of stevia when administered orally and intravenously (Ferri et al. 2006; Jeppesen et al. 2003; Kujur et al. 2010). Stevia, however, did not show any

significant effect on the blood pressure in normotensive individuals (Chan et al. 1998; Jeppesen et al. 2003).

Vascular smooth muscle cell relaxation in response to opening of K^+ and/or blocking of Ca^{2+} channels plays an important role in the control of blood pressure. Studies on healthy rat's isolated aortic rings showed that stevia has vasodilatory effects on the blood vessel from both intact and endothelium-denuded tissues (Bornia et al. 2008). Nitric oxide synthase acts as a key mediator in stevia-induced vasorelaxation when the endothelium is intact, since pretreatment of the aorta with N^G-nitro L-arginine (L-NAME) diminished the relaxation caused by stevia (Bornia et al. 2008). In endothelium denuded aortic preparations, the suggested dilatory mechanism of stevia may be through the blockade of Ca^{2+} channels (Bornia et al 2008; Lee et al 2001).

In addition to vascular dysfunction, diabetes and hypertension contribute to gastromucosal damage, gastrointestinal dysmotility and impaired nerve signalling in the gut (Rayner et al. 2001; Samsom et al. 1997). Stevia extract showed a protective effect against gastric mucosal damage induced by histamine in the rainbow trout (Shiozaki et al. 2006). Stevia prevented increased contractility of the gastrointestinal smooth muscle evidenced by its antispasmodic effect in guinea pig ileum and reduced gizzard erosion in broiler chickens fed dietary histamine (Shiozaki et al. 2006; Shiozaki et al. 2004; Takahashi et al. 2001). Moreover, stevia was suggested to be gastroprotective by regulating gastric acid secretion and preventing gastric ulcer formation in a similar way to that of calcium channels blockers. Studies showed that CCBs are well established gastric acid regulator and protect the gut from ulcer (Shiozaki et al. 2004).

Chronic cardiovascular disease leads to arterial stiffness which in turn increases pulse pressure causing a greater myocardial load and increased oxygen demand (Chue et al. 2010). Increased loads and greater oxygen demand initiate maladaptive structural and functional changes of the cardiac myocytes and vascular walls (Chue et al. 2010; Tedesco et al. 2004). Electrophysiological studies in human and animal models showed that action potential prolongation was directly associated with cardiac remodelling and heart failure (Milberg et al. 2011a; 2011b; Stams et al. 2011). Intracellular Na^+ and Ca^{2+} concentrations were found to be altered in chronic heart failure (CHF), causing a prolonged action potential in the failing myocardium

(Milberg et al. 2011a; 2011b). This resulted in an increased Ca^{2+} channel reopening time and enhanced release of calcium from the sarcoplasmic reticulum (Milberg et al. 2011a; 2011b).

Stevia is capable of improving vascular smooth muscle contractility through a calcium channel antagonist-like action on vascular smooth muscle cells (Lee et al. 2001; Wong et al. 2004a). However, no published reports on cardiac electrophysiological studies of stevia on human or animal models were found in the literature. The current paper will: (i) investigate the action of stevia on the electrophysiological and contractile properties of cardiac tissues, and (ii) establish the mechanism of action of stevia in aortic and mesenteric vessels, and isolated ileum from healthy Wistar rats.

4.2 Materials and Methods

Eight-week old male Wistar rats (weighing ≈300g) were obtained from the animal resource centre in Perth, Western Australia and housed in the animal housing facility at CQUniversity. Animals were acclimatized and housed at a constant temperature (22 ± 2 ºC) on a twelve-hour light/darkness cycle to simulate a normal living environment. They had unlimited access to water and chow pellets. Animals were euthanized via an intraperitoneal overdose of pentobarbitone sodium (Lethabarb® - 60 mg i.p.). All experiments conformed to the guidelines of the National Health and Medical Research Council of Australia and were approved by CQUniversity Animal Ethics Committee (AEC# A06-190), Rockhampton, Australia.

Experiments were performed on the eight-week old healthy Wistar rats. Cardiac electrophysiological measurement and recording of single-cell microelectrode were described in Chapter 3, section 3.2.1. Vascular responses to stevia, verapamil, noradrenaline, acetylcholine, L-NAME, 4-AP and sodium nitroprusside were measured for thoracic aortic rings (Chapter 3, section 3.2.2, section 3.2.4.) and for mesenteric arteries (section 3.2.3., section 3.2.4). The function of isolated ileum to electrical field stimulation (Chapter 3, section 3.2.5), carbachol and pilocarpine were also assessed (Chapter 3, section 3.2.5.).

Statistical Analysis

All data are presented as mean ± SEM with n values representing the number of animals that contributed to the experimental mean. The concentration of carbachol required to produce 50% maximum contractile response (EC_{50}) were interpolated from semi-logrithmic plots of individual concentration-response curve for carbachol. The effects of pilocarpine on EFS-mediated contraction are expressed as the ratio of contraction in the presence of drug to the contraction in the absence of drug. Results were analysed using two-way ANOVA and Student's t tests where appropriate. When ANOVA showed a significant treatment effect, Bonferroni's multiple comparison tests was used to compare individual means. A $p<0.05$ was considered statistically significant.

4.3 Results

4.3.1 Effect of stevia on thoracic aorta preparations

Stevia induced a significant relaxation response in noradrenaline (3×10^{-3} M) pre-contracted aortic tissue comparable to that of verapamil and acetylcholine (Figure 4.1). Pre-treatment of the tissues prior to the noradrenaline-induced contraction with verapamil (1×10^{-3} M), caused an increase in the relaxation response to stevia (Figure 4.1). Incubation with 4-aminopyridine (4-AP) (1×10^{-3} M), a K^+-channel blocker, prior to pre-contraction with noradrenaline, demonstrated no significant differences ($p>0.05$) in the relaxation response to stevia (Figure 4.1). Whereas, incubation with L-NAME (1×10^{-3} M) completely diminished the vasorelaxation response caused by stevia in noradrenaline precontracted aortic tissue (Figure 4.1).

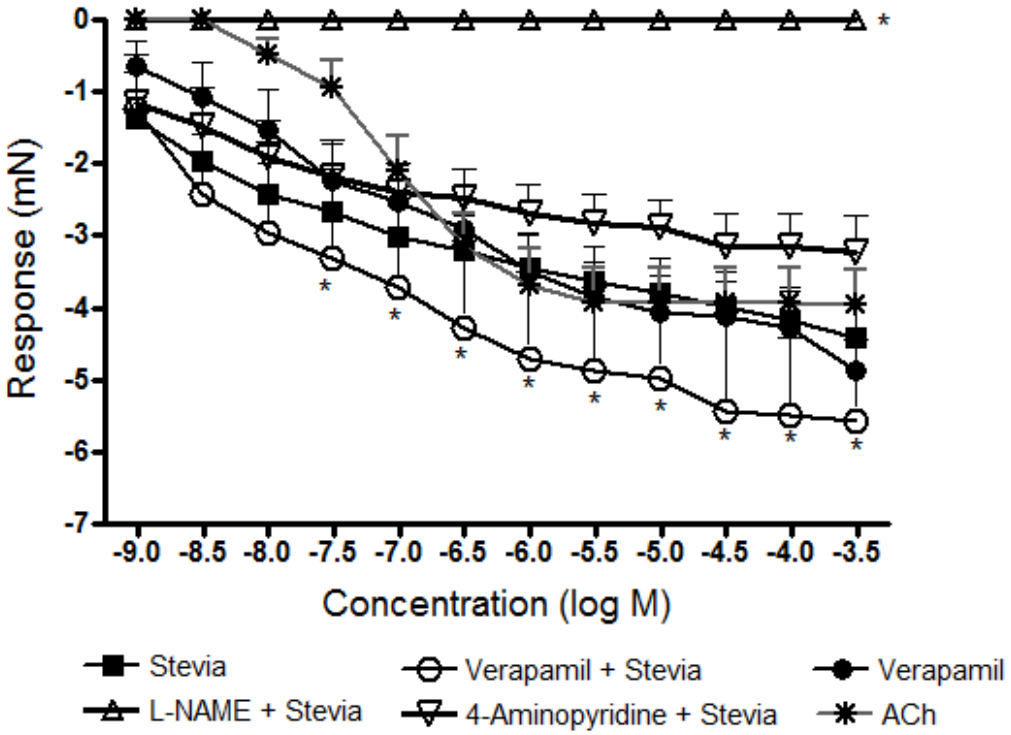


Figure 4.1: Effects of stevia on noradrenaline precontracted thoracic aortic preparations.Results are presented as mean ± SEM. n=15 for all groups. .*P<0.05 vs stevia (two-way ANOVA with Bonferroni's multiple comparisons tests).

4.3.2 Effect of stevia on mesenteric artery preparations

Stevia demonstrated a concentration-dependent relaxation effect on mesenteric arteries, but not to the extent exerted by verapamil (Figure 4.2). However, incubation of mesenteric arteries with verapamil (3 x 10^{-3} M) reduced the dilatory response to stevia (Figure 4.2). The dilatory response to stevia was significantly increased in presence of the potassium channel blocker 4-amino pyridine (4-AP) (Figure 4.2). Incubation with L-NAME did not show any significant effect on the relaxation response of stevia in noradrenaline precontracted mesenteric arteries (Figure 4.2).

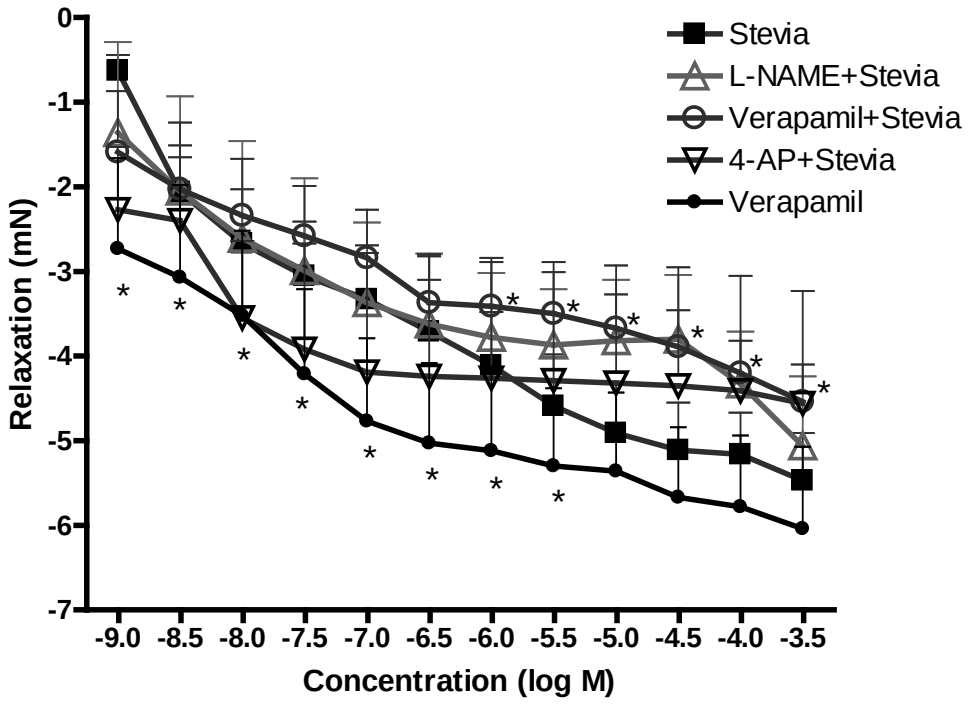


Figure 4.2: Effects of stevia on the noradrenaline precontracted mesenteric arteries. Results are presented as mean ± SEM. n=15 for all groups. .*P<0.05 vs stevia (two-way ANOVA with Bonferroni's multiple comparisons tests).

4.3.3 Electrical field stimulation (EFS) on isolated rat ileum

EFS produced frequency dependent contraction of gastrointestinal smooth muscle on the isolated ileum (Figure 4.3). Incubation of the tissues for 15 minutes with stevia ($3x10^{-4}$ M) significantly reduced the frequency dependent contraction compared to that of control tissues (Figure 4.3).

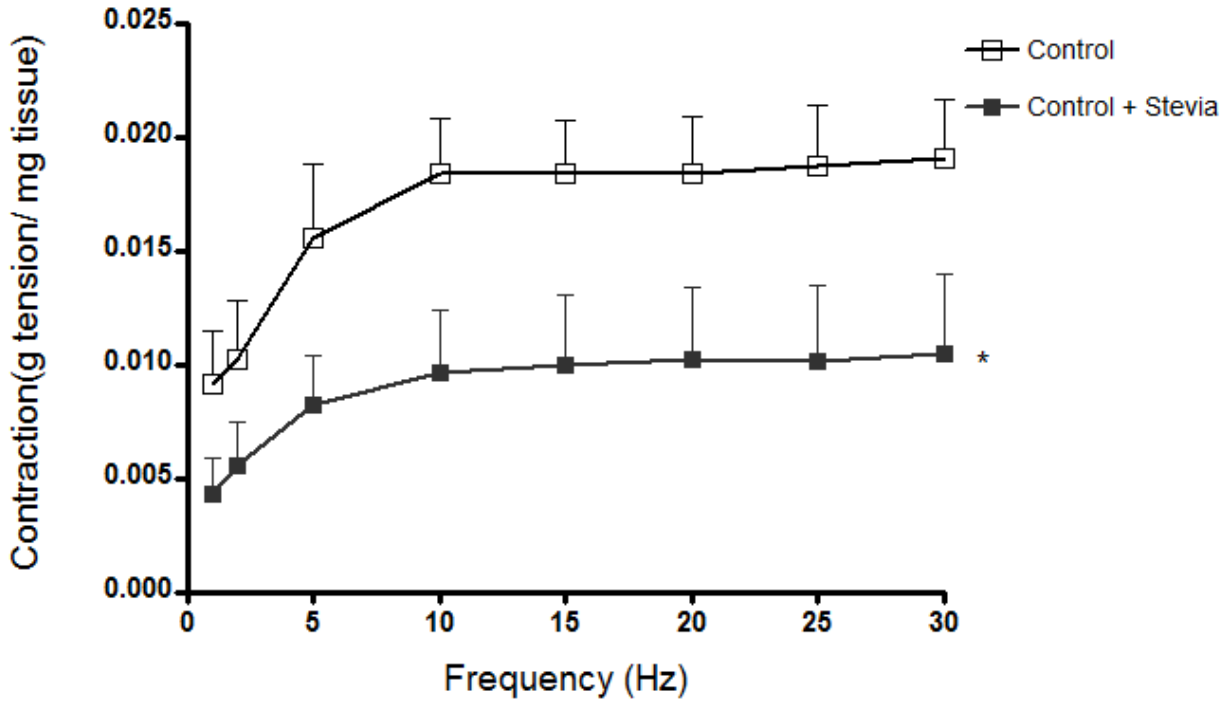


Figure 4.3: Effects of stevia on electrical field stimulation (EFS) on gastrointestinal smooth muscle. The maximum responses to EFS-induced contraction of tissues incubated with stevia were significantly lower than those of control rats.Results are expressed as the increase in g tension built up above the baseline per mg of tissue and are presented as mean ± SEM. n=7-8 for all groups. *P<0.05 vs control (two-way ANOVA with Bonferroni's multiple comparisons tests).

4.3.4 Carbachol-induced contractile responses of isolated rat ileum

Incubation of the ileum for 15 minutes with stevia ($3x10^{-4}$ M) significantly reduced carbachol-induced contractions of isolated ileum (Figure 4.4). Carbachol-induced contractions were significantly reduced in the tissues treated with stevia (3×10^{-4} M) indicating a decrease in postjunctional M_3 muscarinic receptor function (Figure 4.4). However, ileum sensitivity to carbachol remained unaffected (Table 4.1).

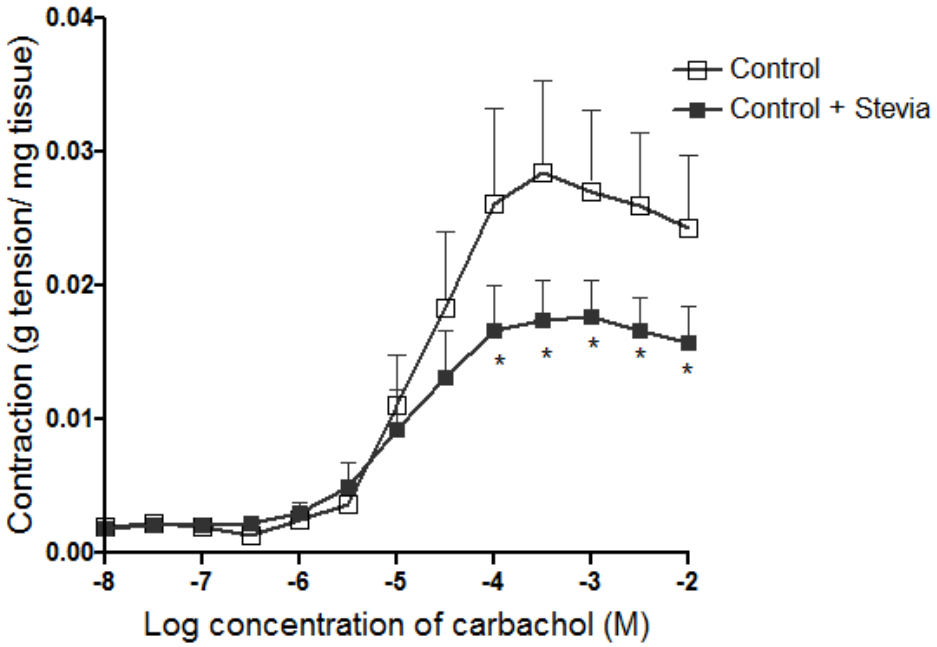


Figure 4.4: Effects of stevia on Carbachol-induced contraction on gastrointestinal smooth muscle. The maximum responses to carbachol of tissues incubated with stevia were significantly lower than those of control rats. Results are expressed as the increase in g tension built up above the baseline per mg of tissue and are presented as mean ± SEM. n=6-8 for all groups. *P<0.05 vs control (two-way ANOVA with Bonferroni's multiple comparisons tests).

Table 4.1: Change in the sensitivity (-log EC_{50}) and maximal response (R_{max}) to carbachol in isolated ileum incubated with stevia (for 15 minutes; $3x10^{-4}$ M).

Treatment	-log EC_{50}	R_{max} (% to carbachol)
Control	4.32 ± 1.2	92.87 ± 21.88
Control + Stevia	4.25 ± 1.2	89.79 ± 23.93

Results are presented as mean ± SEM. n=7-8 for all groups.

4.3.5 Effects of stevia on pilocarpine-induced relaxation responses in rat isolated ileum

Pilocarpine inhibited EFS contractions in a concentration dependent manner, demonstrating that presynaptic M_2 muscarinic receptors inhibit EFS contractions

(Figure 4.5). The inhibition of EFS contraction by pilocarpine was greater in stevia ($3x10^{-4}$ M) incubated tissues, showing an increase in M_2 muscarinic receptor response in these preparations to pilocarpine (Figure 4.5).

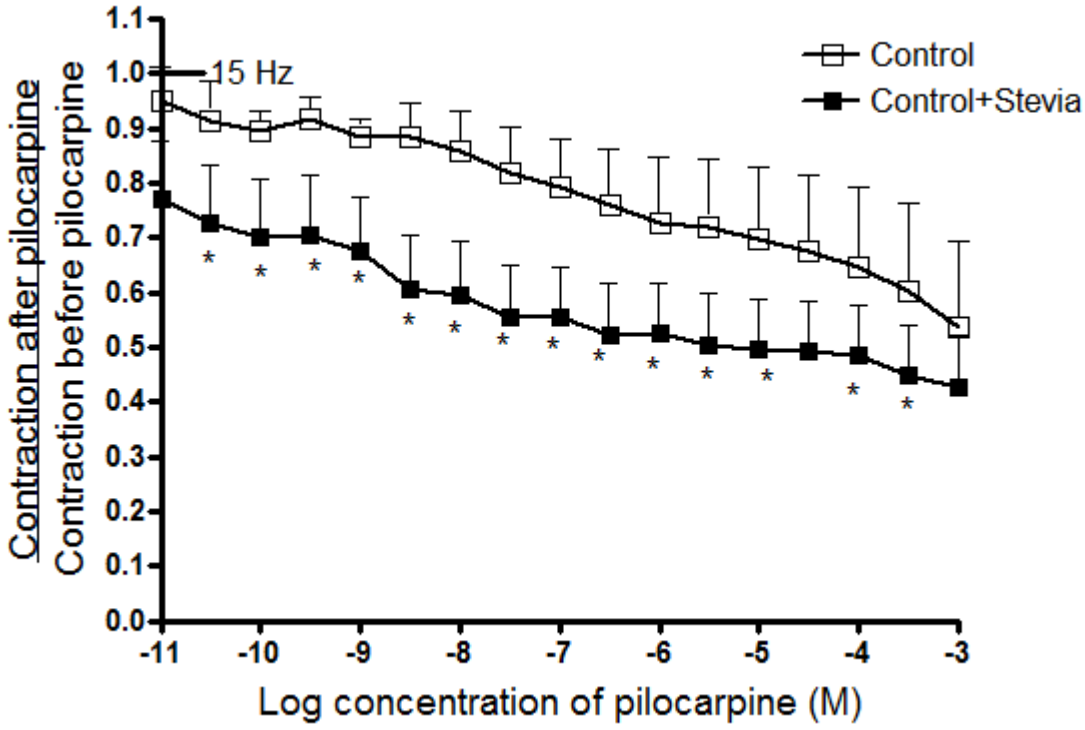


Figure 4.5: Effects of stevia on pilocarpine-induced inhibition *in vitro* in response to electrical field stimulation (EFS, 15 Hz, 100 V, 0.2 ms pulse duration for 5 s at 30s intervals) on isolated rat ileum. Incubation with stevia shifted the pilocarpine concentration response curves to the left representing stevia significantly potentiates the inhibition of pilocarpine to the gastrointestinal smooth muscle. Results are presented as mean ± SEM. n=6-8 for all groups. *P<0.05 vs control (two-way ANOVA with Bonferroni's multiple comparisons tests).

4.3.6 Effect of stevia on cardiac electrophysiological parameters in left ventricular papillary muscle preparations

In cardiac myocytes, stevia (3×10^{-4} M) attenuated the force of contraction (FOC), shortened the action potential (APD) at all phases of repolarization and decreased the average peak amplitude (APA) of the initial cell depolarization (Table 4.2). Buffer containing a high concentration of Ca^{2+} (3×10^{-7} M) diminished the effects of stevia in terms of cardiac contractile force, but no change was observed in APD and APA (Table 4.2). Subsequent addition of stevia (3×10^{-4} M) into the high concentration

Ca^{2+} solution (3 x10^{-7} M) did not significantly shorten APD or decrease force of contraction (FOC) (Table 4.2). Similarly, addition of verapamil (3 x10^{-5} M), a highly potent Ca^{2+}-channel antagonist, did not decrease these parameters to any extent (Table 4.2).

Table 4.2: Effects of stevia on electrophysiological parameters on left ventricular papillary muscles (LVPM) from Wistar rats.

Parameter	**Control** (n=9)	**Stevia** (n=9)	**$CaCl_2$** (n=9)	**$CaCl_2$ + Stevia** (n=8)	**$CaCl_2$ + Verapamil** (n=7)	**Verapamil** (n=8)
RMP (mV)	-55.2 ± 4.0	-55.2 ± 2.9	-54.9 ± 2.8	-55.9 ± 4.7	-55.5 ± 4.5	-60.0 ± 4.8
APA (mV)	62.9 ± 2.9	50.2 ± 4.6*	52.8 ± 3.4*	51.8 ± 4.6*	49.0 ± 3.9*	55.9 ± 4.6*
APD_{20} (ms)	13.9 ± 0.9	10.3 ± 0.5*	10.3 ± 0.7*	9.9 ± 0.8*	9.4 ± 0.2*	9.9 ± 0.5*
APD_{50} (ms)	22.5 ± 2.3	14.8 ± 0.9*	14.8 ± 1.3*	14.1 ± 1.4*	13.1 ± 0.4*	13.8 ± 0.8*
APD_{90} (ms)	53.7 ± 5.5	34.4 ± 2.2*	39.8 ± 4.5	37.4 ± 6.5	35.0 ± 2.6*	39.0 ± 3.5*
FOC (mN)	1.7 ± 0.4	0.8 ± 0.1*Δ	3.5 ± 0.7*	3.7 ± 0.8*Δ	3.9 ± 0.6*	3.2 ± 0.4*
TR_{90} (ms)	160 ± 14	145 ± 12	121 ± 9	122 ± 11	114 ± 5	112 ± 4

The electrophysiological measurements of the LVPM muscles were recorded for the following treatments: (i) normal Tyrode's solution (control) (ii) effect of stevia addition (3 x 10^{-4} M) (iii) effect of Ca^{2+}-enhanced solution (3 x 10^{-7} M) (iv) effect of stevia (3 x 10^{-4} M) in Ca^{2+}-enhanced solution (3 x 10^{-7} M) (v) effect of Ca^{2+}-channel blocker verapamil (3 x 10^{-5} M) in Ca^{2+}-enhanced solution (3 x 10^{-7} M) (vi) effect of the Ca^{2+}-channel blocker verapamil (3 x 10^{-5} M). Results are presented as mean ± SEM. n=8-9 for all groups.*P<0.05 vs control. Δ P<0.05 ($CaCl_2$ + Stevia) vs Stevia (two-way ANOVA with Bonferroni's multiple comparisons tests). RMP: resting membrane potential; APD: action potential duration at 20%, 50% and 90% of repolarisation (APD_{20}, APD_{50}, APD_{90}); APA: action potential amplitude; FOC: force of contraction; TR90: time to 90% relaxation of force.

4.4 Discussion

Stevia caused vasorelaxation in noradrenaline precontracted aortic preparations in line with previous studies showing stevia's vasorelaxation ability in large and small blood vessels (Lee et al. 2001; Wong. et al. 2004a). Earlier studies indicated that stevia-induced vasorelaxation might occur via inhibition of calcium influx into the blood vessel (Lee et al. 2001; Wong et al. 2004a). In this study, verapamil potentiated the relaxation of aortic tissue induced by stevia, which suggests that stevia's vasorelaxation may partly occur via blockade of calcium channels. Interestingly, L-NAME had the opposite effect by completely blocking the action of stevia in the aorta. These data suggest that the actions of nitric oxide release in combination with selective calcium channel blocking activity are responsible for the vasorelaxation activity of stevia in large vessels. However, Wong et al. (2004) indicated that release of nitric oxide might not be the mechanism of action of stevia for aortic relaxation (Wong et al. 2004a). Interestingly, stevia was capable of relaxing aortic tissue both with and without endothelium, pre-contracted with either noradrenaline or potassium chloride (Bornia et al. 2008). This would indicate that in large vessels, part of the relaxation mechanisms of stevia is via the NO synthase/guanylate cyclase pathway which only works when the endothelium is present. The other component of vasorelaxation is not solely dependent on a functional endothelium; rather it showed that stevia's response is dependent on antagonism of calcium channels. However, in the current study we have not assessed the possibility of other mechanisms of relaxation which may be contributing to the response such as endothelium derived relaxing factor (EDRF) and endothelium derived hyperpolarizing factor (EDHF). The presence of 4-aminopyridine partially inhibited the relaxation of aortic tissues induced by stevia, indicating that potassium channels may be involved in the relaxation process of stevia. A previous study showed that the opening of K^+ channels plays an important role in the vascular smooth muscle relaxation and antihypertensive effects of smooth muscle (Waldron & Cole 1999). Hyperpolarization caused by outward-directed K^+ currents leads to closing of voltage-gated Ca^{2+} channels which ultimately reduces intracellular Ca^{2+} and induces vasorelaxation (Berg 2003; Torill 2002). Therefore, stevia can cause release of EDHF which relaxes vascular smooth muscle through hyperpolarization following opening of potassium channels.

Our results showed that stevia also caused vasorelaxation in mesenteric arteries. The relaxation responses of stevia in the mesenteric arteries were significantly smaller (10^{-9} to 10^{-6} M; $P<0.05$) compared to that of verapamil alone. We demonstrated in the aortic tissue that the relaxation responses appeared to be synergistic when stevia and verapamil were administered together to the aortic tissues. On the contrary, in mesenteric vessels, stevia's relaxation effects were not potentiated by verapamil. In contrast to the aortic responses, NOS inhibition with L-NAME in mesenteric vessels did not inhibit stevia's response. Rather, it would seem that the mesenteric vasodilatory effect induced by stevia occurs through a mechanism involving calcium channels. These findings are unique and suggest stevia has a dual vasodilatory role acting as both an endothelium dependant vasodilator (via eNOS actions to constitutively produce NO) and by blocking calcium channels in a similar way to verapamil. In our study, there also appears to be some tissue selectivity with a larger component of calcium channel blockade occurring in the small resistance vessels (mesenteric) and the endothelium-dependant vasodilatation occurring in the larger conduit vessels.

The effects of stevia on cardiac action potential characteristics are as yet unknown. In this study we showed that acute administration of stevia to isolated left ventricular papillary muscles attenuated the force of contraction and shortened the repolarisation phase of the action potential at 20%, 50% and 90% (APD_{20}, APD_{50} and APD_{90}) by 25%, 34% and 36% respectively and decreased the average peak amplitude of the initial cell depolarisation. These data suggest that stevia works by lowering the intracellular Ca^{2+} concentration of cardiomyocytes, thereby attenuating the force of contraction, shortening the repolarisation phase of the action potential and decreasing the average peak amplitude of the initial cell depolarization (Melis 1992; Melis & Sainati 1991; Shiozaki et al. 2006). An animal study on a non-ischemic CHF rabbit model showed that verapamil's anti-arrhythmic effect is mediated by antagonism of inward L-type calcium channels (Ica-L) and is associated with shortening of beat to beat variability of action potential duration (Milberg et al. 2011a; Stams et al. 2011). Accordingly, the infusion of a high concentration Ca^{2+} solution diminished the effects of stevia on cardiac contractile force and restored the normal repolarisation state (APD_{90}). Since resting membrane potential was not altered in our study, this suggests

that the cardiac Na^+ current (I_{Na}) was not affected by stevia administration (Milberg et al. 2011b). Stevia reduced the action potential duration which potentially reduces the likelihood of arrhythmias and may also be linked to the selective activation of cardiac M_2 muscarinic receptor-mediated blockade of intracellular calcium currents. A recent study showed that pilocarpine (a selective muscarinic receptor agonist) caused a marked reduction in APD_{50} and APD_{90} on mice atrial myocardium and ventricular preparations (Abramochkin et al. 2012). Our finding corroborates with this report as stevia was found to activate M_2 - muscarinic receptors on healthy gastrointestinal tissues. However, subsequent introduction of stevia into the high concentration Ca^{2+} solution did not significantly shorten APD or decrease FOC, suggesting a saturation effect was occurring. These promising results showed that stevia may indeed have the potential to improve cardiac electrophysiological parameters.

Stevia reduced isolated ileum contractions induced by both electrical field stimulation (EFS) and the muscarinic M_3 agonist carbachol. Previous studies also demonstrated similar muscle relaxing results with verapamil, a calcium channel blocker, in isolated rat and guinea pig ileum preparations (Hurwitz et al. 1980; Thaina, Poonpanang & Sawangjaroen 2005). EFS persisted in producing contractions in isolated rat intestinal smooth muscle in the presence of propranolol, a beta blocker. However, the contraction was completely blocked by the application of atropine, a muscarinic receptor antagonist, which indicated that the EFS contraction was produced by via activation of acetylcholine triggered pathways. Acetylcholine, as well as carbachol, generates gastrointestinal smooth muscle contraction mediated by muscarinic M_3 receptors. Voltage-gated calcium channels cause the influx of intracellular Ca^{2+} thereby increasing the $[Ca^{2+}]_i$. In our study, incubation with stevia significantly reduced the gut smooth muscle contraction induced by EFS and carbachol. This result indicates that stevia reduced acetylcholine release from the parasympathetic nerves of the ileum and therefore could act as a putative antispasmodic agent. Therefore, the antispasmodic effect of stevia is mediated by the inhibition of calcium influx through voltage gated L-type calcium channels (Coulson, Jacoby & Fryer 2004). Activation of M_2 and M_4 receptors causes an inhibition of voltage-gated Ca^{2+} channels by reducing adenyl cyclase and cyclic AMP signaling in gut smooth muscle cells (van Koppen & Kaiser 2003). Furthermore, to establish the mechanism of action of stevia we

measured the activity of the M_2 receptor agonist pilocarpine to inhibit EFS-induced contraction of the isolated ileum. Pilocarpine showed a significantly greater inhibitory effect in the ileum treated with stevia compared to that of the controls. Therefore, our results suggest that stevia works through activating M_2 or M_4 receptors and blockade of Ca^{2+} channels in healthy ileum tissue *in vitro*, and has the potential to be used as an antispasmodic and antidiarrheal agent (Shiozaki et al. 2006; Thaina, Poonpanang & Sawangjaroen 2005). These changes are in addition to stevia's direct vasodilatory effects on the blood vessels, causing a synergistic reduction in blood pressure. However, further study is needed to establish involvement of other relaxing factors such as EDRF and EDHF in the vasculature.

4.5 Conclusion

This study is the first to show the effectiveness of stevia in altering the cardiac action potential to reduce the likelihood of arrhythmia. In short, our results suggested that the mechanism of action of stevia is multimodal since stevia showed beneficial modulatory effects on cardiovascular and gastrointestinal tissues which can be related to calcium channel inhibition, activation of M_2-muscarinic receptor function, and via enhanced NO activity. Therefore, extracts of stevia are found to be more than just a calcium channel blocker and can be used as a cardio-protective, a vasorelaxant and an antispasmodic agent.

Chapter 5 Stevia and verapamil prevent cardiovascular remodelling following hypertension

5.1 Introduction

Hypertension is considered the most prevalent and primary risk factor for the development of cardiovascular disease (CVD) (Gu et al. 2009). Poorly controlled hypertension increases the occurrence of atherosclerosis, left ventricular hypertrophy, endothelial dysfunction, renal damage and heart failure in both humans and animal models of high blood pressure (Bing et al. 2002; Boluyt & Bing 2000; Weber, K. 2002). This study was designed to investigate the cardiovascular complications following a genetic model of hypertension using spontaneously hypertensive rats (SHR) in an attempt to explore the effect stevia has on preventing cardiovascular dysfunction.

