# INVESTIGATION OF PHYTONUTRIENTS IN THE TREATMENT OF CARDIO-METABOLIC DISEASE

A thesis submitted by

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## ABSTRACT

This thesis investigated the effect of different foods such as spices (green cardamom and black cardamom), vegetables (beetroot), cereal grains (purple maize), fruits (chokeberry and Queen Garnet plum) and bioactive molecules (sodium nitrate and cyanidin 3-glucoside) on risk factors for metabolic syndrome using a diet-induced obese rat model. Obesity, insulin resistance, impaired glucose tolerance, atherogenic dyslipidaemia, hypertension and endothelial dysfunction are the major components of metabolic syndrome. Maintaining a healthy lifestyle including regular exercise together with a healthy and balanced diet has been recommended as the first-line of defence to prevent metabolic syndrome. However, with the growing prevalence of obesity worldwide, this complex disorder is considered to be a clinical challenge and an important public health concern. While current pharmaceutical drug therapies for obesity show some benefits, there are also multiple side effects. Hence, complementary and alternative therapies have become popular to reduce the incidence of metabolic syndrome with the aim of decreasing future health risks.

Nature has provided us with an immense diversity in the form of plants and herbs, which contain a wide range of chemicals, produced to protect the plants from environmental stress factors and pathogenic infections. These foods have been used since antiquity as the therapeutic interventions in traditional health care systems. However, the effectiveness of many of these plants and their bioactive molecules in the treatment of human diseases has yet to be fully explored.

Hence, in this thesis, I have examined these food sources in a validated diet-induced rat model of metabolic syndrome mimicking most of the changes in the human syndrome. High-carbohydrate, high-fat diet induced obesity, impaired glucose tolerance, dyslipidaemia, hypertension, cardiovascular remodelling including ventricular dilatation, cardiomyocyte hypertrophy and cardiac fibrosis, reduced ventricular function, hepatic steatosis, hepatic inflammation and portal fibrosis and increased plasma markers of liver damage.

A comparison of green and black cardamom showed that black cardamom ameliorated the changes in cardiovascular and hepatic structure and function and decreased visceral adiposity whereas green cardamom worsened both liver function and cardiovascular structure and function. Both beetroot, naturally high in nitrates, and the same dose of sodium nitrate improved cardiovascular and hepatic structure and function as well as glucose metabolism but failed to reduce visceral adiposity. Anthocyanin-rich chokeberry, purple maize and Queen Garnet plum reduced body weight gain, visceral adiposity, improved liver

enzymes and improved cardiovascular and hepatic structure and function. Cyanidin 3-glucoside, the most common anthocyanin, showed similar effects as the Queen Garnet plum at the same dose. As similar responses were observed in cyanidin 3-glucoside and Queen Garnet rats, it is proposed that cyanidin 3-glucoside is the key bio-active molecule to reduce the diet-induced metabolic, cardiovascular and liver changes.

Anthocyanin-rich Queen Garnet plums gave the most beneficial effects in the obese rat model, so this intervention was chosen to proceed with a 12 week clinical translation study in obese or over-weight humans. These subjects showed a marked decrease in blood pressure and fasting blood glucose concentrations, but no change was observed in body weight, total body fat mass and plasma lipids.

These studies from the thesis clearly indicate that these natural food sources have the potential to reverse or attenuate most of the risk factors associated with metabolic syndrome. In contrast, green cardamom exacerbated adiposity, decreased liver function and worsened cardiovascular structure and function. The most likely mechanism of action of these natural sources is the prevention of infiltration of inflammatory cells into organs and tissues. From these studies, it is evident that dark-coloured foods are very effective in the attenuation of cardio-metabolic risk factors associate with metabolic syndrome.

## CANDIDATE DECLARATION

I, Maharshi Bhaswant Cheethirala, declare that the PhD thesis entitled "Investigation of phytonutrients in the treatment of cardio-metabolic disease" is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

**Signature:**  **Date: 19/02/2016**

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## PUBLICATIONS AND PRESENTATIONS DURING CANDIDATURE

Book chapter

- Panchal S K, **Bhaswant M**, and Brown L. Obesity and Metabolic Syndrome, in In Vivo Models for Drug Discovery. (eds J. M. Vela, R. Maldonado and M. Hamon), Wiley-VCH Verlag GmbH & Co. KGaA. 2014.

Peer-reviewed publications

- **Bhaswant M**, Poudyal H, Brown L. Mechanisms of enhanced insulin secretion and sensitivity with n-3 unsaturated fatty acids. J Nutr Biochem, 2015; 26(6): 571-584.
- **Bhaswant M**, Poudyal H, Mathai M, Ward L, Mouatt P, Brown L. Green cardamom and black cardamom in a diet-fed rat model of metabolic syndrome. Nutrients, 2015; 7(9): 7691-7707.
- **Bhaswant M**, Fanning K, Netzel M, Mathai M, Panchal S, Brown L. Cyanidin 3-glucoside improves diet-induced metabolic syndrome in rats. Pharm Res, 2015; 102: 208-217

Manuscripts in preparation

- **Bhaswant M**, Shafie SR, Mathai M, Mouatt P, Brown L. Anthocyanin-containing chokeberry and purple maize reverses diet-induced metabolic syndrome in rats. Journal of Functional Foods.
- **Bhaswant M,** Brown L, McAinch A, Mathai M. Effect of inorganic nitrates in improvement of cardiovascular remodelling. European Heart Journal.
- **Bhaswant M,** Brown L, Fanning K, Netzel M, Mathai M. Blood pressure lowering effect of Queen Garnet plum juice on mild-hypertensive, obese or over-weight subjects: a double-blind placebo controlled study. Pharmacological Research.

Oral presentations

- Cyanidin 3-glucoside attenuates diet-induced metabolic syndrome in rats. Victorian Obesity Consortium (VOC), Melbourne, Australia (Dec 2015).
- Dietary nitrates in metabolic syndrome. Indian Academy of Biomedical Sciences (IABS), Hyderabad, India (January 2015).
- Effect of cardamom in diet-induced obese rat model. World obesity (ICO), Kuala Lumpur, Malaysia (March 2014).

Poster presentations

- Are dietary inorganic nitrates helpful in metabolic syndrome? Institute of cardiovascular and nutrition (ICAN), Paris, France (December 2014).
- Queen Garnet plums in a rat model of diet-induced metabolic syndrome. Asian Congress of Nutrition (ACN), Yokohama, Japan (May 2015).

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# CHAPTER 1

# 1. Metabolic syndrome, prevalence, relevant animal models and dose translation to humans

## 1.1. Why metabolic syndrome?

Metabolic syndrome is the cluster of metabolic complications such as obesity, dyslipidaemia, impaired glucose tolerance and insulin resistance together with hypertension (1, 2) influenced by environmental factors including lifestyle and diet (3-5). Different organisations have specified different requirements for classifying patients under the definition of metabolic syndrome (6-10). These requirements have been summarised in Table 1.1. Many complications are not included in the definition of metabolic syndrome, such as fatty liver, oxidative stress, inflammation and endothelial dysfunction, but these are frequently found in patients with metabolic syndrome (1, 3). The prevalence of metabolic syndrome has reached a level where it is considered as a worldwide health problem. Table 1.2 summarises the prevalence of metabolic syndrome in different countries according to the different definitions.

The high prevalence of metabolic syndrome makes it extremely important to find effective treatments or preventions. Hence, it is important to understand the mechanisms associated with the development of metabolic syndrome in rodents, the pathophysiological similarities and differences between humans and rodent models and finally the translatability of results from rodent models to humans.

**Table 1.1 Definitions of metabolic syndrome**

| | **WHO (1998) (6)** | **EGIR (1999) (7)** | **NCEP ATP III (2005 revision) (8)** | **IDF (2005) (9)** | **Harmonised definition (2009) (10)** |
|---|---|---|---|---|---|
| Compulsory | Insulin resistance | Hyperinsulinaemia | None | Central obesity | None |
| Requirements | Insulin resistance or diabetes, plus any two of the five criteria | Hyperinsulinaemia, plus any two of the four criteria | Any three of the five criteria | Central obesity, plus any two of the four criteria | Any three of the five criteria |
| Central obesity | Waist:hip ratio (>0.90 in males, >0.85 in females) or BMI >30 kg/$m^2$ | Waist circumference (≥94 cm in males, ≥80 cm in females) | Waist circumference (>102 cm in males, >88 in females) | Waist circumference (≥94 cm in males, ≥80 cm in females) | Population- and country-specific definitions |
| Hyperglycaemia | Impaired glucose regulation, type 2 diabetes or insulin resistance | Hyperinsulinaemia; fasting plasma glucose ≥6.1 mmol/L | Fasting glucose ≥100 mg/dl | Fasting glucose ≥100 mg/dl or previously diagnosed type 2 diabetes | Fasting glucose ≥100 mg/dl |
| Dyslipidaemia (Triglycerides) | Triglycerides ≥150 mg/dl or HDL-cholesterol (<35 mg/dl in males, <39 mg/dl in females) | Triglycerides ≥180 mg/dl or HDL-cholesterol <40 mg/dl or | Triglycerides ≥150 mg/dl or treated for dyslipidaemia | Triglycerides ≥150 mg/dl or treated for dyslipidaemia | Triglycerides ≥150 mg/dl or treated for dyslipidaemia |

| | | | | | |
|---|---|---|---|---|---|
| Dyslipidaemia (Cholesterol) | | treated for dyslipidaemia | HDL-cholesterol (<40 mg/dl in males, <50 mg/dl in females) | HDL-cholesterol (<40 mg/dl in males, <50 mg/dl in females) or treated for dyslipidaemia | HDL-cholesterol (<40 mg/dl in males, <50 mg/dl in females) or treated for reduced HDl-cholesterol |
| Hypertension | ≥160/90 mmHg | ≥140/90 mmHg | >130/85 mmHg or treated for hypertension | >130/85 mmHg or treated for hypertension | ≥130/85 mmHg or treated for hypertension |
| Other criteria | Microalbuminuria (urinary albumin excretion ≥20 μg/min or albumin:creatinine ratio ≥20 mg/g) | | | | |

Abbreviations used: WHO, World Health Organisation; EGIR, European Group for the Study of Insulin Resistance; NCEP ATP III, National Cholesterol Education Program Adult Treatment Panel III; IDF, International Diabetes Federation; BMI, body mass index; HDL, high-density lipoprotein.

**Table 1.2 Prevalence of metabolic syndrome**

| Country of survey | Definition used | Year of survey | Age group | Prevalence |
|---|---|---|---|---|
| Johannesburg metropolitan area, South Africa (11) | Harmonised definition (10) | Started in 1990 | | 29% in African population<br>46% in Asian Indian population |
| Australia (12) | NCEP ATP III | 2004/2005 | 25-74 years | 27.1% in men; 28.3% in women |
| | IDF | | | 33.7% in men; 30.1% in women |
| Kuwait (13) | IDF | 2006 | 20-65 years | 36.2% |
| | NCEP | | | 24.8% |
| Australia (14) | ATP III | 1999-2000 | ≥ 25 years | 22.1% |
| | WHO | | | 21.7% |
| | IDF | | | 30.7% |
| | EGIR | | | 13.4% |
| India (15) | NCEP ATP III | 2010-2011 | ≥ 30 years | 29% in women; 23% in men; overall 25.6% |
| Taiwan (16) | WHO | - | 19-95 years | 18.5% males; 14.7 % females |
| USA (17) | NCEP | 1999-2002 | ≥ 20 years | 33.7% males; 35.4% females; 34.5% overall |

| | | | | |
|---|---|---|---|---|
| | IDF | | | 39.9% males; 38.1% females; 39% overall |
| USA (18) | NCEP ATP III | 2003-2006 | ≥ 20 years | 35.1% in men; 32.6% in women; 34% overall |
| Spain (19) | Revised NCEP ATP III | 2001-2003 | ≥ 20 years | 28.2% in men; 26.3% in women; overall 27.2% |
| | IDF | | | 36.9% in men; 28.1% in women; overall 32.2% |
| | Harmonised definition | | | 38.9% in men; 28.4% in women; overall 33.2% |

Abbreviations used: WHO, World Health Organisation; EGIR, European Group for the Study of Insulin Resistance; NCEP ATP III, National Cholesterol Education Program Adult Treatment Panel III; IDF, International Diabetes Federation

### 1.2. Obesity and insulin resistance

Diabetes is a metabolic disease characterised by hyperglycaemia resulting from defects in insulin secretion, insulin action, or both (20). The International Diabetes Federation estimates that in 2013, there were over 382 million diabetic patients world-wide with an additional 316 million with impaired glucose tolerance, associated with 5.1 million diabetes-related deaths and a burden of USD 548 billion on the world's health-care systems (21).

Depending on the underlying cause, the usual classification is as either type-1 or type-2 diabetes (20). In type-1 diabetes, the destruction of pancreatic β-cells leads to absolute insulin deficiency (20). Type-2 diabetes, which accounts for 90-95% of all diabetes cases, is characterised by varying degrees of insulin resistance with inadequate compensatory increase in insulin secretion (20). In type-2 diabetes, an initial β-cell compensatory hypertrophy and hyperplasia is followed by an insulin secretory defect largely mediated by progressive loss of β-cell functions such as glucose-stimulated insulin secretion ultimately leading to β-cell apoptosis induced by glucotoxicity and lipotoxicity (22, 23). High glucose concentrations down-regulate the expression of insulin, GLUT2, glucokinase, voltage-dependent $Ca^{2+}$ channels, and their transcription factors, all involved in glucose-stimulated insulin secretion (22). Free fatty acids derived from adipose tissue largely contribute to the lipotoxic effects on the β-cell through endoplasmic reticulum (ER) stress and NO-mediated β-cell apoptosis (23).

The pathogenesis of insulin resistance depends on increased ectopic fat deposition in organs such as the liver and muscle from diets rich in saturated fats and lipogenic sugars, rather than from increased circulating free fatty acids (24-27). Fructose is a lipogenic sugar since metabolism to 2-carbon units produces the substrates for synthesis of fatty acids *in vivo*. Further, insulin resistance leads to dysfunctional responses from key insulin-sensitive lipogenic transcriptional factors such as liver X receptors (LXRs) and sterol regulatory element-binding protein 1c (SREBP-1c) (28). However, in an insulin-resistant state, SREBP-1c is paradoxically up-regulated, due to activation of cleavage of SREBP-1c by ER stress leading to the stimulation of insulin-independent lipogenesis in an insulin-resistant liver (29). Other transcriptional factors such PPARα and PPARγ improve insulin sensitivity by decreasing ectopic fat accumulation and enhancing insulin signalling resulting in enhanced blood glucose control (30, 31). Many extra-pancreatic hormones are key regulators of both insulin secretion and sensitivity including glucagon-like peptide 1 (GLP-1) (32), insulin-like growth factor-1 (IGF-1) (33), and apelin (34, 35), as well as classical adipokines including

interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-α), adiponectin, leptin, resistin and visfatin (36, 37).

### 1.3. Adipokines in insulin resistance

Adipose tissue secretes many bioactive factors, known as adipokines, to regulate energy intake and fat storage (38). Increased deposition of fat as white adipose tissue has been postulated to be part of the evolutionary process with the assumption of "survival of the fattest" (3). The circulating concentrations of adipokines such as adiponectin, leptin, resistin and visfatin secreted by adipocytes in white adipose tissue regulate insulin secretion and sensitivity. Increased adipose tissue increases production of cytokines including TNF-α and IL-6, and adipokines including leptin, adiponectin, resistin and visfatin (38). These adipokines have complex roles in the regulation of insulin secretion and sensitivity (Figure 1.1). Further, chronic inflammation associated with obesity could lead to development of insulin resistance in obesity by the increased production of pro-inflammatory cytokine mediators such as TNF-α and IL-6 (39). The increased production of TNF-α and the increased expression of its receptors have been associated with obesity in both animals and humans (38).

Adipokines such as leptin, adiponectin, resistin and visfatin play a role in development of obesity, insulin resistance, diabetes, inflammation and auto-immunity (40) and also control energy intake and expenditure (41). These adipokines exhibit anti-inflammatory effects that could improve insulin resistance and obesity-related diseases (42-44) and they cross the blood-brain barrier to control satiety and hunger by acting on the hypothalamus (41).

Basal concentrations of leptin were higher in obese compared to insulin-resistant patients who had higher concentrations than insulin-sensitive patients (45). Intravenous glucose tolerance tests with tolbutamide showed decreased plasma leptin concentrations in insulin-resistant patients where no changes were observed in insulin-sensitive and obese patients, indicating that elevated plasma leptin concentrations are associated with insulin resistance independent of obesity and insulin sensitivity (45). Calorie restriction for 8 weeks in 162 obese/overweight patients on a weight loss program decreased body weight as well as plasma leptin and insulin concentrations (46). The same study was continued for another 24 weeks where subjects were given general dietary guidelines to maintain the weight loss, but without calorie restrictions or specific follow-up instructions. Subjects who started with

higher plasma leptin and insulin and lower plasma ghrelin concentrations, regained their lost weight (46), confirming the involvement of these hormones in the development of obesity with insulin resistance. In male Sprague-Dawley rats, continuous subcutaneous leptin infusion for 48 hours decreased fasting plasma insulin, glucose, IGF-1 and C-peptide concentrations and improved insulin sensitivity (47), while chronic leptin infusion increased arterial pressure and decreased fasting blood glucose and insulin concentrations (48).

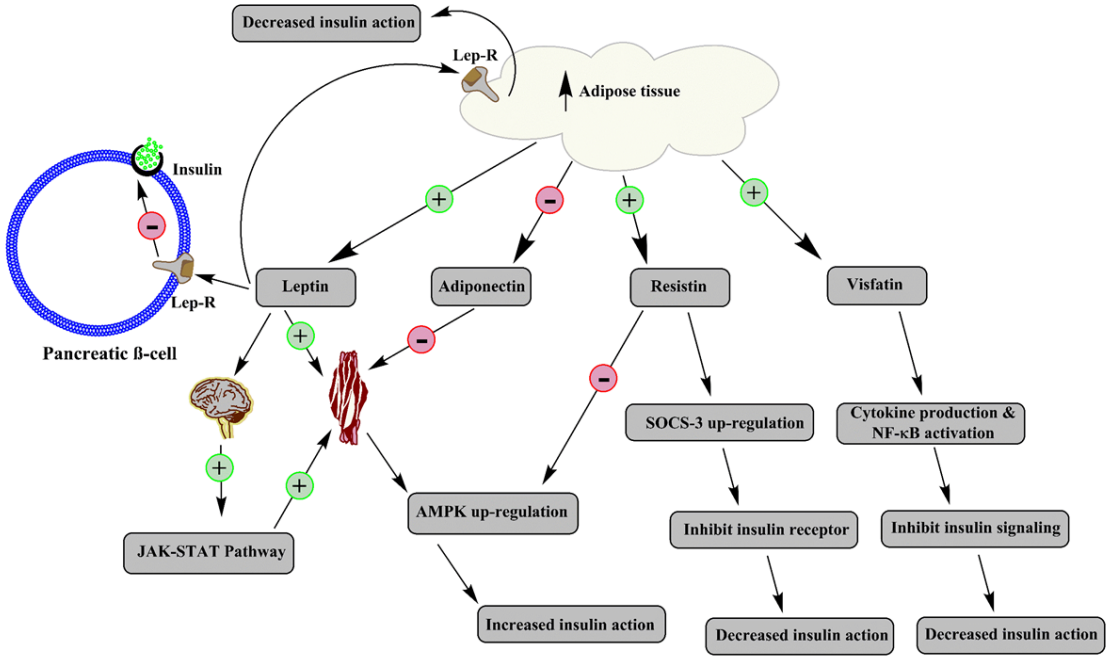



Figure 1.1 The mechanisms of action of adipokines in increasing insulin resistance

Leptin binding to the Lepr-b isoform of leptin receptor (Lep-R) activates the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway (JAK-STAT) in the hypothalamus, directly acting on skeletal muscle to increase AMP-activated protein kinase (AMPK). Whether this action of leptin on skeletal muscle increases or decreases glucose uptake and insulin-stimulated glucose metabolism remains controversial (49-51). Leptin binding to lepr-b has a direct impact on glucose homeostasis, as leptin acts on skeletal muscle increasing glucose uptake and insulin-stimulated glucose metabolism (52, 53), but inhibits insulin secretion from β-cells (54) and glucagon secretion from α-cells (55) and increases expression of GLUT-4 and uncoupling proteins-1 and -3 in adipose tissue (56, 57). Similarly, *in vitro* and *in vivo* studies with chronic leptin treatment particularly in leptin deficiency showed increased glucose uptake and movement of GLUT-4 to plasma membranes with normalized glycogen synthase and glucose oxidation in skeletal muscle and hepatic cell lines (51, 58-61).

Increased concentrations of adiponectin were associated with improved insulin resistance and decreased total body fat mass in an in-patient study on 33 obese adolescents treated for 9 months with moderate physical activity (62). Low plasma adiponectin concentrations were reported in 128 patients with diabetes or diabetic coronary artery disease (63), while weight reduction increased plasma adiponectin concentrations with improved insulin resistance (64). Hypoadiponectinemia may contribute to insulin resistance and may accelerate atherogenesis associated with obesity as serum adiponectin concentrations are lower in obesity and increase after weight loss (65). Increased plasma adiponectin concentrations may improve regulation of insulin sensitivity (66).

Increases in adiponectin concentrations and adiponectin receptors AdipoR1 and AdipoR2 stimulate AMPK phosphorylation and activation in skeletal muscle and liver (67) and increase glucose utilization and fatty-acid metabolism (67). Adiponectin globular domain (ACRP30) enhanced muscle fat oxidation and glucose transport via AMPK activation and acetyl-CoA carboxylase inhibition (68). In transgenic mice, ACRP30 reduced expression of gluconeogenic enzymes such as phosphoenolpyruvate carboxylase (PEPCK) and glucose-6-phosphatase (G6Pase) associated with elevated phosphorylation of hepatic AMPK, which may account for inhibition of endogenous glucose production by adiponectin (69, 70). Adiponectin in gAd Tg, *ob/ob* or ApoE-deficient mice activated PPARα, which increased fatty-acid metabolism and energy consumption with decreased triglyceride content in the liver and skeletal muscle, thereby increasing insulin sensitivity (71).

Resistin increases with an increase in adipose tissue in rodents and also in patients with type 2 diabetes (72), inhibiting insulin-stimulated glucose uptake causing impaired glucose tolerance and metabolism (73). Increased serum resistin concentrations were shown in obese diabetic patients compared with obese non-diabetic controls (74). In obese subjects, concentrations of resistin were increased compared to lean individuals (75) and decreased after weight loss with sibutramine or orlistat treatment for 6 months (76). In 2141 subjects, increased resistin concentrations were measured in women with metabolic syndrome, with men showing no changes in resistin concentrations with or without metabolic syndrome (77). Extremely high resistin concentrations were shown in patients with stable angina pectoris or acute coronary syndrome with positive correlations between resistin concentrations and obesity, serum lipids, inflammatory markers and insulin resistance (78).

Infusion of resistin in male Sprague-Dawley rats induced hepatic insulin resistance (79). In resistin-knockout mice, increased AMPK activity and decreased gluconeogenic

enzyme expression improved glucose homeostasis (80) and also increased cytokine signalling-3 expression (SOCS-3), a negative regulator for insulin with inhibition of insulin receptor (IR) and activation of IRS-1 by phosphorylation contributing to insulin resistance (81). Human hepatoma (HepG2) cells treated with resistin showed decreased phosphorylation of AMPK, GLUT-2 mRNA expression and glycogen synthesis with increased expression of G6Pase and PEPCK leading to hepatic insulin resistance (82).

The roles of visfatin in insulin resistance, insulin-mimetic effects, and diabetes are controversial as some studies showed improved insulin resistance (83-85) while other studies showed negative effects on insulin resistance (86-88). Higher fasting visfatin concentrations were measured in patients with diabetes or impaired glucose tolerance with increased pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β and NF-κB activation (89-91). Patients with long-term type-1 diabetes showed elevated serum visfatin, TNF-α and IL-6 concentrations (86). These increased visfatin concentrations could cause β-cell deterioration (86). Increased plasma visfatin concentrations were observed in obese children and women (92, 93) whereas other studies in obese subjects showed lower plasma visfatin concentrations (94, 95). Increased visfatin concentrations caused smooth muscle inflammation with impaired endothelial function (96), with increased risk of coronary artery disease (97, 98) and vascular disease (99). In contrast, visfatin improved insulin resistance (100). Extracellular visfatin in HepG2 cells increased glucose production, mRNA expression of PEPCK and G6Pase and stimulated gluconeogenic enzyme expression via phosphorylation of cyclic AMP-responsive element-binding protein (100). Resistin and visfatin concentrations increased with increased visceral adipose tissue (72, 101), with increased resistin and visfatin concentrations leading to inflammation, increased TNF-α, IL-6, pre-B cell formation and other inflammatory markers, inhibiting insulin signalling therefore decreasing insulin action (91).

### 1.4. Cardiovascular remodelling and hypertension

Cardiovascular remodelling is a process of change in the size, shape and function of the heart as a physiological response to metabolic or hormonal changes in the body (102, 103). These physiological responses lead to the development of molecular and cellular changes, including hypertrophy, necrosis and apoptosis of the myocyte, fibroblast proliferation and fibrosis in the interstitium (103). These changes lead to abnormalities in myocardial function including impaired contractility and relaxation, diminished cardiac pump function, dilatation and increased sphericity of the heart. Ultimately, these changes lead to systolic and diastolic cardiac dysfunction, which forms the basis of heart failure and death

(103). Hypertension appears to be the strongest predictor of cardiovascular mortality out of all the components of metabolic syndrome. The pathological mechanisms of hypertension in metabolic syndrome are not fully understood. Hypertension in metabolic syndrome has multifactorial causes with the following mechanisms thought to play a pivotal role in the pathophysiology of metabolic syndrome hypertension, these include: sympathetic hyperactivation, increased renin-angiotensin-aldosterone activity and endothelial dysfunction (104).

### 1.5. Inflammation

Inflammation is part of the non-specific immune response that occurs in reaction to any injury to the tissues. In some pathological conditions, the inflammatory process becomes continuous and subsequently leads to the development of chronic inflammatory diseases such as obesity (105, 106). Initially, a link between obesity and inflammation was established through the expression of by the increased plasma concentrations of pro-inflammatory markers including cytokines and acute phase proteins such as C-reactive protein (CRP) in obese subjects (107-109). CRP is now considered an independent risk factor for the development of cardiovascular disease (110). Many inflammatory markers present in the plasma of obese individuals originate from adipose tissue (108). Thus, obesity is now defined as the state of chronic low-grade inflammation, which is initiated by the morphological changes in the adipose tissue (111). Some of the pro-inflammatory cytokines from adipose tissue interfere with the signalling pathway for insulin. This ultimately leads to insulin resistance (112). Liver is the other tissue that is affected by excess adipose tissue and pro-inflammatory cytokines produced by adipose tissue. Chronic activation of nuclear factor-$\kappa$B (NF-$\kappa$B) by cytokines leads to the development of insulin resistance in liver (113, 114). The development of hepatic steatosis and non-alcoholic fatty liver disease in presence of insulin resistance has been established (115-118).

### 1.6. Non-alcoholic fatty liver

Non-alcoholic fatty liver disease is a clinical condition that includes a wide spectrum of liver complications ranging from simple steatosis to steatohepatitis, advanced fibrosis and cirrhosis (119). The liver is a metabolic workhorse that performs a range of biochemical functions including metabolism of lipids. Hepatic steatosis develops when there is excess deposition of triglycerides in the hepatocytes. This pathological condition can develop when the total input of fatty acids is more than the total output (120). Sources of fatty acids in the liver include the hepatic free fatty acid uptake from blood and *de novo* lipogenesis. The

output of fatty acids from the liver can be through fatty acid oxidation and fatty acid export within very low-density lipoproteins (120). The disturbances in hepatic input and output of lipids can occur in the conditions of dyslipidaemia, obesity and insulin resistance (119, 121). Steatosis is characterised by excess fat storage and it can progress to steatohepatitis and finally leads to cirrhosis and structural and functional abnormalities of the liver. Some patients only develop steatosis whereas others develop steatohepatitis and fibrosis.

### 1.7. Classical animal models of obesity and metabolic syndrome

Animal experiments have contributed significantly to the understanding of human biochemistry, physiology, pathophysiology and pharmacology. Different models have been developed, characterised and then successfully used for the development of preventions or cures for human diseases. One of the important human conditions is metabolic syndrome and this can be successfully induced in rodents. Many animal models have been used in metabolic syndrome research to mimic the human conditions (122). These animal models have been consistently used to identify both the roles of various regulators in the body that may be responsible for the development of metabolic syndrome in humans and some of the treatment strategies against metabolic syndrome (122-124). Review articles have identified the usefulness of these animal models; however, few models reproduce the range of changes that metabolic syndrome produces throughout the human body (122, 123, 125-133). The animal models currently used include the genetic models that develop metabolic syndrome spontaneously, genetically modified models to induce metabolic syndrome and the models in which the metabolic syndrome is induced using specialised diets.

### 1.8. Genetic models of obesity and diabetes

Genetic models of obesity and diabetes include *db/db* mice, *ob/ob* mice, Zucker diabetic fatty rats, Otsuka Long-Evans Tokushima Fatty rats and Goto-Kakizaki rats. These models are primarily useful in identifying treatments for metabolic syndrome consequent to genetic defects. However, the models also induce other pathological conditions associated with metabolic syndrome in humans (122).

o*b/ob* mice have a mutation in the leptin gene, important since leptin controls energy intake and expenditure. These leptin-deficient mice become obese at a very early age. Obesity accompanies hyperinsulinaemia, hyperglycaemia and impaired glucose tolerance and non-alcoholic fatty liver disease (122, 134). However, they do not develop hypertension or dyslipidaemia (122). *db/db* mice and Zucker diabetic fatty rats have a mutation in the gene

for leptin receptor, impairing leptin signalling (135, 136). Similar to *ob/ob* mice, *db/db* mice and Zucker diabetic fatty rats show signs of early-life obesity, hyperinsulinaemia, hyperglycaemia and cardiovascular complications along with dyslipidaemia (122, 136). However, they fail to develop hypertension (122). These 3 models suggest the importance of leptin in control of metabolism in the body and also the role of leptin or leptin signalling defects in the development of metabolic syndrome.

Otsuka Long-Evans Tokushima Fatty rats have decreased cholecystokinin-1 receptor density. Cholecystokinin is a peptide hormone secreted from L-cells of intestine that regulates digestion and food intake. These rats show progression of obesity with increasing age, correlated with higher food intake due to deficiency of cholecystokinin-1 receptor. These rats also show dyslipidaemia, hyperglycaemia, impaired glucose tolerance and insulin resistance along with hypertension and cardiovascular complications (122). Goto-Kakizaki rats are non-obese and spontaneously diabetic. These rats develop hyperglycaemia at a very early age; they also show cardiac hypertrophy and systolic dysfunction along with signs of kidney damage at later stages of life. The other symptoms of metabolic syndrome observed in this model are impaired glucose tolerance, dyslipidaemia, insulin resistance and hyperinsulinaemia but hypertension is not observed (122, 137). An inbred model for obesity has been identified at the National Institute of Nutrition, Hyderabad, India (138). Both males and females from this strain show similar responses. This strain (WNIN/Ob) develops obesity, probably through hyperphagia, at a very early stage of life and hyperglycaemia and hyperinsulinaemia by 28 days of age. They have lower lean mass and much higher fat mass compared to their lean littermates along with dyslipidaemia. These rats show hyperleptinaemia probably with normal leptin and leptin receptor locus. A defect on chromosome 5 near leptin receptor locus has been suggested as the cause of obesity; however, this has not been confirmed (138, 139).

Although these rodent models provide a reasonably reproducible pathological condition, they do not mimic the actual pathophysiology in humans as the occurrence of genetic defects in either leptin or cholecystokinin receptors leading to metabolic syndrome, obesity and diabetes is quite rare (122, 140).

### 1.9. Artificially induced metabolic syndrome in animals

Metabolic syndrome can be artificially induced in experimental animals, usually mice, through genetic engineering where a particular gene of interest is knocked out or made non-

functional. These genetic models define the role of a particular protein or receptor in the pathophysiology of metabolic syndrome. For example, GLUT-4, IRS-1, IRS-2 and insulin receptor knockout mice have been studied (141-145). These genetically engineered models do not mimic the human condition of metabolic syndrome but they can provide useful information about a particular protein, its receptor and the intracellular pathways involved in the regulation of metabolism (122).

A commonly used strategy to induce metabolic syndrome in animals, especially rodents, is the use of hypercaloric diets. It is argued that the diet-induced models best mimic the human conditions as they share similar mechanisms in the development of metabolic syndrome (122). The diets used to induce metabolic syndrome include fructose, sucrose, animal and plant fats. Different research groups have characterised different combinations of these components in different species and strains of rodents. Some examples of the combinations of diets include high-carbohydrate diets (either fructose or sucrose as carbohydrate), high-fat diets (either animal or plant fats) and combination high-carbohydrate, high-fat diets (122). These diets differ in the contribution of carbohydrate and fat to available calories and the sources of fat. Basically, the aim of these studies is to provide excess energy from diet, which is the major cause of human metabolic syndrome and obesity (129). Most of the diet-induced models show symptoms of metabolic syndrome including central obesity, dyslipidaemia, impaired glucose tolerance, insulin resistance and hypertension along with cardiovascular complications as well as non-alcoholic fatty liver disease (122).