Left ventricular (LV) function is an important predictor of cardiovascular disease and heart failure, both in the general population and in patients with heart disease. Development of left ventricular hypertrophy (LVH) reflects the typical pattern of organ damage following hypertension (Izzo et al. 2011). Pathological LVH is associated with a reduced left ventricular longitudinal strain, along with increased circumferential deformation and torsion (Cappelli et al. 2009). This leads to a compromise in left ventricular pump function. From epidemiological studies, it is now evident that heart failure and arrhythmias, arising as the effect of LVH and fibrosis, are the major causes of cardiovascular disease (Edwards et al. 2009; Fenning et al. 2003). Increased arterial stiffness due to chronic kidney disease and systolic hypertension is a major factor in the development of LVH and fibrosis (Edwards et al. 2009; Fenning et al. 2003; Tonelli et al. 2006). Thus, pathological LVH has a significant impact on the overall survival of patients with cardiovascular disease.

Uncontrolled hypertension leads to an increased, uneven accumulation of fibrillar collagen in the interstitial space of hypertrophied left ventricle and aortic tissues. Thus the disproportionate accumulation of collagen contributes to the abnormal myocardial stiffness and impaired pumping capacity (Brilla, Janicki & Weber 1991; Thiedemann et al. 1983). Enhanced growth of myocytes also potentiates the hypertrophic

syndrome. Earlier studies demonstrated that treatment with adrenergic system inhibitors such as, methyldopa and propranolol induced a marked decrease in myocardial mass in 12 week-old spontaneously hypertensive rats with LVH (Brilla, Janicki & Weber 1991; Sen & Bumpus 1979). Recent studies indicated that increased myocytes stress caused by enhanced production of stress-responsive intracellular signalling proteins is the underlying cause of hypertrophic cardiomyopathy (Aj 2000; Patel et al. 2001). A study using a transgenic rabbit model of human left ventricular hypertrophy demonstrated significantly increased level of extracellular signal-regulated kinase (ERK1/2) in the untreated group compared to the simvastatin treated animals (Patel et al. 2001). This study shows that protein kinase C and calcium-calmodulin system play critical roles in activation of ERK1/2 leading hypertrophy and fibrosis (Lorimer & Lavictoire 2001; Patel et al. 2001).

Endothelial dysfunction is directly involved in the development of atherosclerosis leading to coronary and peripheral vascular diseases. The endothelium functions to regulate vascular tone, platelet activity, thrombosis and leukocyte adhesion (Avogaro, Kreutzenberg & Fadini 2008). The adhesion of monocytes to the endothelium is characterized as the beginning of atherosclerosis. It has been shown that hypertension and hyperglycaemia stimulate monocyte adhesion to the vascular wall (Watada, Azuma & Kawamori 2007). Atherosclerosis is associated with substantial changes in the endothelium of blood vessels whilst increased oxidative stress accounts for a significant amount of damage to the endothelium as oxygen derived free radicals are directly linked to changes in vasomotor function in experimental models of atherosclerosis (Shaikh & Suryakar 2008). Clinical data showed that treatment with antioxidants improves the function of the endothelium in coronary artery disease (CAD) (Heitzer et al. 2001). Quercetin, one of the potent bioflavonoid antioxidants reduced oxidative vascular damage by modulating gene expression leading to suppression of reactive oxygen and nitrogen species production (Luangaram et al. 2007). In phenylhydrazine-induced oxidant stressed rats, quercetin was shown to protect against vascular damage by inhibiting superoxide anion production (Luangaram et al. 2007). All these results are in support of our assumption that stevia could prevent vascular damage through its antioxidant activity in addition to its calcium channels blockade.

Hypertension is also associated with the formation of advance glycation end products (AGEs) which subsequently increases oxidative stress in the vascular wall through an interaction with their receptors (RAGE). The activated receptors (RAGE) then lead to stimulation of transcription factor NF-κB pathway, generation of tumour necrosis factor (TNF), interleukin-1 (IL-1), and induction of interleukin-6 (IL-6), all of which play an important role in the progression of vascular inflammation and oxidative stress (Lin, Park & Lakatta 2009; Méndez. et al. 2010). Evidence showed that RAGE is a key pathogenic factor involved in endothelial cell activation, vascular wall remodelling, and neointimal expansion in atherosclerosis and arterial plaque formation (Méndez et al. 2010). Hypertension often triggers the metabolic syndrome that would further worsen the patency of the vascular wall.

Stevia, the natural non-caloric sweetener causes a fall in blood pressure, reduced renovascular resistance together with a pressure diuresis and natriuresis per millilitre of glomerular filtration rate in rats when measured using a classical clearance technique (Melis & Sainati 1991). Chronic administration of crude extract of stevia in normal Wistar rats increased renal plasma flow (Melis & Sainati 1991; Melis 1995; Lee 2001). Stevia's renal and vascular effects were found to be potentiated by concurrent addition of verapamil (Melis & Sainati 1991). A recent study has measured total phenolic compounds and bioflavonoids in stevia and reported that leaf and callus extracts of stevia contains phenols and flavonoids (Shukla et al. 2011). Also stevia's antioxidant capacity was found to be in the range of 9.66 to 38.24 in the leaves expressed as mg equivalent of gallic acid, ascorbic acid per gram on dry weight basis (Tadhani 2007). Initial animal study with stevia showed a reduction in production of inflammatory mediators such as TNF- ∝ and IL- 1ß and demonstrated immunomodulatory effects in cyclophosphamide treated mice (Bookwanean 2007; Sehar 2008). Recently, nifedipine, one of the most widely used calcium channel blockers (CCBs), has been reported to exert anti-oxidative and anti-AGE-RAGE axis properties (Yamagishi, Nakamura & Matsui 2009). Thus the use of nifedipine, in addition to ensuring blood pressure control, has beneficial effects in preventing cardiorenal damages in patients with hypertension and diabetes (Yamagishi, Nakamura & Matsui 2009). Moreover, CCBs protect the vasculature by stimulating endothelial Nitric Oxide (NO) production and/or restoring normal adiponectin and

high density lipoprotein (HDL) levels in circulation. Furthermore, CCBs were found to increase circulating endothelial progenitor cell levels which play a major role in normal endothelial cell function (Morimoto, Kureishi-Bando & Murohara 2010; Sirmagül et al. 2007). Accordingly, these data suggest that CCB can be used as a vasculo-protective agent in patients with diabetes and hypertension.

The SHR model together with the Wistar-Kyoto (WKY) normotensive rats as the control is the most commonly used experimental model of human essential hypertension (Kodavanti & Costa 2001). This model shares many of the cardiovascular abnormalities observed in essential hypertension in humans (Hughes & Bund 2002). SHRs attain a steady value of increased blood pressure and early hypertension at approximately 8-12 weeks of age with increased vascular resistance (Guinamard et al. 2006). Following this initial hypertensive insult, SHR hearts develop pathological cardiac remodelling (LVH) with progressive increases in perivascular and intestinal fibrosis, thus the SHR represents a realistic model of human cardiac hypertrophy (Boluyt & Bing 2000; Guinamard et al. 2006). During the compensated stage after LVH development, the SHR's hearts show an increase in myocyte contractility, amplitude of the intracellular Ca^{2+} transient and action potential duration (APD) with no changes in resting membrane potential (Guinamard et al. 2006; Weisser-Thomas et al. 2007). A prolonged QT interval leads to APD prolongation on the ECG and is mainly due to a reduction of the transient outward current (Ito) density (Guinamard et al. 2006; Shipsey, Bryant & Hart 1997; Weisser-Thomas et al. 2007). In addition to this, SHRs' ventricular myocytes were found to show a higher incidence of delayed-after-depolarization (DAD) and associated-after-contractions compared to WKY rats. As DADs are generated by intracellular Ca^{2+} waves released from intracellular stores, SHR cardiomyocytes have a greater incidence of presenting Ca^{2+} waves (Guinamard et al. 2006). In the SHR, vascular structural alterations represented by a larger medial volume and a higher number of smooth muscle cell layers are observed at the age of 3-4 weeks, before a significant increase of the blood pressure takes place (Gao et al. 2008). This is an important finding and shows that it is not just the haemodynamic changes in this model but many humoral factors which play a significant role in the SHR. A greater contractile response in SHR arteries and an elevated peripheral vascular resistance are developed

from these changes in blood vessel structure. Therefore, the SHR vessel walls are subjected to greater shear stress compared with those from the normotensive WKY which causes the functional impairment of the endothelium (Gao et al. 2008). These changes then contribute to the systemic increases in oxidative stress and inflammation with a reduced ability to release and manufacture endothelial derived nitric oxide.

5.2. Methods

A total of 50 male spontaneously hypertensive rats (SHR) and 50 Wistar-Kyoto (WKY) control rats were used in this study (CQUniversity Australia's Animal Ethics Committee number #A06-190). Both SHR and WKY animals were obtained at 8 weeks age as indicated in chapter 3 of this thesis. The animals were sacrificed at 16 weeks of age with the treated rats receiving stevia (200 mg/kg/day) or verapamil (4 mg/kg/day) from 0 to 8 weeks of experimental period. Experimental groups were:-

- WKY untreated control
- WKY control treated with stevia
- WKY treated with verapamil
- SHR untreated disease model
- SHR disease model treated with stevia
- SHR disease model treated with verapamil

Animals of each species were randomly assigned to either treatment (stevia or verapamil) or non-treatment groups. Systolic blood pressure and heart rate were measured in the selected animals by the tail-cuff method described in Chapter 3; section 3.7.2. Cardiac structure and function were assessed by the isolated Langendorff technique (Chapter 3, section 3.7.8.), electrophysiological measurement and recording of single-cell microelectrode experiments (Chapter 3, section 3.7.7) and terminal organ weights as discussed in section 3.7.4. During terminal experiments, plasma was taken to measure serum nitrate/nitrite level (Chapter 3, section 3.7.5-*B*), lipid peroxidation (Chapter 3, section 3.7.5.-*A*), total antioxidant capacity (Chapter 3, section 3.7.5.-*C*) and serum IL-6 levels (Chapter 3, section 3.7.5.-*D*). Vascular responses to noradrenaline, acetylcholine and sodium nitroprusside were measured for thoracic

aortic rings (Chapter 3, section 3.7.6-*A*.) and for mesenteric arteries (Chapter 3, section 3.7.6-*B*.). The function of the isolated ileum to electrical field stimulation (Chapter 3, section 3.7.9), carbachol (Chapter 3, section 3.7.9.) and pilocarpine (Chapter 3, section 3.7.9.) were also assessed.

All results are presented as mean ± SEM. The results were analysed using two-way ANOVA with Bonferroni's Multiple Comparison Test. A Student's t-test was used to compare and evaluate two group means via paired/ unpaired where appropriate. All standard curves were formulated using Graphpad Prism 4.0 to create a four parameter logic curve from which unknown values were determined. *$p<0.05$ vs control (WKY) and $^{\Delta,}$ **$p<0.05$ vs SHR were considered statistically significant.

5.3 Results

Weekly measurement of body weight and water intake from 8 to 16 weeks of age demonstrated that spontaneously hypertensive rats (SHR) failed to gain weight and showed only a very small increase in water consumption compared to the WKY control rats (Figures 5.1 & Figure 5.2). The SHR initially showed a slight decrease in body weight; however, by the end of 8 weeks, SHR demonstrated a significant reduction in body weight compared to the WKY controls (Figure 5.1). Stevia treatment partially normalized the body weight in the hypertensive rats whereas verapamil treatment failed to improve body weights in the treated SHR (Figure 5.1). Body weights remained unchanged in the stevia and verapamil treated control animals (Figure 5.1). Stevia treatment significantly increased water intake close to normal in the hypertensive rats, however a decrease in water intake (values are not significant) was observed in the stevia treated WKY rats compared to the control animals (Figure 5.2). Dosing with verapamil showed a slight increase in water intake in the SHR (Figure 5.2). Verapamil treatment did not show any change in water intake in the WKY rats (Figure 5.2).

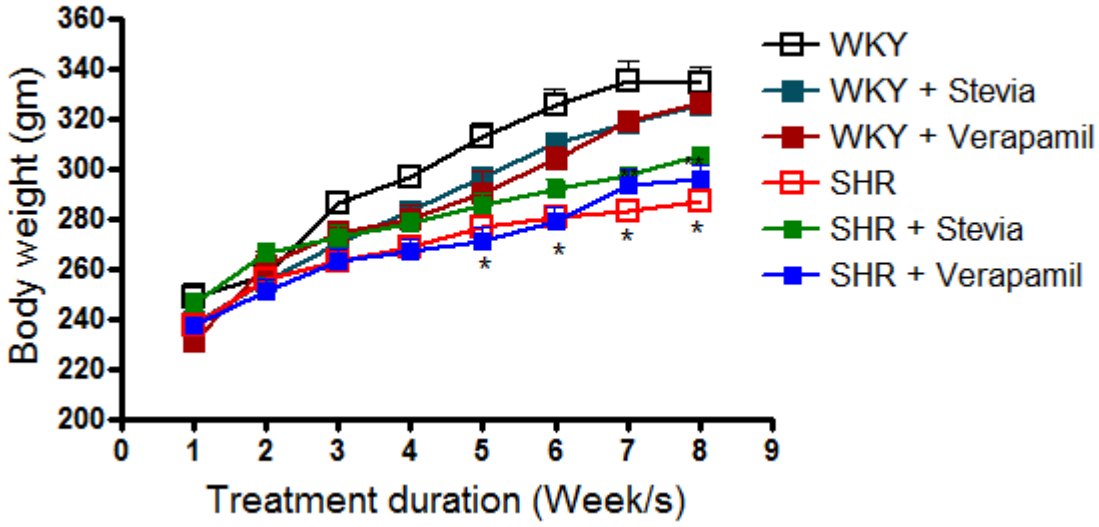


Figure 5.1: Weekly body weight for WKY, WKY + stevia, WKY + verapamil, SHR, SHR + stevia (200 mg/kg/day, p.o.) and SHR + verapamil (4 mg/kg/day, p.o.). Results are presented as means ± s.e.m. n=15 for all groups. *P<0.05 vs WKY and **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).

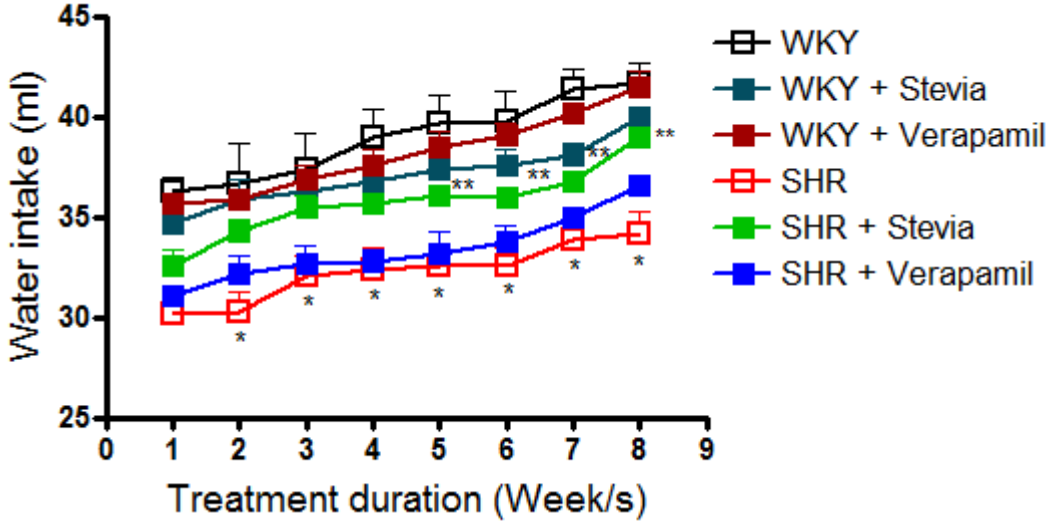


Figure 5.2: Weekly water intake for WKY, WKY + stevia, WKY + verapamil, SHR, SHR + stevia (200 mg/kg/day, p.o.) and SHR + verapamil (4 mg/kg/day, p.o.). Results are presented as means ± s.e.m. n=15 for all groups. *P<0.05 vs WKY and **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).

The spontaneously hypertensive rats (SHR) showed a marked elevation in systolic blood pressure from 10-12 weeks of age which continued to increase till 16 weeks of

age compared to the age-matched WKY rats (Figure 5.3). However, the WKY demonstrated a slightly elevated blood pressure at the end of the experimental period (Figure 5.3). Eight weeks of stevia treatment reduced the blood pressure by 23% in the SHR animals (Figure 5.3). Treatment with a calcium channel blocker, verapamil decreased the blood pressure within a typical normal range (Figure 5.3). SHRs also showed increased heart rates at 12 weeks of age peaking at 16 weeks of age (Figure 5.4). Stevia treatment prevented the increased heart rate in the treated SHR rats compared to the untreated animals (Figure 5.4). On the other hand, dosing with verapamil showed the highest reduction in heart rate compared to other treated and untreated animals (Figure 5.4).

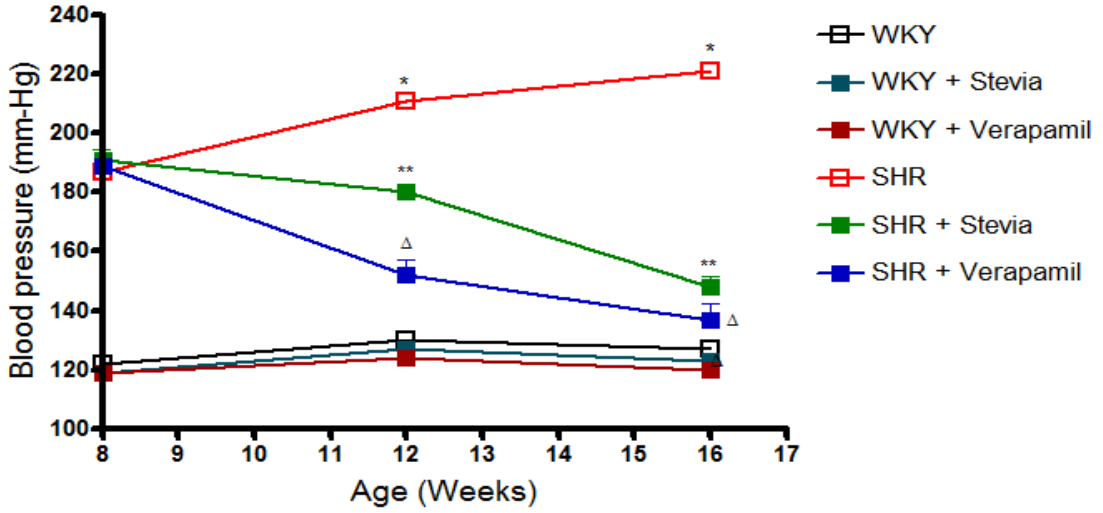


Figure 5.3: Systolic blood pressure measurement for WKY, WKY + stevia, WKY + verapamil, SHR, SHR + stevia (200 mg/kg/day, p.o.) and SHR + verapamil (4 mg/kg/day, p.o.) at 8 weeks, 12 weeks and 16 weeks of age. Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs WKY and $^{\Delta,}$ **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).

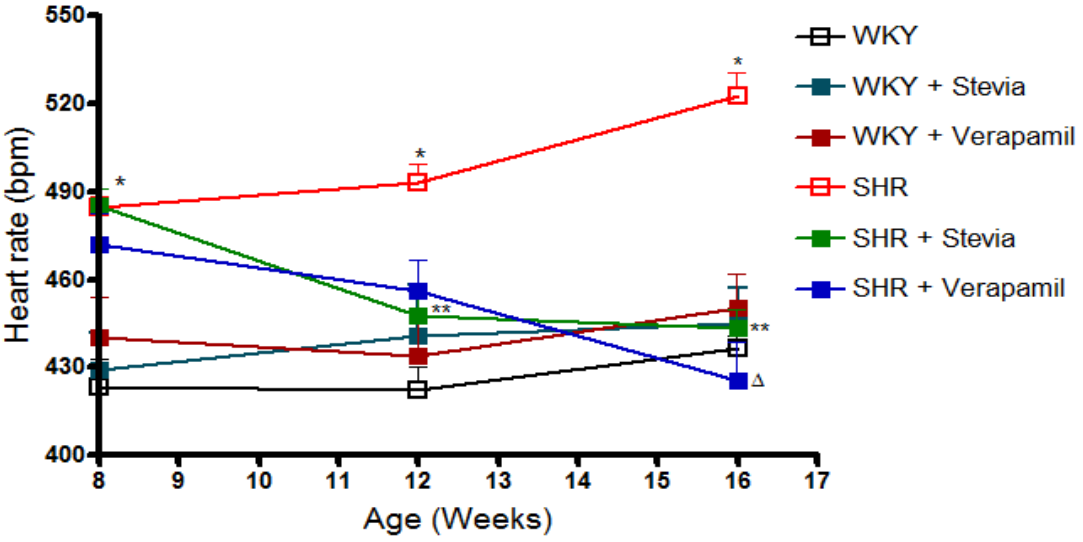


Figure 5.4: Resting heart rate measurement for WKY, WKY + stevia, WKY + verapamil, SHR, SHR + stevia (200 mg/kg/day, p.o.) and SHR + verapamil (4 mg/kg/day, p.o.) at 8 weeks, 12 weeks and 16 weeks of age. Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs WKY and $^{\Delta,}$ **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).

The spontaneously hypertensive rats showed a reduced cardiac contractility and impaired cardiac function indicating left ventricular hypertrophy compared to the WKY control rats. This cardiac dysfunction was evidenced by increased terminal left ventricular wet weight to body weight and to tibial length ratio and by reduced cardiac force of contraction and decreased end systolic pressure in the untreated SHRs (Tables 5.1, 5.2). These were accompanied by a slight increase in diastolic stiffness in the untreated SHRs (Table 5.2).

Treatment with stevia and verapamil showed a small improvement in left ventricular weight/body weight ratio (Table 5.1). However, both treatments significantly normalised the increased left ventricular weight / tibial length ratio in the hypertensive animals (Table 5.1). Both stevia and verapamil restored cardiac contractility and end systolic pressure to the normal range (Table 5.2). Mean coronary blood flow normalized to ventricular weight was calculated as a measure of coronary vasodilator reserve and the results showed a marked reduction in SHRs compared with hearts from WKY control rats (Figure 5.5). After 8 weeks of stevia treatment, coronary blood flow significantly increased in the SHR animals (Figure 5.5). Dosing with verapamil normalized the coronary blood flow in the SHRs (Figure 5.5). The values of coronary blood flow were slightly increased in the WKY animals treated with stevia and verapamil (Figure 5.5).

Terminal weights of the right ventricle, kidney, liver and spleen were all found to be elevated in the SHRs compared to the WKY control rats (Table 5.1). Stevia treatment normalised right ventricle and liver weights and significantly reduced kidney weights in the hypertensive animals (Table 5.1). Stevia did not alter the organ weights in the normotensive controls (Table 5.1). There was a significant increase in tibial length in the stevia treated SHRs (Table 5.1). Verapamil treatment normalised increased organ weights in the SHRs (Table 5.1). Verapamil also reduced right ventricle and kidney weights in the WKY rats (Table 5.1). Plasma glucose concentrations were not different among the SHR and WKY animals (Table 5.1). Treatment with stevia and verapamil did not alter this parameter (Table 5.1).

At 16 weeks of age, the SHR hearts showed a significant reduction in developed pressure and a msrked decrease in rate of contraction compared to the WKY animals (Table 5.2). The SHR rats showed a slight increase in diastolic stiffness compared to

the age-matched WKY controls (Table 5.2). Both Stevia and verapamil treatment normalised the developed pressure and restored normal rates of contraction and relaxation in the SHRs (Table 5.2). Stevia treatment significantly reduced diastolic stiffness in the treated SHR and WKY rats (Table 5.2). Verapamil treatment normalised these parameters and prevented further decreases in cardiac function (Table 5.2). The cardiac parameters remained unchanged in the stevia and verapamil treated WKY controls (Table 5.2).

Table 5.1 Comparison of biometric parameters

Parameters	WKY	WKY + Stevia	WKY + Verapamil	SHR	SHR + Stevia	SHR + Verapamil
Blood glucose (mmol/l)	9.9 ± 0.6	8.8 ± 0.9	9.2 ± 1.1	9.6 ± 1.5	9.3 ± 0.7	9.8 ± 0.8
LV wet weight (mg/g body weight)	2.29 ± 0.17	2.2 ± 0.09	2.3 ± 0.26	3.0 ± 0.06*	2.7 ±0.05	2.8 ± 0.07
LV wet weight (mg/ mm tibial length)	23.2 ± 0.71	22.1 ± 1.2	23.1± 0.96	26.4 ± 0.59*	23.2 ± 0.59**	23.6 ± 0.8$^{\Delta}$
RV wet weight (mg/g body weight)	0.48 ± 0.03	0.49 ± 0.02	0.5 ± 0.05	0.65 ± 0.05*	0.54 ± 0.02	0.48 ± 0.02$^{\Delta}$
RV wet weight (mg/mm tibial length)	4.39 ± 0.22	4.43 ± 0.21	4.47 ± 0.25	5.9 ± 0.48*	4.58 ± 0.13**	4.18 ± 0.20$^{\Delta}$
Liver wet weight (mg/g body weight)	32.3 ± 0.85	30.0 ± 0.72	29.2 ± 0.94	37.4 ± 0.73*	32.6 ± 0.55**	33.7 ± 0.74$^{\Delta}$
Spleen wet weight (mg/g body weight)	1.7 ± 0.05	1.81 ± 0.08	1.7 ± 0.14	1.9 ± 0.07	1.8 ± 0.07	1.88 ± 0.05
Kidney wet weight (mg/g body weight)	6.2 ± 0.43	6.0 ± 0.15	6.4 ± 0.10	7.6 ± 0.08*	6.6 ± 0.12**	6.8 ± 0.18
Tibial length (mm)	35.2 ± 0.36	34.1 ± 1.52	31. ± 1.42	31.8 ± 0.34	36.5 ± 0.9**	34.2 ± 0.4$^{\Delta}$

WKY control and spontaneously hypertensive rats (SHR) after 8-week treatment with stevia (200 mg/kg/day, p.o.) and verapamil (4 mg/kg/day.,p.o.). LV= Left ventricle, RV= Right ventricle. Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs WKY and $^{\Delta,}$ **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).

Table 5.2: Comparison of functional parameters measured in isolated hearts.

Parameters	WKY	WKY + Stevia	WKY + Verapamil	SHR	SHR+ Stevia	SHR+ Verapamil
Diastolic stiffness	23.0 ± 0.8	23.5 ± 1.2	23.3 ± 0.7	26.5 ± 0.5	23.1 ± 0.9	24.5 ± 1.2
Developed pressure (mmHg)	126 ± 7.5	132 ± 4	129 ± 4	89 ± 9*	108 ± 5	133 ± 7Δ
Maximum +dP/dt (mmHg/sec)	2049 ± 64.7	2428 ± 85	2391 ± 65	1409 ± 144*	1790 ± 44**	2462 ± 134Δ
Maximum -dP/dt (mmHg/sec)	-1701 ± 29	-1696 ± 78	-2125 ± 428	-1163 ± 172	-1713 ± 115**	-1839 ± 112Δ
End systolic pressure (mmHg)	135 ± 9	141 ± 5	141 ± 4	101 ± 8*	112 ± 3	130 ± 15Δ

WKY control and spontaneously hypertensive rats (SHR) after 8-week treatment with stevia (200 mg/kg/day, p.o.) and verapamil (4 mg/kg/day, p.o.). Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs WKY and Δ, **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).

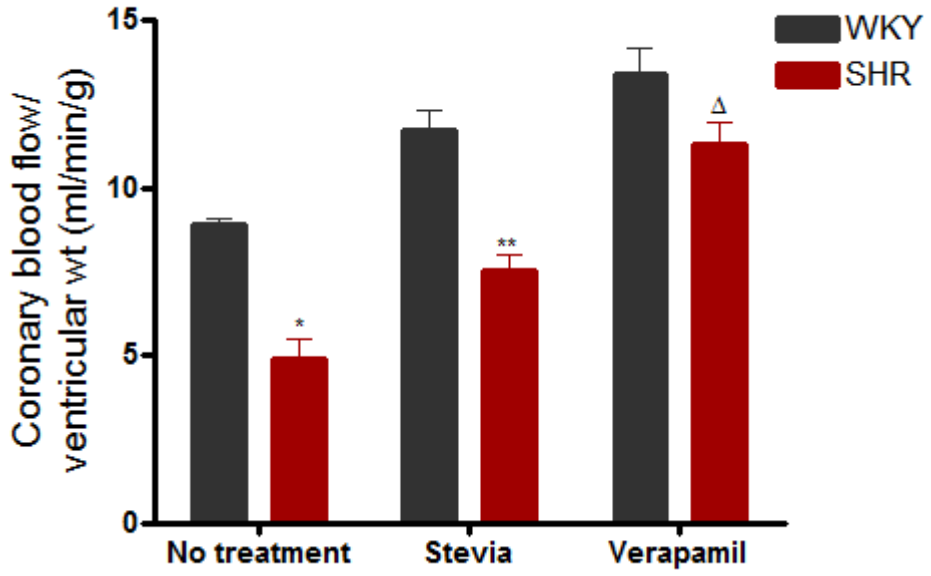


Figure 5.5: Coronary blood flow (CBF) measured in isolated hearts normalized to ventricular weight (VW) for 16 weeks old WKY, WKY + stevia, WKY + verapamil, SHR, SHR + stevia (200 mg/kg/day, p.o.) and SHR + verapamil (4 mg/kg/day, p.o.) animals. Results are presented as means ± SEM. n=10 for all groups. *P<0.05 vs WKY and Δ, **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).

The SHRs demonstrated increased levels of oxidative stress and inflammation as measured by reduced serum antioxidant capacity (TAC), elevated malondialdehyde levels (MDA) and increased IL-6 concentrations at 16 weeks of age compared to the age-matched WKY controls (Table 5.3).

Table 5.3: Comparison of serum markers

Serum inflammatory markers	WKY	WKY + Stevia	WKY + Verapamil	SHR	SHR + Stevia	SHR + Verapamil
TAC (mmol/L)	1.6 ± 0.8	1.8 ± 0.3	2.1 ± 0.2	0.96 ± 0.5	1.9 ± 0.2	2.0 ± 0.6
MDA (pmol/mg)	13.1 ± 5.2	9.4 ± 1.7	12.0 ± 2.8	51.8 ± 3.7*	21.5 ± 8.9**	29.9 ± 8.4$^{\Delta}$
IL-6 (pg/mL)	39.4 ± 1.0	37.9 ± 1.6	37.6 ± 1.4	61.6 ± 3.8*	39.9 ± 0.8**	38.2 ± 1.5$^{\Delta}$

WKY, WKY + stevia, WKY + verapamil, SHR, SHR + stevia (200 mg/kg/day, p.o.) and SHR + verapamil (4 mg/kg/day, p.o.) at 16 weeks of age.**TAC**:Serum total antioxidant capacity, **MDA**: malondialdehyde and **IL-6**: interleukin-6. Results are presented as means ± SEM. n=8 for all groups. *P<0.05 vs WKY and $^{\Delta,}$ **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).

Both stevia and verapamil treated SHRs partially restored total antioxidant capacity in the hypertensive rats (Table 5.3). Stevia and verapamil significantly reduced serum MDA levels in the SHR (Table 5.3). Dosing with both stevia and verapamil normalized the concentration of IL-6 in the SHR serum (Table 5.3).

Electrophysiological parameters from isolated left ventricular papillary muscles showed no significant difference in resting membrane potential and action potential amplitude in the SHR rats compared to WKY rats (Table 5.4). The SHRs demonstrated a significant increase in action potential duration at 20%, 50% and 90% of repolarisation compared to the WKY rats (Table 5.4). Stevia treatment significantly reduced action potential duration at 20%, 50% and 90% repolarisation in the SHRs which was similar to the effect of verapamil observed in the SHRs (Table 5.4). Dosing with verapamil also reduced the action potential duration at 50% and 90% of repolarisation in the WKY controls (Table 5.4). Electrophysiological assessment of the left ventricular papillary muscles of the SHR showed a significant decrease in force of contraction compared to the age-matched WKY rats (Table 5.4). Treatment

with stevia and verapamil partially prevented the decrease in force of contraction in the SHRs (Table 5.4).

Table 5.4: Comparison of electrophysiological parameters measured in isolated left ventricular papillarty muscle.

Parameters	WKY	WKY + Stevia	WKY + Verapamil	SHR	SHR + Stevia	SHR + Verapamil
Resting membrane potential (mV)	-65.1 ± 3.5	-66.7 ± 1.8	-62.6 ± 1.2	-66.2 ± 2.6	-63.6 ± 2.2	-64.6 ± 2.5
Action potential amplitude (mV)	65.3 ± 2.5	70.2 ± 3.3	66 ± 1.4	67.7 ± 3.9	69.2 ± 2.7	70.2 ± 2.7
Action potential duration at 20% of repolarisation (msec)	13.3 ± 0.6	14.2 ± 0.7	12.7 ± 0.6	17.4 ± 1.6*	14.7 ± 0.9	15.2 ± 0.9
Action potential duration at 50% of repolarisation (msec)	22.5 ± 1.5	22.7 ± 1.4	19.5 ± 1.9	29.4 ± 2.4*	22.5 ± 2.1**	23.4 ± 1.7$^{\Delta}$
Action potential duration at 90% of repolarisation (msec)	58.8 ± 4.6	57.5 ± 9.3	54.7 ± 6.2	85.4 ± 8.6*	57.8 ± 6.7**	67.9 ± 10.2$^{\Delta}$
Force of contraction (mN)	8.9 ± 2.1	5.6 ± 0.7	9.6 ± 0.9	3.6 ± 0.6*	3.9 ± 0.5	4.2 ± 0.9
TR90 (ms)	95.2 ± 5.2	109.8 ± 4.4	105.3 ± 9.4	110.4 ± 5.9*	105.8 ± 5.6	87.4 ± 4.2$^{\Delta}$

WKY, WKY + stevia, WKY + verapamil, SHR, SHR + stevia (200 mg/kg/day, p.o.) and SHR + verapamil (4 mg/kg/day, p.o.) at 16 weeks of age. Results are presented as means ± SEM. n=13 for all groups. *P<0.05 vs WKY and $^{\Delta}$,**P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests). TR90 = time to 90% relaxation of force.

Isolated thoracic aorta demonstrated a clear indication of vascular endothelial dysfunction in the SHR rats. Hypertension caused a decreased response to noradrenaline, acetylcholine and sodium nitroprusside in the aortic rings compared to age-matched WKY control animals (Figures 5.6, 5.8 & 5.10). Moreover, a decreased responsiveness of thoracic aorta to acetylcholine in the SHR rats was observed by a reduction in potency (-log EC_{50}) without an increase in acetylcholine efficacy (R_{max})

(Table 5.6). The –log EC_{50}s were found to be similar to that of control rats in both stevia and verapamil treated hypertensive rats (Table 5.6). Stevia and verapamil did not alter EC_{50}s in the control rats (Table 5.6). The EC_{50}s and maximum responses for sodium nitroprusside and noradrenaline in the aorta were found to be comparable among all the groups (Tables 5.5, 5.7). Stevia restored the maximal contractile response of the aorta to noradrenaline in the SHRs (Figure 5.6). Dosing with verapamil also prevented the decreased contractile response of aorta to noradrenaline in the SHRs (Figure 5.7). Treatment with stevia and verapamil partially restored the response of aorta to acetylcholine in the SHRs (Figures 5.8, 5.9). In noradrenaline precontracted isolated aorta, hypertension significantly decreased endothelium-independent relaxation to sodium nitroprusside as observed in the 16 weeks old SHRs (Figures 5.10, 5.11). Eight weeks of treatment with stevia and verapamil increased the relaxation response to sodium nitroprusside in the SHR animals, however these treatments could not normalise this response (Figure 5.10). Overall, stevia and verapamil both improved blood vessel reactivity to both vasoconstrictors and vasodilators. This could be due to generalised improvements in smooth muscle cell function and given that it occurred for both drugs could be via enhanced cellular performance.

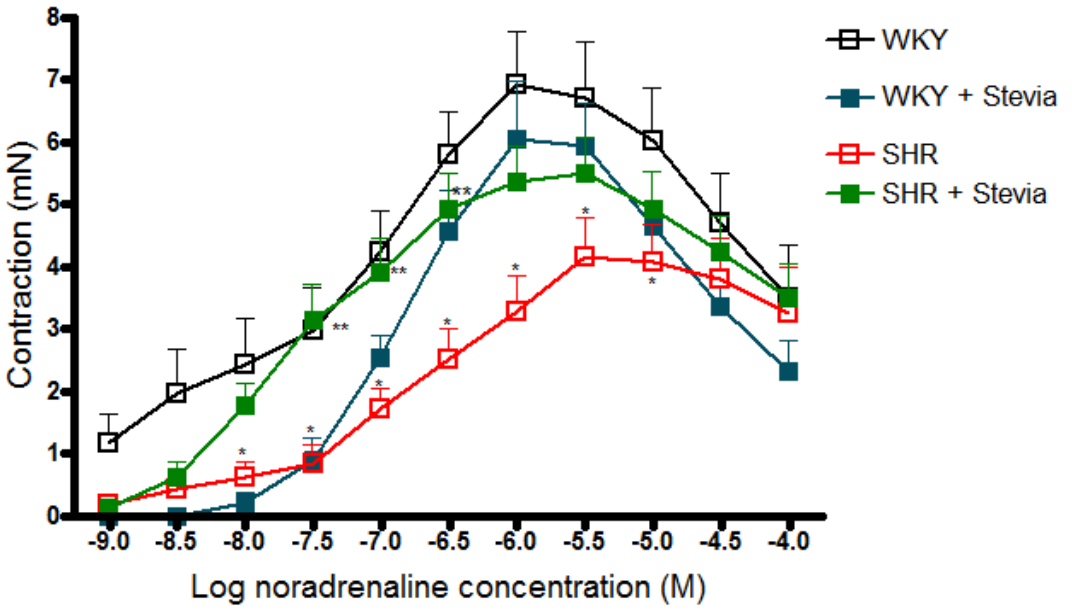


Figure 5.6: Cumulative-concentration contractile response to noradrenaline in isolated thoracic aortic preparations from WKY, WKY + stevia, SHR, SHR + stevia (200 mg/kg/day, p.o.) at 16 weeks of age. Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs WKY and $^{\Delta,}$ **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).

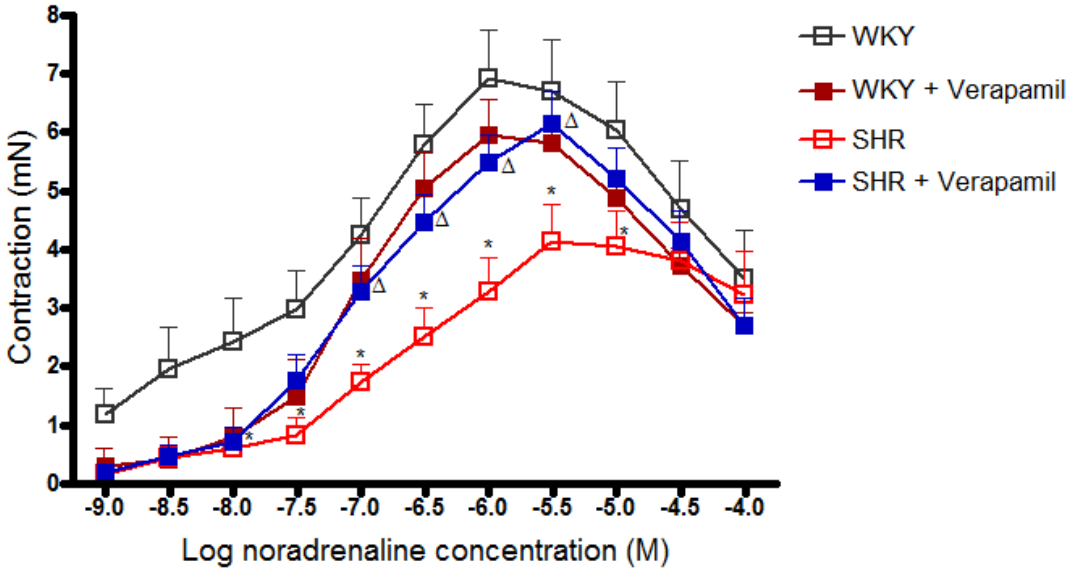


Figure 5.7: Cumulative-concentration contractile response to noradrenaline in isolated thoracic aortic preparations from WKY, WKY + verapamil, SHR, and SHR + verapamil (4 mg/kg/day, p.o.) at16 weeks of age. Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs WKY and $^{\Delta,}$ **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).

Table: 5.5: -Log EC50 and maximum contractile responses to noradrenaline in isolated thoracic aorta

Parameters	WKY	WKY + Stevia	WKY + Verapamil	SHR	SHR + Stevia	SHR + Verapamil
-Log EC_{50}	5.7 ± 0.2	5.7 ± 0.1	5.7 ± 0.2	5.5 ± 0.2	5.7 ± 0.07	5.5 ± 0.1
R_{max} (% to noradrenaline)	92 ± 4.7	95 ± 2.4	95 ± 2.2	91 ± 3.3	94 ± 2.2	94 ± 3

WKY, WKY + stevia, WKY + verapamil, SHR, SHR + stevia (200 mg/kg/day, p.o.) and SHR + verapamil (4 mg/kg/day, p.o.) at16 weeks of age. Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs WKY and $^{\Delta,}$ **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).

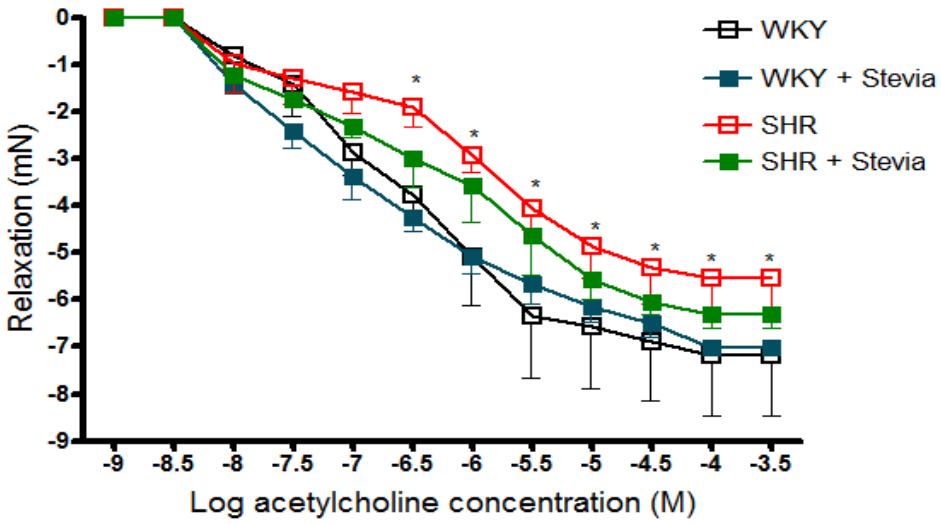


Figure 5.8: Cumulative-concentration relaxation response to acetylcholine (10^{-9} to 10^{-4} mol/L) in isolated thoracic aortic preparations from WKY, WKY + stevia, SHR, SHR + stevia (200 mg/kg/day, p.o.) at 16 weeks of age. Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs WKY and $^{\Delta,}$ **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).

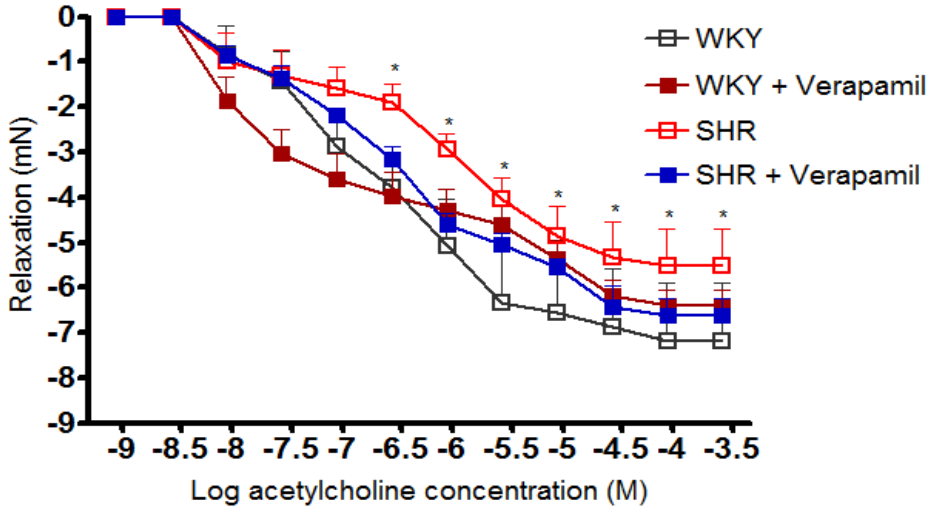


Figure 5.9: Cumulative-concentration relaxation response to acetylcholine (10^{-9} to 10^{-4} mol/L) in isolated thoracic aorta from WKY, WKY + verapamil, SHR, and SHR + verapamil (4 mg/kg/day, p.o.) at 16 weeks of age. Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs WKY and $^{\Delta,}$ **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).

Table: 5.6: -Log EC50 and maximum responses to acetylcholine in isolated thoracic aorta

Parameters	WKY	WKY + Stevia	WKY + Verapamil	SHR	SHR + Stevia	SHR + Verapamil
-Log EC_{50}	6.5 ± 0.3	6.5 ± 0.2	6.3 ± 0.4	5.5 ± 0.1	5.9 ± 0.1	6.0 ± 0.2
R_{max} (% to acetylcholine)	100 ± 0.0	99 ± 0.9	95 ± 2.3	99 ± 0.6	98 ± 1.0	96 ± 4

WKY, WKY + stevia, WKY + verapamil, SHR, SHR + stevia (200 mg/kg/day, p.o.) and SHR + verapamil (4 mg/kg/day, p.o.) at16 weeks of age. Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs WKY and $^{\Delta,}$ **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).

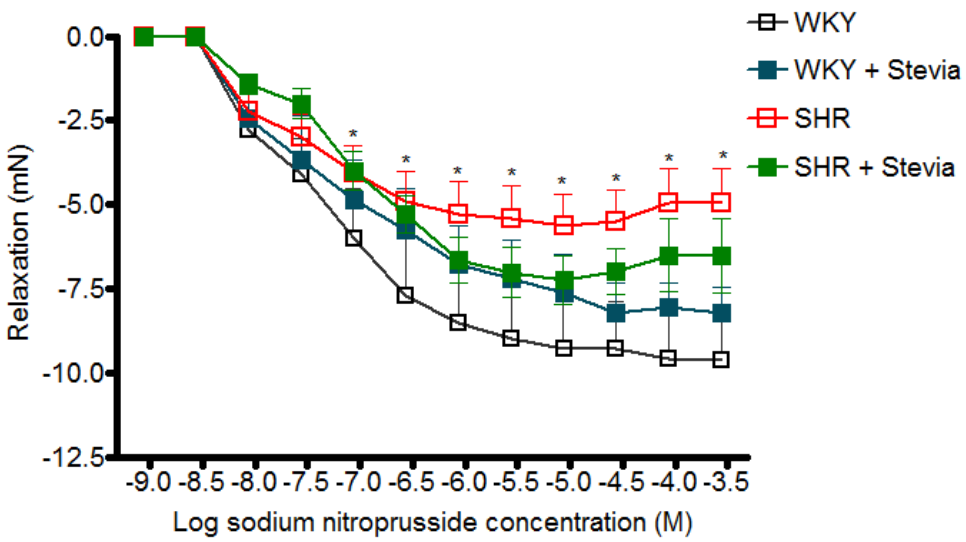


Figure 5.10: Cumulative-concentration relaxation to sodium nitroprusside (10^{-9} to 10^{-4} mol/L) in isolated thoracic aortic preparations from WKY, WKY + stevia, SHR, SHR + stevia (200 mg/kg/day, p.o.) at 16 weeks of age. Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs WKY and $^{\Delta,}$ **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).

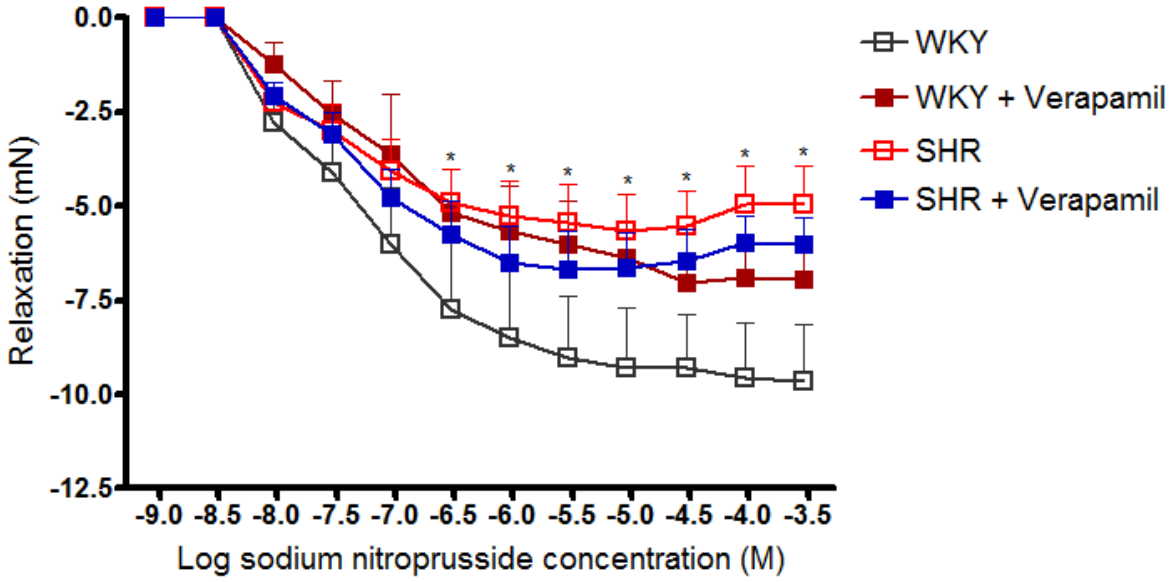


Figure 5.11: Cumulative-concentration relaxation to sodium nitroprusside (10^{-9} to 10^{-4} mol/L) in isolated thoracic aortic preparations from WKY, WKY + verapamil, SHR, SHR + verapamil (4 mg/kg/day, p.o.) at 16 weeks of age.Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs WKY and $^{\Delta,}$ **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).

Table: 5.7: -Log EC50 and maximum responses to sodium nitroprusside in isolated thoracic aorta

Parameters	WKY	WKY + Stevia	WKY + Verapamil	SHR	SHR + Stevia	SHR + Verapamil
-Log EC_{50}	6.2 ± 0.2	6.5 ± 0.3	6.1 ± 0.3	6.4 ± 0.2	6.0 ± 0.2	6.6 ± 0.5
R_{max} (% to sodium nitroprusside)	96 ± 0.7	98 ± 2.3	98 ± 0.8	97 ± 1.4	96 ± 1.0	98 ± 0.7

WKY, WKY + stevia, WKY + verapamil, SHR, SHR + stevia (200 mg/kg/day, p.o.) and SHR + verapamil (4 mg/kg/day, p.o.) at16 weeks of age. Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs WKY and $^{\Delta,}$ **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).

In small mesenteric arteries, hypertensive rats did not show any significant change in the contractile response to noradrenaline compared to the normotensive animals (Figure 5.12). Again, stevia and verapamil treatment increased this response in the SHR rats (Figures 5.12, 5.13). Endothelium-dependent relaxation response to acetylcholine and endothelium-independent relaxation to sodium nitroprusside were reduced in SHR rats which were partially prevented by eight weeks of stevia treatment (Figures 5.14, 5.16). Stevia normalised the endothelium- independent relaxation response to sodium nitroprusside and partially prevented the endothelium-dependent relaxation response to acetylcholine (Figures 5.14, 5.16).

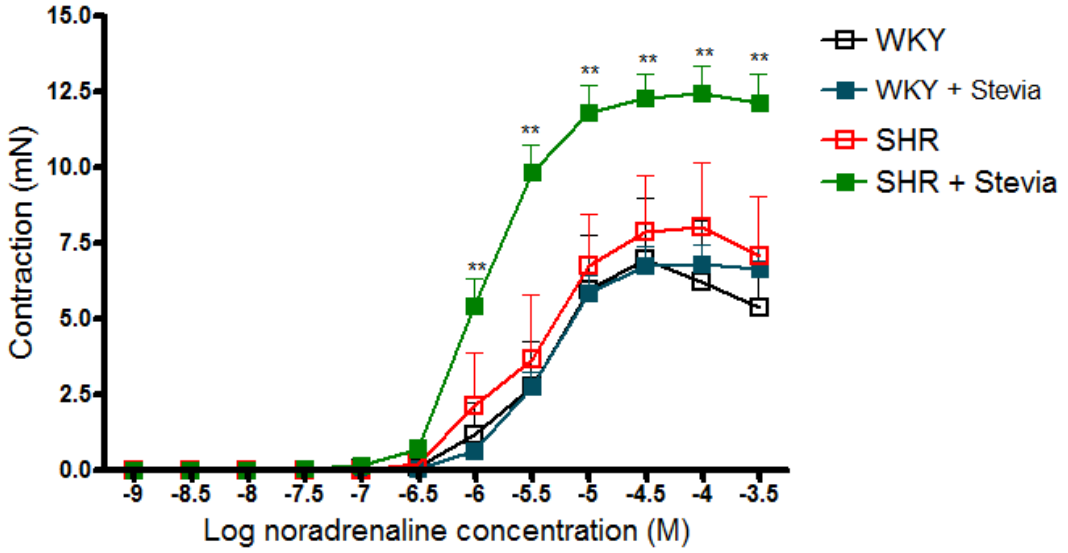
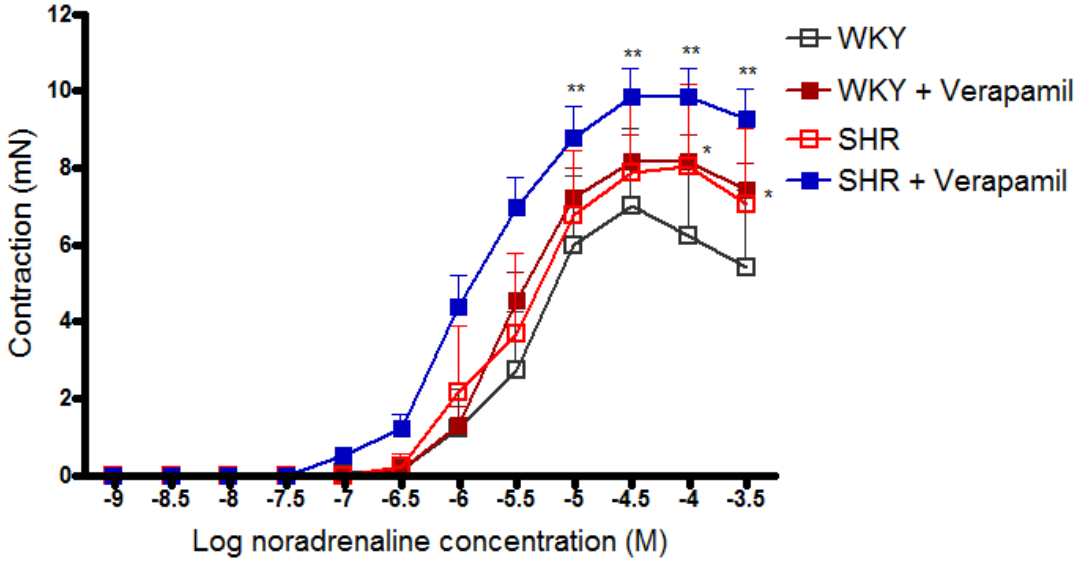


Figure 5.12: Cumulative-concentration contractile response to noradrenaline (10^{-9} to 10^{-4} mol/L) in mesenteric arteries from WKY, WKY + stevia, SHR, SHR + stevia (200 mg/kg/day, p.o.) at 16 weeks of age. Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs WKY and $^{\Delta,}$ **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).



Figuren 5.13: Cumulative-concentration contractile response to noradrenaline ((10^{-9} to 10^{-4} mol/L) in mesenteric arteries from WKY, WKY + verapamil, SHR, and SHR + verapamil (4 mg/kg/day, p.o.) at 16 weeks of age. Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs WKY and $^{\Delta,}$ **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).

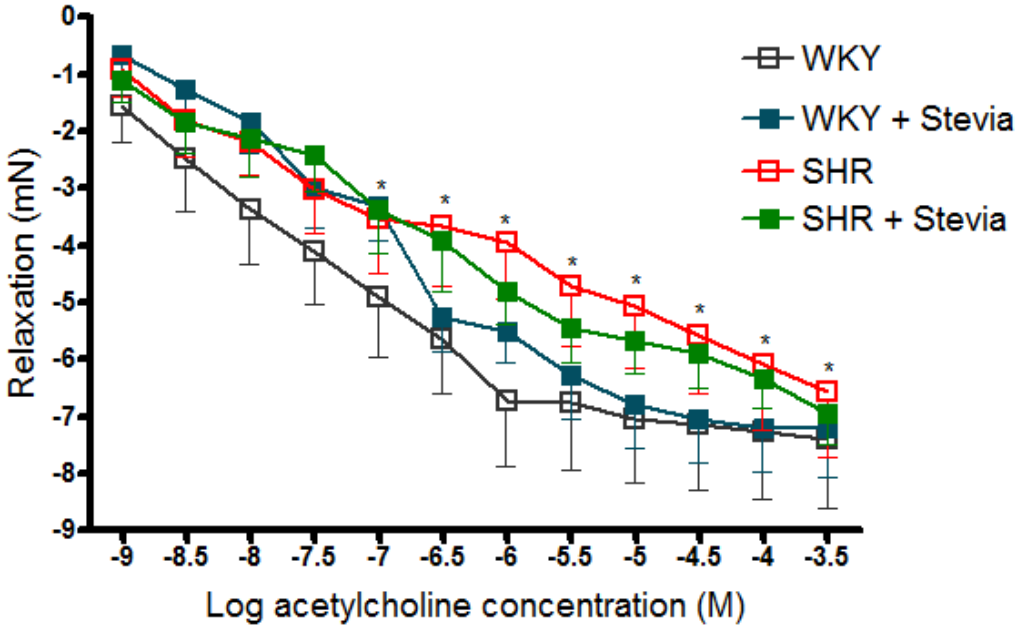


Figure 5.14: Cumulative-concentration relaxation to acetylcholine in mesenteric arteries from WKY, WKY + stevia, SHR, and SHR + stevia (200 mg/kg/day, p.o.) at 16 weeks of age. Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs WKY and $^{\Delta,}$ **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).

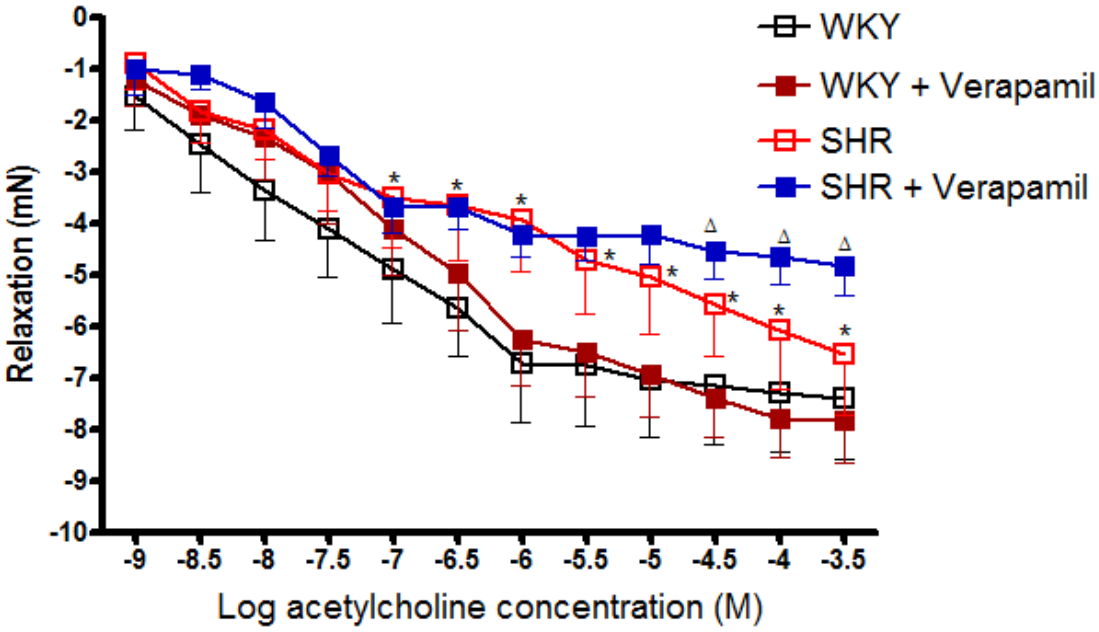


Figure 5.15: Cumulative-concentration relaxation to acetylcholine in mesenteric arteries from WKY, WKY + verapamil, SHR, and SHR + verapamil (4 mg/kg/day, p.o.) at 16 weeks of age. Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs WKY and $^{\Delta,}$ **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).

Endothelium-independent relaxation to sodium nitroprusside of the precontracted mesenteric arteries remained unaffected by eight weeks verapamil therapy (Figure 5.17). In addition to the endothelial dysfunction, the hypertensive rats showed a significant reduction in serum nitrate levels and serum nitrite/nitrate levels (Figure 5.18). Stevia treatment partially prevented the decrease in nitrate concentration following hypertension (Figure 5.18). Verapamil increased serum nitrate concentration both in SHR and WKY rats (Figure 5.18).

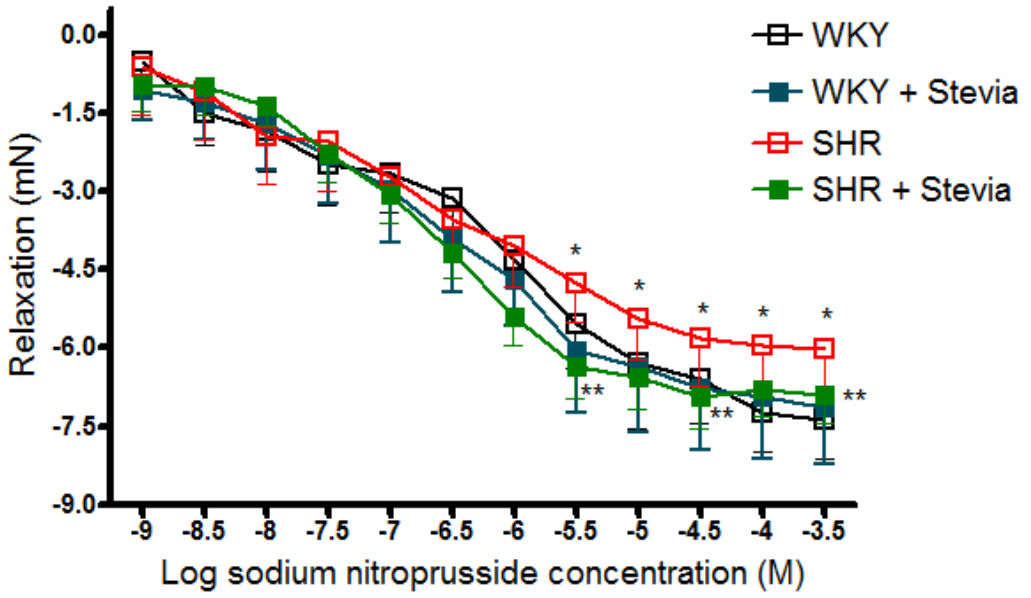


Figure 5.16: Cumulative-concentration relaxation to sodium nitroprusside in mesenteric arteries from WKY, WKY + stevia, SHR, SHR + stevia (200 mg/kg/day, p.o.) at 16 weeks of age. Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs WKY and $^{\Delta,}$ **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).

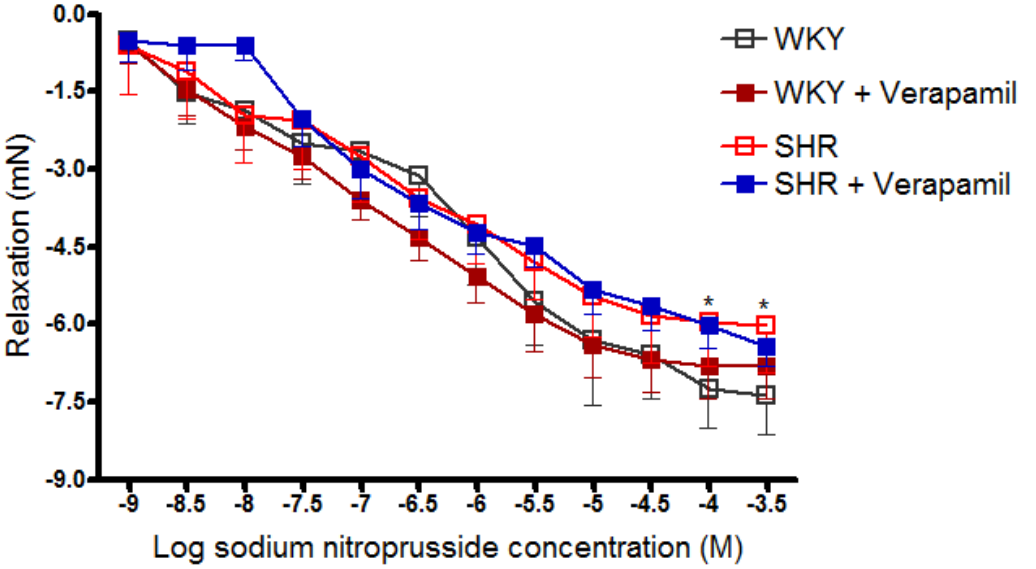


Figure 5.17: Cumulative-concentration relaxation to sodium nitroprusside in mesenteric arteries from WKY, WKY + stevia, WKY + verapamil, SHR, SHR + stevia (200 mg/kg/day, p.o.) and SHR + verapamil (4 mg/kg/day, p.o.) at 16 weeks of age. Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs WKY and $^{\Delta,}$ **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).

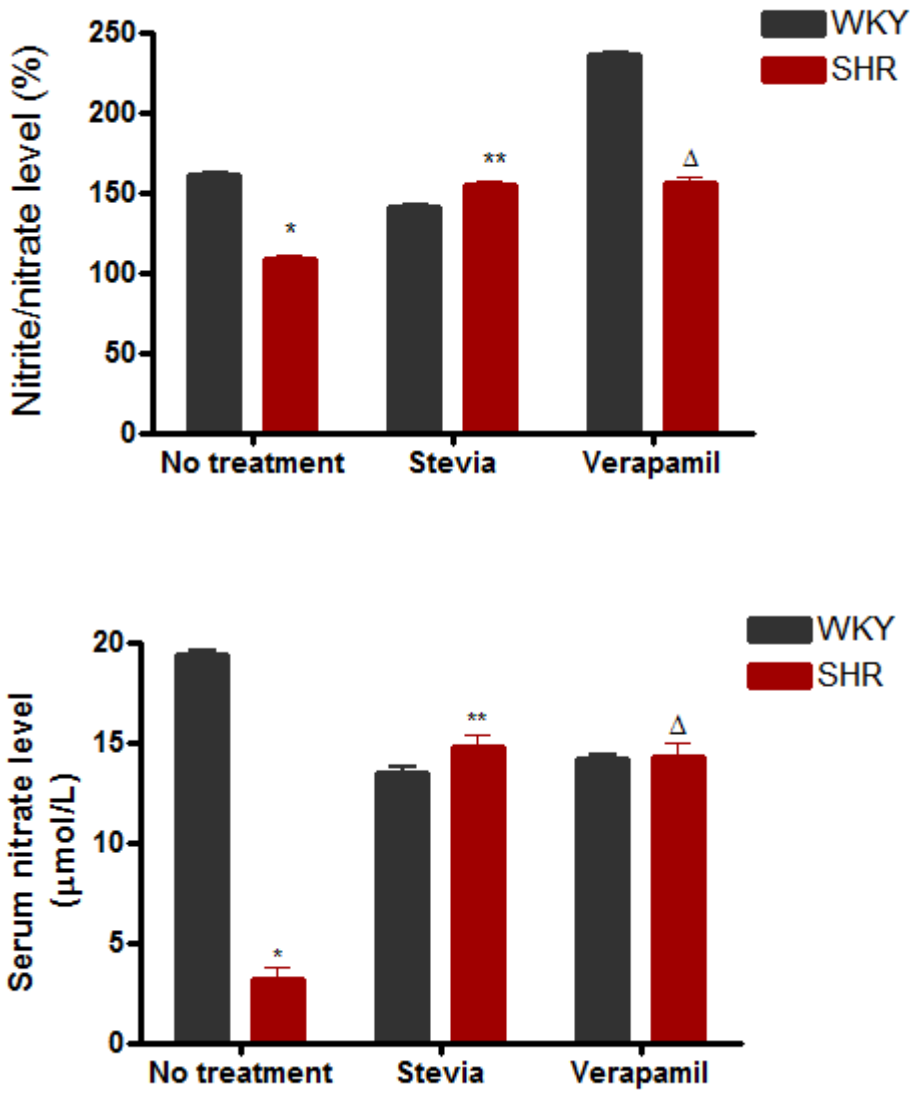


Figure 5.18: Serum nitrate levels and total nitrite/nitrate ratios for WKY, WKY + stevia, WKY + verapamil, SHR, SHR + stevia (200 mg/kg/day, p.o.) and SHR + verapamil (4 mg/kg/day, p.o.) at 16 weeks of age. Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs WKY and **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).