It is argued that a combination of simple sugars and animal fat serves as the best model to mimic metabolic syndrome in rodents (122). This type of diet mimics the Western/cafeteria diet that is rich in fructose, sucrose and animal fat. Also, the prevalence of metabolic syndrome is very high in developed countries (*Table 1.2*). This may directly reflect the role of high-carbohydrate, high-fat diets in the development of metabolic syndrome. Based on this assumption, we have characterised an animal model with complex diet composition including fructose, condensed milk and beef tallow, which induced a range of complications that are generally found in metabolic syndrome patients (146). These complications included hyperinsulinaemia, impaired glucose tolerance, central obesity, non-alcoholic fatty liver disease, cardiac remodelling, hypertension and endothelial dysfunction along with mild renal damage and increased pancreatic islet mass (146). The high-carbohydrate, high-fat diet mimics the full range of metabolic syndrome changes occurring in either vegetarian or non-vegetarian populations, depending on the source of the increased

fats. However, the complexity and variability of the human diet cannot be mimicked by a fixed rodent diet on a day-to-day basis. An advantage of the rat model is the shortened time period to develop the syndrome of months rather than years or decades as in humans. This model has been used earlier to test natural products for the attenuation of metabolic syndrome (147-149). The different responses shown by these interventions clearly suggest that this model is capable of responding to the pharmacological interventions.

*Monosodium glutamate-induced obesity:* Monosodium glutamate given by subcutaneous injection induces obesity in mice and rats (150-152) together with glucose tolerance, dyslipidaemia, liver dysfunction and hyperinsulinaemia (151). Monosodium glutamate in rats and mice did not affect body weight when it was given in diet (up to 20% of diet) or in drinking water (2%) (153). It has been argued that monosodium glutamate does not reflect human obesity in rats and mice (153, 154) since, in humans, monosodium glutamate is taken in foods and not as an injection. Thus, results with injected monosodium glutamate in mice and rats cannot be extrapolated to oral ingestion in humans.

*Intrauterine growth restricted rats:* This is also a very recently developed model based on the Barker hypothesis (155). The rat model was developed through bilateral ligation of uterine artery to reduce the blood flow to the foetus (156). At birth, rats showed lower insulin concentrations and body weight compared to control rats. After 7 weeks of age, fasting blood glucose and insulin concentrations were higher in these rats than control. After 15 weeks of age, the growth restricted rats showed lower insulin with higher fasting blood glucose concentrations than their normal controls. By 26 weeks of age, rats showed obesity and very high fasting blood glucose concentrations, a characteristic of type 2 diabetes (156, 157). A similar model has been developed by restricting the diet to the pregnant mothers (158).

Although there are differences between the physiology of humans, rats and mice, appropriate animal models can provide an excellent initial point to study either the causes or treatment strategies for metabolic syndrome. This provides the basic understanding of the intervention strategy before going into human trials. At this point, *in vitro* assays may help provide more information on the possible effects of the proposed interventions.

In this thesis, the high-carbohydrate, high-fat diet-induced model of the metabolic syndrome in rats was used (146). This diet mimicked the human diet associated with the development of metabolic syndrome. It produced changes in metabolic, cardiovascular and

hepatic structure and functions such as excessive abdominal fat deposition with increases in body weight of 10-15%, impaired glucose tolerance (pre-diabetes), elevated blood pressure by 30-40 mmHg and increased plasma lipid concentrations together with increased plasma liver enzymes compared with corn starch diet-fed rats (146). Histological evaluation of heart and liver showed increased infiltration of inflammatory cells and fat vacuoles in the liver. This was accompanied by increased left ventricular stiffness and decreased aortic reactivity (146). Corn starch is a slowly digestible complex carbohydrate (159) and functions as a control for the HCHF diet where the primary carbohydrate is fructose. Unlike fructose, corn starch does not increase blood glucose, plasma insulin or free fatty acid concentrations (159, 160). This model would be an appropriate model of the human metabolic syndrome (146).

### 1.10. Human experimental models

Human trials are essential in the development of drug therapies. New compounds are firstly identified in animal studies as potential human treatments. Experimental parameters such as dosage, dietary composition and exercise can be controlled in animal studies, but are much more difficult to control in outpatient studies in humans. Table 1.3 describes some of the human trials for interventions against the symptoms of metabolic syndrome.

### 1.11. Translation to clinical practice: difficulties and limitations

Translation of results from rodent studies to human trials remains a problem. For example, quercetin attenuated the symptoms of metabolic syndrome in rodent models (161, 162). It is one of the most commonly found flavonoids in the human diet, yet prevalence of metabolic syndrome is increasing in the community. Two well-studied compounds against metabolic syndrome are curcumin and resveratrol with an immense literature available for these compounds *in vitro* and *in vivo* (163-165). However, the successful translation of these interventions to humans has not yet been reported.

Relevant human doses have been estimated from rodent doses (166, 167), but these doses assume high oral bioavailability for compounds given in the food while drug metabolism may vary between rats and humans. Further, the food matrix is a well-known variable affecting absorption of food components. The length of dosing is important, with the average life-span of laboratory rats of about 2 years being much less than the average human life-span of 75-80 years in many countries. Thus, 8 week interventions in rats are approximately 6 year interventions in humans, based on life-span. Longer interventional

studies in humans, at higher doses, may be necessary to show therapeutic benefits. This may impose safety issues in the use of new interventions in humans.

**Table 1.3 Treatments used in humans against metabolic syndrome**

| Subject | Study type and model | Study duration | Treatment and dose | Exclusion criterion | Effects |
|---|---|---|---|---|---|
| 19 females; 11 males (18-75 years age) (168) | Randomised, double-blind, 2-arm, parallel-group, placebo-controlled; in type 2 diabetes patients | 2 weeks | 5µg exenatide (1st week); 10µg exenatide (2nd week) | clinically important medical conditions or had used sulfonylureas, meglitinides, a-glucosidase inhibitors, pramlintide, exogenous insulin, or weight-loss drugs within the prior 2 months; fasting triglycerides >4.5 mmol/L, >1 episode of severe hypoglycaemia within 6 months, treatment with corticosteroids within 2 months, treatment with an investigational drug within 30 days, or current treatment with drugs known to affect gastrointestinal motility | Lowerd post-prandial glucose and triglyceride excursion; no change in free fatty acids |
| 24 females; 5 males (18-30 years age) (169) | Randomised crossover; obese subjects (BMI ≥30) | 2 study visits; at least 7 days apart | Cereal prepared with 6g ground cassia cinnamon during study visit | Allergy to wheat, cinnamon and sucralose; pregnancy; type 1 or type 2 diabetes | Lowered postprandial glucose |

| | | | | | |
|---|---|---|---|---|---|
| 875 patients (55-80 years age); normal (n=282); overweight (n=405); obese (n=150); severely obese (n=38) (170) | Essential hypertension; double-blind treatment with losartan compared to atenolol | LIFE trial; cardiovascular death/fatal or non-fatal myocardial infection/stroke as endpoint | Losartan & atenolol | Underweight (BMI <18.5 kg/$m^2$) | Shift from concentric to eccentric hypertrophy in both the treatment groups; higher cardiovascular mortality despite antihypertensive treatment in obese subjects |
| 7447 subjects (57% females); (55-80 years age) (171) | PREDIMED trial; Parallel group, multicentre, randomised; Type 2 diabetes or any 3 of following: smoking. Hypertension, higher LDL-c, lower HDL-c, obesity, family history of coronary heart | Primary end-points: myocardial infarction, stroke and death from cardiovascular causes; Secondary end-points: stroke, myocardial infarction, death | Mediterranean diet with extra-virgin olive oil (1L/week) or Mediterranean diet with nuts (30g/day) | - | Mediterranean diet without energy-restriction reduces the risk of major cardiovascular events among high-risk persons |

| | | | | | |
|---|---|---|---|---|---|
| | disease | from cardiovascular causes and death from any cause | | | |
| 35 males (mean age: 53.8 ± 5.8 years) (172) | Mild hypercholesterolaemia | 18 weeks (6 weeks treatment followed by 6 weeks gap followed by 6 week treatment) | Chokeberry juice, 250 mL/day | No earlier pharmacological treatment | Decreased serum triglycerides, serum total/LDL-c, improved endothelial function, |
| 42 males; 54 females (25-65 years age); BMI 25-35 kg/$m^2$ (173) | Double-blind, randomised, placebo controlled crossover trial; central obesity and high serum triglycerides | 6 weeks treatment followed by 5 week washout | Quercetin, 150 mg/day | Smoking; insulin-dependent diabetes mellitus; liver, gastrointestinal, or inflammatory diseases; a history of cardiovascular events; abnormal thyroid function; use of anti-obesity medications, dietary supplements, or anti-inflammatory drugs; cancer; recent major surgery or illness; pregnancy or breast-feeding; alcohol abuse; participation in a current weight loss program; necessity for a medically | apoE3 genotype- decreased blood pressure, serum TNFα, no change in serum total cholesterol, triglyceride, glucose; apoE4 genotype- reduction in HDL, serum TNFα, no change in serum total cholesterol, triglyceride, glucose, no change in |

| | | | | | |
|---|---|---|---|---|---|
| | | | | supervised diet; >5 kg weight loss within the 3 month prior to the study | blood pressure |
| 30 males;<br>12 females<br>(18-75 years age)<br>(174) | Randomised, double-blind crossover trial;<br>Blood pressure: systolic 140-170mmHg & diastolic 90-105mmHg | 4 weeks | Potassium bicarbonate or potassium chloride (potassium, 6.4 mmol/day) | Impaired renal function, secondary cause of hypertension, chronic diarrhoea, history of ulcer disease, previous stroke, ischaemic heart disease, heart failure, diabetes mellitus, malignancy, liver disease, pregnancy or breastfeeding, oral contraceptive pills | Improved endothelial function, reduced cardiovascular risk factors; potassium bicarbonate – improved calcium and bone metabolism |
| 338 males;<br>352 females<br>(25-64 years age)<br>(175) | Randomised control trial | 6 months | Increase in consumption of fruits and vegetables | Cardiovascular disease other than hypertension, gastrointestinal disease, cancer, serious psychiatric disorders, hypercholesterolaemia, recent traumatic events | Reduction in systolic and diastolic blood pressure by 4mmHg and 1.5mmHg, respectively |
| 4 males;<br>44 females<br>(mean age: 50 ± 3 years) (176) | Randomised controlled study | 8 weeks | Freeze-dried blueberries, 50g/day | <21 years age; taking medications for hypoglycaemic, hypolipidemic, anti-inflammatory or steroidal medications; liver, renal or thyroid disorders; anaemia; consuming antioxidants or fish oil supplements regularly; smokers; | Decrease in blood pressure<br>Decreased LDL & lipid peroxidation<br>Trend towards decrease in body weight |

| | | | | | |
|---|---|---|---|---|---|
| | | | | consuming alcohol regularly; pregnant or lactating females | |
| 289 subjects; males (45-74 years age); females (55-74 years age) (177) | Randomised, double-blind, parallel trial; carotid intima–media thickness (0.7-2.0 mm on at least one side) | 18 months | Pomegranate juice, 240 mL/day | Coronary heart disease, diabetes, BMI>40kg/$m^2$, hepatic disease, cancer in previous 2 years, HIV, hepatitis B or C, uncontrolled hypertension, untreated or unstable hypothyroidism | Rate of carotid intima–media thickness progression was slowed |
| 45 subjects (69-80 years age) (178) | Randomised, placebo-controlled, double-blinded study | 3 months | Pomegranate juice, 240 mL/day | History of stroke or transient ischemic attack; myocardial infarction during the preceding 6 weeks; surgically untreated left main coronary artery lesion with >50% diameter narrowing; coronary revascularization procedure during the preceding 6 months; current unstable angina pectoris; abnormal lung uptake on previous scintigram or positron emission tomogram; class IV congestive heart failure; or ejection fraction <30% at time of study entry; significant co-morbidity; current use of tobacco | Decreased myocardial ischaemia and improved myocardial perfusion; no negative effects on lipids, blood glucose, haemoglobin A1c, body weight or blood pressure |

| | | | | | |
|---|---|---|---|---|---|
| | | | | products; or alcohol or drug abuse | |
| 28M; 28F, (30-60 years age) (179) | Randomised double-blind, placebo-controlled parallel trial; BMI≥30kg/$m^2$; Stable hypertension with systolic <160mmHg and diastolic <100mmHg for 6 months | 3 months | 379mg green tea extract (including 208mg of epigallocatechin-3-gallate) | Secondary hypertension and/or secondary obesity; diabetes; history of coronary artery disease; stroke congestive heart failure; malignancy; history of use of any dietary supplements within 3 months before the study; current need for modification of antihypertensive therapy; abnormal liver, kidney or thyroid gland function; clinically significant inflammatory process within respiratory, digestive or genitourinary tract, or in the oral cavity, pharynx or paranasal sinuses; history of infection in the month before the study; nicotine or alcohol abuse | Decreased blood pressure, total plasma cholesterol, triglycerides and LDL-c with increase in HDL-c; decreased inflammatory markers like TNFα and C-reactive protein; improved insulin resistance |

## 1.12. Natural products in treatment of metabolic syndrome

Ayurveda and Chinese medicine have been treating diseases using natural products for several millennia and age-old anecdotal reports strongly suggest a role for diet in both preventive and therapeutic medicine (180, 181). Indian spices have a traditional history of use as both preventive and therapeutic medicines (181). Several reviews have discussed the use of herbal medicines including spices in the treatment of the symptoms of the metabolic syndrome such as diabetes (181, 182), insulin resistance (183), hypertension and other cardiovascular diseases (184) and inflammation (185).

Cardamom is a perennial herb, indigenous to the Indian subcontinent. Phytochemical studies of cardamom revealed a wide variety of compounds, including α-terpineol, myrcene, subinene, limonene, cineol, α-phellandrene, menthone, α and β-pinene (186), cis/trans-linalol oxides, trans-nerolidol (187), β-sitostenone, γ-sitosterol, phytol, eugenyl acetate (188), bisabolene, borneol, citronellol, p-cymene, geraniol, geranyl acetate, stigmasterol and terpinene (189). Cardamom essential oil traditionally has been used as a tonic to the digestive system, as well as a component of many sensual aphrodisiac blends. The oil has the aroma of freshly dried cardamom pods, far superior to the comparatively flat steam-distilled variety of this oil. Cardamom oil may relieve spasm, possibly making it beneficial for colitis, irritable bowel syndrome, indigestion and cramps. In addition, cardamom oil can relieve nausea and may be useful for morning sickness in pregnancy. *In vitro* studies suggest that cardamom inhibited platelet aggregation when induced with agents such as ADP, epinephrine, collagen and the calcium ionophore, A 23187 (190). Furthermore, cardamom reduced blood pressure in rats probably by acting through cholinergic and calcium antagonist mechanisms (191). There is no clear literature evidence that intervention with cardamom, either black or green, decreases the signs of the metabolic syndrome, although improvements in individual signs have been published.

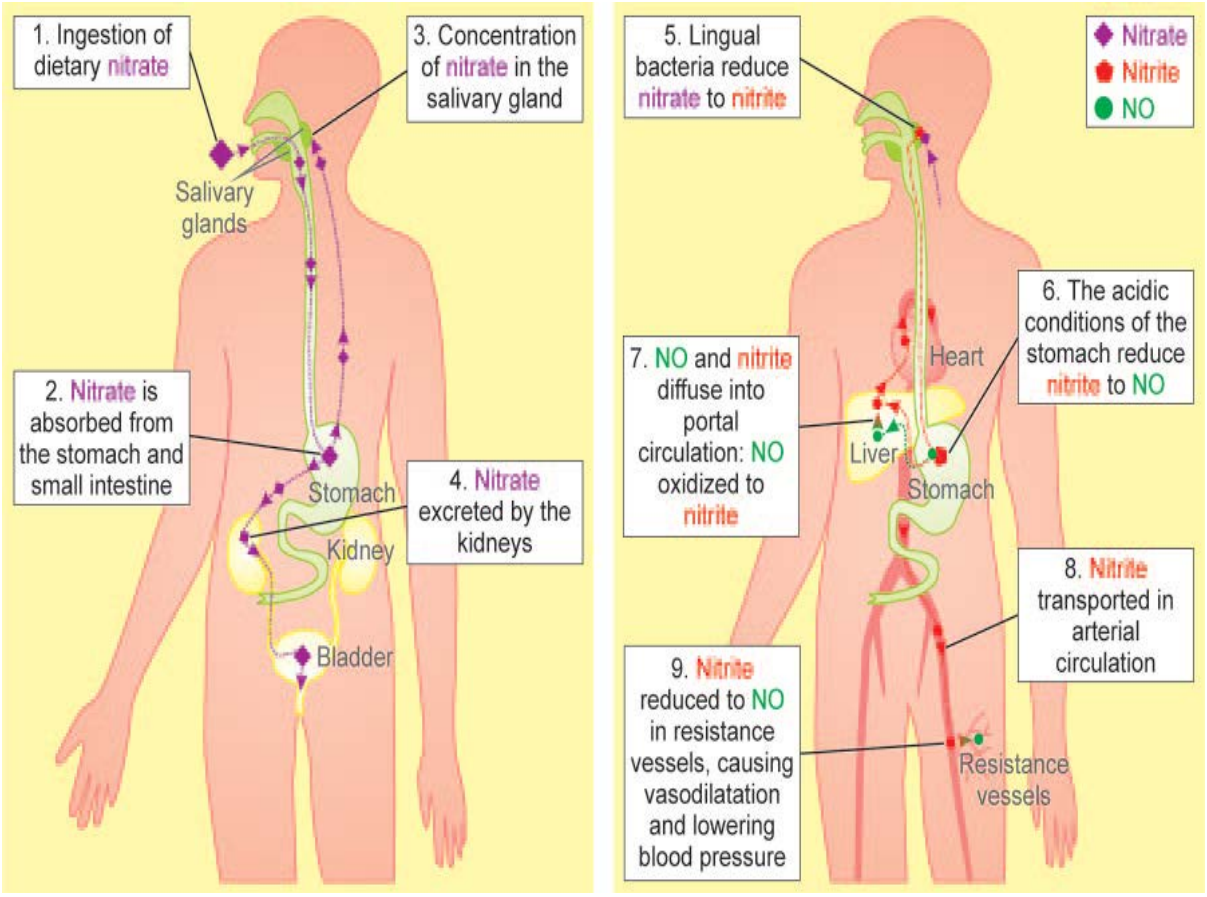



Figure 1.2 The fate of dietary nitrate, derived from consuming beetroot juice. Systemically absorbed nitrate is concentrated 10-fold in the salivary glands (left panel) and undergoes an enterosalivary circulation where it is reduced to nitrite by bacterial nitrate reductases on the dorsal surface of the tongue, and swallowed into the stomach providing a source of systemically available nitrite/NO. Right panel, Nitrite is transported in the arterial circulation to resistance vessels, where lower O2 tension favors the reduction of nitrite to NO, causing vasodilatation, with consequent lowering of BP (adapted from Webb J. A; 2008).