The effect of high blood pressure on gastrointestinal smooth muscle and muscarinic receptor function were measured by the isolated ileum organ bath preparation. Addition of pilocarpine, a neuronal M_2 receptor agonist to the organ bath inhibited EFS-induced response in a dose-dependent manner (Figures 5.19 and 5.20) reflecting functional M_2 muscarinic receptors in the ileum. In SHR rats, the concentration

response curves to pilocarpine were shifted slightly to the right indicating a decrease in M_2 muscarinic receptor activity compared to the WKY control rats (Figures 5.19, 5.20). Hypertensive rats treated with stevia demonstrated a further shifting of the dose response curves to the right indicating decreased inhibition of pilocarpine to EFS-induced contraction and reduced activity of M_2 receptor functions compared to the SHR rats (Figure 5.19). Stevia also decreased the inhibition of pilocarpine to EFS-induced contraction in the WKY control rats (Figure 5.19). Treatment with verapamil did not show any effects in the SHR and WKY control animals (Figure 5.20). Electrical field stimulation (EFS) of isolated ileum demonstrated a frequency-dependent contraction of the gastrointestinal smooth muscle (Figures 5.21, 5.22). Ileum from the SHR rats showed significantly less contraction in response to electrical field stimulation compared to the WKY controls (Figures 5.21, 5.22). Eight weeks of stevia treatment increased the contractile response to EFS in the both SHR and WKY control animals (Figure 5.21).

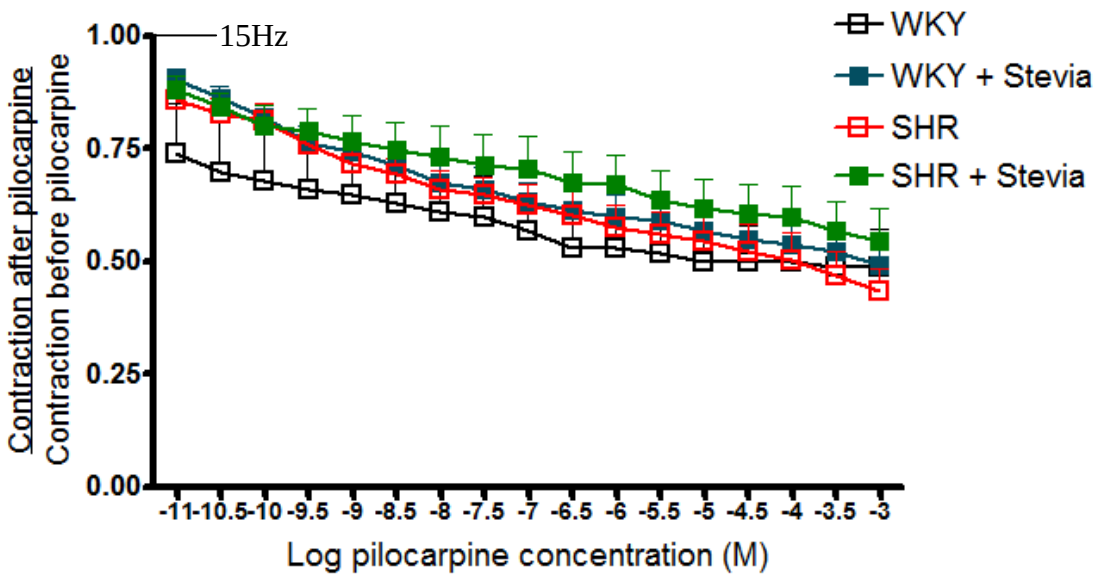


Figure 5.19: Inhibitory response to pilocarpine to electrical field stimulated (EFS, 15 Hz, 100 V, 0.2 ms pulse duration for 5 s at 30s intervals) contractions of isolated ileum from WKY, WKY + stevia, SHR, SHR + stevia (200 mg/kg/day, p.o.) at 16 weeks of age. Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs WKY and **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).

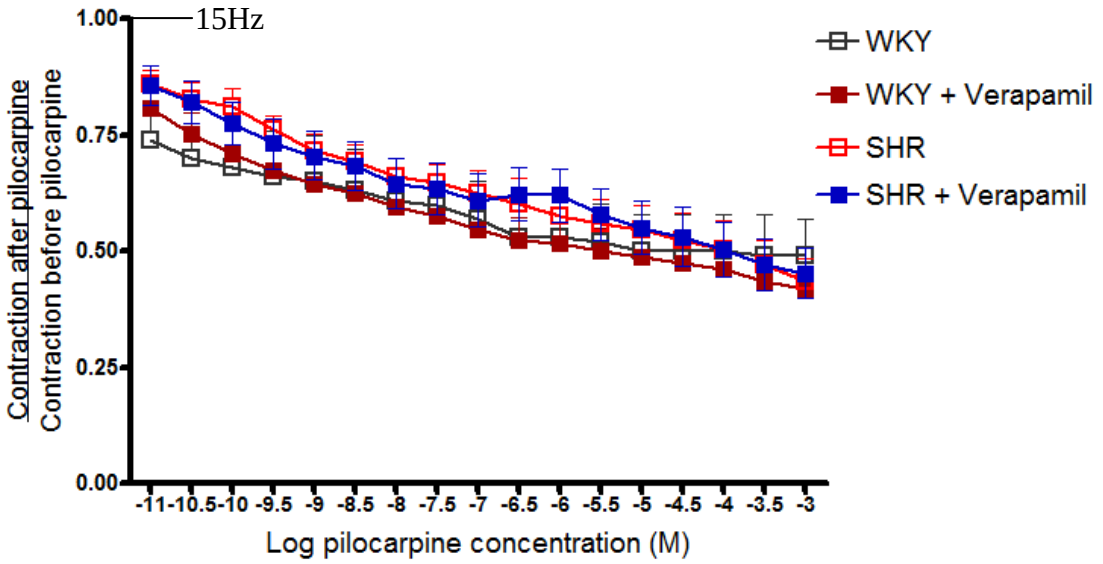
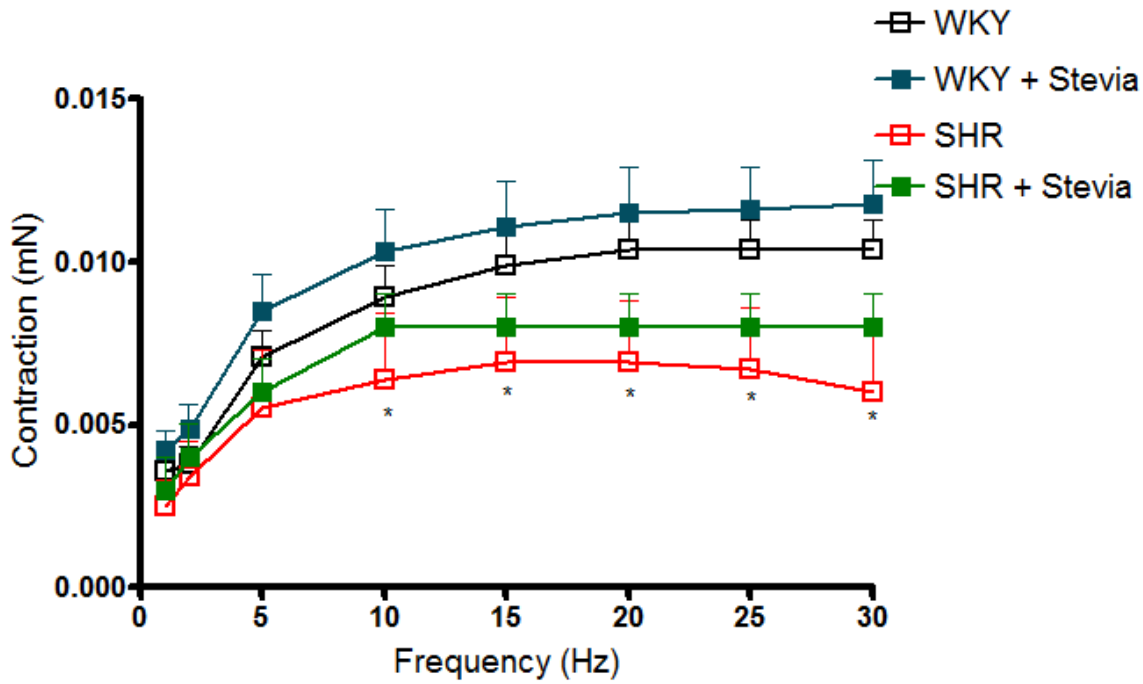


Figure 5.20: Inhibitory response to pilocarpine electrical field stimulated (EFS, 15 Hz, 100 V, 0.2 ms pulse duration for 5 s at 30s intervals) contractions of isolated ileum from WKY, WKY + verapamil, SHR, and SHR + verapamil (4 mg/kg/day, p.o.) at 16 weeks of age. Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs WKY and **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).



FFigure 5.21: Response to electrical field stimulation of isolated ileum from WKY, WKY + stevia, SHR, SHR + stevia (200 mg/kg/day, p.o.) at 16 weeks of age. Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs WKY and $^{\Delta,}$ **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).

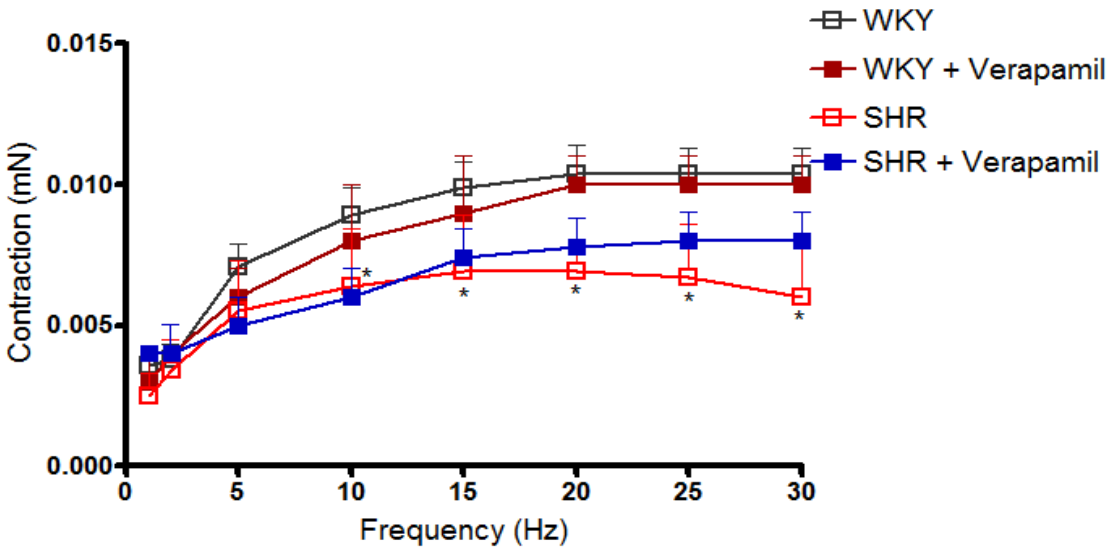


Figure 5.22: Response to electrical field stimulation of isolated ileum from WKY, WKY + verapamil, SHR, and SHR + verapamil (4 mg/kg/day, p.o.) at 16 weeks of age. Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs WKY and $^{\Delta,}$ **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).

Dosing with verapamil failed to improve the contractile response to EFS in the SHR rats (Figure 5.22). Verapamil showed no change in the response to EFS in the WKY control rats (Figure 5.22). Carbachol demonstrated a concentration-dependent contraction of the isolated ileum (Figures 5.23, 5.24). The hypertensive rat's ileum showed a marked decrease in the contraction in response to carbachol (Figures 5.23, 5.24). Stevia treatment normalized the reduced response to carbachol in the SHR group (Figure 5.23). Stevia treatment prevented the decrease in contraction to carbachol (Figure 5.23); however verapamil failed to improve the decreased contraction in the SHR rats (Figure 5.24). Carbachol induced contraction were found to be similar among the WKY controls, control treated with stevia and verapamil (Figure 5.24). In the isolated ileum, neither the EC_{50}s nor the maximum responses were different in among the SHR, control and stevia or verapamil treated groups (Table 5.8).

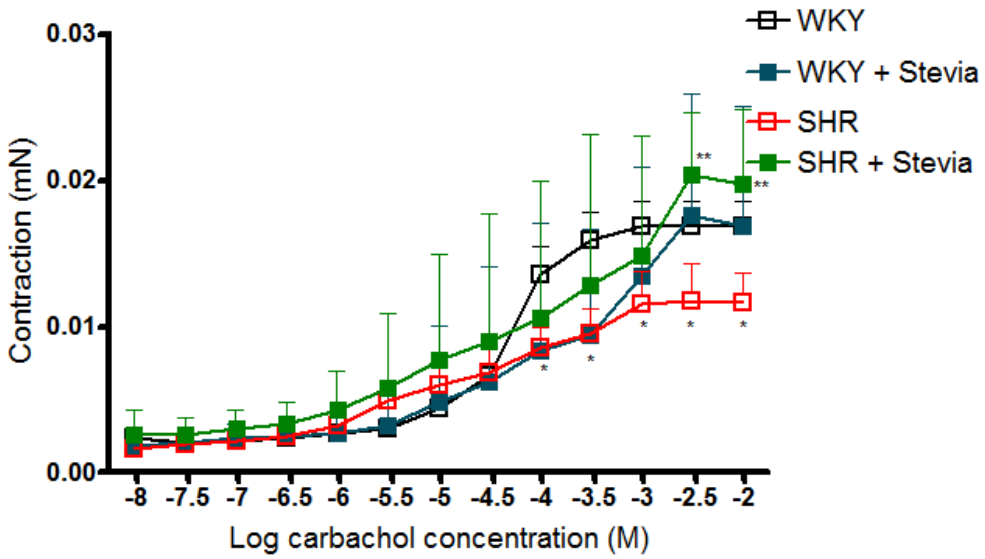


Figure 5.23: Contractile response to carbachol of isolated ileum from WKY, WKY + stevia, SHR, SHR + stevia (200 mg/kg/day, p.o.) at 16 weeks of age. Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs WKY and **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).

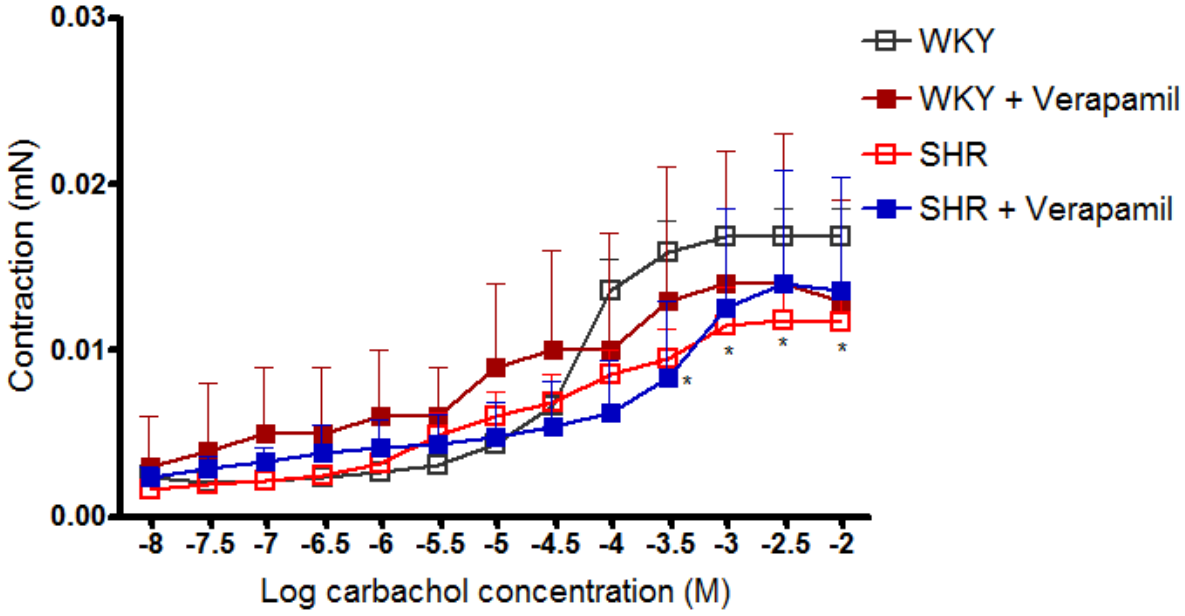


Figure 5.24: Contractile response to carbachol of isolated ileum from WKY, WKY + verapamil, SHR, and SHR + verapamil (4 mg/kg/day, p.o.) at 16 weeks of age. Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs WKY and **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).

Table: 5.8: -Log EC50 and Maximum responses to carbachol in isolated ileum from WKY, WKY + verapamil, SHR, and SHR + verapamil (4 mg/kg/day p.o.) at 16 wks of age.

Parameters	**WKY**	**WKY + Stevia**	**WKY + Verapamil**	**SHR**	**SHR + Stevia**	**SHR + Verapamil**
-Log EC_{50}	4.1 ± 0.1	3.8 ± 0.2	4.2 ± 0.2	4.3 ± 0.4	3.7 ± 0.3	3.9 ± 0.2
R_{max} (%to Carbachol)	95 ± 2.1	92 ± 3.4	92 ± 2.3	92 ± 5.8	92 ± 2.5	97 ± 2.4

Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs WKY and $^{\Delta,}$ **P<0.05 vs SHR (two-way ANOVA with Bonferroni's multiple comparisons tests).

5.4 Discussion

The current study encompasses a detailed investigation on the electrophysiological, functional, mechanical and immunochemical changes relating to cardiovascular disease in SHR and WKY from 8 to 16 weeks of age.

In this study, eight weeks of oral stevia treatment (200 mg/kg/day) showed a significant reduction in blood pressure and demonstrated an overall increase in body weights and water intake in the spontaneously hypertensive rats. This finding is supported by studies that showed stevia lowered blood pressure in spontaneously hypertentensive rats when administered intravenously (Chan et al. 1998; Sehar et al. 2008). The same studies also indicated that stevia reduced diastolic blood pressure in a dose dependent manner in SHR following intravenous doses of 50, 100, and 200 mg/kg (Chan et al. 1998; Sehar et al. 2008). Intraperitoneal injection of stevioside at 25 mg/kg also demonstrated an antihypertensive effect in the SHR (Hsu et al. 2002). Supporting these findings, a study on healthy mongrel anesthetized dogs confirmed the antihypertensive effects of stevioside and suggested that its hypotensive mechanism may be due to inhibition of Ca^{2+} influx (Liu et al. 2003). In addition, Melis & Sainati (1991) showed that stevia reduced blood pressure and decreased renal vascular resistance without any effects on glomerular filtration rate indicating stevia relaxes afferent and efferent arterioles (Melis & Sainati 1991). This result shows the potential of stevia for use in cardiovascular disorders which involve the kidney. Our results clearly showed that kidney weights were reduced by stevia and the mechanism behind this change need to be studies further. In human subjects with mild essential hypertension, stevia reduced blood pressure and prevented development of left ventricular hypertrophy (Paul et al. 2000). Our results also showed that tibial length was increased in stevia treated SHR. Studies reported that hypertension has influence on bone density mediated by angiotesin II on bone cells (Bastos et al. 2010; Shimizu 2008). Evidence suggested that this mediator suppressed the differentiation of osteoblastic cells and, accordingly, reduced bone formation by these cells (Shimizu 2008). Therefore, our result that stevia increased tibial length in the SHR indicated that stevia might have some effect in regulating angiotensin II and subsequent increase in the activity of osteoblastic cells

Dosing with verapamil (4 mg/kg/day) normalised blood pressure in the SHR. Verapamil, a calcium channel blocker has well established vasodilatory and blood pressure lowering effects. Published data reported that verapamil decreased blood pressure, reduced cardiac hypertrophy and prolonged overall survival in SHR (Lederballe et al.1982). In the current study, verapamil reduced blood pressure more significantly than stevia which indicates verapamil is a more potent vasodilator than stevia. However, both stevia and verapamil did not show any effect on blood glucose levels in the hypertensive rats. This is also a very interesting finding. In our diabetic study, stevia only reduced blood glucose levels in the STZ rats indicating its specificity for lowering blood glucose only if hyperglycaemia was present. The SHR animals did not have elevated blood glucose levels with stevia producing the expected result. Several studies verified that SHR models represent human insulin-resistance syndrome manifested by high plasma insulin level and dysfunction in fatty acid metabolism (Collison et al. 2000; Reaven et al. 1989). While stevia showed no change in plasma glucose levels in the SHR, increased insulin sensitivity and anti-hypoglycaemic effects were observed in the stevia treated STZ-induced diabetic rats (Baskaran et al. 1990; Jeppesen et al. 2000).

The 16-week old SHR showed a clear manifestation of left ventricular hypertrophy and impaired renal function as evidenced by increased left ventricular weight/body weight and left ventricular weight/tibial length ratios and kidney weight/body weight ratios. Stevia treatment significantly reduced left ventricular hypertrophy and improved renal function as measured by a reduction in the left ventricular weight/body weight and LV weight/tibial length ratios and kidney weight/body weight ratio. Previous studies demonstrated that stevioside increased renal plasma flow and caused a greater glomerular filtration rate (GFR) constant indicating vasodilatations in both afferent and efferent arterioles in hypotensive and normotensive rats (Melis 1992). Moreover, in both hypertensive and normotensive rats, stevioside enhanced natriuresis and diuresis (Melis 1992). These positive results indicate that stevia has anti-hypertrophic effects and improves renal function following hypertension. This finding is in line with the published data that showed stevia reduced blood pressure and prevented development of left ventricular hypertrophy in human subjects with mild essential hypertension (Paul et al. 2000).

Verapamil showed less regression in ventricular hypertrophy compared to stevia treated SHR. The possible reason for the insignificant effects of verapamil on left ventricular and kidney weights may be that verapamil works by direct relaxant effects on cardiomyocytes by blocking Ca^{2+} entry into the cells and not by anti-hypertrophic actions on vascular cells. Also verapamil may not have the same vascular profile or antioxidant and anti-inflammatory effects as stevia in the kidney. However verapamil is capable of reducing central blood pressure more effectively than stevia but failed to prevent the increase in left ventricular hypertrophy to the same extent as stevia. This result leads us to consider that stevia does have more humoral effects than verapamil and works through different pathways such as blocking calcium channels, reduction in oxidative stress and inflammation followed by decreased collagen deposition in the ventricular myocytes. Although a reduction in blood pressure is correlated with the decrease in ventricular mass, previous studies showed that prevention and/or attenuation of left ventricular hypertrophy (LVH) is not always dependent on blood pressure (Bombig et al. 1996; Bregagnollo et al. 2005; Filho et al. 2010). Several studies on spontaneously hypertensive rats showed that many anti-hypertensive drugs do not have effects on LVH such as hydralazine and minoxidil but still reduced blood pressure. On the other hand, captopril, methyldopa, beta blockers and calcium channel antagonists were found to reduce left ventricular hypertrophy in addition to their antihypertensive effects (Bombig et al. 1996; Filho et al. 2010). This phenomenon suggests that the mechanisms of these drugs differ in the activity of neural and humoral pathways and that stevia also follows this pattern.

Measurement of heart rate evaluates the autonomic function of the heart reflecting the propensity for arrhythmic events. Tucker & Johnson (1984) demonstrated an increase in resting heart rate in SHR indicating that a hyperactive sympathetic nervous system could be an early manifestation of genetically developed hypertension (Tucker & Johnson 1984). Human studies have shown that sudden cardiac death is closely associated with an altered autonomic nervous system output (Pinar et al. 1998; Vaage-Nilsen & Rasmusssen 1998; Zipes 1990). Particularly, in post-MI (Myocardial infarction) patients, sympathetic activation led to fatal cardiac arrhythmias and conversely vagal stimulation demonstrated cardio-protective effects (Schwartz, La Rovere & Vanoli 1992). In our study, the SHRs showed an increase in the basal heart

rate. Eight weeks of stevia treatment normalized the increased heart rate in the hypertensive group to control levels. Previous studies indicated that intravenous stevia administration caused a slight decrease in heart rate in the SHRs and that the effects appeared to be dose dependent which are in agreement with our findings (Chan et al. 1998).

Verapamil demonstrated a significant reduction in heart rate in the SHRs which supported the earlier study that verapamil significantly reduced heart rate, blood pressure and ratio of ventricular weight to body weight in SHRs (Ruskoaho & Savolainen 1985). By slowing the heart rate, improving coronary blood flow and decreasing sympathetic stimulation, verapamil demonstrated anti-arrhythmic effects (Vaage-Nilsen & Rasmusssen 1998). Lower heart rates and increased heart rate variability (HRV) are indicators of good cardiac function. Verapamil was found to improve HRV in post-MI patients with the autonomic balance shifted to parasympathetic predominance (Pinar et al. 1998; Vaage-Nilsen & Rasmusssen 1998). After MI, decreased HRV is representative of reduced vagal activity and increased sympathetic drive increasing the risks of sudden death and being a predictor of CVD (Pinar et al. 1998; Vaage-Nilsen & Rasmusssen 1998).

Resting heart rate is an independent risk factor for coronary artery disease. In the Coronary Artery Surgery Study (CASS) on 24 913 men and women with suspected or proven CAD, resting heart rate was found strongly associated with cardiovascular mortality irrespective of sex, age, hypertension, cardiac function, body weight, presence of diabetes, or use of betablockers (Diaz et al. 2005; Hjalmarson 2007). Beta blockers are found to be effective in controlling coronary arterial disease with the degree of CAD and improvements post treatment, directly associated with the grade of resting heart rate (Cordero et al. 2011). The current study clearly indicates that stevia has anti-arrhythmic effects and can be effectively used in CAD as it normalized heart rate in the SHR. Calcium channel blockers (CCBs) are considered as the second-line treatment option after beta-blockers in CAD patients (Cordero et al. 2011). This study showed that verapamil dramatically reduced heart rate in comparison to stevia, indicating its effectiveness in patients with CAD, however, fatal cardiac events and death may occur due to severe cardiac depression (Cordero et al. 2011). On the other hand, verapamil did not reduce blood pressure and heart rate in the normotensive

WKY rats indicating that verapamil has specific anti-hypertensive properties rather than just vasodilatation in hypertensive subjects. Therefore the negative inotropic effect of verapamil becomes less prominent in the normal rats following chronic treatment. Moreover, a greater number of calcium channels and a high proportion of inactivated channels were found in the vasculature of hypertensive rats which also explains the increased activity of verapamil during the disease state which might also extend to the effects of stevia in this setting (Godfraind 2005).

Chronic high blood pressure is a major contributor to maladaptive vascular changes, heart failure and death. Cardiovascular remodelling is manifested by impaired endothelial function and reduced endothelium dependent vasodilation (M Gómez-Roso, et al. 2009). Spontaneously hypertensive rats (SHR) showed a structural alteration of the aortic walls such as increased medial thicknesses and cross sectional areas (Vaja et al. 2009; van Gorp et al. 2000). Functional changes associated with the aortas from SHR include reduced distensibility and decreased compliance of the thoracic aorta prior to the development of hypertension (Vaja et al. 2009; van Gorp et al. 2000) In this study, hypertension showed characteristic features of endothelial dysfunction as manifested by reduced contractile responses of isolated aorta from the SHR to noradrenaline, acetylcholine and sodium nitroprusside.

Stevia treatment normalized the contractile response to noradrenaline and partially prevented the altered vascular responses to endothelium-independent (sodium nitroprusside; NaN) relaxation and endothelium-dependant (acetylcholine) pathways in the spontaneously hypertensive rats. Previous studies demonstrated that stevia induces vasorelaxation in both normotensive and hypertensive animals (Chan et al. 1998; Wong et al. 2004a) and the suggested mechanism may be via the opening of small conductance calcium-activated potassium channels (SK_{Ca}) and ATP-sensitive potassium (K_{ATP}) channels (Wong et al. 2004a). Selective opening of potassium channels with a subsequent reduction in Ca^{2+} concentration causing vasorelaxation could be one of the mechanisms of how isosteviol reduces blood pressure (Wong et al. 2004a). Moreover, isosteviol showed a dose-dependent vasorelaxation of vasopressin-induced vasoconstriction in isolated aortic rings with or without endothelium which was also consistent with the report of opening of potassium channels (Topouzis, Schott & Stoclet 1991; Wong et al. 2004a).

This study results showed that chronic treatment with verapamil normalized contractile response of aorta to noradrenaline and significantly enhanced endothelium-dependent and endothelium-independent relaxation to acetylcholine and sodium nitroprusside. A short-term (seven day) study with calcium channel antagonists demonstrated that verapamil was effective in restoring contractile response to KCl but did not affect acetylcholine-induced dilator responses in the aorta of hypertensive rats (Vaja et al. 2009). Similar results were found in a long-term study which showed that hypertension-induced structural changes of the aorta could be prevented completely with chronic verapamil treatment (Koffi et al. 1999). The results strengthened these findings by showing that chronic verapamil administration improves vascular function by increasing contractile and relaxation responses following hypertension. Moerover, stevia and verapamil both improved blood vessel reactivity to both vasoconstrictors and vasodilators. This could be due to generalised improvements in smooth muscle cell function and given that it occurred for both drugs could be via enhanced cellular performance. The EC_{50}s for noradrenaline were the same between SHR, verapamil and stevia treatments therefore it has not increased potency, just the maximal response.

Mesenteric arteries from the SHR showed decreased responses to noradrenaline, acetylcholine and sodium nitroprusside compared to the WKY control rats. These findings corroborate with previous studies that showed that chronic hypertension led to contractile dysfunction and reduced responsiveness in small mesenteric arteries (Cox & Rusch 2002; Lin, Y. et al. 2010; Wu, B. et al. 2007a). Mesenteric arteries play important roles in maintaining smooth muscle contractility and regulating the diameter of resistance arteries (Nelson & Quayle1995). The activity of large conductance Ca^{2+} activated K^{+} channels in the arterial smooth muscle is important in maintaining normal vascular contractility. Hypertension reduced expression of large conductance Ca^{2+}activated K^{+} channels and voltage dependent Ca^{2+} channels causing an increase in cytosolic [Ca^{2+}] and enhanced cell proliferation leading to vascular remodelling (Lin, Y. et al. 2010). The data showed that stevia treatment increased the contractile response of mesenteric arteries to noradrenaline and normalised the reduced response sodium nitroprusside in the SHRs. In this study, stevia successfully prevented the decreased vascular response to noradrenaline and sodium nitroprusside

in both aorta and mesenteric arteries but the extent of endothelium-independent relaxation was less dramatic in mesenteric arteries. These results indicate that stevia has dual roles acting as both an endothelium dependant vasodilator via eNOS to produce NO and by blocking calcium channels in a similar way to verapamil. Previous data suggested that stevioside's vasodilating effect is analogous to verapamil which is a specific inhibitor of calcium channels in cardiac and vascular muscle (Melis 1991, 1992a, 1994).

Dosing with verapamil significantly increased the contractile response to noradrenaline. Conversely, verapamil was found to decrease relaxation in response to sodium nitrpoprusside. However it did not show any change in the response to sodium nitroprusside. Increased Ca^{2+} influx plays a key role in altered vascular smooth muscle activity in hypertension as previous studies demonstrated increased Ca^{2+} channel activation in vascular smooth muscle cells from rat mesenteric arteries in young pre-hypertensive SHRs compared to the age-matched WKY control animals (Kubo, Taguchi & Ueda 1998; Wilde, Furspan & Szocik 1994). In addition to greater Ca^{2+} influx, vascular smooth muscle beds also showed an elevated agonist (norepinephrine) sensitivity and selectivity leading to hypertension and vascular dysfunction (Bohr & Webb 1988; Kubo, Taguchi & Ueda 1998). An increased expression of L-type calcium channels was demonstrated in hypertension by whole-cell voltage-clamp experiments from SHR vascular smooth muscle cells (Kubo, Taguchi & Ueda 1998). Augmented expression of L-type calcium channels followed, with increases in agonist and antagonist binding sites in the L-type calcium channels found in SHRs (Kubo et al. 1998). This was confirmed by greater inhibition of Ca^{2+} current by nifidipine in the SHR but verapamil did not potentiate relaxation in the hypertensive rats compared to the control animals (Kubo, Taguchi & Ueda 1998). This means that the hypertensive state promotes an increase in the specific targets for calcium channel antagonists and similar agents such as stevia may have more pronounced effects.

It is observed that the effect of stevia on the mesenteric arteries in response to noradrenaline, acetylcholine and sodium nitroprusside were greater than those of verapamil. These findings suggest that stevia's vasodilation effects are mainly mediated by the blockade of calcium channels and stevia has a larger affinity for

mesenteric smooth muscle and/or there may be an increase in stevia binding sites in L-type calcium channels on the mesenteric arteries of SHRs compared to the aorta. The results from the healthy Wistar rats' mesenteric artery showed that L-NAME partially blocked the stevia-induced relaxation reflecting that stevia also works through increasing the function and signalling of NO. Moreover, increased responsiveness of mesenteric arteries to noradrenaline in the stevia treated rats were observed without an increase in noradrenaline potency (-log EC_{50}) indicating that stevia works beyond the receptor-agonist interaction. To the best of my knowledge, this study provides the first direct evidence that stevia prevents vascular remodelling in the mesenteric arteries in hypertension and is more effective than a calcium channel blocker verapamil. However despite these potential direct effects of stevia, vascular function may have also been improved via the increased levels of NO and TAC and decreased concentrations of circulating cytokines and reactive oxygen species.

One hypothesis initiating this research was whether stevia has the capacity to prevent or reverse vascular dysfunction following chronic hypertension. This hypothesis remained uphold as the results in the large arteries and conduit vessels from stevia treated hypertensive animals showed a clear picture of better vascular function than the untreated animals. Another index of normal vascular activity is serum total nitrite/ nitrate ratio. An increase in serum nitrite/nitrate ratio indicates greater production of bioactive NO from eNOS which was demonstrated in our study in the stevia treated hypertensive rats (Wu, L. et al. 2004). In hypertension and cardiovascular disease states, protein kinase C (PKC) becomes activated leading to uncoupling and malfunctioning of eNOS (Wu, L. et al. 2004). As a result of uncoupling, eNOS produces more reactive oxygen species (ROS) than nitric oxide in vascular beds (Wu, L. et al. 2004). This study showed that stevia restored NO production in the treated hypertensive rats evidenced by increased serum nitrate concentration, high nitrite/nitrate ratio and enhanced vasodilatation to acetylcholine compared to the untreated SHRs.