Further, beetroot as a rich source of nitrates has also shown beneficial effects in treating metabolic syndrome risk factors (192). Ingestion of beetroot juice and conversion of nitrates to nitric oxide is illustrated in Figure 1.2. Nitric oxide (NO) is one of the most important signalling molecules in our body (192). Although NO is involved in virtually every organ system within our body, it is known primarily for maintaining normal blood pressure and blood flow to tissues and protecting the cardiovascular system from insult and injury (193). A deficiency in NO production or availability is a hallmark of several disease conditions. It is evident from animal models that high-carbohydrate, high-fat diet causes endothelial dysfunction (146) and dietary nitrate consumption improved endothelial function along with decrease in visceral fat (194). However, there is no evidence from dietary interventions that beetroot juice will produce similar effects to inorganic nitrates and the pleiotropic effects of nitrates are unclear.

Further, colour fruits and vegetables rich in bioactive compounds, such as flavonoids and polyphenols, have also shown potential health benefits (164, 195). Anthocyanins are one group of widely-available polyphenols as water-soluble natural pigments responsible for red blue or purple colours in fruits and vegetables (196). Cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin are the major types of dietary anthocyanins (197). Major dietary sources include coloured fruits and vegetables such as berries (blueberry, bilberry, chokeberry), purple carrot, black currant, red radish, purple maize, red cabbage and purple sweet potato (196, 198).

Epidemiological studies show that increased consumption of diets rich in anthocyanins reduce the risk of developing cardiovascular diseases (199). Animal and cell culture studies using a variety of anthocyanins as dietary interventions suggest that high anthocyanin intake may prevent the increase in blood glucose, blood pressure, serum lipids, adiposity, oxidative stress and inflammation. Purple carrot juice (5% of the diet) reduced abdominal obesity, blood pressure, plasma lipids, hepatic steatosis, cardiac fibrosis and inflammation and improved glucose tolerance in high-carbohydrate, high-fat fed rats (200). Chokeberry fruit juice (5, 10 and 20 ml/kg body weight) for 30 days reduced total cholesterol, LDL-C and triglycerides in 4% cholesterol-containing diet (201). Chokeberries (100 and 200 mg/kg body weight) also reduced visceral adiposity, blood glucose, serum triglyceride, cholesterol and LDL-cholesterol and increased in fructose-fed rats (202). In the same study, anthocyanin supplementation increased plasma adiponectin concentrations and inhibited the expression of pro-inflammatory cytokines such as TNF-α and IL6 in the adipose tissue along with down-regulation of other adipogenic markers such as Gsk3β, PPAR-γ, Fabp4, Fas and Lpl expression with up-regulation of the important intermediates of the insulin signalling cascade (IRS1, IRS2, PI3K, Glut1, Glut4 and Gys1) (202). In diabetes induced by high-fructose diet and simultaneous single injection of streptozotocin (20 mg/kg), dietary supplementation with chokeberry fruit extract (0.2%; ~400mg/g of anthocyanin glycosides in the extract) decreased antioxidant status of vital organs, total plasma cholesterol and blood glucose (203). In Zucker rats fed a high fat diet, 2% dietary blueberry or 1% whole tart cherry powder supplementation reduced plasma triglycerides, fasting insulin, HOMA-IR, glucose tolerance, abdominal fat mass and increased adipose and skeletal muscle PPAR-alpha and PPAR-gamma activity along with reductions in TNF-α, IL-6 and nuclear factor-$\kappa$B in the plasma and the adipose tissue (204, 205). At the same dosage after 90 days, tart cherries reduced fasting blood glucose, hyperlipidaemia, hyperinsulinaemia and fatty liver with

increased hepatic PPAR-alpha expression in Dahl salt-sensitive rat (206). Anthocyanins from back soybean seed coats (cyanidin 3-glucoside, delphinidin 3-glucoside, and petunidin 3-glucoside; 10-100 μg/mL) inhibited TNF-alpha-mediated VCAM-1 expression in human umbilical vein endothelial cells (207). In the same cell line, delphinidin inhibited oxLDL-induced cell viability loss primarily by up-regulating proteins involved in cellular anti-oxidative systems including Bcl-2 and Bax protein (208). These results suggest that the responses to anthocyanins may be mediated by their anti-inflammatory and anti-oxidative properties and up-regulation of the insulin signalling cascade.

Anthocyanins also have direct protective effects on the heart. Hearts from male Wistar rats fed on a diet based on anthocyanin-containing maize kernels for 8 weeks were more resistant to regional ischaemia and reperfusion insult induced in an isolated heart preparation (209). This diet also reduced the infarct size in coronary occlusion and reperfusion model (209). Anthocyanin extract from black rice (5 g/kg diet) lowered body weight gain, serum triglyceride, raised hepatic CPT-1 expression and inhibited plate hyperactivity suggested by decreased thromboxane $A_2$, the thrombogenic ratio of thromboxane $A_2$ and prostacyclin, serum calmodulin and soluble P-selectin expression in high fat-fed rats (210).

Human studies concur with the therapeutic responses produced in animal models to dietary anthocyanins. In patients with metabolic syndrome, chokeberries extract (3 x 100 mg/day) for two months decreased both systolic and diastolic blood pressure, endothelin-1, total cholesterol, LDL-c, triglycerides, TBARS, catalase activity and induced superoxide dismutase activity (211). In hypercholesterolaemic patients, 320 mg/day of purified anthocyanins isolated from bilberries and blackcurrants increased brachial artery flow-mediated dilatation, cGMP and HDL-cholesterol concentrations and decreased the serum soluble vascular adhesion molecule-1 and LDL cholesterol concentrations (212). Anthocyanins also improved endothelial function in these patients as well as in isolated rat aortic rings and these effects were abolished by NO-cGMP inhibitors suggesting the role of NO-cGMP signalling pathway in anthocyanin-mediated vasodilation (212).

In view of the potentially beneficial effects of these natural foods, anthocyanin-containing foods are a promising potential therapeutic strategy to treat the major risk factors of metabolic syndrome. However, to date there are no studies that have evaluated the efficacy in treating all the signs of metabolic syndrome using these natural products despite an increase in demand for natural product-based therapies. Hence, I have studied the two

varieties of cardamom, beetroot and different sources of anthocyanins in a high-carbohydrate high-fat rat model, mimicking most of the signs of metabolic syndrome. Additionally, I have also studied Queen Garnet plum juice, as this intervention attenuated most of the risk factors of metabolic syndrome in rats, in the treatment of obese or overweight human volunteers to characterise the translational effects.

# CHAPTER 2

# 2. Aims and Hypothesis

## 2.1. Animal studies

The first aim was to determine whether different food sources when added to a high-carbohydrate, high-fat diet, can attenuate metabolic syndrome disorders including central obesity, elevated blood pressure, dyslipidaemia and elevated blood glucose concentrations in a rat model of human metabolic syndrome disorders.

For interventions with cardamom, beetroot, sodium nitrate, chokeberry, purple maize, cyanidin 3-glucoside and Queen Garnet plums in rats fed a high-carbohydrate, high-fat diet, the hypotheses to be tested are:

- Following 8 weeks administration of these foods, there will be a reduction in body weight gain, systolic blood pressure and fasting blood glucose concentrations as well as improvements in blood lipid profile compared to rats fed a high-carbohydrate, high-fat diet only.
- Following 8 weeks administration of these foods, there will be a decrease in total body fat and improvement in hepatic and cardiovascular function and structure.
- Following 8 weeks administration of these foods, there will be a reduction in the plasma insulin and leptin concentrations.

## 2.2. Translation to humans

The second aim of this thesis is to study and explore the translational effect of Queen Garnet plums, in mild-hypertensive obese or over-weight human volunteers

The specific hypotheses tested were that:

- Following 12 weeks administration of Queen Garnet plum juice, there will be a reduction in body weight gain, systolic blood pressure and fasting blood glucose levels as well as an improvement in blood lipid profile compared to placebo group.
- Following 12 weeks administration of Queen Garnet plum juice, there will be a decrease in total body fat mass and increase in muscle mass.

# CHAPTER 3

# 3. General methods and materials

## 3.1. Animal experiments

### *3.1.1. Rats*

The experimental groups consisting of male Wistar rats (aged 8–9 weeks; weight 330-340 g) were obtained from Animal Resource Centre, Murdoch, WA, Australia and individually housed at the University of Southern Queensland's Animal House Facility. All experimental protocols were approved by the Animal Experimentation Ethics Committee of the University of Southern Queensland under the guidelines of the National Health and Medical Research Council of Australia. The composition of the corn starch (C) and high-carbohydrate, high-fat (H) diets is given in Table 3.1 (200). C and H rats received their diets for 16 weeks and C+treatment and H+treatment rats received C and H diets for first 8 weeks while, during the final 8 weeks, both diets were supplemented with treatments by replacing equivalent amounts of water. The drinking water in all H diet-fed groups was augmented with 25% fructose for the duration of the study. Body weight and food and water intakes were measured daily and feed efficiency (%) was calculated (149) using the following equation:

$$\text{feed conversion efficiency (\%)} = \frac{\text{increase in body weight(\%)}}{\text{daily energy intake(kJ)}} \times 100$$

Increase in body weight (%): body weight difference between day 56 (week 8) and day 112 (week 16); daily energy intake: average of daily energy intake from week 8 to week 16.

**Table 3.1 Composition of C and H diets**

| Ingredient, g/kg | C | H |
|---|---|---|
| Corn starch | 570.0 | - |
| Powdered rat feed | 155.0 | 155.0 |
| HMW salt mixture | 25.0 | 25.0 |
| Fructose | - | 175.0 |
| Beef tallow | - | 200.0 |
| Condensed milk | - | 395.0 |
| Water | 250.0 | 50.0 |

| | | |
|---|---|---|
| Energy, kJ/g | 11.23 | 17.93 |

The sample size was determined using Resource Equation Method (213). The degree of freedom for the error term used to test the adiposity change in this study is over 20 (ideally between 10 to 20), which means sample size of 8 per group is more than the number necessary to achieve the scientific objective. Rats were divided into 4 groups of 10-12 rats for each study: (i) corn starch (C), (ii) high carbohydrate, high fat (H), (iii) C + treatment (n = 10-12), (iv) H + treatment (n = 10-12). All experimental groups were housed in a temperature-controlled (about 21-23ºC), 12-h light/dark cycle environment with access to water and food *ad libitum*. Parameters [systolic blood pressure (SBP), oral glucose tolerance test (OGTT) and dual energy X-ray absorptiometric (DXA)] were measured. The experimental design for rat studies has been outlined in Figure 3.1.

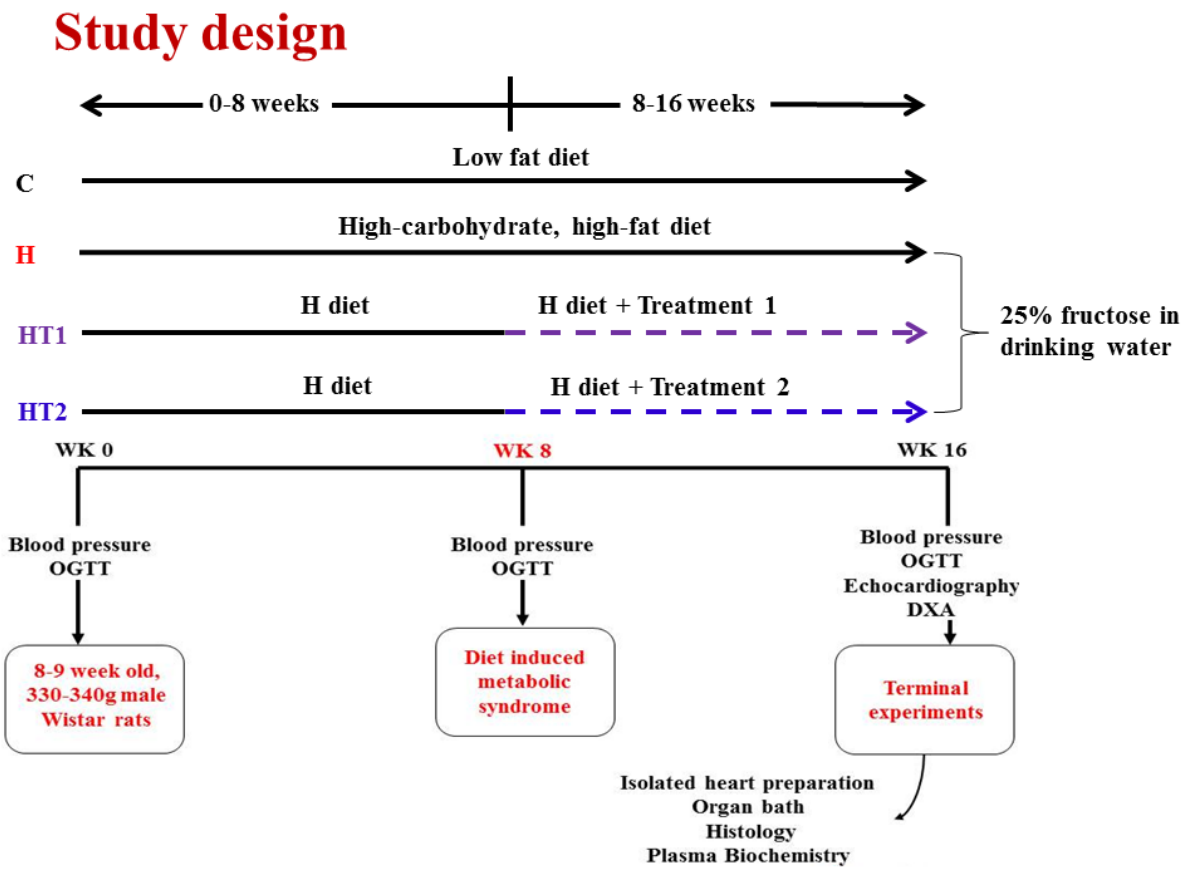



Figure 3.1 Experimental protocol

### *3.1.2. Body Composition Measurements*

For rats, dual-energy X-ray absorptiometric (DXA) measurements using a Norland XR36 DXA instrument (Norland Corp., Fort Atkinson, WI, USA) were performed after 16 weeks of feeding, 2 days before rats were killed for pathophysiological assessments under anaesthesia with Zoletil (tiletamine 25 mg/kg and zolazepam 25 mg/kg) and Ilium Xylazil (xylazine 15 mg/kg) via intraperitoneal injection. DXA scans were analysed using the manufacturer's recommended software for use in laboratory animals (Small Subject Analysis Software, version 2.5.3/1.3.1; Norland Corp.) (146). The precision error of lean mass for replicate measurements, with repositioning, was 3.2%. Visceral adiposity index (%) was calculated from wet weights of fat pads at euthanasia (214).

### *3.1.3. Echocardiography*

Echocardiography was performed by trained cardiac sonographers at the Medical Engineering Research Facility, The Prince Charles Hospital, Brisbane, Australia. Rats were anaesthetised via intraperitoneal injection with Zoletil (tiletamine 10 mg/kg, zolazepam 10 mg/kg; Virbac, Peakhurst, NSW, Australia) and Ilium Xylazil (xylazine 6 mg/kg, IP; Troy Laboratories, Smithfield, NSW, Australia). Echocardiographic images were obtained using the Hewlett Packard Sonos 5500 (12MHz frequency fetal transducer) at an image depth of 3 cm using two focal zones. Measurements of left ventricular posterior wall thickness and internal diameter were made using two-dimensional M-mode taken at mid-papillary level. Left ventricular M-mode measurements at the level of the papillary muscles were used to define wall thicknesses and internal diameters at systole (s) and diastole (d). The measured wall thicknesses were the posterior wall (LVPW) and the interventricular septum (IVS). The left ventricular internal diameter in diastole (left ventricular end-diastolic diameter) is abbreviated as LVIDd while the corresponding end-systolic measurement is abbreviated as LVIDs. Fractional shortening (FS;%) was defined as (LVIDd - LVIDs) / LVIDd x 100 (16). The left ventricular end-diastolic dimension, ventricular wall thickness and its fractional shortening were used as a measurement of cardiac geometry and systolic function. Other measurements included early diastolic mitral flow velocity (E), peak mitral flow velocity at atria1 contraction (A), deceleration time and the period between mitral valve closure and mitral valve opening (MC-MO) (215).

### *3.1.4. Oral glucose tolerance test*

Oral glucose tolerance tests were performed after 0, 8 and 16 weeks of diet. After 12 h of food deprivation, including replacement of 25% fructose in water with tap water, blood glucose concentrations were measured in blood samples taken from the tail vein. Subsequently, each rat was treated with glucose (2 g/kg) via oral gavage. Tail vein blood samples were taken every 30 minutes up to 120 minutes following glucose administration. The blood glucose concentrations were analysed with a Medisense Precision Q.I.D glucose meter (Abbott Laboratories, Bedford, MA, USA) (160).

### *3.1.5. Blood pressure and abdominal circumference*

In rats, systolic blood pressure was measured after 0, 8 and 16 weeks under light sedation with intraperitoneal injection of Zoletil (tiletamine 10 mg/kg, zolazepam 10 mg/kg), using an MLT1010 Piezo-Electric Pulse Transducer (ADInstruments) and inflatable tail-cuff connected to a MLT844 Physiological Pressure Transducer (ADInstruments) and PowerLab data acquisition unit (ADInstruments, Sydney, Australia). The tail cuff was inflated to inhibit the pulse signal and pump was slowly released to enable the return of the pulse signal. The pressure where the first pulse signal returned was recorded as the systolic pressure. The pressure was recorded at least 5 times per rat and the mean value of the readings was taken. Abdominal circumference was measured using a standard measuring tape under light sedation (160).

### *3.1.6. Isolated heart preparation*

The diastolic stiffness constant and the contractility of the hearts of the rats were assessed using the Langendorff heart preparation. Rats were euthanized with an intraperitoneal injection of pentobarbitone sodium (Lethabarb, 100 mg/kg, Virbac, Peakhurst, NSW, Australia). Once anaesthetised, heparin (200 IU, Sigma-Aldrich Australia, Sydney, NSW, Australia) was injected into the right femoral vein. The heart was stunned in ice-cold crystalloid perfusate (modified Krebs-Henseleit bicarbonate buffer (KHB) containing [in mM]: NaCl 119.1; KCl 4.75; $MgSO_4$ 1.19; $KH_2PO_4$ 1.19; $NaHCO_3$ 25.0; glucose 11.0 and $CaCl_2$ 2.16) upon removal. The aorta was then isolated and cleared of extraneous fat and cannulated via the dorsal root (with the tip of the cannula positioned immediately above the coronary ostia of the aortic stump) and perfused in a non-recirculating Langendorff manner at 100 cm of coronary perfusion pressure. The buffer was bubbled with carbogen (95% O2/ 5% $CO_2$), giving a pH of 7.4 and the temperature maintained at 36.9 ± 0.5°C. The hearts were

punctured at the apex with a small piece of polyethylene tubing to facilitate Thebesian drainage. The isovolumetric ventricular function was measured by inserting a latex balloon into the left ventricle via the mitral orifice connected to a Capto SP844 MLT844 physiological pressure transducer and Chart software on a Maclab system. All left ventricular end-diastolic pressure values were measured by pacing the heart at 250 beats per minute using an electrical stimulator by touching two electrodes to the surface of the right atrium. End-diastolic pressure was obtained starting from 0 mmHg up to 30 mmHg. The right and left ventricles were separated and weighed.

To assess myocardial stiffness, stress (δ, dyne/$cm^2$) and tangent elastic modulus (E, dyne/$cm^2$) for the midwall at the equator of the left ventricle were calculated by assuming spherical geometry of the ventricle and considering the midwall equatorial region as representative of the remaining myocardium. Myocardial diastolic stiffness was calculated as the diastolic stiffness constant (k, dimensionless), the slope of the linear relation between E and δ (216). To assess contractile function, developed pressure [systolic pressure (mmHg) - diastolic pressure (mmHg)], maximal +dP/dT (rate of positive rise of pressure) and maximal -dP/dT (rate of negative rise of pressure) were calculated at a diastolic pressure of l0 mmHg (217).

### *3.1.7. Organ bath studies*

Contractility of the thoracic aorta was assessed using organ bath studies. Thoracic aortic rings (4 mm in length) were dissected out and suspended in an organ bath chamber with a resting tension of 10 mN. Tissues were bathed in a modified Tyrode's solution containing [in mM]; NaCl 136.9; KCl 5.4; MgCl 1.05; $CaCl_2$ 1.8; $NaHCO_3$ 22.6; $NaH_2PO_4$ 0.42; glucose 5.5; ascorbic acid 0.28 and sodium ethylenediaminetetra-acetic acid (EDTA) 0.1 (Sigma-Aldrich Australia). The Tyrode's solution was bubbled with carbogen (95% O2/ 5% $CO_2$) and the temperature maintained at 35 ± 0.5°C. Force of contraction was measured isometrically with Grass FT03C force transducers connected via amplifiers to a Macintosh computer via a MacLab system. The aortic rings were allowed to settle and equilibrate for approximately 60 min, with regular washing every 15 min. Cumulative concentration-response (contraction) curves were measured for noradrenaline (Sigma-Aldrich Australia, NA; from $1x10^{-8}$ to $lx10^{-4}$ M) to examine changes to the force of contraction; concentration-response (relaxation) curves were measured for acetylcholine (Sigma-Aldrich Australia, ACh; from $1x10^{-8}$ to $lx10^{-4}$ M) or sodium nitroprusside (Sigma-Aldrich Australia, SNP; from $1x10^{-8}$ to $lx10^{-4}$ M) in the presence of a submaximal contraction to noradrenaline (146, 217).

Results were analysed as the maximal increase or decrease in force of contraction, in mN, for each drug concentration (160).

### *3.1.8. Organ weights*

The right and left ventricles were separated after perfusion experiments and weighed. Following removal of the heart, the liver, retroperitoneal, epididymal and omental fat pads were collected and blotted dry for weighing. Organ weights were normalised relative to the tibial length at the time of their removal (in mg/mm) (160).

### *3.1.9. Histology of heart and liver*

Immediately after removal, heart and liver tissues were fixed in 10% buffered formalin with three changes of formalin every third day to remove traces of blood from the tissue. The samples were then dehydrated and embedded in paraffin wax. Thin sections (5-7 μm) of left ventricle and the liver were cut and stained with haematoxylin and eosin stain for determination of inflammatory cell infiltration. Sections of 5-7 μm thickness of the left ventricle were cut and floated onto glass slides for staining of inflammatory cells. Before staining, sections were cleared of paraffin by immersion in xylene (three 2-minutes changes). Sections were then hydrated with 100% (twice), 90% and 70% ethanol (2 minutes each). After brief wash with distilled water, sections were placed in haematoxylin stain (100 mL 1% aqueous haematoxylin, 75 mL 5% aluminium sulphate, 25 mL Lugol's iodine, 8 mL acetic acid glacial and 50 mL glycerol) for 6 min. Sections were then washed in the water bath for 2 minutes then immersed in 70% ethanol for approximately 2 minutes, followed by eosin stain (1 g eosin powder and 100 ml 90% ethanol; diluted 1:1 with 90 % ethanol) for 6 min. The sections were then dehydrated in 95% and 100% (three times) ethanol (2 minutes each) and sections were then cleared in xylene, mounted in Depex mounting medium and cover-slipped. Slides were scanned Olympus microscope (Olympus-Australia, Notting Hill, VIC, Australia) for imaging and analysis.

Collagen distribution was observed in the left ventricle following picrosirius red staining. These sections followed the same deparaffinisation and hydration process as described above. Sections were then transferred to distilled water for a brief wash and then bathed in phosphomolybdic acid (0.2% in distilled water) for 2 minutes to inhibit background autofluorescence and non-specific staining. Later, sections were placed in the collagen-selective stain picrosirius red (0.1% Sirius Red F3BA in saturated picric acid) for 90 min. The sections were then washed in 0.1 N HCl for 2 min, followed by dehydration with 95%

and 100% (three times) ethanol (2 minutes each). Sections were then mounted as described above. Laser confocal microscopy (Nikon A1R+ upright Confocal Microscope, Tokyo, Japan) was used to determine the extent of collagen deposition in selected regions (146, 160).

#### *3.1.10. Plasma analyses*

In rats, blood was collected from the abdominal aorta following euthanasia and centrifuged at 5000x *g* for 15 minutes within 30 minutes of collection into heparinised tubes. Plasma was separated and transferred to Eppendorf tubes for storage at −20°C before analysis. Plasma concentrations of total cholesterol, triglycerides, non-esterified fatty acids (NEFA), activities of plasma alanine transaminase (ALT) and aspartate transaminase (AST) were determined using kits and controls supplied by Olympus using an Olympus analyser (AU 400 Tokyo, Japan). Plasma insulin and leptin concentrations (ALPCO, USA) were estimated using a commercial ELISA kit according to manufacturer-provided standards and protocols (146, 160).

#### *3.1.11. Statistical analysis*

For rat experimental studies, all data sets were represented as mean ± standard error of mean (SEM). Comparisons of findings between groups were made via statistical analysis of data sets using one-way and two-way analysis of variance (ANOVA). When interaction and/or the main effects were significant, means were compared using Newman-Keuls multiple-comparison post hoc test. A p-value of $<0.05$ was considered as statistically significant. All statistical analyses were performed using Graph Pad Prism version 6.00 for Windows.

### 3.2. Clinical trial

#### *3.2.1. Study outline and recruitment of volunteers*

The human study was a randomised, double-blinded, placebo-controlled trial, involving adult volunteers residing in Melbourne. The trial was conducted at Victoria University Nutrition Clinic, Melbourne, Australia. Potential volunteers were recruited from the general public by newspaper advertisement and staff members at Victoria University by University-wide emails and posters after attaining study approval from Victoria University Human Research Ethics Committee (HRE14281) and registered with ANZCTR (Australia New Zealand Clinical Trial Registry). Human volunteers with a BMI greater than 25kg/m$^2$ or a waist circumference > 94 cm (male) or > 80 cm (female) along with systolic blood pressure 130-150 mmHg and diastolic blood pressure 85-100 mmHg were recruited.

After recruitment, participants were coded and allocated into groups based on their physical characteristics including age, body weight and BMI. Stratified randomisation was used to ensure all baseline variables associated with the outcome were evenly distributed. Treatment and placebo were packed in opaque brown bags and the principal investigator implemented the allocation sequence and assigned the participants into their groups. Due to minimal similarities in taste and colour between treatment juice and placebo juice, participants were informed that they are involved in a research project testing the effects of two different fruit drinks. At the beginning of the study, all eligible volunteers were informed about the details of the study including that they would be randomly assigned into a drink 1 or drink 2 groups. Formal consent was obtained from all participants. Staff and participants involved in the intervention process of the trial were blinded to group assignment. The randomisation code was broken only after data collection and analysis was completed. The inclusion and exclusion criteria for the study are presented in Figure 3.2.

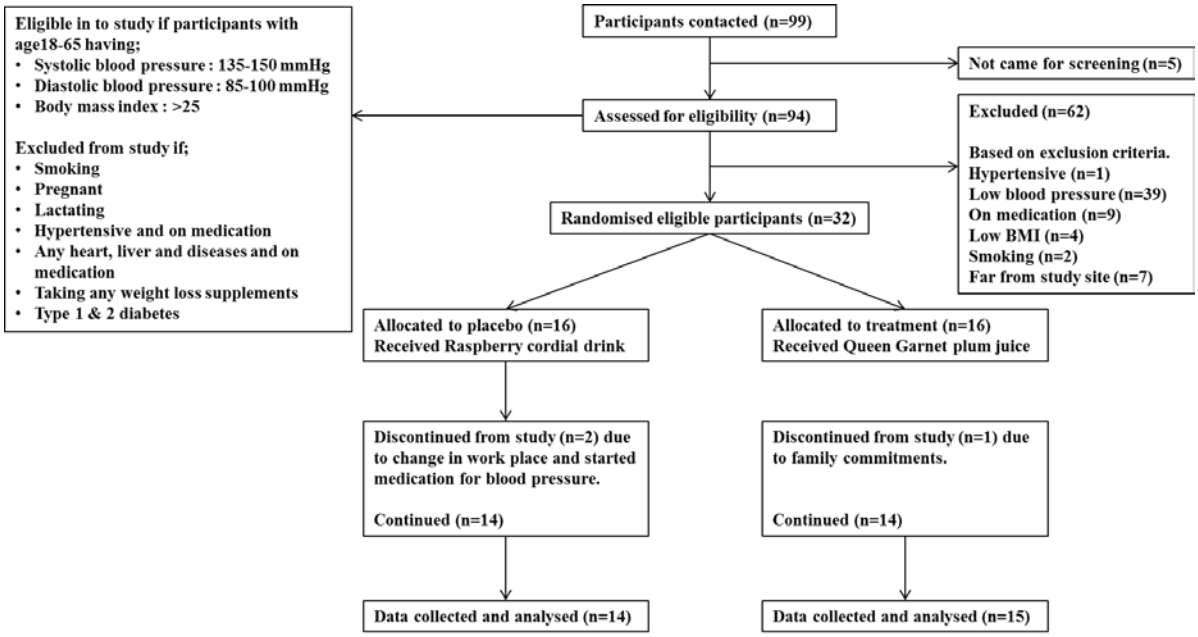



Figure 3.2 Eligibility criteria and study design

### *3.2.2. Administration of juice*

Treatment and placebo juice was packed in 2 L plastic bottles. All participants received 2 x 2 L treatment or placebo drink packed in opaque brown bags with blinded label for 12 weeks and participants were requested to drink 250 ml every morning. The ingestion of the drinks was monitored fortnightly during the general consultations and a drink calendar was also handed out along with the drinks and collected every fortnight for12 weeks. The dietary intake was monitored through 3-day food diaries collected fortnightly. Along with

food diaries, participation in physical activity was also recorded and submitted during fortnightly visits. The food diaries and physical activity records were analysed using Food Works Professional 2009, version 6 (Xyris Software, QLD, Australia Pty Ltd).

### *3.2.3. Anthropometric measurements*

Anthropometric measures were taken during fortnightly consultations in accordance with standard equipment and technique. Measurements were taken thrice with the mean of the measurements used as the final reading. Height was measured after the removal of shoes using a stadiometer to the nearest millimetre. Body weight was taken using digital scales (Tanita Inner Scan, BC-545, Cloverdale, WA, Australia) when heavy clothing was removed. BMI was calculated using the following formula: BMI = weight (kg)/height$^2$ (m) (218). Waist circumference was measured to the nearest 0.1 cm at the midway point between the lowest costal border and the iliac crest in a horizontal plane (above the umbilicus). Hip circumference was measured in a horizontal plane at the maximum posterior protuberance of the buttocks. Waist to hip ratio (WHR) was calculated using the following formula: WHR = Waist circumference (cm)/hip circumference (cm).

### *3.2.4. Blood pressure measurement*

Blood pressure was measured in a seated position using Omron HEM-7320 (OMRON HEALTHCARE Co., Ltd., Kyoto, Japan), an automated digital blood pressure monitor, where the inflatable cuff of the sphygmomanometer was positioned at the brachial artery in the right upper extremity of each subject. Blood pressure was measured thrice with the final blood pressure reading obtained by calculating the mean of the three readings. Heart rate was also recorded using the automated digital blood pressure monitor.

### *3.2.5. Body composition*

Dual-energy X-ray absorptiometric (DXA) measurements were performed on all participants at the beginning and end of the study using a GE-Lunar iDXA sxcanner (Silverwater, NSW, Australia). Participants were requested to wear light clothing and remove all the jewellery before the scan was performed. Scans were analysed using the manufacturer's recommended software.

### *3.2.6. Basal metabolic rate*

Participants were recommended to fast overnight for at least 10h and basal metabolic rate (BMR) measured using a metabolic cart (closed circuit spirometry). The gas exchange

was measured by indirect calorimetry by comparing room air with exhaled air samples. Participants were asked to rest for 15-25 minutes while the BMR test was performed. The participant breathes through a mouthpiece and valve attached to the volume displacement spirometer. The equipment consists of a closed system because the subjects will rebreathe only the gas in the spirometer. A canister of soda lime in the breathing circuit absorbs the carbon dioxide in exhaled air. A drum attached to the spirometer revolves at a known speed to record oxygen uptake from the changes in the system's volume.