Verapamil also clearly demonstrated vasculoprotective effects observed by increased serum nitrate level and elevated nitrite/nitrate ratio in the SHRs. Previous studies support this result as verapamil was found to restore normal level of nitric oxide in mice with cocaine induced oxidative stress (Bhattacharya et al. 2009). In this study

stevia increased nitrite/nitrate ratio more significantly than verapamil. Serum nitrate is endogenously produced from the oxidation of nitric oxide initiated by oxyhaemoglobins (Wennmalm et al. 1992). Nitric oxide was found to be an important signalling and effector molecule required for several physiological processes and also involved in many pathological events that mediates vascular remodelling (Bhattacharya et al. 2009). Nitric oxide is synthesized by the enzyme nitric oxide synthase (NOS) which has three distinct isoforms and all are expressed in kidney. Constitutive endothelial and neuronal NOS produce small (pM) transient bursts of NO by following calcium dependent pathway. The inducible isoform iNOS is present most conspicuously in the medullary thick ascending limb and inner medullary collecting duct and is stimulated by inflammatory cytokines (Bhattacharya et al. 2009). Hypertension induces chronic subclinical inflammation which causes damage to the heart that initiates cardiac remodelling (Zipes 1997). Endothelial dysfunction in hypertension is also associated with production of increased free radicals and oxidative stress (Zipes 1997). Serum from the 16 weeks old SHRs showed a significant reduction in total antioxidant capacity (TAC) and an increase in serum malondialdehyde (MDA) levels. In myocardial ischemia, cellular damage leads to increased lipid peroxidation (Grech et al. 1996; Haiyun et al. 2004). Serum malondialdehyde, produced from degradation of lipid peroxides is a powerful indicator of myocardial cell damage and altered membrane potential following myocardial ischemia (Grech et al. 1996; Haiyun et al. 2004).

Both stevia and verapamil treatment decreased MDA levels to normal values. Stevia marginally reduced serum MDA levels in WKY control rats. SHRs demonstrated a marked increase in serum IL-6 levels which were normalized by dosing with both stevia and verapamil. Stevia was found to have anti-inflammatory and antioxidant activities which might also explain the beneficial effects of stevia in cardiovascular and metabolic complications (Boonkaewwan, Toskulkao & Vongsakul 2006; Chatsudthipong & Muanprasat 2009). A study on obese insulin resistant mice showed that stevia treatment increased insulin signalling and total antioxidant defence in the vascular wall (Geeraert 2010). Another study on scopolamine treated rats showed pre-treatment with stevia reduced scopolamine-induced oxidative stress levels in brain cells (Sharma 2010). A recent study demonstrated a direct association between

circulating inflammatory markers with large blood pressure variability in hypertensive patients (Kai et al. 2009). Here elevated cytokines increased blood pressure which was a similar finding to the current study. Stevioside attenuates synthesis of inflammatory mediators in LPS-stimulated THP-1 cells by interfering with the IKKβ and NF-κB signalling pathway and induces TNF-α secretion which is partially mediated through TLR4 (Boonkaewwan, Toskulkao & Vongsakul 2006). Isosteviol (1-100 µmol/l) acts by inhibiting angiotensin-II-induced DNA synthesis and endothelin-1 secretion as observed in cultured rat aortic smooth muscle cells (Wong et al. 2006). Evidence showed that increased angiotensin II levels leads to production of reactive oxygen species in Angiotensin-converting enzyme 2 (ACE2)-deficient hearts by inhibiting metabolism of angiotensin II into angiotensin 1-7 (Kassiri et al. 2009). This provides the hypertensive state with many avenues for the generation of inflammation and oxidative stress (Kassiri et al. 2009).

Previous studies showed that verapamil has antioxidant effects as it enhanced sluggish peripheral coronary flow which is also known as “no reflow” after a myocardial infarction (Okamoto et al. 2004). The “no reflow’’ observation was due to neutrophil plugging and increased oxidative stress initiated at the site of reperfusion injury (Okamoto et al. 2004). In this study, SHRs showed a marked decrease in coronary blood flow potentiating cardiovascular dysfunction in the hypertrophic heart. Stevia significantly increased coronary blood flow reflected by a reduction in coronary vascular resistance which plays a key role to supply blood to the ischemic hypertrophied myocardium. Verapamil enhanced coronary blood flow more significantly than stevia. These results suggest that verapamil causes a stronger and more progressive vasorelaxation effect on coronary artery tone. Moreover, a recent study showed that a high level of phenolic components is present in the leafy extract of stevia (Shukla et al. 2011). It is now well established that bioflavonoid and phenolic components are capable of scavenging free radicals (Shukla et al. 2011), inhibiting platelet aggregation and low density lipoprotein (LDL) peroxidation (De Whalley et al. 1990; Shukla et al. 2009), increasing vasodilating effects and restoring the normal functions of cardiac muscle (Duarte, J. et al. 1993; Haiyun et al. 2004),

Spontaneously hypertensive rats showed a marked decrease in NO-mediated vasodilator response when treated with verapamil and diltiazem indicating that nitric

oxide mediated signal transduction processes are mainly dependent on Ca_{VS}^{2+} - channels (Lewis, S. et al. 2005). Our study suggests that resistance arteries from hypertensive rats show increased activity of $Cavs^{2+}$ channels with an underlying mechanism possibly through cGMP-dependent protein kinase C (Lewis, S. et al. 2005). Our results showed that verapamil did not show any effect on the vasodilator action of acetylcholine in the SHR and WKY rats. This finding is in line with the data showing verapamil lacks effects on the vasorealaxing effects of L-S-nitrocysteine and acetylcholine indicating that acetylcholine (releases L-S-nitrocysteine and EDRF) produces vasorelaxation through mechanisms other than the closure of Ca^{2+} channels (Lewis, S. et al. 2005).

Left ventricular hypertrophy is regarded as a powerful indicator of cardiovascular disease and mortality (Duarte, D. R. et al. 2009; Filho et al. 2010). The occurrence of ventricular arrhythmias is very common in patients with cardiac hypertrophy. Previous studies have showed that Sprague-Dawley rats with left ventricular hypertrophy induced by aortic banding demonstrated a decrease in developed pressure, reduced coronary flow and a severe reduction in oxygen consumption (Raya et al. 1989). Similar results were established in SHR rats which showed increased collagen deposition and fibrosis in the myocardium leading to increased diastolic stiffness observed as early as 8-9 weeks of age (Filho et al. 2010). In the current study isolated Langendorrf hearts demonstrated a mild diastolic stiffness in the SHRs which was normalised by stevia treatment. SHRs also showed compromised systolic cardiac function with decreased developed pressure, reduced rates of contraction and relaxation. Chronic stevia treatment significantly improved cardiac rates of contraction and increased developed pressure and end systolic pressure although the results were not significant. Therefore, stevia significantly increased coronary blood flow and prevented the reduced cardiac function in hypertensive rat hearts.

Similarly, chronic verapamil dosing prevented cardiac dysfunction by significantly improving developed pressure and rates of contraction in the hypertensive hearts. However, verapamil failed to normalize diastolic stiffness in the SHR rats. A previous study showed that acute and chronic verapamil treatment improved cardiac function and post-ischemic recovery of hypertrophied hearts following hypertension induced by aortic stenosis (Buser et al. 1989). Earlier studies demonstrated that untreated

hypertrophied hearts showed significantly lower coronary blood flow and oxygen consumption compared to the normal hearts (Buser et al. 1989). The reduced coronary flow and a corresponding decrease in oxygen consumption lead to increased cardiac rate-pressure output and a shift in metabolism in cardiomyocytes (Buser et al. 1989). This would help to promote cardiometabolic stress and reactive oxygen species production promoting remodelling in the left ventricle. In our study, verapamil was found to improve coronary blood flow more significantly than stevia. This increased blood flow might play a major role in the improvement of developed pressure and rates of contractions by verapamil observed in the isolated Langendorff experiments. Therefore, it has been clearly demonstrated that verapamil exerts protective effects on the hypertrophied myocardium. Similarly, it was shown that verapamil protected even the normal myocardium in the event of ischemia (Buser et al. 1989; da Luz et al. 1980; Kajiyama et al. 1987).

Cardiac electrophysiological studies in animal model of chronic hypertension and hypertrophy offers a clear understanding of these events and associated mechanism of arrhythmias (Guinamard et al. 2006; Hasenfuss 1998). The cardiac action potential duration was significantly prolonged in the hypertensive rats at 20%, 50% and 90% of repolarization following 16 weeks of hypertension. Similar results were published in a study using electrophysiology to assess *in vivo* left ventricular hypertrophy where impaired cardiac function has been shown to reduce resting membrane potential, action potential amplitude and prolong the cardiac acrtion potential at 90% of repolarisation in pigs with tachycardia-induced cardiomyopathy (Zipes 1997). Recent studies have showed that increased heart rates, particularly ventricular rate changes even for a short duration can initiate and influence the development of cardiac arrhythmias (Zipes 1990, 1997). Human studies also showed atrial remodeling following tachycardia with refectory periods decreased from the normal rate (Zipes 1997). The possible mechanism of the reduced action potential may be a decrease in Ito and Ica currents (Zipes 1997). Human subjects with chronic atrial fibrillation demonstrated a decrease in the outward K^+ current density and down regulation of Kv1.5 (Ausma et al. 1997; Zipes 1997). It was also observed that atrial fibrillation caused a shortened refractoriness in human subjects (Ausma et al. 1997; Zipes 1997).

Chronic stevia treatment normalized the action potential duration at 50% and 90% of repolarization therefore showed antiarrhythmic effects in the treated SHR rats. To the best of our knowledge, this is the first cardiac electrophysiological study in the hypertensive rats following chronic stevia administration. Verapamil also decreased prolonged action potential duration but the results were not significant.

SHR rats showed expression of non-selective cationic channels (NSC_{Ca}) in their ventricular cardiomyocytes which demonstrated no/ weak pearmeability to calcium, sensivity to intracellular calcium, equal permeability to all monovalent cations. The cardiac NS_{ca} may be stimulated by TRPMS, a calcium-sensitive cationic channel which is not inhibited by ATP (Guinamard et al. 2006). The SHR cardiomyocytes showed an enhanced TRPM4 current activity which may be due to increased production of PKC stimulation TRPM4 protein expression (Guinamard et al. 2006). Activation of TRPM4 leads to delayed after depolarization (DAD) as it has equal sensitivity to Na^+ and K^+ thereby produce a combination between Na+ and K^+ . DADs are produced because of a calcium dependent current known as transient inward current (I_{ti}) (Guinamard et al. 2006). In this current study, the positive results demonstrated by stevia in shortening the action potential duration were possibly by down regulating the function and expression of Ca^{2+} channels.

Verapamil was found to restore cardiac electrophysiology in dogs with multiple organ dysfunction syndrome (MODS) characterized by prolongation of action potential associated with altered L-type calcium current activities (Hou et al. 2010). Verapamil shortened the action potential prolongation and decreased early delayed afterdepolarization (Hou et al. 2010). A study on patients living with systolic hypertension highlighted that a combination of calcium channel blockers and ACE inhibitor is more effective in decreasing fatal and nonfatal cardiovascular events in hypertensive patients with diabetes.

Atrial fibrillation is a common form of arrhythmia causing a significant cardiac remodelling (Tieleman et al. 1997). The concept of electrical remodelling was described in normal, chronically instrumented goats where artificial maintenance of atrial fibrillation caused a significant reduction in refectory periods (Tieleman et al. 1997). Increased intracellular calcium levels in the cardiac myocytes are directly

related with rapid irregular rhythm (Tieleman et al. 1997). Administration of verapamil during atrial fibrillation prevented cardiac remodelling and decreased arterial systolic dysfunction which indicates that L-type calcium channel and an intracellular calcium level plays important role cardiac remodelling. Rapid and irregular depolarization can increase intracellular calcium in cardiomyocytes (Tieleman et al. 1997). A human study showed high frequencies of depolarization in arterial myocytes led to upregulation of calcium currents and a substantial increase in calcium flow and subsequent opening of calcium dependent potassium or chloride channels (Tieleman et al. 1997).

To explore the parasympathetic nerve function in the gastrointestinal smooth muscle, EFS-induced contractions were measured in all rats. The SHR showed a marked decrease in the EFS-induced contraction indicating that hypertension reduces parasympathetic nerve activity in the gastrointestinal tract. This finding agrees with the results by Patten et al. (2005) that SHR rats showed a decreased response to ileal contraction induced by postanoid (Patten et al. 2005). SHRs also showed an increased expression of inhibitory muscarinic M_2 receptors in rostral ventrolateral medulla explaining the possible neural inhibition of this area and subsequent rise of blood pressure (Gattu et al. 1997). However, our results showed no significant difference in the inhibitory effects of pilocarpine to EFS-induced contraction in the hypertensive rats which suggested no increase in the expression of M_2 receptor in gut smooth muscle in hypertension. The SHRs demonstrated a reduction in carbachol-induced contraction reflecting a possibility of decrease in post junctional M_3 receptors in hypertension. Stevia partially prevented reduced parasympathetic function evidenced by improved EFS-induced contraction in the treated SHRs. Stevia also restored the hypertension-induced reduction in M_3 receptor function which was supported by the study of Pattel et al. (2005) that fish oil increased acetylcholine-induced contraction in the gastrointestinal smooth muscle of the SHR (Patten et al. 2005). Results from the diabetic rats in this study showed a decrease in autonomic nerve function mediated by increased M_2 receptor function leading to motility dysfunction and stevia prevents the decreased motility in the gut.

Chapter 6 Effects of stevia and verapamil on diabetes induced cardiovascular changes.

6.1. Introduction

Increasing prevalence of diabetes and associated complications are posing serious threats to the health and wellbeing of the world population. The global prevalence of diabetes was predicted to grow from 366 million in 2011 to 552 million by the year 2030 (International Diabetes Federation [IDF] 2011). Diabetes increases the occurrence of cardiovascular disease with data indicating that people with diabetes are two to four times more prone to develop CVD than the people without diabetes (Stamler et al. 1993). Statistical data illustrated that cardiovascular disease accounted for 80% of all deaths following diabetes (UKPDS 1998). Therefore, it is important to begin the treatment of diabetes and control blood glucose levels and insulin resistance as early as possible to improve health outcomes. Unfortunately, diabetes often remains unnoticed unless any of its primary pathological complications such as increased hunger and thirst, neuropathy, retinopathy, renal failure, arrhythmia and stroke develop (Wei et al 2003).

Diabetes leads to vascular dysfunction, left ventricular hypertrophy and congestive heart failure, neuro-endocrine imbalance and neuropathy (Wachirawadee Malakul 2008; Zozulinska & Wierusz-Wysocka 2006) Vascular dysfunction in diabetes is characterized by functional and structural changes leading to enhanced arterial stiffness and reduced distensibility of the vessels (Rahman et al. 2007). Increased arterial stiffness and reduced flexibility lead to increased pulse wave and greater systolic pressure which directly contributes to the development of left ventricular hypertrophy in diabetes (Nicolaides & Jones 2002; Rahman et al. 2007).

Diabetic cardiomyopathy, a common cardiac dysfunction in diabetes, demonstrates compromised diastolic and neural dysfunction and requires timely attention and treatment (Akula et al. 2003; Davies 2000; Sowers, Epstein & Frohlich 2001). Moreover, diabetic cardiomyopathy potentiates atherosclerosis of the coronary arteries and increases the risk of autoimmune neuropathy playing an important role in the high occurrence of cardiovascular disease in diabetes (Chiquette & Chilton 2002).

Due to its nature as a metabolic disease, increased inflammation and oxidative stress have long been considered to be associated with diabetes. Diabetes enhanced the formation of advanced glycation end products (AGEs), increased production of reactive oxygen species (ROS), reduced nitric oxide (NO) synthesis, activation of protein kinase C (PKC) and renin-angiotensin system (RAS) (Brownlee 2001; Méndez et al. 2010). Different epidemiological studies demonstrated that hyperglycaemia is the single most important factor in the onset and progression of diabetic complications (Jakus & Rietbrock 2004; Méndez. et al. 2010). These metabolic and hemodynamic derangements contribute to the characteristic histopathological changes following chronic diabetes (Brownlee 2001; Méndez et al. 2010). It is also demonstrated that AGEs and advanced lipoxidation end-products (ALEs), accelerated micro- and macrovascular damage observed in diabetic patients (Jakus & Rietbrock 2004; Sebeková et al. 2002).

AGEs increase oxidative stress in vascular wall cells through an interaction with their receptors (RAGE) and subsequent activation of nuclear factor (NF)- κB pathways. Activation of transcription factor NF-κB leads to production of tumour necrosis factor (TNF), interleukin-1 (IL-1), and the induction of interleukin-6 (IL-6) mRNA expression thus plays an important role in vascular inflammation (Lin, Park & Lakatta 2009; Méndez et al. 2010). Evidence showed that RAGE is a key pathogenic factor involved in endothelial cell activation, vascular wall remodelling, and neointimal expansion in diabetic vascular disease as well as in atherosclerosis and arterial plaque formation (Méndez. et al. 2010).

Diabetic gastroparesis characterized by gastric emptying delay, diarrhoea, constipation and abdominal pain frequently occurs in many diabetic patients. These gastrointestinal changes are directly associated with diabetic neuropathy (Okada et al. 2009). Ultimately diabetes causes reduced motility of the gut by disrupting the balance between cholinergic and adrenergic signalling within the enteric nerves (Okada et al. 2009).

Stevia has been used since ancient times for the treatment of diabetes. Physicians in Paraguay were found to prescribe tea made with stevia for the treatment of hyperglycemia (Oveido et al. 1970; Melis 1995). Several successive studies in animal and humans, confirmed that stevia has blood glucose lowering and insulinotropic

effects (Curi et al. 1986; Jeppesen et al. 2000, 2002). Stevia reduced blood glucose levels in a dose dependent manner confirmed by the studies in alloxan-induced diabetic rats (Kujur et al. 2010) and on beta cells from the normal mouse (Jeppesen et al. 2000). Both stevioside and rebaudioside A, the two major diterpenoid glycosides of stevia, were found to have antihyperglycemic effects in diabetes (Kujur et al. 2010; Saravanan, Vengatash babu & Ramachandran 2012). Moreover, the diterpenoid glycosides present in stevia have anti-oxidant and antihypertensive properties and may improve cardiovascular function (Chan et al. 1998; Shukla et al. 2011).

Evidence showed that stevioside has calcium channel antagonist-like action in vascular tissues leading to a direct relaxation of blood vessels (Lee et al. 2001; Wong, et al. 2004b). However, the exact mechanism of action of stevia in modulation of vascular tone and blood pressure is still not completely established (Lee et al. 2001; Wong et al. 2004a).

Both animal and human studies suggest that stevia is capable of reducing inflammatory markers and improving the blood flow to the kidneys (Mizushina et al. 2005; Xu et al. 2008). Thus stevia shows potential beneficial effects in chronic disease states such as diabetes and hypertension. In addition, stevioside also showed anti-inflammatory and immunomodulatory activities which might also explain the beneficial effects of stevia in modulating the cardiovascular and metabolic complications (Boonkaewwan, Toskulkao & Vongsakul 2006; Chatsudthipong & Muanprasat 2009). Stevia was found to have gastro-protective effects against histamine-induced gastro-mucosal damage in rainbow trout and broiler chicks. Stevia also showed spasmolytic effects on guinea pig ileum indicating that stevia could be used as a gastromodulatory agent to improve diabetic gastroparesis.

Calcium channel blockers (CCBs) have pleiotropic effects on the vasculature, thereby restoring endothelial dysfunction and suppressing the progression of atherosclerosis both in experimental and clinical settings (Morimoto, Kureishi-Bando & Murohara 2010). CCBs showed vasculo-protective effects by enhancing endothelial NO production and/or normalising circulatory adiponectin and HDL levels (Morimoto, Kureishi-Bando & Murohara 2010; Sirmagül et al. 2007). Furthermore, CCBs increased circulating endothelial progenitor cells which play an important role in maintaining normal endothelial function (Morimoto, Kureishi-Bando & Murohara

2010; Sirmagül et al. 2007). Accordingly, these data suggest that CCBs have vasculo-protective action and can be used in patients with diabetes and hypertension.

It is also evident that pathological cardiac remodelling can be prevented by CCBs, however a reduction in heart size can be achieved by just reducing blood pressure (Afzal et al. 1988). This statement has been supported by the evidence that verapamil (2, 4, or 8 mg/kg) was found to show a preventive effect on diabetes-induced myocardial changes even at the lower dose without dramatically altering blood pressure. Verapamil also reversed the diabetes-induced alterations in myocardial high-energy phosphate stores and ultrastructural damage without affecting their hyperglycemic status (Afzal et al. 1988).

The steptozotocin-induced rat model (STZ) is the most widely used diabetic model which mimics the pathophysiology of the human disease (Wei et al. 2003). Recent studies have demonstrated that the STZ rat represents a typical characteristic of gastrointestinal dysfunction similar to human diabetes (Narimatsu et al. 2007; Shinbori et al. 2006). The STZ-induced diabetic rat model demonstrates a reliable approximation of human diabetes along with left ventricular hypertrophy, diastolic dysfunction, retinopathy and neuropathy (Akula et al. 2003; Loganathan et al. 2006). This characteristic develops without hypertension and is directly related to a form of diabetic cardiomyopathy. However, unlike human diabetes, STZ rats do not develop hypertension and atherosclerosis; rather they remain normotensive at least over a 24-week study period (Wei et al. 2003). Therefore STZ-induced diabetes is an excellent model to examine the complications of diabetes in isolation without co-variables such as elevated blood pressure and atherosclerosis (Wei et al. 2003). This allows for changes in blood glucose, oxidative stress and inflammation to be fully assessed and to establish if potential treatments mitigate these processes. The development of evident nephropathy is exceedingly slow in STZ-induced diabetic rat models, although increase in albuminuria, mesangial matrix expansion and other substitute end points are observed earlier (Bidani et al. 2007).

Thus the current study used STZ-induced diabetic rat models to investigate the extent of the cardiovascular and end organ damage following 8 weeks of STZ-induced diabetes and to unravel the preventive effects of stevia and verapamil in these pathophysiological conditions.

6.2. Methods

A total of total of 50 male streptozotocin-induced diabetic rats (STZ) and 50 Wistar (control) rats were used in this study under the approval from CQUniversity Australia's Animal Research Ethics Committee (ethics number #A06-190).

Male Wistar animals were obtained at 8 weeks age from the animal resource centre in Western Australia and housed in the CQUniversity animal house facility and sacrificed at the 8-week time point following the 2-month treatment protocol. The treated rats received stevia (200 mg/kg/day) or verapamil (4 mg/kg/day) from 0 to 8 weeks of experimental period. Experimental groups were:-

- Wistar untreated control
- Wistar control treated with stevia
- Wistar treated with verapamil
- STZ untreated disease model
- STZ disease model treated with stevia
- STZ disease model treated with verapamil

Animals of each species were randomly assigned to either treatment (stevia or verapamil) or non-treatment groups. Systolic blood pressure, heart rate and development of tactile allodynia were measured in the selected animals by the tail-cuff method (Chapter 3, sections 3.7.1, 3.7.2 and 3.7.3). Cardiac structure and function was assessed by the isolated Langendorff technique (Chapter 3, section 3.7.8.), electrophysiological measurement and recording of single-cell microelectrode experiments (Chapter 3, section 3.7.7.) and terminal organ weights as discussed in Chapter 3, section 3.7.4. During terminal experiments, plasma was taken to measure serum nitrate/nitrite level (Chapter 3, section 3.7.5; *B*), lipid peroxidation (Chapter 3, section 3.7.5; *A*), total antioxidant capacity (Chapter 3, serum 3.7.5; *C*) and serum IL-6 levels (Chapter 3, section 3.7.5; *D*). Vascular responses to noradrenaline, acetylcholine and sodium nitroprusside were measured for thoracic aortic rings (Chapter 3, section 3.7.6) and for mesenteric arteries (Chapter 3, section 3.7.6). The function of isolated

ileum to electrical field stimulation (Chapter 3, section 3.7.9.), carbachol (Chapter 3, section 3.7.9.) and pilocarpine (Chapter 3, section 3.7.9.) were also assessed.

All results are presented as mean ± SEM. The results were analysed using two-way ANOVA with Bonferroni's Multiple Comparison Test. A Student's t-test was used to compare and evaluate two group means via paired/ unpaired where appropriate. All standard curves were formulated using Graphpad Prism 4.0 to create a four prameter logic curve from which unknown values were determined. A p value of less than 0.05 was considered significant. *$p<0.05$ vs control and **$p<0.05$ vs STZ were considered statistically significant.

6.3. Results

Eight weeks post streptozotocin administration led to significant manifestation of diabetes characterized by increased plasma glucose concentration, greater water intake and loss of body weight ($P<0.05$ vs control) (Table 6.1, and Figures 6.1, 6.2). Eight weeks of stevia treatment significantly improved all of these parameters ($P<0.05$ vs STZ) in the diabetic rats (Table 6.1, and Figures 6.1, 6.2). However, eight weeks of verapamil treatment only partially prevented the increased water intake and plasma glucose concentrations in the diabetic rats (Table 6.1, and Figure 6.2). Body weights were slightly increased (values are not significant) in the STZ animals following eight weeks of verapamil treatment (Figures 6.1). Stevia and verapamil treatment did not show any differences in body weights, water intake and plasma glucose levels in the control rats compared to the untreated age-matched control animals (Table 6.1, and Figures 6.1, 6.2).

No significant change in blood pressure was observed across any of the treatment groups except for the untreated diabetic rats which showed a slight but significant increase in blood pressure at the end of the 8-week experiment (Figure 6.3).

The STZ-diabetic animals demonstrated a significant increase in the terminal organs weights relative to body weight and in tibial length relative to body weight compared to the age-matched control rats (Table 6.1). Particularly, the STZ-animals demonstrated evidence of left ventricular hypertrophy characterized by a significant increase in the weight of the left ventricle relative to body weight and tibial length ratios (Table 6.1). However, the diabetic rats' right ventricle showed an insignificant increase in weight relative to body weight and tibial length ratios (Table 6.1). Stevia treatment prevented the increase in weights of both left and right ventricles in the diabetic animals but did not show any effects on these organ weights in the control rats (Table 6.1).

Verapamil treatment prevented left ventricular hypertrophy observed in diabetes by significantly reducing left ventricular weight ($P<0.05$ vs STZ) compared to tibial length (Table 6.1). Verapamil also prevented the increased liver weights normalized to body weights in the diabetic rats. Verapamil did not show any significant changes on the organ weights in the control groups (Table 6.1). Mean tibial lengths were markedly

reduced in the diabetic groups compared to the controls rats (Table 6.1). Both stevia and verapamil treatment normalized the tibial lengths in the diabetic groups (Table 6.1).

Terminal weights of the kidney (significant; $P<0.05$ vs control) , liver (significant; $P<0.05$ vs control) and spleen (not significant) were all found to be elevated in the STZ rats compared to the Wistar control animals (Table 6.1). Stevia treatment significantly reduced liver and kidney weights ($P<0.05$ vs STZ) in the diabetic animals (Table 6.1). Stevia treatment showed no significant change in liver, kidney and spleen weights in the controls (Table 6.1). Verapamil treatment prevented the increased liver and kidney weights in the STZ animals (Table 6.1). Verapamil showed no significant change in liver, kidney and spleen weights in the control rats (Table 6.1).

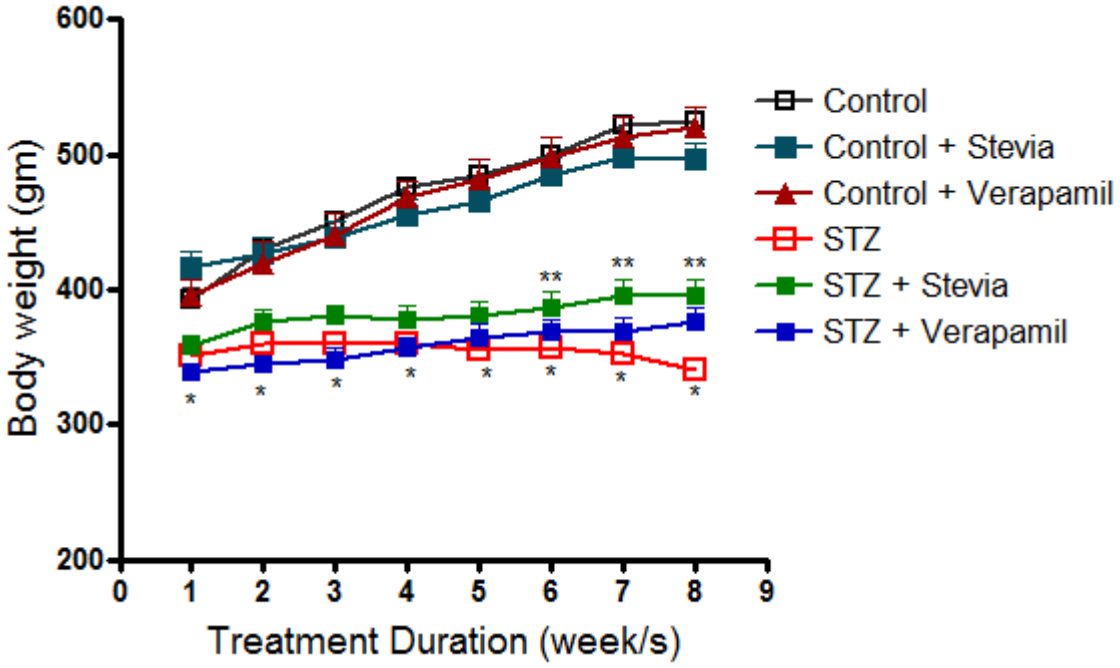


Figure 6.1: Weekly body weight for control, control + stevia, control + verapamil, streptozotocin (STZ)- diabetic rats and STZ + stevia (200 mg/kg/day, p.o.) and STZ+ verapamil (4 mg/kg/day, p.o.). Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs control and $^{\Delta,}$ **P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests).

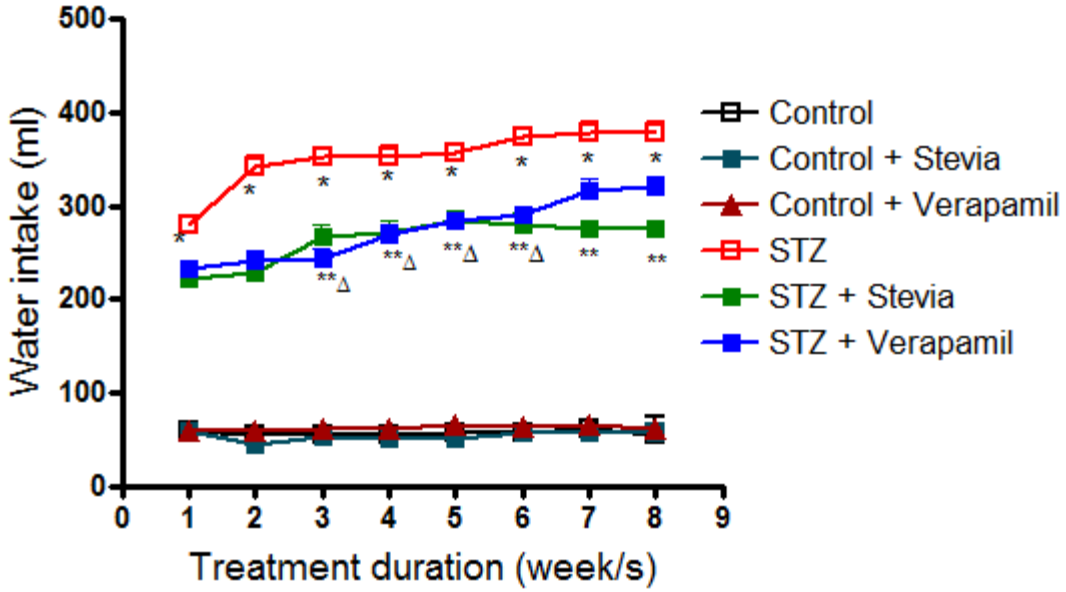


Figure 6.2: Weekly water intake for control, control + stevia, streptozotocin (STZ)- diabetic rats, STZ + stevia (200 mg/kg/day, p.o.) and STZ+ verapamil (4 mg/kg/day, p.o.). Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs control; $^{\Delta,}$**P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests).

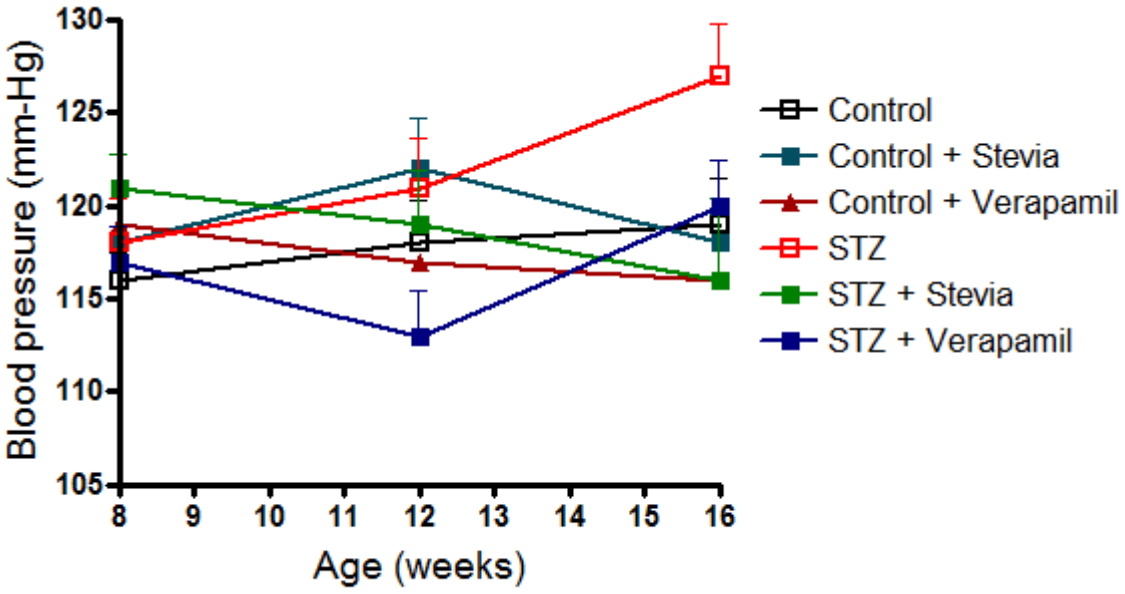


Figure 6.3: Weekly blood pressure for control, control + stevia, streptozotocin (STZ)-diabetic rats, STZ + stevia (200 mg/kg/day, p.o.) and STZ+ verapamil (4 mg/kg/day, p.o.). Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs control; $^{\Delta,}$ **P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests).