### *3.2.7. Blood collection and plasma analysis*

Following an overnight fast of at least 10 h, 10 ml of forearm venous blood was collected using the ethylenediaminetetraacetic acid (EDTA) Vacutainer system at baseline and post-intervention (BD vacutainer tubes, Becton, Dickinson and Company). Using Samsung LABGEO$^{PT10}$ Analyser (Point of Care Diagnostics, Sydney, Australia), blood concentrations of glucose (GLU), total cholesterol (CHOL), triglycerides (TGA), high-density lipoproteins (HDL) and low-density lipoproteins (LDL) and enzyme activities gamma-glutamyl transferase (GGT), alanine transaminase (ALT) and aspartate transaminase (AST) were determined by adding 75 µl of blood to Samsung Labgeo PT10 Biochem 9 test cartridge. The remaining blood was then centrifuged for 15 minutes at 3000 *g* at 4 ◦C. Plasma was collected into aliquots and frozen at −80°C for further analysis.

### *3.2.8. Statistics*

For the clinical trial, data were presented as the mean and standard deviation and were analysed using SPSS package, version 22 (SPSS, Chicago, IL, USA). Target sample size was calculated to include a minimum of 14 participants per group to detect significant differences in systolic blood pressure, one of the main measured outcomes with 90 % power (n= $2*20^2/25^2$ *10.51), based on the previous findings (211). One way ANOVA was performed to compare all baseline data between groups. Mixed model ANOVA was used to analyse the effects of the intervention, time, and the interaction (time*group) between the intervention and time with pairwise comparisons (adjusted for multiple comparisons by Bonferroni's *post-hoc* test). When the interaction and/or the main effects were significant, means were compared using Tukey's multiple comparison *post-hoc* test. The significance level was set as $p < 0.05$. The precision of the primary and secondary outcomes for each group were calculated using 95% confidence intervals.

# CHAPTER 4

# 4. Green and black cardamom in a diet-induced rat model of metabolic syndrome

## 4.1. Summary

Both black (B) and green (G) cardamom are used as flavours during food preparation. This study investigated the responses to B and G in a diet-induced rat model of human metabolic syndrome. Male Wistar rats were fed either a corn starch-rich diet (C) or a high-carbohydrate, high-fat diet with increased simple sugars along with saturated and *trans* fats (H) for 16 weeks. H rats showed signs of metabolic syndrome leading to visceral obesity with hypertension, glucose intolerance, cardiovascular remodelling and non-alcoholic fatty liver disease. Food was supplemented with 3% dried B or G for the final 8 weeks only. The major volatile components were the closely related terpenes, 1,8-cineole in B and α-terpinyl acetate in G. HB rats showed marked reversal of diet-induced changed with decreased visceral adiposity, total body fat mass, systolic blood pressure and plasma triglycerides, and structure and function of the heart and liver. In contrast, HG rats increased visceral adiposity and total body fat mass, and increased heart and liver damage, without consistent improvement in the signs of metabolic syndrome. These results suggest that black cardamom is more effective in reversing the signs of metabolic syndrome than green cardamom.

## 4.2. Introduction

Spices are used to flavour foods but they may also be effective as functional foods to improve health or decrease the risk of disease (219-221). In particular, spices may decrease the metabolic syndrome defined as the cluster of obesity, hypertension, diabetes and non-alcoholic fatty liver disease (10). These metabolic perturbations lead to chronic changes in the structure and function of the heart, liver, kidneys and pancreas (222). The prevalence of metabolic syndrome is high in both developing and developed countries including USA (34%), India (25.6%), Kuwait (24.8%) and Australia (22.1%) (12, 13, 15, 223, 224).

Cardamom is a well-known spice with both green (*Elettaria cardamomum* Maton*)* and black (*Amomum subulatum* Roxburgh) varieties, both in the family Zingiberaceae, used in culinary and traditional medicine practices. Black cardamom is grown in the north-eastern Indian state of Sikkim as well as in neighbouring Nepal and Bhutan (224) while green cardamom is grown in the southern Indian states of Tamil Nadu, Kerala and Karnataka (225)

with Guatemala as the other major source. Dry pods of cardamom contain volatile oils, phenolic acids, lipids and sterols (224, 225). Both black and green cardamom contains terpenes in the essential oils, with 1,8-cineole and $\alpha$-terpineol found in black cardamom and α-terpinyl acetate and 1,8-cineole in green cardamom (224, 225).

Green cardamom has been used since the 4$^{th}$ century BC by Indian Ayurvedic practitioners and ancient Greek and Roman physicians for the treatment of indigestion, bronchitis, asthma and constipation, and to stimulate appetite in anorexia (226-228); other indications include diarrhoea, dyspepsia, epilepsy, hypertension, cardiovascular diseases, ulcers, gastro-intestinal disorders and vomiting (191, 229, 230). Similarly, black cardamom is used by Ayurvedic and Unani practitioners for many ailments including indigestion, vomiting, rectal diseases, dysentery, liver congestion, gastrointestinal disorders and genitourinary complaints (228, 231).

Rats fed with high-carbohydrate, high-fat diet for 8 weeks developed visceral adiposity, impaired glucose tolerance with increase plasma insulin concentrations, increased systolic blood pressure, structural damage to the heart and liver and elevated plasma lipid concentrations (146). Therefore, I have compared the cardiovascular, liver and metabolic responses to green and black cardamom in a high-carbohydrate, high-fat diet-fed rat model of human metabolic syndrome (146). These measurements included systolic blood pressure, echocardiography, vascular reactivity, cardiac collagen deposition, stiffness, plasma biochemistry and histology for structural changes on heart and liver. Addition of black cardamom to the diet improved the signs of metabolic syndrome much more effectively than green cardamom. Further, green cardamom may worsen heart and liver structure.

## 4.3. Materials & methods

### *4.3.1. Analysis of green cardamom and black cardamom*

100mg of black or green cardamom was extracted in 3 mL of 100% ethanol by sonication for 10 minutes. After centrifugation, an aliquot of the supernatant was transferred to a vial and injected into a HP 6890 GC and 5973 MS (Agilent Technologies, Mulgrave, Victoria, Australia). The analysis was performed using on a HP-5MS GC column (Agilent 19091S-433), 30 m x 0.25 µm, with a flow rate of 0.9 mL/minute helium at an average velocity of 35 cm/second. The oven settings were an initial 50°C held for 5 minutes, with a ramp of 10°C per minute up to 250°C, a total run time of 30 minutes. Inlet temperature was 250°C with an injection of 1 µL and split ratio of 50:1. MS setting were EM voltage 71,

source 230 and quadrupole 150, with a scan for masses between 35 and 350 amu. Constituents were identified by comparison of peak MS spectra with GC MS libraries of NIST, Adams and Wiley with threshold match of >95%. Powdered black and green cardamom were analysed for protein, fat, total carbohydrates and energy value by Symbio Alliance, Brisbane, QLD, Australia.

### *4.3.2. Rats and diets*

The experimental group consisting of 72 male Wistar rats (9-10 weeks old; weighing 335-340 g) were randomly divided into 6 experimental groups (n=12 each) and fed with corn starch (C), corn starch + black cardamom (CB), corn starch + green cardamom (CG), high-carbohydrate, high-fat (H), high-carbohydrate, high-fat + black cardamom (HB) or high-carbohydrate, high-fat + green cardamom (HG). CB, CG, HB and HG rats were fed with a basal C and H diet for the first 8 weeks of the protocol and for the next 8 weeks, these rats were treated with the same diet supplemented with 3% green or black cardamom (30 g/kg replacing 30 ml/kg water in the food).

Experimental procedures such as body composition, oral glucose tolerance, echocardiography, blood pressure, isolated heart preparation, organ bath, plasma analysis and histology were conducted as described in Chapter 3.1.

## 4.4. Results

### *4.4.1. Cardamom analysis*

Black cardamom contained 1,8-cineole as the major volatile constituent (>65%) while green cardamom contained α-terpinyl acetate (>72%) that was not present in black cardamom (Table 4.1). Black cardamom had increased carbohydrate content but decreased fat content compared to green cardamom (Table 4.1).

**Table 4.1 Cardamom analysis**

| Variable | Green cardamom | Black cardamom |
|---|---|---|
| **GC-MS (area %)** | | |
| α-terpinyl acetate | 72.73 | -* |
| 1,8-cineole | 10.61 | 65.52 |
| α-terpineol | 0.86 | 3.29 |

| | | |
|---|---|---|
| Limonene | 0.38 | 3.59 |
| α-pinene | 1.50 | 2.84 |
| β-pinene | 0.23 | 3.43 |
| **Composition** | | |
| Energy (KJ/100 g) | 1557 | 1477 |
| Protein (% w/w) | 10.8 | 9.3 |
| Total fat (% w/w) | 10.3 | 1.7 |
| Moisture (% w/w) | 12.2 | 9.4 |
| Total carbohydrate (%) | 58.4 | 73.9 |

Values are represented as mean of duplicate analysis; * not detected by GC-MS.

### *4.4.2. Metabolic parameters*

Food and water intake was decreased in H, HB and HG rats compared to C, CB and CG rats, respectively (Table 4.2). Rats fed with C, CG, CB group received plain drinking water and H, HG and HB rats received 25% fructose in the drinking water, thereby increasing the cumulative energy intake in all H diet-fed rats. CG and HB rats showed a significant increase in water intake, compared to C and H rats respectively, where the cumulative energy intake was not altered. (Table 4.2). Feed conversion efficiency was increased in H rats compared to C rats but reduced by black cardamom (CB, HB) compared to green cardamom (CG, HG) (Table 4.2). Black cardamom groups showed decreased body weight gain and abdominal circumference compared to green cardamom and high-carbohydrate, high-fat diet-fed groups (Table 4.2). Body mass and visceral adiposity indices were reduced in HB rats only (Table 4.2). Bone mineral density was increased in H rats compared to C rats and normalised by black cardamom treatment only. Total body lean mass increased in H and HB rats compared to C, CB and CG rats. In HG rats, total body lean mass decreased but total body fat mass increased (Table 4.2). Black cardamom reduced total body fat in both CB and HB groups (Table 4.2). These changes in total body fat are consistent with abdominal fat measurements where black cardamom decreased and green cardamom increased abdominal fat pads (Table 4.2).

Plasma lipid concentrations were increased in H rats compared to C rats (Table 4.2). CB, CG and HB rats showed decreased plasma lipid concentration, in contrast to HG rats.

Plasma insulin concentrations almost quadrupled in H rats compared to C rats (Table 4.2); these concentrations were decreased by both black and green cardamom. Oral glucose tolerance test showed improved glucose metabolism in C rats compared to H rats, while no significant changes were seen either with green or black cardamom treatment (Table 4.2).

**Table 4.2 Dietary intakes, body composition and anthropometrics, organ wet weights, changes in glucose tolerance test, plasma insulin and plasma biochemistry in C, CG, CB, H, HG and HB diet-fed rats (n=8 rats/group)**

| Variable | C | CG | CB | H | HG | HB | P values | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Diet | Treatment | Interaction |
| Food intake (g/d) | 33.8±0.7$^{a}$ | 35.1±0.7$^{a}$ | 34.6±0.8$^{a}$ | 26.9±0.7$^{ab}$ | 24.2±0.5$^{b}$ | 25.0±0.6$^{ab}$ | <0.0001 | 0.55 | 0.0157 |
| Water intake (mL/d) | 23.9±1.4$^{b}$ | 36.8±2.0$^{a}$ | 27.2±2.0$^{b}$ | 26.6±1.2$^{b}$ | 28.0±1.0$^{b}$ | 35.0±1.3$^{a}$ | 0.65 | <0.0001 | <0.0001 |
| Cardamom intake (g/d) | 0.0±0.0$^{c}$ | 1.1±0.0$^{a}$ | 1.1±0.0$^{a}$ | 0.0±0.0$^{c}$ | 0.7±0.0$^{b}$ | 0.8±0.0$^{b}$ | <0.0001 | <0.0001 | <0.0001 |
| Cumulative energy intake from water (kJ) | 0.0±0.0$^{c}$ | 0.0±0.0$^{c}$ | 0.0±0.0$^{c}$ | 6231.7±696.5$^{b}$ | 6446.6±347.4$^{b}$ | 7950.8±492.9$^{a}$ | <0.0001 | 0.0554 | 0.0554 |
| Cumulative energy intake from food (kJ) | 21637±732 | 22440±689 | 22151±417 | 24761±672 | 24681±129 | 24893±670 | 0.0001 | 0.88 | 0.85 |
| Cumulative energy intake (kJ) | 21637±732$^{b}$ | 22440±689$^{b}$ | 22151±417$^{b}$ | 30993±725$^{a}$ | 31127±142$^{a}$ | 32844±929$^{a}$ | <0.0001 | 0.40 | 0.51 |
| Feed conversion efficiency (%) | 1.6 ±0.3$^{c}$ | 3.1±0.2$^{c}$ | 1.3±0.5$^{c}$ | 8.4±1.2 $^{a}$ | 8.1±0.5 $^{a}$ | 4.8±1.2$^{bc}$ | <0.0001 | 0.0029 | 0.0202 |
| Initial body weight (g) | 336.4±2.5 | 337.8±2.1 | 337.1±1.9 | 336.3±1.9 | 338.8±2.4 | 336.2±1.1 | >0.99 | 0.59 | 0.89 |
| Body weight at 8 weeks (g) | 392.1±7.4$^{b}$ | 401.3±9.6$^{b}$ | 389.8±4.7$^{b}$ | 477.3±15.3$^{a}$ | 488.3±14.3$^{a}$ | 462.4±11.9$^{a}$ | <0.0001 | 0.25 | 0.78 |
| Body weight at 16 weeks (g) | 417.1±8.3$^{c}$ | 434.9±8.8$^{c}$ | 408.7±5.8$^{c}$ | 560.6±17.9$^{a}$ | 574.1±19.2$^{a}$ | 506.3±10.8$^{b}$ | <0.0001 | 0.0023 | 0.15 |
| Body weight gained (8-16 weeks) (%)* | 6.4±1.3$^{b}$ | 8.3±1.2$^{b}$ | 4.8±1.2$^{b}$ | 17.2±1.3$^{a}$ | 17.6±1.1 $^{a}$ | 9.5±2.4$^{b}$ | <0.0001 | 0.0003 | 0.0578 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Visceral adiposity index (%) | 4.5±0.2$^{b}$ | 4.2±0.4$^{b}$ | 4.4±0.2$^{b}$ | 7.2±0.6$^{ab}$ | 8.7±0.5$^{a}$ | 5.7±0.4$^{b}$ | <0.0001 | 0.0057 | 0.0015 |
| Abdominal circumference (cm) | 20.0±0.2$^{b}$ | 21.5±0.3$^{ab}$ | 18.3±0.2$^{c}$ | 23.9±0.4$^{a}$ | 23.1±0.3$^{a}$ | 20.7±0.3$^{b}$ | <0.0001 | <0.0001 | 0.0011 |
| Body mass index (kg/m$^{2}$) | 5.6±0.2$^{b}$ | 5.9±0.2$^{b}$ | 5.6±0.1$^{b}$ | 6.7±0.2$^{a}$ | 7.1±0.1$^{a}$ | 5.9±0.1$^{b}$ | <0.0001 | 0.06 | 0.78 |
| Bone mineral content (g) | 12.7±0.4$^{c}$ | 13.1±0.6$^{c}$ | 12.0±0.3$^{c}$ | 16.0±0.7$^{ab}$ | 17.9±0.4$^{a}$ | 13.8±0.6$^{c}$ | <0.0001 | <0.0001 | 0.0223 |
| Total body lean mass (g) | 295±7$^{b}$ | 297±7$^{b}$ | 297±7$^{b}$ | 329±9$^{a}$ | 267±10$^{c}$ | 311±6$^{b}$ | 0.36 | 0.0019 | 0.0008 |
| Total body fat mass (g) | 100±7$^{c}$ | 112±19$^{c}$ | 77±8$^{d}$ | 203±19$^{b}$ | 270±15$^{a}$ | 140±17$^{c}$ | <0.0001 | <0.0001 | 0.0108 |
| **Tissue wet weight (mg/mm)** | | | | | | | | | |
| Retroperitoneal adipose tissue | 171±10$^{c}$ | 153±16$^{c}$ | 137±9$^{c}$ | 375±48$^{b}$ | 488±43$^{a}$ | 234±28$^{c}$ | <0.0001 | 0.0002 | 0.0011 |
| Epididymal adipose tissue | 119±8$^{b}$ | 109±12$^{b}$ | 95±9$^{b}$ | 225±23$^{a}$ | 268±28$^{a}$ | 144±15$^{b}$ | <0.0001 | 0.0008 | 0.0113 |
| Omental adipose tissue | 85±7$^{b}$ | 96±10$^{b}$ | 100±8$^{b}$ | 190±19$^{a}$ | 227±27$^{a}$ | 137±13$^{b}$ | <0.0001 | 0.0282 | 0.0129 |
| Liver | 261.8±10.1$^{b}$ | 248.6±11.3$^{b}$ | 226.3±4.7$^{c}$ | 336.3±12.3$^{a}$ | 345.9±10.4$^{a}$ | 282.4±11.0$^{b}$ | <0.0001 | <0.0001 | 0.15 |
| **Glucose metabolism and plasma biochemistry** | | | | | | | | | |
| OGTT-AUC (mmol/L min) | 659±13$^{c}$ | 722±18$^{b}$ | 715±28$^{bc}$ | 799±9$^{a}$ | 818±19$^{a}$ | 763±7$^{a}$ | <0.001 | 0.0574 | 0.0364 |
| Plasma insulin (μmol/L) | 2.0 ±0.2$^{b}$ | 0.9±0.1$^{b}$ | 1.3±0.2$^{b}$ | 5.7±1.3$^{a}$ | 2.2±0.5$^{b}$ | 2.8±0.5$^{b}$ | <0.001 | 0.0014 | 0.11 |
| Plasma leptin (ng/ml) | 3.3±0.5$^{b}$ | 5.3±0.8$^{b}$ | 1.9±0.5$^{b}$ | 7.9±1.0$^{a}$ | 9.1±0.6$^{a}$ | 3.4±0.5$^{b}$ | <0.001 | <0.0001 | 0.07 |
| Plasma ALP (U/L) | 131±7$^{cd}$ | 170±13$^{cd}$ | 113±4 | 214±18$^{b}$ | 261±23$^{a}$ | 178±15$^{c}$ | <0.001 | 0.0001 | 0.66 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Plasma ALT (U/L) | 30.1±2.0$^{c}$ | 34.8±2.3$^{bc}$ | 25.5±0.8$^{c}$ | 39.0±3.8$^{a}$ | 38.3±1.6$^{bc}$ | 35.4±2.4$^{bc}$ | 0.0003 | 0.0376 | 0.35 |
| Plasma AST (U/L) | 61.5±1.5$^{b}$ | 67.2±3.5$^{b}$ | 59.2±1.7$^{b}$ | 90.2±5.6$^{a}$ | 62.2±2.0$^{b}$ | 59.3±1.2$^{b}$ | 0.0024 | <0.0001 | <0.0001 |
| Plasma total cholesterol (mmol/L) | 1.8±0.1$^{bc}$ | 1.5±0.1$^{c}$ | 1.7±0.1$^{c}$ | 2.2±0.1$^{a}$ | 2.0±0.1$^{ab}$ | 1.8±0.1$^{bc}$ | <0.001 | 0.0042 | 0.0487 |
| Plasma triglycerides (mmol/L) | 0.9±0.1$^{b}$ | 0.7±0.1$^{b}$ | 0.5±0.1$^{b}$ | 2.2±0.4$^{a}$ | 2.3±0.3$^{a}$ | 1.1±0.2$^{b}$ | <0.001 | 0.0032 | 0.1 |
| Plasma NEFA (mmol/L) | 3.8±0.6$^{bc}$ | 2.1±0.2$^{c}$ | 2.2±0.2$^{c}$ | 6.6±0.8$^{a}$ | 6.2±0.4$^{a}$ | 4.1±0.5$^{bc}$ | <0.001 | 0.0008 | 0.1 |

Each value is a mean±S.E.M. Means within a row with unlike superscripts differ, P<0.05. ALP, alkaline phosphatase; ALT, aspartate transaminase; AST, aspartate transaminase; NEFA, non-esterified fatty acids.

* Body-weight gain calculated as percentage of body weight increase from 8 weeks to 16 weeks (8 weeks)

### *4.4.3. Cardiovascular structure and function*

Compared to C rats, H rats increased left ventricular weight and internal diameter in diastole as a sign of eccentric hypertrophy, without changes in relative wall thickness, increased stroke volume or cardiac output (Table 4.3). H rats also showed impaired cardiac function seen as increased systolic blood pressure and diastolic stiffness with increased diastolic, systolic and stroke volumes and decreased fractional shortening, developed pressure and dP/dt (Table 4.3). Green cardamom rats showed impaired cardiac function seen as decreased fractional shortening, increased wall stress, increased diastolic stiffness, decreased developed pressure and decreased dP/dt in HG rats. Additionally, diastolic, systolic and stroke volumes and cardiac output were elevated with green cardamom supplementation. HB rats showed normalised volumes, no signs of eccentric hypertrophy and normalised estimated left ventricular mass and hence improved cardiac function (Table 4.3). HB increased the heart rate and therefore the cardiac output. However, HB rats showed decreased systolic blood pressure, LV wet weight and diastolic stiffness constant when compared to H and HG rats; no significant changes were observed in CB rats (Table 4.3).

The LV of H rats showed greater infiltration by inflammatory cells (Figure 4.1D) as well as increased interstitial collagen deposition (Figure 4.1J) compared to C rats (Figure 4.1A and G, respectively). Black cardamom normalised the inflammatory state and markedly reduced collagen deposition in HB rats (Figure 4.1F and L, respectively). The reduction in LV fibrosis is consistent with the reduced diastolic stiffness constant in black cardamom rats. Green cardamom rats showed greater inflammatory cell infiltration (Figure 4.1B and E) and increased collagen deposition (Figure 4.1H and K) with hypertrophied cardiomyocytes in HG rats compared to H and HB rats and in CG rats compared to C and CB rats. No significant differences were observed between C and CB rats (Figure 4.1C and I).

H rats showed diminished vascular contraction to noradrenaline in isolated thoracic aortic rings compared to C rats (Figure 4.2A). Additionally, H rats showed decreased smooth muscle-dependent and endothelium-dependent relaxant responses to sodium nitroprusside and acetylcholine, respectively (Figure 4.2B and C). Black cardamom rats showed increased vascular contraction to noradrenaline as well as increased smooth muscle-dependent and endothelium-dependent relaxant responses to sodium nitroprusside and acetylcholine, while HG rats failed to improve aortic function (Figure 4.2). These effects were associated with normalised systolic blood pressure in HB rats (Table 4.3).

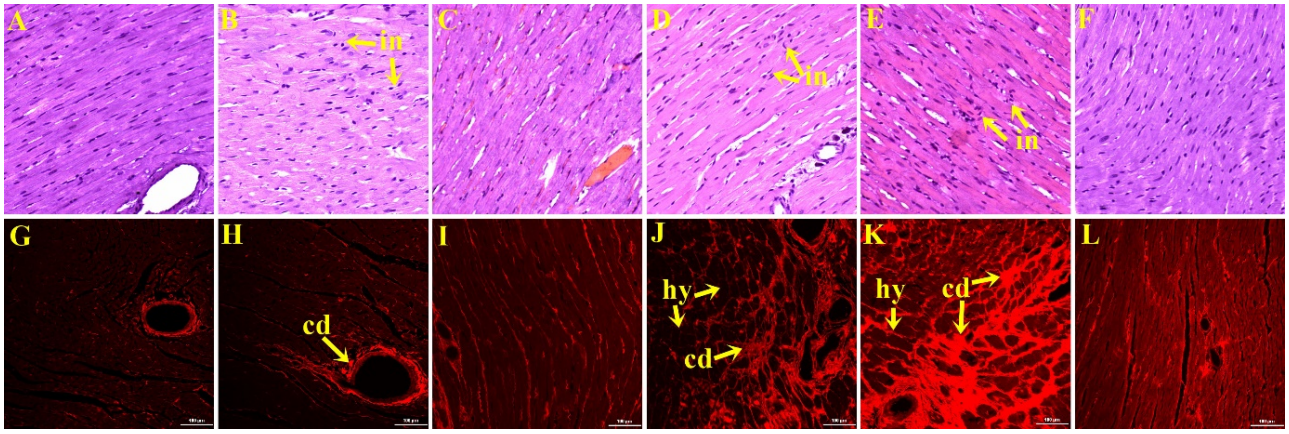
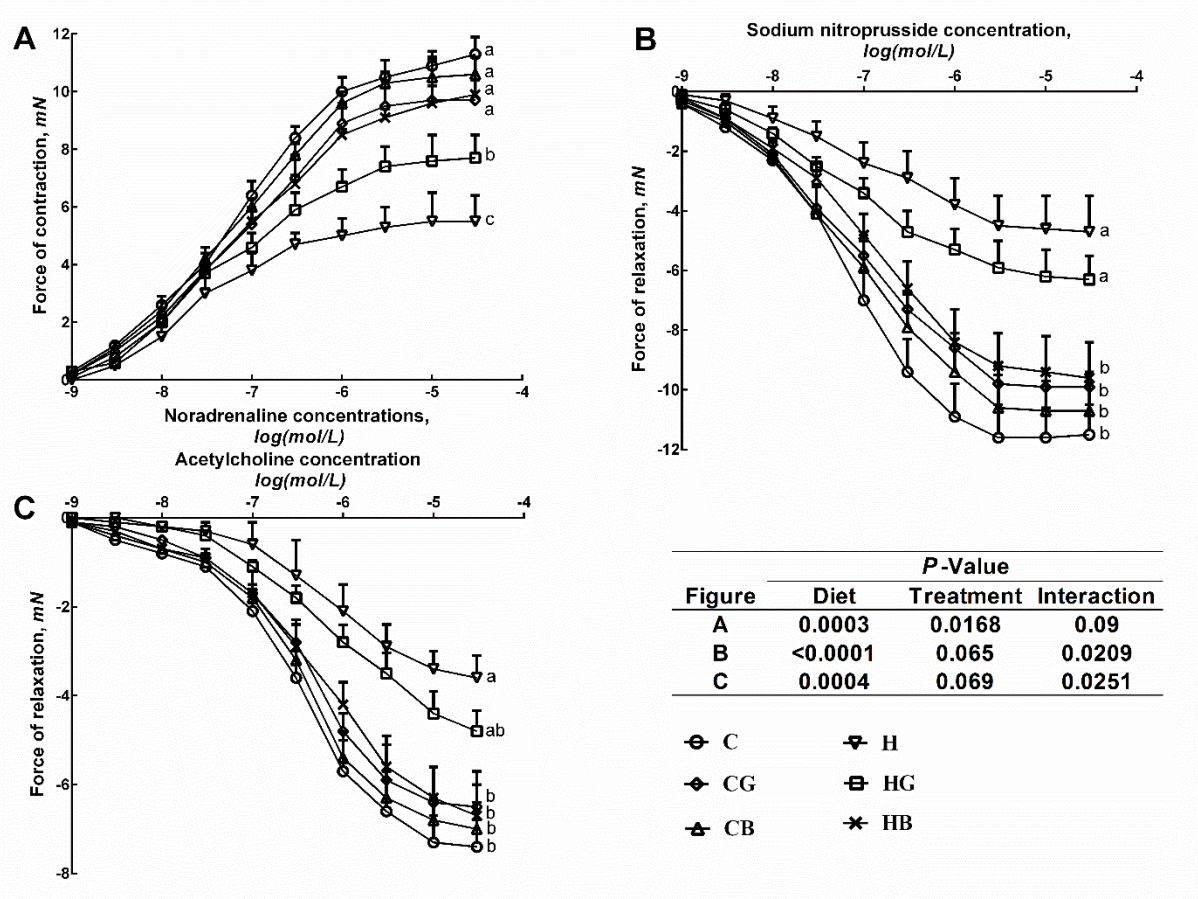



Figure 4.1 Haematoxylin and eosin staining of left ventricle (original magnification ×20) showing inflammatory cells (marked as "in") as dark spots outside the cardio myocytes in rats fed the C (A), CG (B), CB (C), H (D), HG (E) and HB (F) diet. Picrosirius red staining of left ventricular interstitial collagen deposition (original magnification ×20) in rats fed the C (G), CG (H), CB (I), H (J), HG (K) and HB (L) diet. Collagen deposition is marked as "cd" and hypertrophied cardiomyocytes are marked as "hy".




| Figure | P-Value | | |
|---|---|---|---|
| | Diet | Treatment | Interaction |
| A | 0.0003 | 0.0168 | 0.09 |
| B | <0.0001 | 0.065 | 0.0209 |
| C | 0.0004 | 0.069 | 0.0251 |

Figure 4.2 Cumulative concentration-response curves for noradrenaline (A), sodium nitroprusside (B) and acetylcholine (C) in thoracic aortic rings from rats fed the C, CB, CG, H, HB and HG diet. Data are shown as means ± S.E.M. significantly different end-point means indicated by different letters, P<0.05 and n=8/group.

**Table 4.3 Changes in cardiovascular structure and function in C, CG, CB, H, HG and HB diet-fed rats (n=8 rats/group)**

| Variable | C | CG | CB | H | HG | HB | P values | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Diet | Treatment | Interaction |
| Heart rate (bpm) | 268±21[b] | 236±10[b] | 299±22[b] | 352±22[a] | 255±19[b] | 317±19[ab] | 0.0164 | 0.0024 | 0.18 |
| LVIDd (mm) | 6.61±0.27[b] | 7.81±0.16[a] | 7.07±0.18[b] | 7.86±0.36[a] | 8.11±0.16[a] | 7.24±0.26[b] | 0.006 | 0.003 | 0.06 |
| LVIDs (mm) | 3.46±0.12[b] | 4.44±0.18[a] | 3.35±0.16[b] | 4.20±0.16[a] | 4.45±0.28[a] | 3.48±0.24[b] | 0.08 | <0.0001 | 0.15 |
| Fractional shortening (%) | 53.0±1.5[a] | 43.3±1.5[b] | 55.3±1.9[a] | 51.4±3.6[ab] | 45.0±3.3[b] | 50.4±2.3[a] | 0.44 | 0.0016 | 0.42 |
| (+)dP/dt (mmHg/S) | 1298±56[a] | 1200±63.[a] | 1265±54 [a] | 842±42 [b] | 883±59 [b] | 1186±61 [a] | <0.0001 | 0.0045 | 0.0063 |
| (-)dP/dt (mmHg/S) | -858±38[a] | -687±42[c] | -734±27[cb] | -437±38[d] | -532±39 [d] | -712±38[cb] | <0.0001 | 0.0125 | <0.0001 |
| Diastolic stiffness (k) | 22.7±0.7[b] | 23.6±0.9[b] | 22.2±0.8[b] | 28.5±0.5[a] | 27.3±0.5[a] | 24.1±0.8[b] | <0.0001 | 0.0014 | 0.0286 |
| Diastolic volume (µL) | 313±36[b] | 504±29[a] | 377±29[b] | 530±68[a] | 562±33[a] | 408±42[b] | 0.0043 | 0.0037 | 0.07 |
| Systolic volume (µL) | 44±5[b] | 95±11[a] | 42±6[b] | 80±9[a] | 100±16[a] | 49±10[b] | 0.06 | <0.0001 | 0.24 |
| Stroke volume (µL) | 269±38[b] | 409±22[ab] | 335±28[b] | 445±63[a] | 463±31[a] | 359±37[b] | 0.0092 | 0.06 | 0.11 |
| Cardiac output (mL/min) | 71.9±13.0[b] | 96.0±5.5[b] | 101.2±10.6[b] | 157.2±23.7[a] | 119.6±16.1[ab] | 115.4±14.4[ab] | 0.0017 | 0.88 | 0.0451 |
| Estimated LV mass, Litwin (g) | 0.80±0.03[b] | 1.04±0.04[a] | 0.90±0.04[ab] | 1.15±0.07[a] | 1.15±0.06[a] | 1.03±0.07[a] | <0.0001 | 0.0364 | 0.06 |
| LV+septum wet weight (mg/mm tibial length) | 17.9±1.7[b] | 19.6±0.7[ab] | 16.6±0.5[b] | 21.9±0.7[a] | 22.8±1.0[a] | 18.0±0.8[b] | 0.0009 | 0.001 | 0.41 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Relative wall thickness | 0.56±0.04 | 0.48±0.01 | 0.53±0.01 | 0.52±0.03 | 0.48±0.01 | 0.56±0.02 | 0.86 | 0.0122 | 0.32 |
| Systolic blood pressure (mmHg) | 132±3[c] | 135±2[c] | 132±2[c] | 161±3[a] | 151±2[b] | 136±1[c] | <0.0001 | <0.0001 | <0.0001 |
| Systolic wall stress (mmHg) | 80.4±5.1[b] | 111.5±5.7[a] | 72.9±5.2[b] | 105.5±8.1[a] | 112.8±9.2[a] | 81.2±7.2[b] | 0.047 | <0.0001 | 0.22 |

Each value is a mean ± S.E.M. Means within a row with unlike superscripts differ, P<0.05.

### *4.4.4. Liver structure and function*

In comparison to C rats, H rats had elevated plasma ALP, ALT and AST activities with increased liver weights. Black cardamom supplementation improved liver function, indicated by the decreased plasma activities of these enzymes. Green cardamom decreased plasma ALT and AST activity, but increased plasma ALP activity (Table 4.2).