Table 6.1: Comparison of biometric parameters

Parameter	Control	Control + Stevia	Control + Verapamil	STZ	STZ + Stevia	STZ + Verapamil
Blood glucose (mmol/l)	10.5 ± 1.2	9.7 ± 1.2	9.4 ± 1.4	32.0 ± 1.0*	22.2 ± 1.7**	27.8 ± 2.2
LV wet weight (mg/g body weight)	1.8 ± 0.10	1.6 ± 0.1	1.4 ± 0.12	2.7 ± 0.1	1.9 ± 0.1	1.9 ± 0.05
LV wet weight (mg/mm tibial length)	28.3 ± 1.6	23.9 ± 1.6	23.0 ± 1.5	31.2 ± 1.9	18.7 ± 1.6**	20.1 ± 1.4$^{\Delta}$
RV wet weight (mg/g body weight)	0.39 ± 0.03	0.38 ± 0.05	0.39 ± 0.05	0.57 ± 0.06	0.39 ± 0.03	0.41 ± 0.03
RV wet weight (mg/mm tibial length)	5.5 ± 0.5	5.2 ± 0.8	6.4 ± 0.6	7.4 ± 0.4	3.9 ± 0.6**	4.4 ± 0.4
Liver wet weight (mg/g body weight)	29.6 ± 1.4	27.5 ± 1.9	28.3 ± 0.63	46.0 ± 1.7*	37.4 ± 1.1**	38.8 ± 0.9$^{\Delta}$
Spleen wet weight (mg/g body weight)	2.2 ± 0.1	1.6 ± 0.2	1.2 ± 0.08	2.6 ± 0.3	2.0 ± 0.2	2.1 ± 0.08
Kidney wet weight (mg/g body weight)	5.9 ± 0.3	4.7 ± 0.20	4.0 ± 0.2	11.5 ± 0.5*	7.9 ± 0.2**	8.4 ± 0.3$^{\Delta}$
Tibial length (mm)	33.1 ± 0.5	33.4 ± 2.5	30.3 ± 1.8	31.2 ± 1.3	38.2 ± 0.3**	39.6 ± 2.1$^{\Delta}$

Control and streptozotocin (STZ)-diabetic rats after 8-week treatment with stevia (200 mg/kg/day, p.o.) and verapamil (4 mg/kg/day, p.o.). Results are presented as means ± SEM. LV= Left ventricle, RV= Right ventricle. n=13 for all groups. *P<0.05 vs control and $^{\Delta,}$**P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests).

In the diabetic groups, tactile allodynia developed after one week of STZ administration and continued until end of the experiment (Figure 6.4). Stevia significantly reduced tactile allodynia by increasing the pain threshold levels in the diabetic rats compared to the untreated STZ animals after 8 weeks of treatment (Figure 6.4). No change in pain threshold levels were observed in the control rats (Figure 6.4). Interestingly, verapamil administration showed a significant improvement in tactile allodynia in the diabetic rats from the first week of dosing and the effect was comparable to that of stevia (Figure 6.4). Verapamil did not show any change in pain threshold levels in the control animals (Figure 6.4).

Heart rate (HR) was measured to determine any changes in autonomic function of the cardiac cycle (Figure 6.5). Heart rates were found to be significantly lowered in diabetic rats at 12 weeks of age with a further decrease in heart rate at 16 weeks of age compared to the controls (Figure 6.5). Stevia treatment partially normalized HR (values are significant; $P<0.05$ vs STZ) in the diabetic animals (Figure 6.5) but did not show any effects on the control animals (Figure 6.5). Verapamil also prevented the decrease in heart in the diabetic rats at the end of eight week of treatment (Figure 6.5). Heart rate variability (HRV) was found to be significantly lower in diabetic rats (Figure 6.6). Both stevia and verapamil treatment significantly enhanced HRV in the diabetic animals (Figure 6.6).

Isolated Langendorff hearts from the diabetic rats demonstrated an initiation of left ventricular fibrosis manifested by a significant increase in diastolic stiffness of the diabetic hearts (Table 6.2). Increased stiffness of the heart muscle represented a decrease in left ventricular compliance indicating an increase in left ventricular fibrosis in the diabetic rats. Treatment with both stevia and verapamil showed a reduction in diastolic stiffness compared to the untreated diabetic rats (Table 6.2). In addition to increased diastolic stiffness, diabetic hearts also showed a significant decrease in maximum force of contraction and relaxation and reduced end systolic pressures compared to the control animals (Table 6.2). Stevia treatment increased maximal rates of contraction and relaxation in both diabetic and control animals (Table 6.2). Stevia also normalized end systolic pressures and developed pressures ($p<0.05$) in diabetic treated animals (Table 6.2) compared to the untreated controls. Stevia treatment slightly decreased but could not normalize the increased diastolic

stiffness in diabetes (Table 6.2). Treatment with verapamil reduced diastolic stiffness and improved cardiac function compared to untreated diabetic rats (Table 6.2). We also measured mean coronary blood flow normalized to ventricular weight in the treated and untreated diabetic rats (Figure 6.7). Our results showed that development and progression of diabetes significantly reduced mean coronary blood flow indicating a marked reduction in the coronary vasodilator reserve following diabetes (Figure 6.7). After 8 weeks of stevia treatment, coronary blood flow significantly improved in the STZ animals (Figure 6.7). Dosing with verapamil normalized the coronary blood flow in the diabetic rats (Figure 6.7). Coronary blood flow in the control groups remained unchanged irrespective of stevia and verapamil treatment by 16 weeks of age (Figure 6.7).

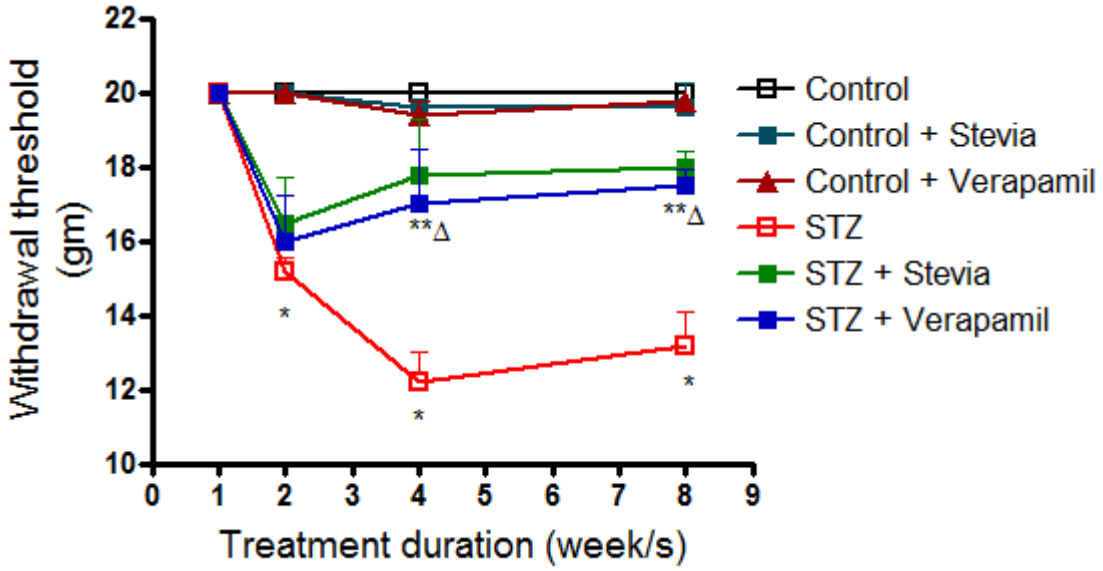


Figure 6.4: Von Frey measurement for tactile allodyna in control, control + stevia, streptozotocin (STZ) - diabetic rats and STZ + stevia (200 mg/kg/day, p.o.) and STZ + verapamil (4 mg/kg/day, p.o.). Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs control; $^{\Delta,}$**P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests).

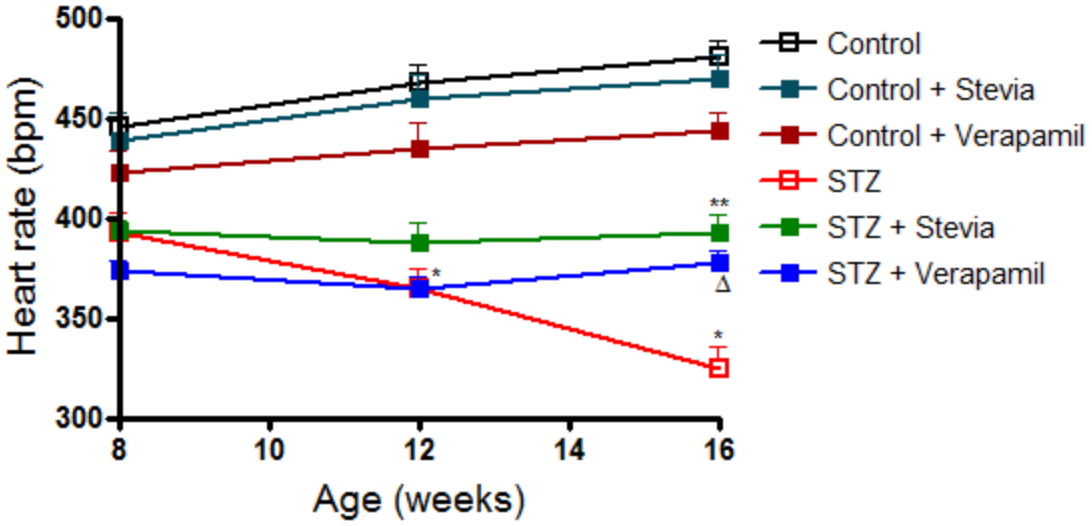


Figure 6.5: Weekly heart rate measurement for control, control + stevia, streptozotocin (STZ)- diabetic rats, STZ + stevia (200 mg/kg/day, p.o.) and STZ + verapamil (4 mg/kg/day, p.o.). Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs control; $^{\Delta,}$**P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests).

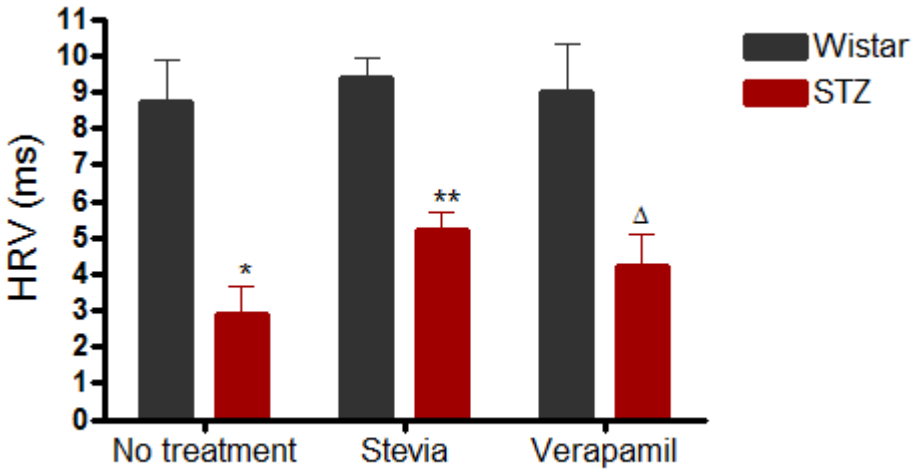


Figure 6.6: Heart rate variability measurement for control, control + stevia, streptozotocin (STZ)- diabetic rats, STZ + stevia (200 mg/kg/day, p.o.) and STZ + verapamil (4 mg/kg/day, p.o.). Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs control; $^{\Delta,}$**P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests).

Table 6.2: Comparison of functional parameters in isolated hearts

Parameter	Control	Control + Stevia	Control + Verapamil	STZ	STZ + Stevia	STZ + Verapamil
Diastolic stiffness	22.8 ± 0.7	23.8 ± 0.7	24.6 ± 0.6	26.6 ± 0.9*	24.6 ± 0.9	24.1 ± 0.8
Developed pressure (mmHg)	107 ± 4.4	109 ± 0.6	107 ± 2.6	76 ± 0.6 *	111 ± 2.8**	115 ± 1.8$^{\Delta}$
Maximum +dP/dt (mmHg/sec)	1926 ± 92	1907 ± 131	1906 ± 60	1723 ± 140.	1982 ± 59	2072 ± 41
Maximum -dP/dt (mmHg/sec)	-1354 ± 62	-1405 ± 83	-1428 ± 33	-1098 ± 115*	-1326 ± 45**	-1478 ± 45$^{\Delta}$
End systolic pressure (mmHg)	116 ± 4.4	114 ± 7.5	116 ± 3.7	87 ± 7.3*	121 ± 3**	127 ± 1.6$^{\Delta}$

Control and streptozotocin (STZ)-diabetic rats after 8-week treatment with stevia (200 mg/kg/day, p.o.) and verapamil (4 mg/kg/day, p.o.). Results are presented as means ± SEM. n=8-10 for all groups. *P<0.05 vs control and $^{\Delta,}$**P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests).

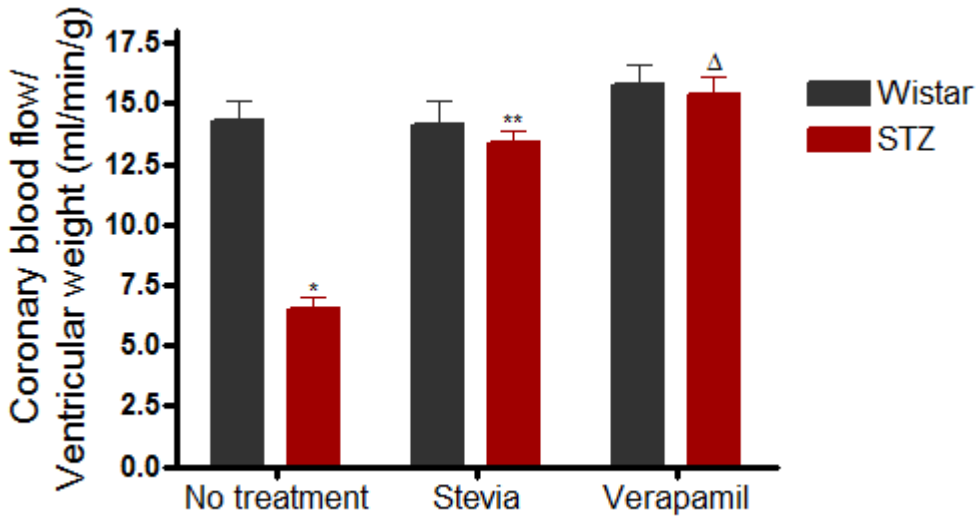


Figure 6.7: Coronary blood flow (CBF) normalized to ventricular weight (VW) after 8-week treatment with stevia (200 mg/kg/day, p.o.) and verapamil (4 mg/kg/day, p.o.) for control and streptozotocin (STZ)-diabetic rats. Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs control; $^{\Delta,}$**P<0.05 vs STZ and ΔP<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests).

Electrophysiological recordings of left ventricular papillary muscle from the diabetic rats, demonstrated a reduction in depolarised resting membrane potential (Table 6.3). Treatment with stevia improved the resting membrane potential in STZ diabetic rats (Table 6.3). Verapamil did not show any improvement in the resting membrane potential in the diabetic rats (Table 6.3). Eight weeks of untreated diabetes led to a significant prolongation of action potential duration at 20%, 50%, and 90% of repolarisation along with an increased force of contraction in the isolated papillary muscle (Table 6.3). Stevia significantly prevented the prolongation of action potential duration at 20%, 50% and 90% of repolarisation (Table 6.3). The Ca^{2+} channel antagonist, verapamil, prevented the prolongations of action potential duration at 20%, 50% and 90% of repolarisation and reduced the increased force of contraction in the diabetic rats. Verapamil demonstrated more promising effects on the electrophysiological parameters in the diabetic heart such as reduction in force of contraction and APD compared to stevia (Table 6.3). Dosing with stevia and verapamil did not alter any of these parameters in the control animals (Table 6.3).

Table 6.3: Comparison of electrophysiological parameters

Parameters	Control	Control + stevia	Control + verapamil	STZ	STZ + stevia	STZ + verapamil
Resting membrane potential (mV)	-67.0 ± 1.6	-69.2 ± 1.5	-66.0 ± 1.2	-65.2 ± 2.4	-72.2 ± 2.5	-64.3 ± 3.1
Action potential amplitude (mV)	65.2 ± 3.1	68.5 ± 2.8	64.6 ± 2.7	73.2 ± 2.8	73.6 ± 2.2	69.3 ±3.3
Action potential duration at 20% of repolarisation (msec)	14.1 ± 0.6	17.4 ± 0.5	15.5 ± 0.6	27.0 ± 2.3*	20.5 ± 1.5**	20.3 ± 1.8$^{\Delta}$
Action potential duration at 50% of repolarisation (msec)	24.4 ± 1.3	29.8 ± 1.3	25.3 ± 2.2	60.1 ± 8.1*	39.3 ± 4.6**	34.4 ± 5.8$^{\Delta}$
Action potential duration at 90% of repolarisation (msec)	57.0 ± 1.8	64.8 ± 6.2	56.2 ± 5.1	137.3 ±11.0*	107.9± 7.3**	92.1 ± 11.8$^{\Delta}$
Force of contraction (mN)	2.9 ± 0.2	4.7 ± 0.9	2.5 ± 0.22	4.0 ± .09	5.4 ± 0.5	3.3 ± 1.9
Time to 90% relaxation of force (ms)	159.7 ± 12.9	153.4 ± 13.5	119.1 ± 4.3	129 ± 4.3*	128 ± 4.2	136.4 ± 16.0

Control and streptozotocin (STZ)-diabetic rats after 8-week treatment with stevia (200 mg/kg/day, p.o.) and verapamil (4 mg/kg/day, p.o.). Results are presented as means ± SEM. n=8-10 for all groups. *P<0.05 vs control and $^{\Delta}$,**P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests).

Eight weeks of diabetes induced a maladaptive functional change in vascular beds observed by vascular responses in the isolated aortic ring preparation. Diabetes caused a significantly greater response to noradrenaline in aortic tissues from the diabetic rats compared to the control groups (Figure 6.8). This was in contrast to the dilator responses whereby endothelium dependant relaxation to acetylcholine and endothelium independent relaxation to sodium nitroprusside were reduced in isolated aortic tissues from the diabetic rats (Figures 6.10, 6.12). Moreover, a decreased responsiveness of thoracic aorta to acetylcholine in the diabetic rats was observed by a reduction in R_{max} without an increase in acetylcholine potency (-log EC_{50}) (Table 6.5). Eight weeks of stevia administration showed improvement in theses contraction

and relaxation responses and thereby prevented the altered vascular function of the isolated aortic rings induced by diabetes (figures 6.8, 6.10, 6.12). Verapamil showed a significant increase in contractile response to noradrenaline compared to both the untreated diabetic rats and control animals (Figure 6.9, 6.11, 6.13). Dosing with verapamil caused a marked decrease in endothelium-dependent relaxation to acetylcholine and endothelium-independent relaxation to sodium nitroprusside (Figures 6.11, 6.13). The responses of verapamil treated group were greater than those of stevia in the aorta of the diabetic rats (Figures 6.8, 6.9, 6.10, 6.11, 6.12, 6.13).

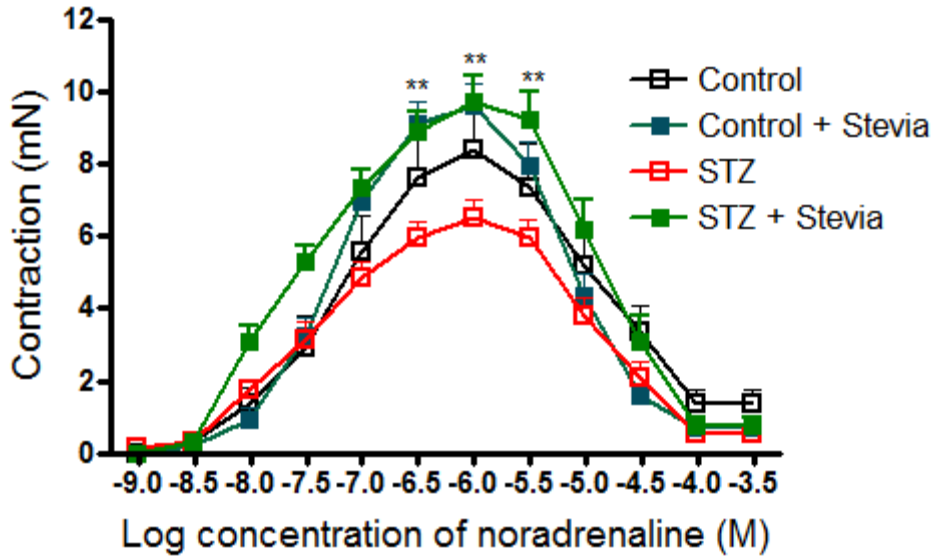


Figure 6.8: Cumulative-concentration contractile response to noradrenaline in isolated thoracic aortic rings from control, control + stevia, streptozotocin (STZ)- diabetic rats and STZ + stevia (200 mg/kg/day, p.o.). Results are presented as means ± SEM. n=13 for all groups. *P<0.05 vs control and $^{\Delta}$**P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests).

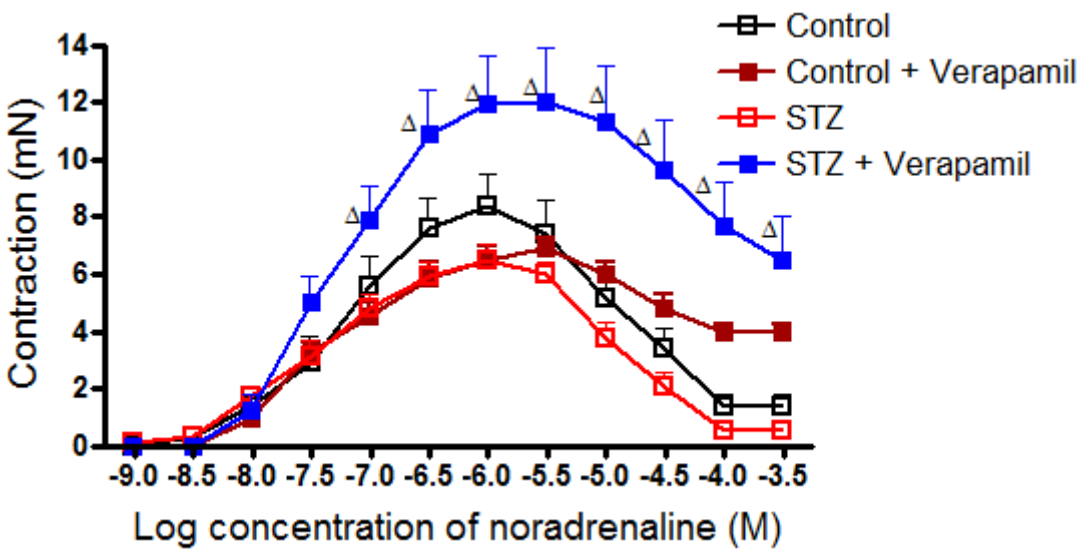


Figure 6.9: Cumulative-concentration contractile response to noradrenaline in isolated thoracic aortic rings from control, control + verapamil, streptozotocin (STZ)-diabetic rats and STZ + verapamil (4 mg/kg/day, p.o.). Results are presented as means ± SEM. n=13 for all groups. *P<0.05 vs control and $^{\Delta}$**P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests).

Table 6.4: Maximum responses and –Log EC_{50} values for noradrenaline in the isolated aorta

Parameters	Control	Control + Stevia	Control + Verapamil	STZ	STZ + Stevia	STZ + Verapamil
-Log EC_{50}	6.6 ± 0.2	6.3 ± 0.08	7.0 ± 0.1	7.4 ± 0.09	6.8 ± 0.1	6.8 ± 0.1
R_{max} (% to noradrenaline)	91 ± 4.3	98 ± 0.9	98 ± 2.2	95 ± 0.3	98 ± 2.0	97 ± 1.1

Control and streptozotocin (STZ)-diabetic rats after 8-week treatment with stevia (200 mg/kg/day, p.o.) and verapamil (4 mg/kg/day, p.o.). Results are presented as means ± SEM. n=10 for all groups. *P<0.05 vs Control and $^{\Delta}$**P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests)

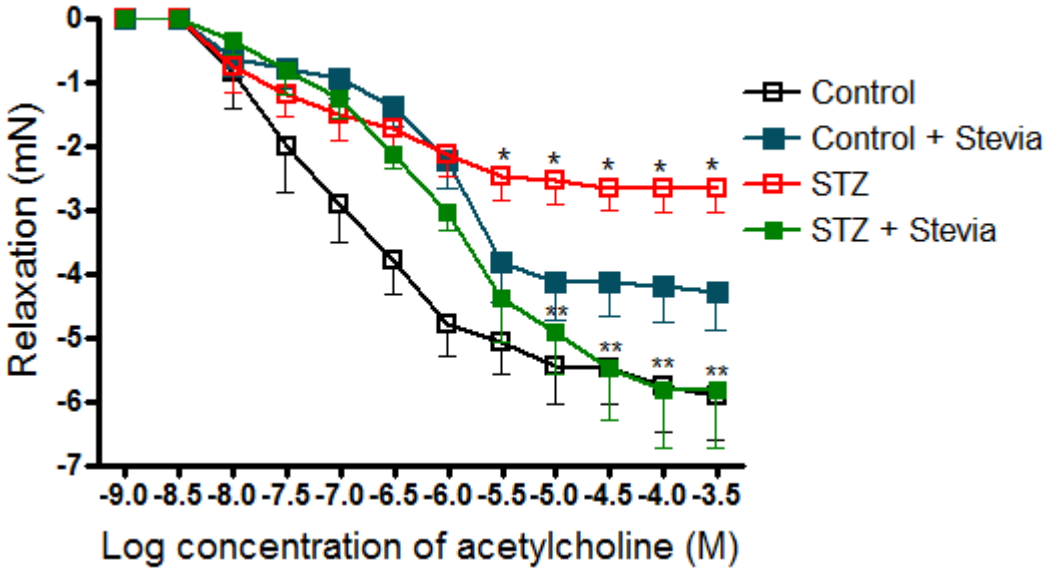


Figure 6.10: Endothelium-dependent relaxation by acetylcholine of noradrenaline-precontracted thoracic aortic preparations from control, control +stevia, streptozotocin (STZ)-diabetic rats and STZ + stevia(4 mg/kg/day, p.o.). Results are presented as means ± SEM. n=13 for all groups. *P<0.05 vs control and $^{\Delta}$,**P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons test).

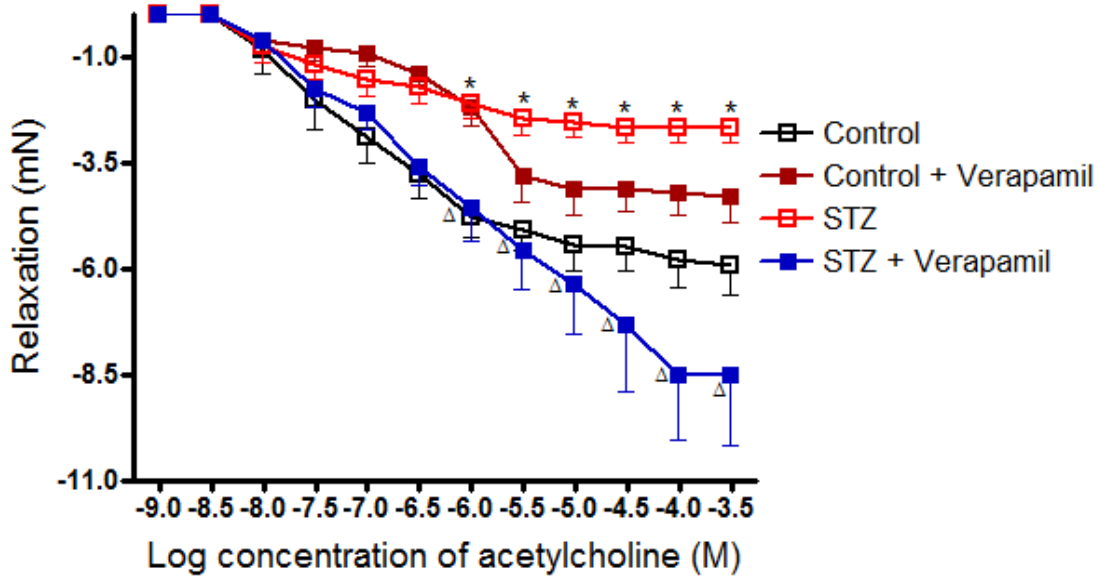


Figure 6.11: Endothelium-dependent relaxation by acetylcholine of noradrenaline-precontracted thoracic aortic preparations from control, control + verapamil, streptozotocin (STZ)- diabetic rats and STZ + verapamil (4 mg/kg/day, p.o.). Results are presented as means ± SEM. n=13 for all groups. *P<0.05 vs control and $^{\Delta}$,**P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests).

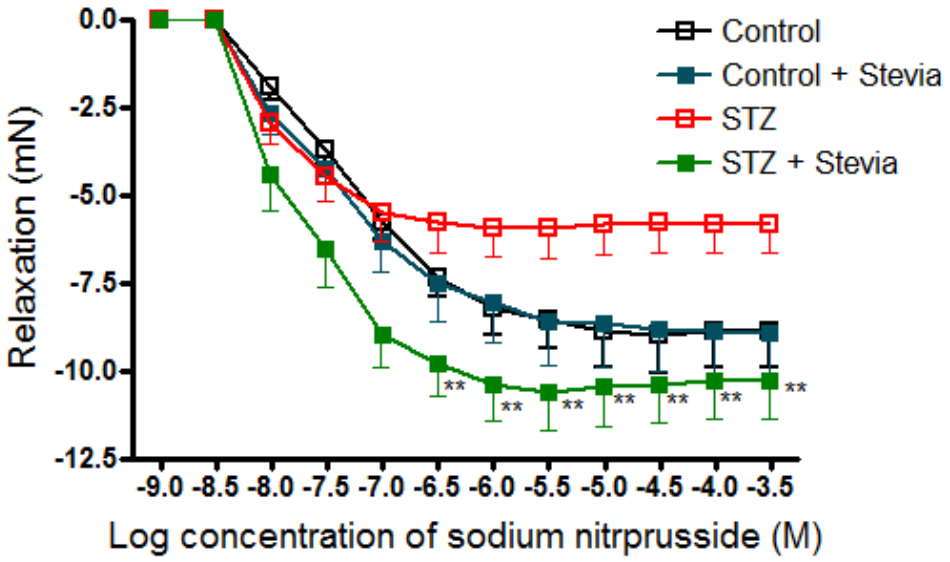


Figure 6.12: Endothelium-independent relaxation by sodium nitroprusside of noradrenaline precontracted thoracic aortic preparations from control, control + stevia, streptozotocin (STZ) - diabetic rats, STZ + stevia (200 mg/kg/day, p.o.). Results are presented as means ± SEM. n=13 for all groups. *P<0.05 vs control and $^{\Delta}$**P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests).

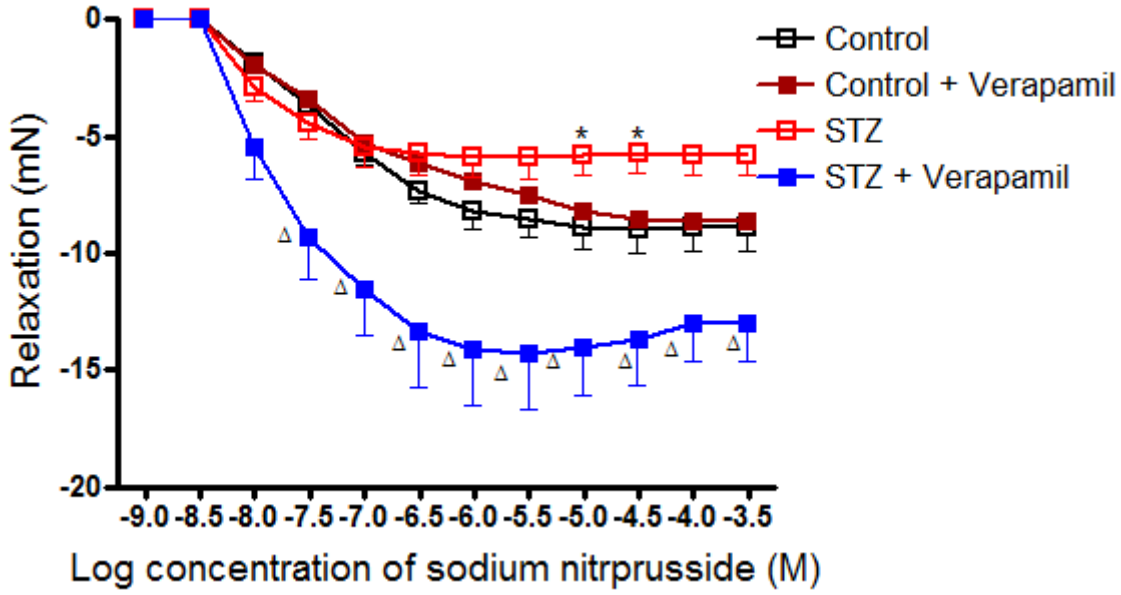


Figure 6.13: Endothelium-independent relaxation by sodium nitroprusside of noradrenaline precontracted thoracic aortic preparations from control, control + verapamil, streptozotocin (STZ) - diabetic rats and STZ + verapamil (4 mg/kg/day, p.o.). Results are presented as means ± SEM. n=13 for all groups. *P<0.05 vs control and $^{\Delta}$**P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests).

Table 6.5: Maximum responses and –log EC_{50} values for acetylcholine in the isolated aorta

Parameters	Control	Control + Stevia	Control + Verapamil	STZ	STZ + Stevia	STZ + Verapamil
-Log EC_{50}	6.3 ± 0.2	5.9 ± 0.1	5.8 ± 0.1	5.9 ± 0.2	5.9 ± 0.2	5.8 ± 0.1
R_{max} (% to acetylcholine)	96 ± 2.2	94 ± 5.6	94 ± 6.6	85 ± 5.9*	97 ± 2.1**	100 ± 0.0$^{\Delta}$

Control and streptozotocin (STZ)-diabetic rats after 8-week treatment with stevia (200 mg/kg/day, p.o.) and verapamil (4 mg/kg/day, p.o.). Results are presented as means ± SEM. n=10 for all groups. *P<0.05 vs Control and $^{\Delta}$,**P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests)

In small resistance arteries, diabetes reduced contractile response to noradrenaline (Figure 6.14). Eight weeks of stevia treatment caused a significant increase in the contraction to noradrenaline in mesenteric arteries (Figure 6.14). Treatment with verapamil normalised the reduced contractile response of the mesenteric arteries to noradrenaline in the diabetic rats at the eight-week treatment point (Figure 6.15). Relaxant responses to acetylcholine and sodium nitroprusside were also reduced in the diabetic groups compared to the age-matched control animals (Figures 6.16, 6.18). Once again both stevia and verapamil prevented the maladaptive changes of the small arteries induced by diabetes (Figures 6.14, 6.15, 6.16, 6.17, 6.18, 6.19). The vasculo-endothelial dysfunction was supported by a significant reduction in serum nitrate levels and serum nitrite/nitrate ratios that we observed after 8-weeks post STZ administration (Figure 6.20). Stevia treatment significantly increased serum nitrate levels in the diabetic groups compared to the control rats (Figure 6.20). Verapamil showed a small but significant increase in serum nitrate levels in the STZ-animals (Figure 6.20). In this study, stevia was found to have more prominent effects in preventing the reduced NO synthesis than verapamil following 8-weeks of diabetes (Figure 6.20).

Quantitative measurement of serum malondialdehyde (MDA) levels acts as an indicator of lipid peroxidation and oxidative stress. Chronic diabetes showed a significant increase in serum MDA levels compared to the control rats (Table 6.6). Stevia treatment reduced plasma malondialdehyde levels in the STZ rats (Table 6.6).

Interestingly verapamil treatment demonstrated a significant reduction in serum MDA levels in STZ rats whereas a slight increase in MDA levels was observed in the control rats (Table 6.6). In addition to the reduced MDA levels, the STZ rats demonstrated increased levels of inflammation and oxidative stress as measured by enhanced IL-6 concentrations and decreased total antioxiadant capacity (TAC) at 16 weeks of age compared to the controls (Table 6.6). Diabetes caused a marked increase in serum IL-6 levels compared to the age-matched Wistar rats (Table 6.6). Dosing with both stevia and verapamil significantly reduced the concentration of IL-6 in the diabetic rats. Stevia treatment normalized total antioxidant capacity in the STZ rats (Table 6.6). Verapamil also increased the total antioxidant capacity in the diabetic group (Table 6.6).

Reduced gastrointestinal motility and diabetic gastroparesis are two major complications of diabetes. In the isolated ileum, electrical field stimulation (EFS) caused frequency-dependent contraction of the smooth muscle (Figures 6.21, 6.22). In our study, ileum tissues from the diabetic rats showed a significant decrease in the contractile responses to EFS compared to the control animals reflecting reduced nerve function in diabetes (Figures 6.21, 6.22). Treatment with stevia partially normalized the contractile responses indicating stevia has the ability to improve altered nerve function in diabetes (Figure 6.21). Verapamil showed only a slight improvement in the contractile response to EFS (Figure 6.22).

Pilocarpine, a muscarinic receptor agonist inhibited EFS-mediated contraction in the gastrointestinal smooth muscle characterising muscarinic M_2-receptors function (Figure 6.23, 6.24). Diabetes caused a left-shift of the concentration-response curve to pilocarpine (Figure 6.23, 6.24). This result represented an augmentation in the M_2 receptors response to pilocarpine. In stevia-treated rats, significant improvement was observed in the inhibition of pilocarpine concentration-response curve suggesting stevia can prevent the increased M_2 receptor response in diabetes (Figure 6.23). On the other hand, verapamil-treated rats showed a small shifting of the pilocarpine-response curve to left compared to the untreated diabetic rats (figure 6.24) indicating verapamil has little or no effects on increased M_2-receptor function following diabetes. Moreover, carbachol induced contraction were significantly reduced in the diabetic rats compared to the control animals (Figure 6.25, 6.26). Treatment with stevia

restored the carbachol-induced contraction back to normal (Figure 6.25). Whereas, verapamil demonstrated only a little improvement in the contractile response to carbachol (Figure 6.26) compared to the untreated diabetic rats.

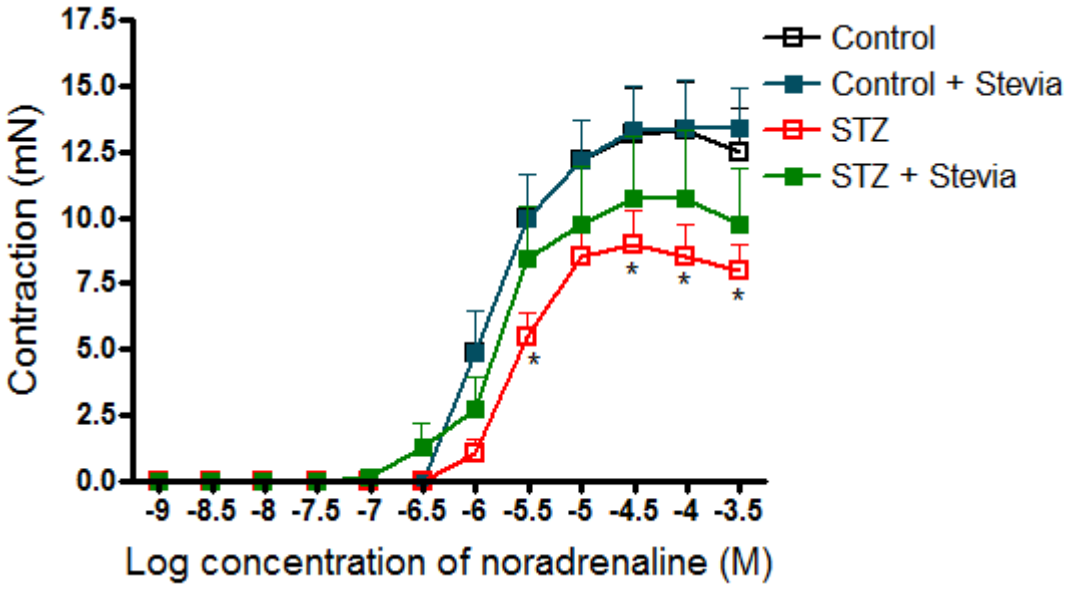


Figure 6.14: Cumulative-concentration contractile response to noradrenaline in isolated mesenteric arteries from control, control + stevia, streptozotocin (STZ) - diabetic rats, STZ + stevia (200 mg/kg/day, p.o.). Results are presented as means ± SEM. n=13 for all groups. *P<0.05 vs control and $^{\Delta}$**P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests).

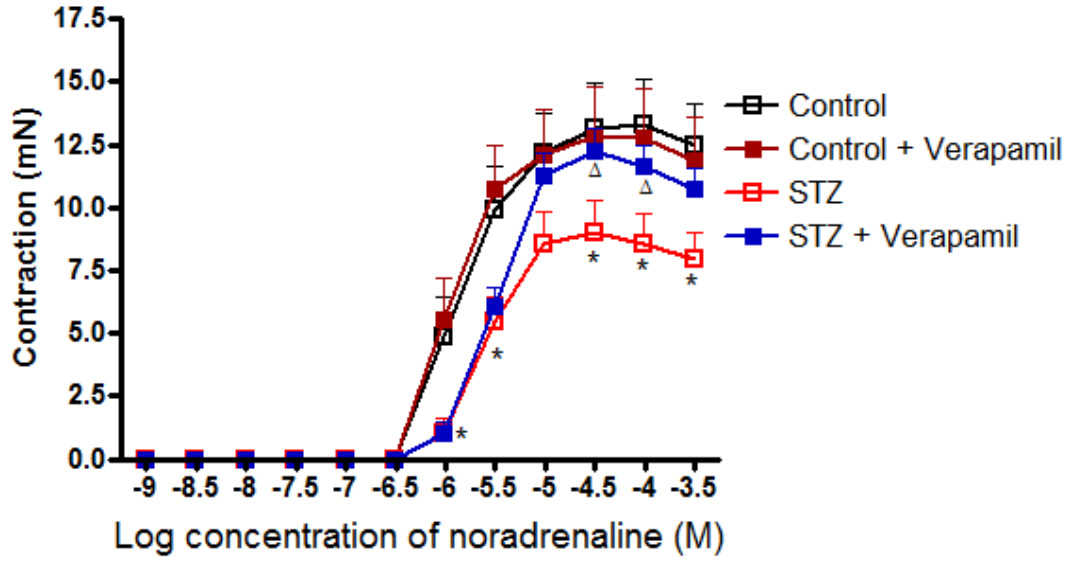


Figure 6.15: Cumulative-concentration contractile response to noradrenaline in isolated mesenteric arteries from control, control + verapamil, streptozotocin (STZ)-diabetic rats and STZ+ verapamil (4 mg/kg/day, p.o.). Results are presented as means ± SEM. n=13 for all groups. *P<0.05 vs control and $^{\Delta}$**P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests).

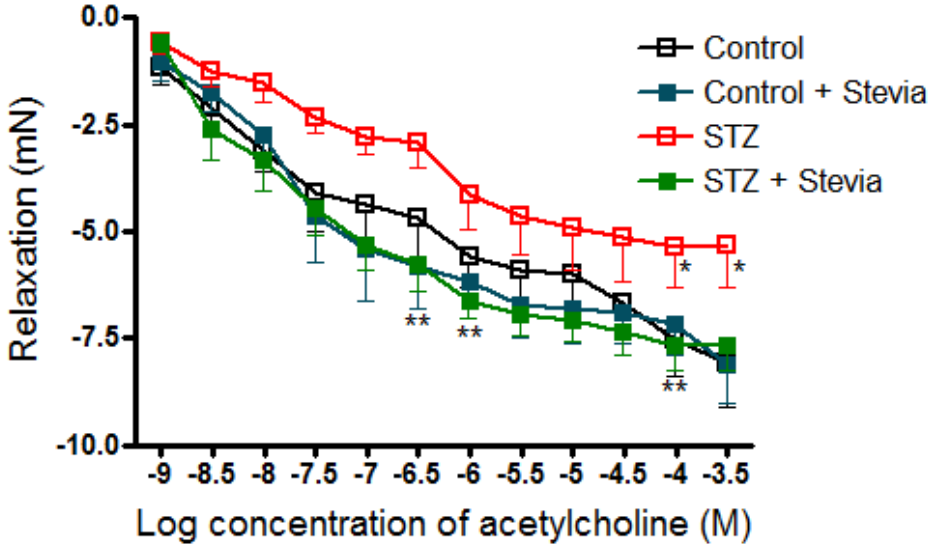


Figure 6.16: Endothelium-dependent relaxation by acetylcholine of noradrenaline precontracted mesenteric arteries from control, control + stevia, streptozotocin (STZ)-diabetic rats, STZ + stevia (200 mg/kg/day, p.o.). Results are presented as means ± SEM. n=13 for all groups. *P<0.05 vs control and $^{\Delta}$,**P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests).

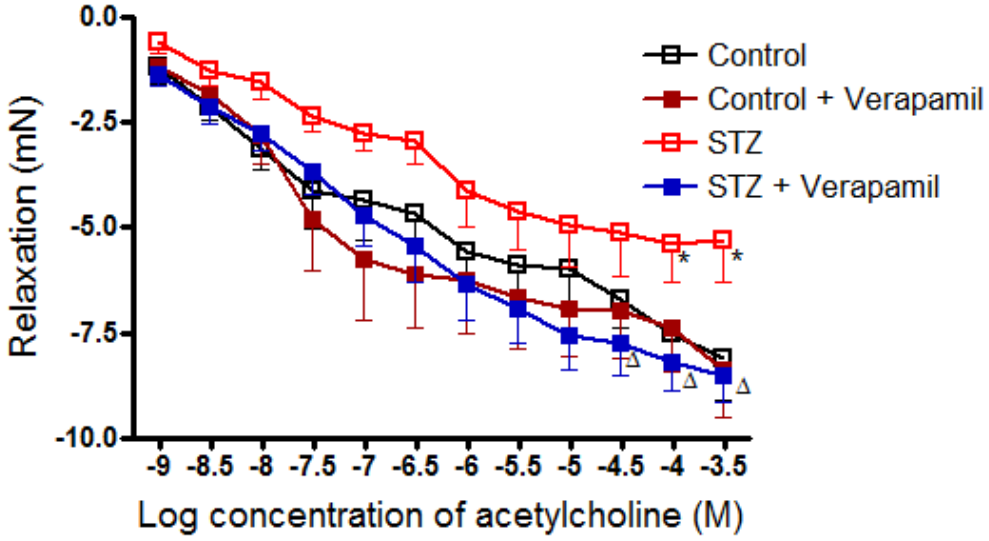


Figure 6.17: Endothelium-dependent relaxation by acetylcholine of noradrenaline precontracted mesenteris arteries from control, streptozotocin (STZ) - diabetic rats and STZ + verapamil (4 mg/kg/day, p.o.). Results are presented as means ± SEM. n=13 for all groups. *P<0.05 vs control and $^{\Delta}$,**P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests).

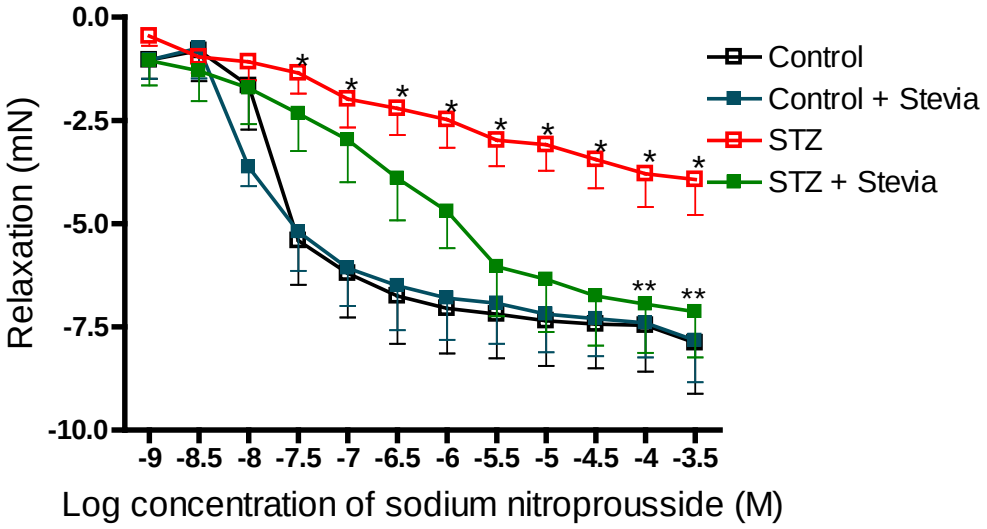


Figure 6.18: Endothelium-independent relaxation by sodium nitroprusside of noradrenaline precontracted mesenteric arteries from control, control + stevia, streptozotocin (STZ)-diabetic rats, STZ + stevia (200 mg/kg/day, p.o.). Results are presented as means ± SEM. n=13 for all groups. *P<0.05 v s control and $^{\Delta}$**P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests).

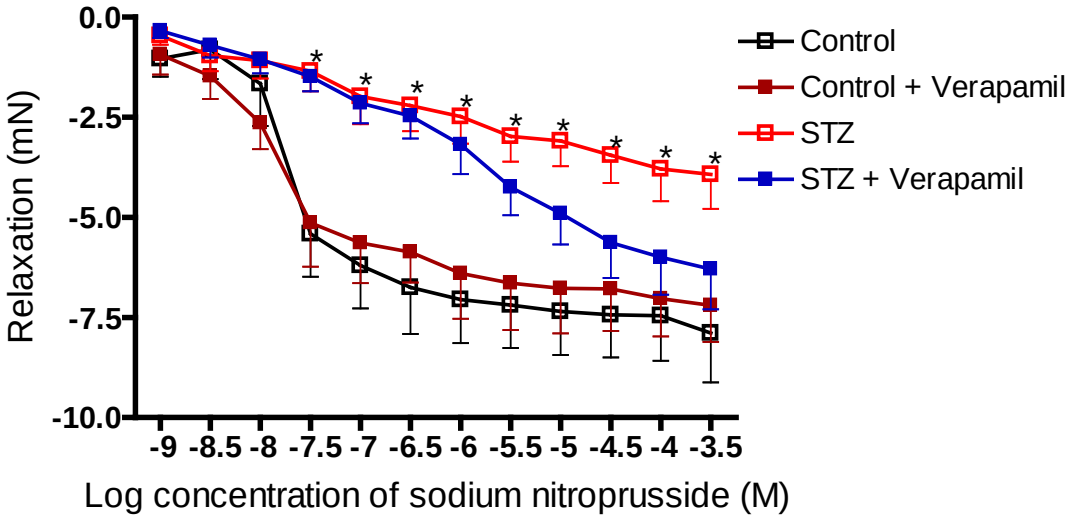


Figure 6.19: Endothelium-independent relaxation by sodium nitroprusside of noradrenaline precontracted mesenteric arteries from control, control + verapamil, streptozotocin (STZ)-diabetic rats, STZ-diabetic rats treated with verapamil (4 mg/kg/day, p.o.). Results are presented as means ± SEM. n=13 for all groups. *P<0.05 vs control and $^{\Delta}$**P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests).

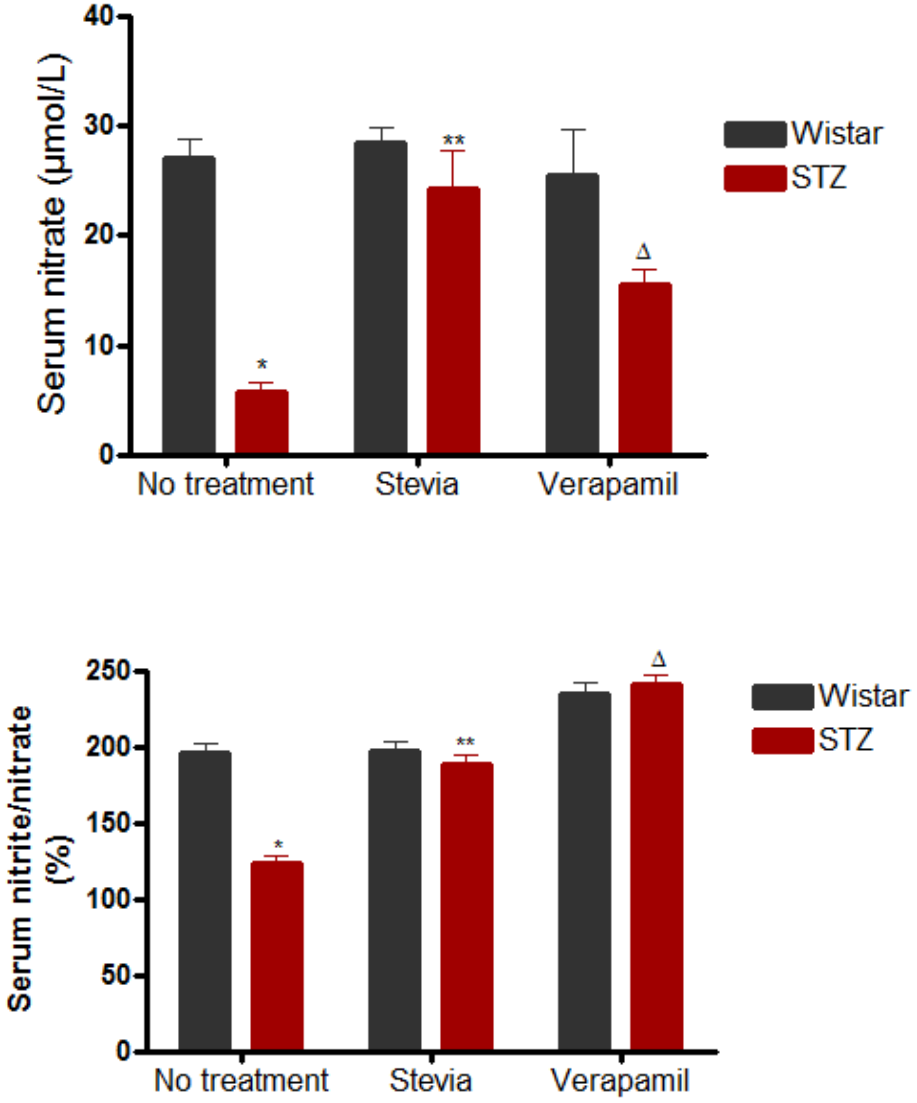


Figure 6.20: Serum nitrate levels and total nitrite/nitrate ratios for 16 weeks old control, control + stevia, streptozotocin (STZ) - diabetic rats, STZ + stevia (200 mg/kg/day, p.o.) and STZ+ verapamil (4 mg/kg/day, p.o.). Results are presented as means ± SEM. n=15 for all groups. *P<0.05 vs control; $^{\Delta,}$**P<0.05 vs STZ and ΔP<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests).

Table 6.6 Comparison of serum markers

Serum inflammatory markers	Control	Control + Stevia	Control + Verapamil	STZ	STZ + Stevia	STZ + Verapamil
TAC (mmol/L)	2.7 ± 0.4	2.6 ± 0.3	2.7 ± 0.2	0.97 ± 0.2*	2.7 ± 0.5**	2.1 ± 0.4
MDA (pmol/mg)	17.4 ± 5.8	19.2 ± 3.7	22.3 ± 4.4	38.5 ± 3.9*	18.9 ± 4.4**	11.9 ± 3.7$^{\Delta}$
IL-6 (pg/mL)	49.2 ± 5.1	50.6 ± 4.9	52.2 ± 3.9	237.6 ± 24*	138.2 ± 12**	139.2 ± 14$^{\Delta}$

Control and streptozotocin (STZ)-diabetic rats after 8-week treatment with stevia (200 mg/kg/day, p.o.) and verapamil (4 mg/kg/day, p.o.). Results are presented as means ± SEM. n=8 for all groups. *P<0.05 vs control and $^{\Delta}$,**P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests). **TAC**: Serum total antioxidant capacity, **MDA**: malondialdehyde and **IL-6**: interleukin-6 levels for 16 weeks of age.

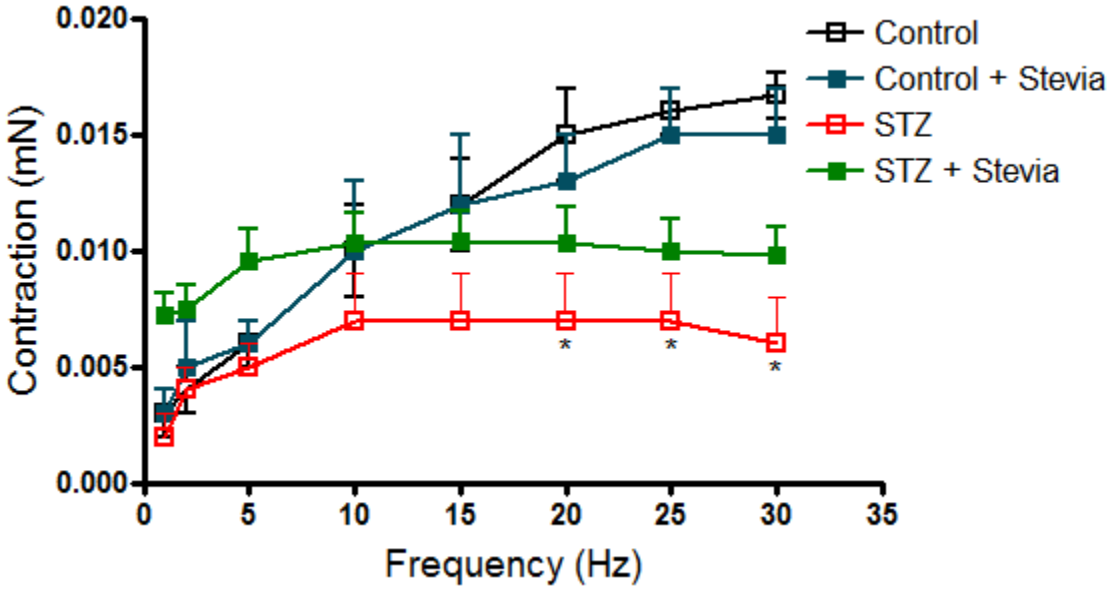


Figure 6.21: Contractile response to electrical field stimulation (EFS) on gastrointestinal smooth muscle from control, control + stevia, streptozotocin (STZ) - diabetic rats, STZ + stevia (200 mg/kg/day, p.o). Results are expressed as the increase in g tension built up above the baseline per mg of tissue and are presented as mean ± SEM. n= 10-15 for all groups. *P<0.05 vs control and $^{\Delta}$,**P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests).

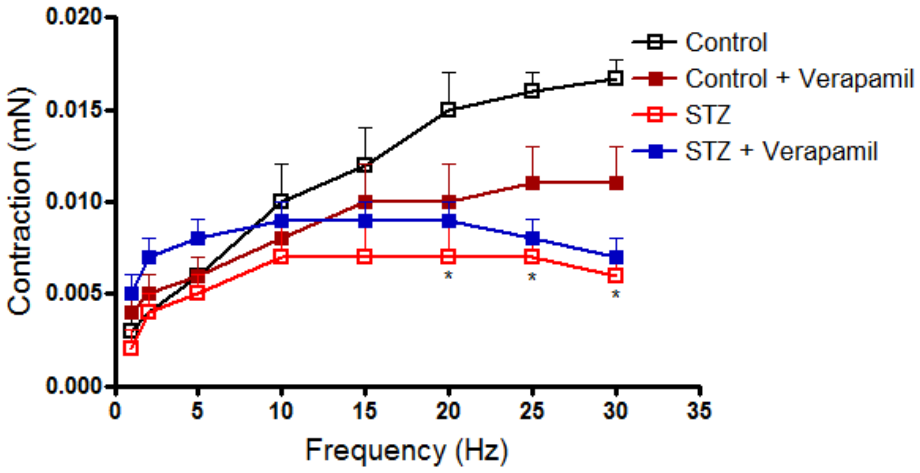


Figure 6.22: Contractile response to electrical field stimulation (EFS) on gastrointestinal smooth muscle from control, control +verapamil, streptozotocin (STZ)-diabetic rats, STZ + verapamil (4 mg/kg/day, p.o). Results are expressed as the increase in g tension built up above the baseline per mg of tissue and are presented as mean ± SEM. n= 10-15 for all groups. *P<0.05 vs control and $^{\Delta,}$**P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests).

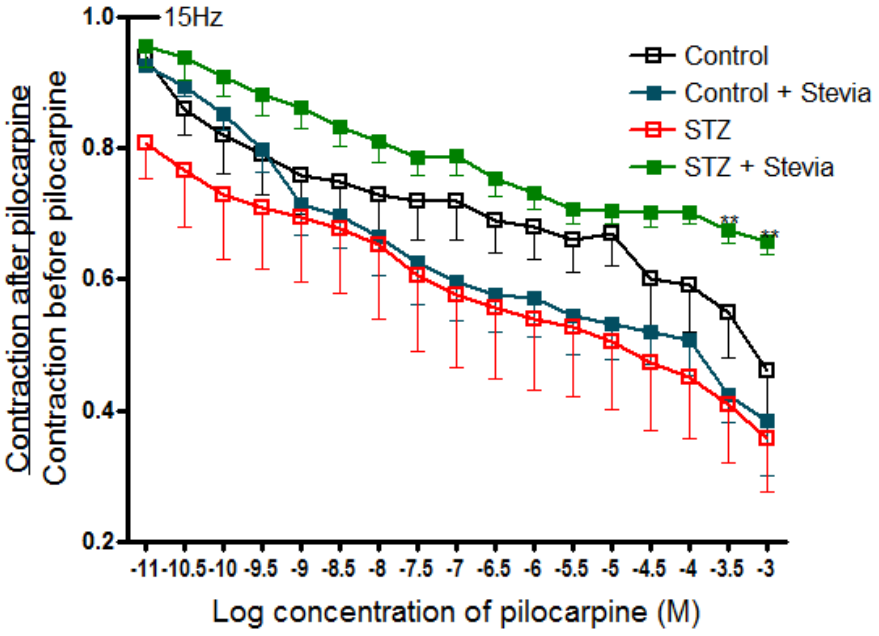


Figure 6.23: Pilocarpine-induced inhibition in response to electrical field stimulation (EFS, 15 Hz, 100 V, 0.2 ms pulse duration for 5 s at 30s intervals) on isolated rat ileum from control, control + stevia, streptozotocin (STZ)-induced diabetic rats, STZ + stevia (200 mg/kg/day, p.o). Results are expressed as the increase in g tension built up above the baseline per mg of tissue and are presented as mean ± SEM. n= 10-15 for all groups. *P<0.05 vs control and $^{\Delta,}$**P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests).

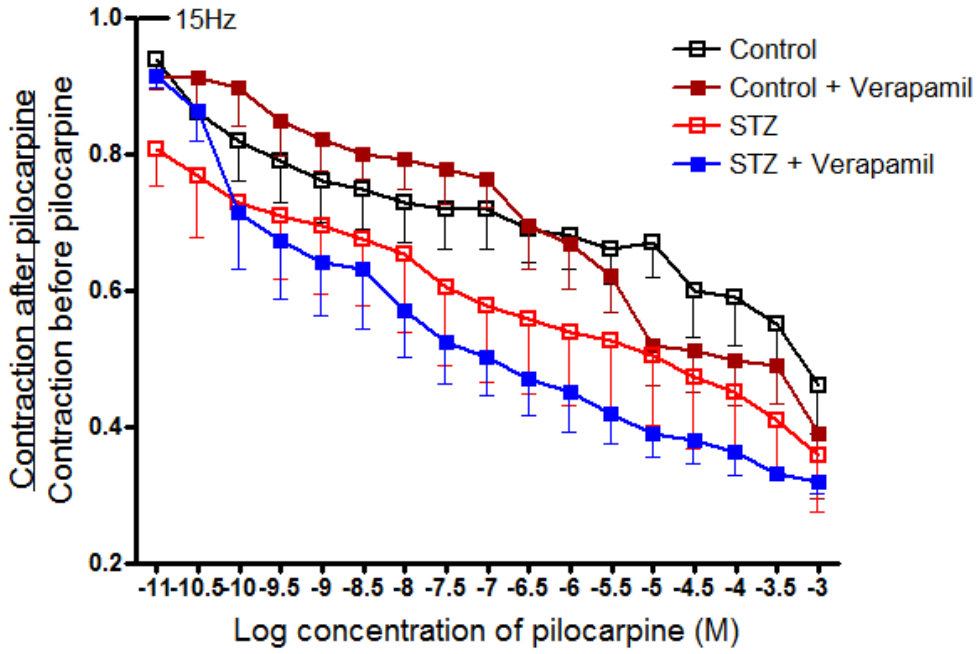


Figure 6.24: Pilocarpine-induced inhibition in response to electrical field stimulation (EFS, 15 Hz, 100 V, 0.2 ms pulse duration for 5 s at 30s intervals) on isolated rat ileum from control, control +verapamil, streptozotocin (STZ)-induced diabetic rats, STZ + verapamil (4 mg/kg/day, p.o). Results are expressed as the increase in g tension built up above the baseline per mg of tissue and are presented as mean ± SEM. n= 10-15 for all groups. *P<0.05 vs control and $^{\Delta,}$**P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests).

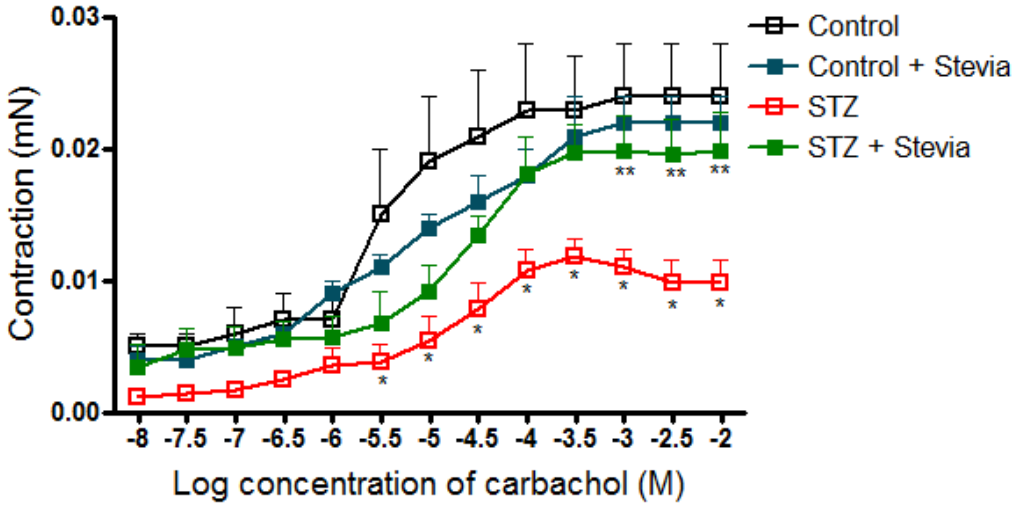


Figure 6.25: Carbachol-induced contraction on gastrointestinal smooth muscle from control, control + stevia, streptozotocin (STZ)-induced diabetic rats, STZ + stevia (200 mg/kg/day, p.o). Results are expressed as the increase in g tension built up above the baseline per mg of tissue and are presented as mean ± SEM. n= 10-15 for all groups. *P<0.05 vs control and $^{\Delta,}$**P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests).

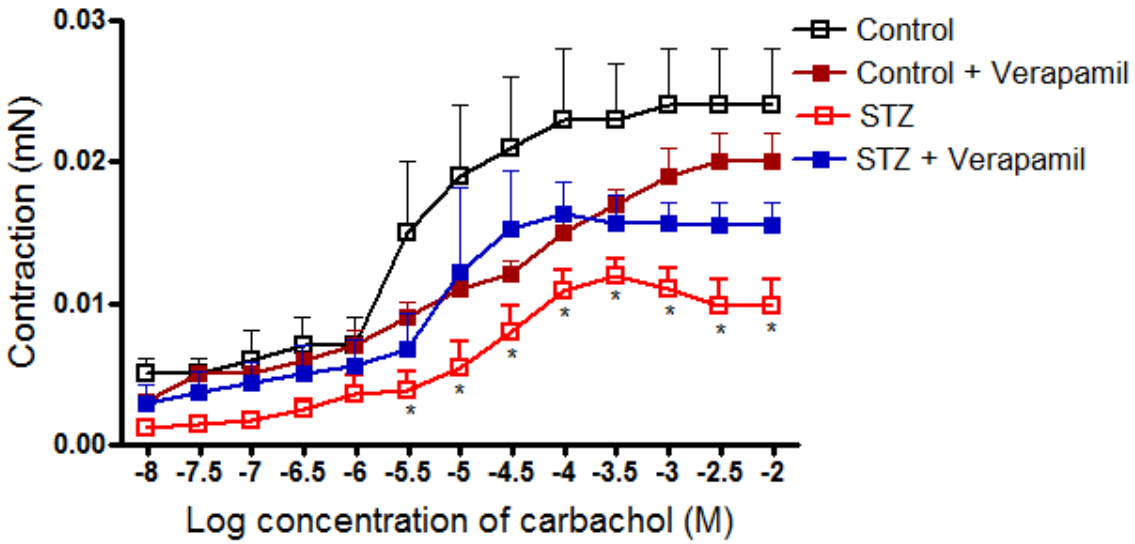


Figure 6.26: Carbachol-induced contraction on gastrointestinal smooth muscle from control, control +verapamil, streptozotocin (STZ)-induced diabetic rats, STZ + verapamil (4 mg/kg/day, p.o). Results are expressed as the increase in g tension built up above the baseline per mg of tissue and are presented as mean ± SEM. n= 10-15 for all groups. *P<0.05 vs control and $^{\Delta,}$**P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests).

Table 6.7: -Log EC_{50} and maximal response (R_{max}) in isolated ileum

Parameters	Control	Control + Stevia	Control + Verapamil	STZ	STZ + Stevia	STZ + Verapamil
-Log EC_{50}	4.6 ± 0.2	4.5 ± 0.2	4.1 ± 0.2	4.3 ± 0.1	4.4 ± 0.1	4.3 ± 0.2
R_{max} (% to carbachol)	96 ± 1.3	98 ± 0.9	98 ± 2.3	95 ± 3.7	97 ± 1.5	92 ± 4.2

Control and streptozotocin (STZ)-diabetic rats after 8-week treatment with stevia (200 mg/kg/day, p.o.) and verapamil (4 mg/kg/day, p.o.). Results are presented as means ± SEM. n=10-15 for all groups. *P<0.05 vs control and $^{\Delta,}$**P<0.05 vs STZ (two-way ANOVA with Bonferroni's multiple comparisons tests).

6.4. Discussion

The STZ-induced diabetic rat model is uniquely positioned from human diabetes in a way that pathological changes following diabetes are independent of hypertension. Also unlike human diabetes, the STZ rat model does not produce atherosclerosis. Therefore, the maladaptive cardiovascular changes in STZ rats are found to be typically associated with hyperglycaemia, cardiomyopathy and alteration of other neurohumoral factors (Choi, K. M. et al. 2002; Howarth et al. 2001, 2005). In this study we have used the STZ rat model to further elucidate the protective effects of stevia and verapamil on cardiovascular remodelling following diabetes. In this study, stevia improved structural and functional changes of the cardiovascular system and decreased peripheral neuropathy observed in the STZ-induced diabetic rat model. Verapamil also demonstrated similar promising results by improving cardiovascular function in the STZ rats.

Eight weeks of stevia treatment (200 mg/kg/day) showed a significant reduction in blood glucose levels in the STZ rats. Stevia demonstrated significant improvement in body weights and prevented polydipsia in the diabetic rats following eight weeks of the disease condition. Previous studies demonstrated that stevia was capable of regulating blood glucose levels by increasing insulin secretion and improving insulin utilization in insulin deficit rats (Chen, T. et al. 2005). Stevioside was found to stimulate insulin secretion via a direct action on INS-1 cells of the pancreas without any influence on ATP sensitive K^+ channel activity, or alteration of cyclic adenosine monophosphate (cAMP) levels in islets of beta cells (Jeppesen et al. 2000, 2003). The insulinotropic effects of both stevioside and steviol were conserved in the absence of extracellular Ca^{2+} (Jeppesen et al. 2000). The current study supports the previous manifestation as stevia markedly decreased blood glucose levels in the diabetic groups without any reduction in the control Wistar rats. Earlier studies also showed that no change in blood glucose levels were observed in control rats following stevioside infusion at euglycemia measured by intra-venous glucose tolerance test (IVGT) (Jeppesen et al. 2002; Suanarunsawat & Chaiyabutr 1997). The likely explanation of the lack of a glucose lowering effect in normal rats may be that stevia's

insulinotropic effect is dependent on the prevailing blood glucose concentration and plasma insulin level since stevia was found to potentiate insulin secretion at or above a blood glucose concentration of 8.3 mmol/l (Jeppesen et al. 2000).

Verapamil showed a small reduction in blood glucose levels in the diabetic rats. Previous studies showed that verapamil improved cardiovascular changes and myopathy in diabetes without changing blood glucose level (Afzal et al. 1988). Moreover, stevia in this study failed to completely normalise the glucose levels in the diabetic rats compared to the age-matched controls. These findings indicate that the vasculo-protective effects of stevia and verapamil demonstrated at a later point in this study were not solely associated with antihyperglycemic effects; rather this protective effect may occur by a combination of a modest drop in blood glucose levels together with inhibition of calcium influx into the smooth muscle and putative oxidant scavenging mechanisms. Besides, improvement of diabetes-induced alterations in heart rates and contraction and relaxation by verapamil, its administration confirms the involvement of Ca^{2+} in diabetic cardiovascular damage (Afzal et al. 1988).

Cardiomyopathy, the major underlying pathophysiology of STZ-induced diabetic rats, is found to be associated with altered heart rhythms and autonomic dysfunction (Howarth et al. 2005). Thus abnormal autonomic activity contributes to contractile dysfunction characterized by a decrease in amplitude and increase in duration of contraction and relaxation in myocytes of STZ-diabetic rats (Howarth et al. 2005). Similarly, in both type 1 and type 2 diabetic patients, autonomic neuropathy frequently develops along with other cardiovascular complications (Kudat et al. 2006; Vinik & Ziegler 2007). STZ-induced diabetic rats develop altered nerve conductance and biochemical dysfunction characteristic of human diabetic neuropathy (Sharma & Thomas 1987; Walker, D. et al. 1999). Other studies have shown that alteration in HR and HRV following diabetes is associated with hyperglycemia, decreased vagal nerve function and increased sympathetic activity leading to cardiac dysfunction (Hicks et al. 1998; Sanyal, Arita & Ono 2002). In our study, diabetic rats showed a clear indication of diabetic neuropathy as evidenced by significant decreases in heart rate and heart rate variability compared to the age-matched control. This was supported by the finding that diabetes caused a progressive autonomic dysfunction manifested by the reduction in resting heart rate and heart rate variability (HRV) particularly in patients

with diabetic complications (Kudat et al. 2006; Yu & McNeill 1992). In addition, human and animal studies demonstrated that altered heart rate and HRV in diabetes is directly associated with a decrease in cardiac β adrenoceptor number in the diabetic myocardium (Thackeray et al. 2011). Therefore, increased sympathetic tone and decreased expression of β adrenoceptors contribute to cardiomyopathy and end-stage heart failure in diabetes (Kostis & Sanders 2005; Thackeray et al. 2011).

Eight weeks of stevia administration restored the decreased HR back to normal and improved HRV in the diabetic rats. The possible mechanism by which stevia exerts these actions may be by the reduction in blood glucose levels, improved nerve conductance and increased sensitivity of β adrenoceptors in the cardiac tissue. Moreover, diabetes leads to a significant change in the myocardium which can be improved by enhanced utilization of glucose and greater transport of glucose and free fatty acid in the heart (Goyal & Patel 2011). Hyperglycemia is directly linked with increased lipid and cholesterol levels contributing to cardiomyopathy in diabetes (Goyal & Patel 2011; Yazaki et al. 1999). Previous studies demonstrated that stevia increased glucose transport across the cell and have lipid-lowering effects in diabetic Zucker rats (Dyrskog et al. 2005; Lailerd et al. 2004). Stevia was found to have no effects on serum catecholamine concentration in the SHR rats (Chan et al. 1998). Stevia (both stevioside and rebaudioside A) demonstrated no change to the heart rate pattern in healthy human patients and control animals (Vasović et al. 2006).

Verapamil also increased heart rate and heart rate variability in the diabetic rats reflecting its cardioprotective effects. This action possibly occurred through a reduction in sympathetic activity and subsequent increase in vagal function. This was supported by the findings that verapamil demonstrated antisympathetic properties and reduced the incidence of cardiac arrhythmias in human subjects (Kailasam et al. 1995; Pinar et al. 1998). Verapamil-mediated reduction of sympathetic outflow, leads to a decreased release of renin which is known to have vagolytic properties (Levine et al. 1982; Niemelä, Airaksinen & Huikuri 1994; Pinar et al. 1998). The suppression of sympathetic outflow may be specific to verapamil since the long acting calcium channel antagonists were found to be associated with increased sympathetic activity and deteriorated HRV parameters (Lopatin, Kirakozov & Statsenko 2003). Afzal et al.

(1988) demonstrated that verapamil increased heart rate and improved maladaptive cardiac changes in diabetic rats (Afzal et al. 1988).

Diabetic rats showed a marked decrease in coronary blood flow indicating a reduced ventricular reserve. This finding is supported by previous studies carried out on human and animal diabetes (Picchi et al. 2011; Zhao et al. 1999). Both stevia and verapamil showed improved coronary blood flow and diastolic dysfunction possibly caused by decreased sympathetic activity and a reduced expression of cytokines, a group of neurohormones which eventually help to prevent heart failure (Sekiguchi et al. 2004; Vinik & Ziegler 2007).

The STZ-rats developed an inflammatory state after 8 weeks of diabetes induction characterized by increased levels of serum IL-6. In addition, an increase in oxidative stress was also demonstrated since diabetic rats showed a significant elevation in serum MDA levels and a decrease in total antioxidant capacity (TAC). These results are supported by animal studies which demonstrated that diabetes leads to an increased inflammation and oxidative stress state (Palsamy & Subramanian 2011; Pitsavos et al. 2007). Evidence also suggested that immune activation and low-level inflammation developed prior to insulin resistance in diabetes (Festa et al. 2000). However, our previous report showed that inflammation was not explicitly present in STZ-induced diabetic rats as a slight increase in serum IL-6 and TNF--α levels were observed. The small sample number may have played some role in these conflicting results for the circulating inflammatory markers.

Eight weeks of stevia treatment significantly reduced serum IL-6 and MDA levels indicating its ability to alleviate inflammation and restore the antioxidant balance. This finding supported the report that stevia extract contains high phenolic compounds contributing to its antioxidant capacity (Shukla et al. 2011). Moreover, stevioside demonstrated significant inhibition of inflammatory ear oedema and tumor-promoting activities in the skin of mice with 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation (Yasukawa, Kitanaka & Seo 2002). This significant antitumor activity along with anti-inflammatory effects renders stevia a potent chemo-preventive agent in cancer as stevia appeared to be non-carcinogenic in F344 rats of both sexes (Toyoda et al. 1997; Yasukawa, Kitanaka & Seo 2002) and non-toxic in

mice, rats and hamsters in both sexes (Toskulkac et al. 1997; Yasukawa, Kitanaka & Seo 2002). A recent study demonstrated that stevia can enhance muscle regeneration following injury by stimulating satellite cell through the nuclear factor (NF)- kappa B pathway (Bunprajun et al. 2012) . This parameter was measured by the presence and extent of muscle inflammation and myofibrillar protein content in the cardiotoxin injured Wistar rats (Bunprajun et al. 2012). Diabetes along with associated cardiovascular complication such as atherosclerotic plaque formation leads to increased lipid peroxidation, oxidative stress and inflammation (Geeraert et al. 2010). Chronic stevioside reduced plaque volume in mice aortic arch by decreasing macrophage activation, oxidation of LDL and lipid content and by subsequent decrease in oxidized LDL levels (Geeraert et al. 2010). Methanolic extracts of stevia showed effective free radical scavenging abilities (Shukla et al. 2009). The metabolic and hemodynamic derangement following diabetes are directly associated with hyperglycemia induced increased production of reactive oxygen species (ROS), impaired nitric oxide (NO) synthesis and enhanced formation of AGEs (Méndez et al. 2010).

Treatment with verapamil also significantly reduced IL-6 and serum MDA levels but showed only a small increase in TAC levels. These findings are in line with the previous report that verapamil demonstrated antioxidant and anti-inflammatory activities by reducing proinflammatory cytokines production in quinolinic acid induced rats (Kalonia, Kumar & Kumar 2011). Verapamil's antioxidant effects were found to be independent of its calcium channel blocking activity. Published data showed that verapamil reduced MDA production in hepatic chromosomal membrane which is devoid of L-type calcium channels suggesting the non-receptor mediated anti-oxidant action of verapamil (Bhattacharya et al. 2009). Calcium channel antagonists are able to modulate physicochemical properties of the lipid bilayer of cell membranes causing a reduction in lipid peroxidation contributing to protective effects of cardiovascular disease (Bhattacharya et al. 2009; Mason et al. 1999). In our study, stevia showed significant potential in reducing inflammation and oxidative stress compared to verapamil which indicates that stevia may participate in biophysical membrane interaction (stevia is sparingly soluble in water) which strengthened its antioxidant potential along with other neurohumoral and calcium channel blockade

activities. The tissue-damaging effect of hyperglycemia, confirmed by a recent study, showed that hyperglycemia leads to increase level of ROS and potentiates cytotoxicity of drugs (Pandey, Chaube & Bhat 2011).

The isolated Langendorff heart showed a significant stiffening of the left ventricle in diabetic rats 8-weeks post STZ injection. Diabetes caused a decrease in left ventricular compliance as evidenced by a marked decrease in end systolic and developed pressures. These results agree with earlier studies which showed diabetes caused impairment to active and passive properties of the heart manifested by altered rates of relaxation and systolic function (Litwin et al. 1994). On the contrary, our results showed only a small reduction in rate of contraction (+dP/dt (mmHg/s)) in the diabetic rats. Stevia treatment improved left ventricular function by normalizing end systolic pressure and systolic reserve represented by developed pressure. It is important to note that the effects of stevia on diabetes- induced left ventricular dysfunction have not been studied thoroughly. However, a number of studies demonstrated that isosteviol showed cardioprotective effects by improving contraction and relaxation of the myocardium following ischemia repurfusion injury of rat and guinea pig heart (Wei et al. 2003; Xu et al. 2006, 2007). Our Langendorff study clearly illustrated the improvement of systolic function by stevia in diabetes-induced impairment of left ventricular function. Moreover, in contrast to the result of Xu et al., 2007, our results demonstrated that chronic stevia treatment increased coronary blood flow indicating stevia's vasodilatory action on the coronary blood vessels. These cardioprotective effects of stevia possibly share a dual mechanisms related to inhibition of binding and release of calcium from troponin C and prevention of decreased heart rate. It can be noted that eight weeks of stevia treatment prevented the decrease observed in resting heart rate in the diabetic rats. Xu et al., 2007 suggested that stevia's cardioprotective effects may partially be impacted by opening of the mitrochondial ATP sensitive K^+ channel in the myocardium following ischemia reperfusion injury since protective action of isosteviol was reduced by pretreatment with selective mitoK_{ATP} antagonist 5-HD, compared with isosteviol alone (Xu et al. 2007). However, we can rule out the association of mitoK_{ATP} channel since our study on healthy rat aorta showed no effect of 4-amino pyridine on stevia induced relaxation (Chapter 4; section 4.5)

Verapamil showed a marked improvement in the systolic and diastolic function following diabetes. Effects of verapamil on systolic function were similar to stevia except verapamil showed a stronger effect in the improvement of rates of contraction and relaxation of the left ventricle. Published data supported this finding by showing that verapamil improved rates of contraction and relaxation, ventricular systolic and diastolic pressures in diabetic cardiomyopathy (Afzal et al. 1988). Verapamil improved systolic and diastolic function and coronary blood flow in the cardiomyocytes of diabetic rats following ischemia reperfusion injury (Yü W 2010). Again, an increased [Ca^{2+}] and a declining Ica-L were found to be associated with ischemia reperfusion injury in diabetes with verapamil having the ability to block Ca^{2+} overload and adjusting Ica-L (Yü et al. 2010) . Thus verapamil improved severe cardiac impairment associated with diabetes (Yü et al. 2010). Another study contradicted this result showing that altered Ca^{2+} handling was not the major cause of reduced contractile force of in diabetic cardiomyopathy (Zhang, L. et al. 2008). This study suggested that increased collagen deposition and decreased sensitivity of myofilament were mostly associated with the altered contractile response in diabetes (Zhang, L. et al. 2008). Our data supported this result as stevia demonstrated a reduction in left ventricular hypertrophy and an improvement in contractile force of the myocardium in diabetic rats.

Similar beneficial effects of stevia have been established following electrophysiological study on left ventricular papillary muscle of the diabetic rats. Diabetic rats showed a significant prolongation of action potential duration at 20%, 50% and 90% of repolarisation. These results are supported by other studies which showed significantly extended action potential duration at 20%, 50% and 90% of repolarisation in the STZ-induced diabetic rats (Shimoni 2001; Wei et al. 2003). Altered potassium channel activity manifested by reduction in transient out K^+ current and a decrease in steady-state outward K^+ current were found in ventricular myocytes from diabetic rats (Shimoni 2001; Shimoni et al. 1994). However further studies showed that activation of cardiac renin-angiotensin system along with other humoral factors are involved in reduction of ventricular K^+ current and subsequent prolongation of action potential duration and Q-T values (Shimoni 2001). Angiotensin II receptor blockers an angiotensin converting enzyme (ACE) inhibitors demonstrated

a significant improvement in K^+ current and reversal of prolonged action potential duration (Gerstein et al. 2000; Shimoni 2001). Moreover, action potential prolongation and Ca^{2+} overload are frequently observed in ventricular tachyarrhythmia following diabetes and hypertension (Milberg et al. 2011a).

Stevia improved left ventricular function by shortening the prolonged action potential duration a 50% and 90% of repolarisation in the diabetic rats. This result indicates that blockage of calcium channels is the key pathway of the anti-arrhythmic effects of stevia. An imbalance in outward and inward current leads to prolongation of action potential duration in ventricular myocardium. Prolonged APD increases the risk of incidence of Torsade de Pointes (TdP) by causing a quick fluctuation in QRS complexes (Vos et al. 2000). Stevia prevention of cardiac dysfunction in diabetic hearts may be through mixed mechanism of blockage of calcium channel, reducing oxidative stress and improving neurohumoral factors in the myocardium. This is supported by a finding of Chen, Su & Hung (2007) that resveratrol, a natural antioxidant from red wine also demonstrated anti-arrhythmic effects by improving action potential duration in rats with ventricular arrhythmias induced by ischemia/reperfusion injury (Chen, Su & Hung 2007). To the best of our knowledge, the electrophysiological effects of stevia on the diabetic myocardium have not been studied before.

Verapamil also showed a significant improvement in electrophysiological activity in the diabetic myocardium as evidenced by shortening of the action potential duration at 20%, 50% and 90% of repolarization. Other study showed that verapamil reduced Q-T values and prevented electric perturbations in the diabetic animals (D'Amico et al. 2001). Verapamil demonstrated an improvement in early after depolarization and shortening of action potential duration in the failing heart (Milberg et al. 2011a). A simulated mathematical model demonstrated that blockade of I_{Ca} is very effective in suppressing ventricular tachyarrhythmia through the reduction of transmural dispersion of repolarization (Milberg et al. 2011a). Verapamil is an effective antiarrhythmic agent by its capacity to lessen Q-T interval and to decrease action potential duration (Oros et al. 2010) and also by a recently demonstrated ability to neutralize spatial dispersion following CHF (Milberg et al. 2011a). However, verapamil is a poor choice in CHF with acute systolic impairment and fractional

dysfunction (Chew et al 1981) because of its negative ionotropic effects and cardiac depression. The choice of ideal antiarrhythmic drugs for a CHF heart is dependent on a balance between antiarrhythmic effects and their hemodynamic patience. Therefore, stevia could be a potential drug to treat cardiac arrhythmias as stevia was found to have multiple mechanisms of action rather than only calcium channel blockage and devoid of serious ventricular depression.

Aorta and mesenteric vascular responses to noradrenaline were found to be increased following both human and animal diabetes, indicating a hypersensitivity to noradrenaline (Baluchnejadmojarad, Roghani & Imani 2004b; Lin, K. Y. et al. 2002). Our results opposed this information as the vascular response to noradrenaline was found to be decreased in the diabetic rats. This finding is supported by a recent study which also showed a decreased response to noradrenaline after 4 weeks of experimental diabetes indicating that early stage of diabetes causes metabolic changes leading to a reduction of vasopressor response (Hong, E., Huang & Villafaña 2010) and these early changes were decreased with the progress of diabetes which is a characteristic feature of impaired vascular function caused by increased sympathetic action (Baluchnejadmojarad, Roghani & Imani 2004b; Lin, K. Y. et al. 2002). Diabetes significantly reduced acetylcholine-induced vasodilation in the aortic rings and coronary blood vessels (Ikubo et al. 2011). Our study produced a similar reduced response to acetylcholine mediated concentration- dependent relaxation in the isolated aorta and endothelium-independent relaxation to sodium nitroprusside.

Eight weeks of stevia treatment improved relaxation response to acetylcholine and normalized the reduced relaxation to sodium nitroprusside in STZ rats. Previous studies have suggested that stevia's vasodilatory response to aortic tissue is mediated by blockade of calcium channels (Melis 2002). A recent study showed that increased oxidative stress in diabetes is associated with up-regulation of low-density lipoprotein receptors and enhanced ICAM -1 expression on aortic endothelial tissues causing endothelial dysfunction (Wang, L. et al. 2008). Therefore, it is conceivable that stevia reduced serum low-density lipoprotein levels and decreased oxidative stress in the diabetic rats to improve vascular responses. Verapamil also improved vascular responses in the isolated aorta following eight weeks of experimental diabetes.

Previous studies showed beneficial effects of verapamil on vascular tissue in diabetes (Küng et al. 1995; Murat, Kalkan & Gidener 1999).

Understanding the patterns of oxidative stress and inflammation is important to explain the causative mechanisms of vascular dysfunction following diabetes. Hyperglycaemia induces an alteration of reactive oxygen species pathways which was demonstrated by an increased mitochondrial superoxide production by the endothelial cells incubated in high glucose conditions (Szabo 2009). An oxidant, peroxynitrite, is formed by the combination of superoxide and nitric oxide generated by eNOS leading to depletion and uncoupling of eNOS (Szabo 2009). Nitric oxide synthesized from the constitutive stimulation of eNOS helps to maintain vascular hemostasis with diabetes causing a significant decrease in expression of phosphorylated eNOS (Ohmasa et al. 2011). The same study showed that, treatment with edaravone, a free radical scavenger, restored the down regulation of phosphorylated eNOS in diabetes (Ohmasa et al. 2011). In light of these findings, it is not unreasonable to suggest that stevia could prevent the down regulation of eNOS following diabetes. However, diabetic rats did not show any change in M_3-muscarinic receptor expression in the vascular bed assessed by real time PCR (Ikubo et al. 2011). Our result showed that diabetes reduced efficacy (lower R_{max}) to acetylcholine but no difference in efficacy was observed in potency (EC_{50}). This result indicates a slight change in sensitivity to M_3-muscarinic receptor mediated vasorelaxation. However, in small vessel like mesenteric arteries, eNOS played limited roles in stevia's vasodilatory responses. Contraction and relaxation responses in the small vessels are mainly mediated by preventing the alteration of calcium channel activities as evidenced by increased action following verapamil treatment.

We assessed the function of parasympathetic nerve activity in the gastrointestinal tract by measuring the electrical field stimulated (EFS) contraction in the diabetic rats. Our results indicated a significant decrease in parasympathetic nerve function following eight weeks post STZ injection as evidenced by a marked decrease in EFS contraction compared to the control rats. Our result is supported by previous studies showing a similar decrease to EFS contraction of the ileum tissues (Coulson, Jacoby & Fryer 2004; Okada et al. 2009; Takeuchi et al. 2005). Decreased acetylcholine release due to an enhanced expression and function of neural M_2-muscarinic receptors are possibly

the mechanisms underlying the reduced contractile response to EFS in diabetes. Coulson et al., (2004) confirmed the increased M_2-muscarinic receptors activity in diabetes by showing that diabetes significantly reduced the inhibition of pilocarpine to EFS-induced contraction and potentiated methoctramine's effects on carbachol induced contraction (Coulson, Jacoby & Fryer 2004). Our study showed similar results with the muscarinic receptor agonist pilocarpine in the ileum tissue of the diabetic rats indicating an increase in M_2-muscarinic receptors. This was also supported by Okada et al. (2009) which showed that both muscarinic M_2 and M_3 receptors are increased in diabetes (Okada et al. 2009). However, other studies demonstrated that the function of M_3 receptor remains unchanged in diabetes (Coulson, Jacoby & Fryer 2002, 2004). On the contrary, some studies showed that gastrointestinal smooth muscle responses to cholinergic agonists were elevated in diabetes (Shinbori et al. 2006; Narimatsu et al. 2007). It was suggested that the conflicting results might have been observed because of the difference in time point and duration of diabetes. This hypothesis can be explained by a phenomenon demonstrating that a diabetic rat's bladder underwent a changeover from compensated to decompensated states between 9 and 12 weeks after STZ injection (Daneshgari et al. 2009).

Stevia treatment reversed the diabetes-induced nerve dysfunction observed by the normalisation of EFS-induced contraction in stevia treated diabetic rats. Simultaneously, the pilocarpine response curve indicated that neuronal M_2-muscarinic function was restored in the diabetic rats. We have seen in the current study that stevia could not normalize the blood glucose levels in the diabetic rats. Other studies showed that stevia reduced insulin resistance and improved insulin sensitivity (Jeppesen et al. 2002). Therefore, we can conclude that stevia could normalize M_2 function and improved cholinergic nerve function by increasing insulin sensitivity and reducing insulin resistance. This was supported by Coulson et al., (2002) which showed insulin insufficiency mainly caused increased M_2 function which ultimately inhibited acetylcholine release from the parasympathetic nerves. Verapamil demonstrated a slight but significant improvement in the EFS induced contraction and on pilocarpine induced inhibition in ileum tissues of the diabetic rats. This result indicates that altered activity of L-type calcium channels also plays roles in diabetic gastropathy.

Diabetes reduced carbachol-induced contraction compared to the control rats. In contrast to our results, previous studies showed muscarinic M_3 receptor functions remained unchanged (Coulson, Jacoby & Fryer 2002) or up-regulated following diabetes as hyperactivity to carbachol was observed (Okada et al. 2009). The contractile response of the rat ileum to muscarinic agonists is mainly mediated by M_3 receptors (Coulson, Jacoby & Fryer 2002, 2004; Narimatsu et al. 2007; Okada et al. 2009), although it has been demonstrated that M_2 receptors along with the M_3 receptor also participate in contraction of the mouse ileum (Unno et al. 2006).

Chapter 7 Conclusion

The primary objective of this study was to mimic the human condition of cardiovascular remodelling in animal models of diabetes and hypertension and to explore the cardioprotective effects of stevia in these chronic disease conditions. The effects of stevia were compared to the effects of the calcium channel antagonist, verapamil. From what has been stated in the previous sections, it is believed that our objectives have been satisfactorily achieved. The major conclusions that can be drawn from this study are discussed below.

This study clearly showed that stevia is effective in treating secondary cardiovascular complications in rat models of both diabetes and hypertension. In this capacity it demonstrated a similar treatment profile to verapamil which was also cardioprotective. An additional outcome of this study was to further define some of the cellular target(s) which might be inhibited or promoted by stevia.

Stevia showed an ability to modify the electrophysiological and mechanical properties of cardiomyocytes and concentration-dependently alter the responses of blood vessels and gastrointestinal smooth muscle in healthy rat tissues. In cardiomyocytes, stevia (3×10^{-4}M) attenuated the force of contraction (FOC), shortened the repolarisation phase of the action potential (APD) – APD_{20}, APD_{50} and APD_{90} and decreased the average peak amplitude (APA) of the initial cell depolarization. Stevia showed a concentration-dependent relaxation response in aortic tissue which was significantly potentiated in the presence of verapamil. This relaxation response was also linked to NOS activity. In mesenteric arteries, incubation with L-NAME failed to block the stevia-induced relaxation indicating the mechanism of action may not be exclusively via NO dependent pathways. Stevia concentration-dependently reduced electrical field stimulated (EFS) and carbachol-induced contractions in the isolated ileum. This study is the first study to show effectiveness of stevia in reducing cardiac action potential duration at 20%, 50% and 90% of repolarisation. In short, our results indicate that the acute mechanism of action of stevia is multimodal, since stevia showed beneficial modulatory effects on cardiovascular and gastrointestinal tissues

which can be related to calcium channel antagonism, activation of M_2 muscarinic receptors, and via enhanced NO signalling activity.

Chronic study using STZ-diabetic rats indicated that the cardioprotective effects of stevia are partially independent of its antihyperglycemic effects. This was highlighted by the fact that stevia improved ventricular function and vascular activity but failed to completely normalize plasma glucose levels in the diabetic rats. Stevia did significantly reduce blood glucose levels but they were still at a significantly high level in the STZ-treated animals. Diabetes induced a reduction in pain threshold, heart rate (HR) and heart rate variability (HRV) which were normalized by stevia treatment. Interestingly, verapamil also increased HR and improved HRV following diabetes. Additionally, both stevia and verapamil treatment improved the maximal contractile and relaxation responses of thoracic aortic rings and mesenteric arteries followed by a significantly increased endothelial NO synthesis in the diabetic rats. Most importantly, chronic diabetes developed a severe form of oxidative stress and inflammation by depleting the antioxidant defences as observed by a reduced serum total antioxidant capacity (TAC), increased malondialdehyde (MDA) and interleukin-6 (IL-6) levels in the diabetic rats. Both stevia and verapamil significantly improved TAC, MDA and IL-6 concentrations following diabetes. Electrophysiological studies showed that stevia prevented prolongation of APD_{20}, APD_{50} and APD_{90} suggesting that stevia might restore sinus rhythm or reduce ventricular arrhythmogenesis following diabetes. This was accompanied by a decrease in left ventricular weight in stevia-treated diabetic rats. Moreover, both stevia and verapamil prevented renal hypertrophy by reducing the increased kidney weights from diabetic animals. Therefore, these studies demonstrate that stevia significantly improved the altered cardiovascular and renal function and reduced peripheral neuropathy observed in the STZ-rat model. Again, as with the mechanistic study, the possible mechanism of actions of stevia was suggested to be through multiple pathways including antagonism of calcium channels, reductions in hyperglycaemia, increased NO synthesis and via a reduction of free radicals synthesis. Similarly, verapamil showed promising results in treating the diabetes-induced vascular dysfunction and altered cardiac electrophysiology through the blockade of calcium channels. This clearly indicates its usefulness in treating the secondary complications of diabetes-induced cardiovascular disease. However, in the

isolated ileum, only stevia prevented the gastrointestinal dysmotility following diabetes. In short, verapamil was found to be more effective in improving vascular response and coronary blood flow with a more potent antioxidant and anti-inflammatory efficacy than stevia. These results were not unexpected given the primary vascular and cardiac targets of verapamil.Whereas, stevia demonstrated a better response in improving biometric parameters and gastrointestinal function than verapamil. Again these results were not totally unexpected given the amount of research completed on the effects of stevia on the gastrointestinal tract.

In the hypertensive rats, chronic stevia treatment prevented maladaptive left ventricular changes, improved systolic and diastolic function and reduced diastolic stiffness caused by hypertension. Stevia also normalised liver and kidney weight compared to body weight and tibial length in the SHR rats. Verapamil normalized the high blood pressure along with significant improvements in both ventricular and vascular function in the SHR group. Clinically, verapamil is indicated for the management of hypertension, angina and atrio-ventricular arrhythmia. In human subjects and animal studies, verapamil was found to reduce cardiac fibrosis and collagen deposition in the extracellular matrix and also prevent renal fibrosis (Bombig et al. 1996; Murat, Kalkan & Gidener 1999; Thaina, Poonpanang & Sawangjaroen 2005). Verapamil significantly reduced aortic wall thickness, cross-sectional area, and the media-to-lumen ratio of the aorta from SHR animals. Our study unravels more beneficial effects of verapamil relating to its antioxidant and anti-inflammatory actions, strong vasodilatory response and prevention of impaired nerve function. Previous studies have demonstrated antioxidant effects of dihydropyridine calcium antagonists, but insufficient data have been published with phenylalkaylamines such as verapamil (Godfrained 2005). This study measured the total profile of oxidative stress and inflammation which was found to be significantly increased in hypertension as well as in diabetes. Both stevia and verapamil successfully reduced oxidative stress and inflammation in the STZ and SHR animals. Previous studies demonstrated stevia's antihypertensive, antidiabetic, immunomodulatory and gastroprotective effects. This research illustrated electrophysiological effects of stevia on cardiomyocytes and confirmed antihypertrophic, anti-oxidant, anti-inflammatory and

gastromodulatory effects of stevia. This study also strengthened stevia's role as a novel antihypertensive agent.

One of the limitations of our study is that the effects of stevia and verapamil have not been tested in the diabetic hypertensive rat model which more accurately represents the majority of human patients. In addition, the length of our study was for 8 weeks, which might not be sufficient to mimic some of the cardiovascular changes in the hypertensive rats. A single dose of verapamil (4 mg/kg/day; once daily; p.o.) and stevia (200 mg/kg/day) were used in this study, as such, the effect of various dosages regimes were not investigated in this project. From the results of this study, it was suggested that stevia is a better option for preventing cardiovascular remodelling without any major side effects. However, the dose of verapamil used was low compared to the high dose (20 mg/kg/day) tested in other animal studies showing cardiovascular benefits (Allen et al. 2000).

In conclusion, the current study showed that stevia has generic pharmacological activity which targets the contractile processes of the myocardium, blood vessels and intestine. Unlike verapamil, stevia did not show any adverse chronotropic or inotropic effects on cardiac function and vascular responses in any of the treatment models. Therefore stevia offers a valuable alternative for the treatment of cardiovascular remodelling following hypertension and diabetes. Our results have also shown that verapamil can uniquely be used as an antioxidant and anti-inflammatory agent in chronic diseases when anti-oxidant defence is compromised in addition to its primary calcium channel blocking role. Finally, we would like to end this thesis with a hope that further kidney function tests and electrophysiological studies on cardiomyocytes and vascular tissue will be carried out to test stevia's function in larger sample sizes and in hypertensive-diabetic and obese models.

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