H rats (Figure 4.3D) showed increased hepatic lipid deposition and inflammatory cell infiltration compared to C rats (Figure 4.3A). Black cardamom decreased the macrovesicular steatosis and portal inflammation in HB rats (Figure 4.3F). In contrast, HG rats showed further increases in hepatic lipid deposition and inflammatory cell infiltration compared to H and HB rats (Figure 4.3E) and CG rats compared to C and CB rats (Figure 4.3B). No changes in tissue morphology, inflammatory cell infiltration or macrovesicular steatosis were seen in CB rats compared to C rats (Figure 4.3C).

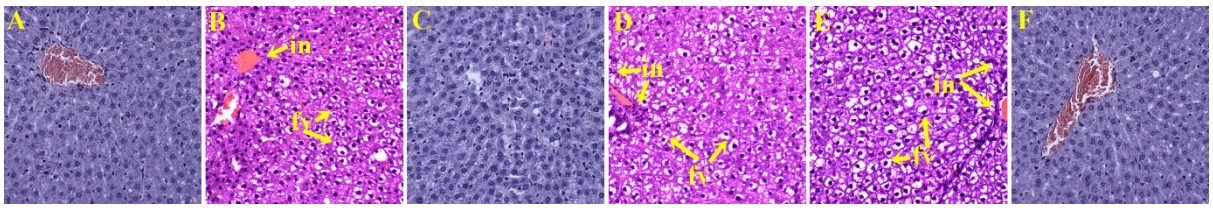



Figure 4.3 Haematoxylin and eosin staining of hepatocytes (original magnification ×20) showing inflammatory cells (marked as "in") and hepatocytes with fat vacuoles (marked as "fv") in rats fed the C (A), CG (B), CB (C), H (D), HG (E) and HB (F) diet.

## 4.5. Discussion

Rats fed on a diet with increased simple sugars such as fructose and sucrose together with increased saturated and *trans* fats developed abdominal obesity, hypertension, endothelial dysfunction and cardiac fibrosis together with an increase in ventricular stiffness, dyslipidaemia, liver inflammation, increased plasma lipid concentrations and impaired glucose tolerance (122, 232-234). These changes closely mimic human metabolic syndrome. Hence, I have used this rat model to investigate whether green or black cardamom can reverse these alterations in metabolic, cardiovascular and liver parameters and data from this study suggests that black cardamom attenuated the symptoms of metabolic syndrome, whereas green cardamom exacerbated adiposity. However, green cardamom improved glucose metabolism and decreased plasma insulin concentrations. These results suggest that green cardamom increased glucose uptake in liver and other organs, converting glucose to fat and thereby increasing fat vacuoles in hepatocytes. Also, given the anti-microbial properties of α-terpinyl acetate rich essential oil (235), it is hypothesised that green cardamom could

have damaged or altered the gut microbiota. Although the microbiota can influence dietary nutrient harvest, the diet can also impact on the microbial community composition and function (236). This suggests that green cardamom could have altered the gut microbiota which regulates intestinal absorption of lipids and metabolism of fatty acids and therefore increased fat absorption, leading to raised plasma lipid concentrations and liver wet weight.

There is no clear literature evidence that intervention with cardamom, either black or green, decreases the signs of the metabolic syndrome, although improvements in individual signs have been published. A green cardamom intake of 3 g/day lowered blood pressure in mildly hypertensive patients (237) and anti-inflammatory effects of green cardamom oil were measured in carrageenan-induced plantar oedema in male albino rats (227). Black cardamom improved alcoholic fatty liver (238), lowered lipids in cholesterol diet-fed rabbits (239, 240), improved glucose metabolism in fructose-fed rats (241) and decreased inflammation in carrageenan-induced paw oedema in rats (231). It is assumed, but not proved, that the volatile oils are the major bioactive principles of cardamom. Further, cardamom contains unknown amounts of phenolic and flavonoid components that may have biological activity. The major constituent of volatile oil from black cardamom, 1,8-cineole, has potential effects in metabolic syndrome as this terpene dose-dependently reduced blood pressure in normotensive rats (242) and in nicotine-induced hypertensive rats (243), and also showed endothelium-dependent vasorelaxation in male Wistar rats (244). Given the few studies reporting the therapeutic effects of green and black cardamom, the aim of this study was to determine the responses to chronic dietary supplementation of α-terpinyl acetate-containing green cardamom and 1,8-cineole-containing black cardamom in rats fed either low-fat, corn starch diet or a high-carbohydrate, high-fat diet as a model of metabolic syndrome. The responses to green and black cardamom were markedly different. Black cardamom reduced visceral adiposity; similarly, male Wistar rats fed with eucalyptus leaf extract containing high amounts of 1,8-cineole showed marked decreases in adipose fat mass (245), leading to a decreased inflammatory cytokine release by adipose tissue (246). This effect of black cardamom on adipose tissue could result from decreased infiltration of inflammatory cells in adipose tissue, as shown here in heart and liver. In contrast, green cardamom further increased visceral adiposity with decreased lean mass confirming that decreased muscle mass increases visceral adiposity (247). In HG rats, increased visceral adiposity could be a source of components of the renin-angiotensin system (RAS), with regulation of their production related to obesity-hypertension (248). Both angiotensin type 1 and 2 receptors have been localized to adipocytes and angiotensin II has been demonstrated to regulate adipocyte

growth and differentiation, lipid metabolism, and expression and release of adipokines and RAS components, and to promote oxidative stress leading to infiltration of macrophages and increase in to cardiac tissue contributing to increase in blood pressure (248).

Black cardamom improved liver function since the liver wet weight and activity of the liver plasma enzymes were lower than high-carbohydrate high-fat diet-fed rats and approximated those of corn starch diet-fed rats. Similarly, improved hepatic function was measured with black cardamom extract in alcohol-induced liver damage (238). Black cardamom may protect the liver by increasing the expression of voltage-dependent anion channels that trigger the opening of mitochondrial membrane permeability transition pores (249). Black cardamom also decreased plasma lipid concentrations in patients with ischaemic heart disease (250) which is consistent with the current study and this action should decrease liver steatosis and insulin resistance, thus improving liver function. However, further studies using black cardamom are warranted to understand the mechanisms underlying these improvements in hepatic function and plasma lipid concentrations.

In contrast, green cardamom increased liver wet weight and increased plasma activities of ALT, AST and ALP as markers of active liver damage (251). In humans, green cardamom extract in Arabic coffee showed no effect on plasma liver enzyme activity (252). From histological evidence, green cardamom further increased infiltration of inflammatory cells in the liver of high-carbohydrate, high-fat-fed rats; in contrast, green cardamom showed anti-inflammatory effects in acute carrageenan-induced plantar oedema in male albino rats (227). Green cardamom did not change the increased plasma lipid profile in H rats, consistent with the presence of increased fat deposition in the liver.

Increased plasma free fatty acid concentrations and liver enzyme activities cause endothelial dysfunction leading to hypertension (253). The high-carbohydrate, high-fat diet led to structural and functional changes in the heart. Cardiovascular abnormalities included increased left ventricular stiffness, increased relative wall thickness, reduced fractional shortening, reduced ejection fraction and increased estimated left ventricular mass (146). Black cardamom normalised plasma free fatty acids concentrations, liver enzyme activities, thoracic aortic ring reactivity and cardiac structure and function. In patients with ischaemic heart disease, black cardamom (3 g/day) improved the plasma lipid profile and enhanced the fibrinolytic activity and antioxidant status, although cardiovascular parameters were not reported (254). Green cardamom showed a smaller decrease in blood pressure, no changes in heart structure or thoracic aortic ring reactivity and increased inflammatory cell infiltration and collagen deposition, consistent with some changes in liver enzyme activities and

insignificant changes in plasma free fatty acid concentrations. Green cardamom at 3 g/day for 12 weeks in mildly hypertensive subjects decreased blood pressure with no changes in plasma cholesterol and triglycerides (237), although liver function and heart structure were not measured. Green cardamom extract with Arabic coffee showed increased total cholesterol and LDL concentration with no effect on blood pressure (252).

The improved cardiovascular function in HB rats could be due to 1,8-cineole, as this compound decreased mean aortic pressure following increased values with hexamethonium, atenolol or methylatropine (242). Further, 1,8-cineole (0.1 mg/kg/day) reduced hypertension induced by chronic nicotine administration and a higher dose (1 mg/kg/day) increased plasma nitrate concentrations (243). Our results suggest that the improved vascular relaxant responses to acetylcholine following black cardamom led to decreased blood pressure. Black cardamom reduced left ventricular infiltration of inflammatory cells, local collagen deposition and left ventricular stiffness. Echocardiographic assessment of HB rats showed improved left ventricular function and decreased left ventricular dimensions. The total wet weight of the heart was also reduced. These results suggest that green and black cardamom produce different responses on cardiac structure and function, and on vascular responsiveness. Black cardamom improved the signs of metabolic syndrome, but the metabolic, cardiovascular and liver responses to the H diet were not improved by green cardamom in this study. Green cardamom (3 g/kg body weight) in mice showed altered energy metabolism, increased oxidative stress and morphological changes in heart structure (255). Further, an increased dose of green cardamom in Arabic coffee may increase cardiovascular risk (252). In this study, 30 g/kg food of either green or black cardamom was used to provide a daily dose of ~1.5 g/kg body weight, half the dose shown to increase oxidative stress in mice (255), which corresponds to ~20 g/d cardamom in a 70 kg human, based on body surface area comparisons between rats and humans (167). This dose would seem too high to be obtained from the diet, suggesting that black cardamom may be useful in a combination with other functional foods to improve the signs of the metabolic syndrome in humans.

### 4.6. Conclusions

Black cardamom attenuated the signs of metabolic syndrome while green cardamom exacerbated adiposity, decreased liver function and worsened cardiovascular structure and function However, green cardamom decreased plasma insulin and the liver enzymes, ALT and AST. These responses suggest that black cardamom containing 1,8-cineole may improve cardiac, hepatic and metabolic parameters, unlike the green cardamom containing α-terpinyl

acetate, which had no effect on heart and liver structure. Further investigations on these closely related terpenes will be necessary to understand their role in the improvement of the signs of metabolic syndrome, which is a limitation of the current study. Components other than the volatile oils such as phenolic and flavonoid constituents may also contribute to the differences in activity. In addition, black cardamom contains increased carbohydrates and this component may improve gastrointestinal function as dietary fibre decreases obesity (256, 257).

### 4.7. Study specific acknowledgements

I thank Mr Parasuram Poudyal (Ranipool, Sikkim, India) and Dr Krishna Kumar (Coimbatore, Tamil Nadu, India), for providing black cardamom and green cardamom respectively. I thank Mr Jason Brightwell, The Prince Charles Hospital, Brisbane, Australia, for the acquisition of echocardiographic images. I thank Dr Kate Kauter, School of Health and Wellbeing, University of Southern Queensland, for her assistance and expert advice in ELISA.

# CHAPTER 5

# 5. Effect of dietary inorganic nitrate on cardio-metabolic risk factors in rats

## 5.1. Summary

Dietary inorganic nitrates improve cardiovascular diseases and dyslipidaemia with anti-inflammatory effects that may inhibit the progression of metabolic syndrome. In this study, we investigate whether dietary inorganic nitrates and a nitrate-rich source such as beetroot improve cardiovascular, liver and metabolic function with changes in mRNA expression. Rats fed with a high-carbohydrate, high-fat diet (H) for 16 weeks develop many of the signs of human metabolic syndrome such as abdominal obesity, changes in cardiovascular and hepatic structure and function, hypertension and impaired glucose tolerance compared to rats fed a corn starch diet (C). We measured cardiovascular, liver and metabolic parameters to compare responses to either sodium nitrate (N) at ~11 mg/kg/day or 5% beetroot juice (B) providing approximately the same dose of sodium nitrate fed during the last 8 weeks. H rats supplemented with either sodium nitrate (HN) or beetroot (HB) showed reduced systolic blood pressure, improved cardiovascular structure and function, plasma triglycerides, plasma lipid profile and plasma liver enzymes ($P<0.05$). Histological examination showed reduced inflammatory cell infiltration in heart and liver of HN and HB rats together with decreased left ventricular fibrosis. N and B treatment exhibited decreased CTGF, MCP1, MMP2, PPARα and AMPKα mRNA expression in left ventricle with no change in TGFβ. Neither treatment altered total fat mass or visceral adiposity, with no change in metabolic gene expression including PPARα and AMPKα in liver and skeletal muscle tissue. Our findings show that inorganic nitrates and beetroot are effective in reversing cardiovascular, liver and metabolic complications and alter left ventricular gene expression in this rat model of human metabolic syndrome, without changing obesity. Importantly, these findings suggest that inorganic nitrates are the most likely bioactive component of beetroot.

## 5.2. Introduction

The prevalence of cardiovascular diseases and mortality is increasing worldwide (258, 259) with an associated increase in the prevalence of obesity (260, 261). Obesity is a major risk factor for most cardiovascular diseases such as hypertension, coronary heart disease and myocardial infarction leading to heart failure (262, 263). In recent years, many countries have focused on improvement of diet, particularly in population groups with an increased risk of cardiovascular disease. Many clinical trials have shown that increased fruit and vegetables in diet reduced blood pressure and other cardiovascular diseases (264, 265), hypothesising that

these protective effects were related to high polyphenol or antioxidant contents in these fruits and vegetables. Consumption of green leafy vegetables and beetroot with increased inorganic nitrates ($NO_3^-$) content may protect against coronary heart disease (265). Recently, a meta-analysis and several clinical studies (266-268) suggest that consumption of dietary inorganic nitrates and beetroot juice reduced blood pressure. These dietary inorganic nitrates reduces to nitrites ($NO_2^-$) and to nitric oxide (NO) via salivary bacteria and in the acidic environment of the stomach (193). NO is produced by NO synthase (NOS) in endothelial cells to regulate vascular dilatation and inhibit inflammatory cells and platelet aggregation (269). Previous studies with beetroot and inorganic nitrates showed decreased blood pressure, and improved endothelial and cardiac function associated with decreased bodyweight (193, 194, 270, 271).

Feeding rats with a high-carbohydrate, high-fat diet induced endothelial dysfunction (146). Endothelial dysfunction leads to hypertension (272), atherosclerosis (273), stroke (274) and cardiovascular structural and functional damage (146). Dietary nitrates can be converted to the pleiotropic molecule NO (275). Although the importance and molecular function of NO have been widely published, there is no clear evidence that dietary inorganic nitrates could also help in cardiovascular protection in metabolic syndrome. Thus, this study has compared beetroot and inorganic nitrate for attenuation of cardiometabolic risks in rats fed a high-carbohydrate, high-fat diet mimicking human metabolic syndrome. Cardiovascular, hepatic and metabolic parameters were measured for this comparison along with heart, liver and skeletal muscle gene expression.

## 5.3. Material and methods

### *5.3.1. Rats and diets*

The experimental group consisted of 72 male Wistar rats (8-9 weeks old were randomly divided into 6 experimental diet groups (n=12 each) and fed with their respective diets for total 16 weeks, either corn starch (C), C + sodium nitrate (CN), C + beetroot juice (CB), high-carbohydrate, high-fat (H), H + sodium nitrate (HN) or H + beetroot juice (HB). Beetroot juice as commercially available BEE IT stamina shots was purchased from local health-food shops and sodium nitrate was purchased from Sigma-Aldrich Australia. C, CB and CN rats were fed with C diet for the first 8 weeks and then with C, C + B and C + N diets for the last 8 weeks. H, HB and HN rats were fed with H diet for the first 8 weeks and then with H, H + B and H + N diets for the last 8 weeks. B 50 ml/kg (containing ~215 mg nitrates) and N 215 mg/kg were supplemented along with the basal diets by replacing equivalent amounts of water.

Experimental procedures such as body composition, oral glucose tolerance, echocardiography, blood pressure, isolated heart preparation, organ bath, plasma analysis and histology were conducted as described in Chapter 3.1.

### *5.3.2. Real-time polymerase chain reaction*

Approximately 5-7 minutes after euthanasia, left ventricle, liver and skeletal muscle portions were snap-frozen in liquid nitrogen and stored at −80°C in a 5-ml cryovial tubes until quantitative analysis of RNA transcripts. Total RNA was extracted from approximately 15 mg of tissue using 1000 mg of ceramic/silica beads in TRIzol® Reagent (Invitrogen, Melbourne, Australia) (276). Extracted RNA concentration was quantified spectrometrically at 260 nm and DNase treated using the commercially available RQ1 RNase-free DNase kit (Promega Corporations, Madison, USA) to ensure the sample was free from DNA contaminates. First strand cDNA was then generated from 0.3 µg of template RNA using the commercially available iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, USA) using random hexamers and oligo dTs as described (276). cDNA was stored at -20°C for subsequent analysis.

Real-time PCR was conducted using MyiQ™ single colour 'real-time' PCR detection system (Bio-Rad Laboratories, Hercules, CA) with iQ™ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, USA) as the fluorescent agent. Forward and reverse oligonucleotide primers for the genes of interest were designed using OligoPerfect™ Suite (Invitrogen, Melbourne, Australia) (Table 5.1). To compensate for variations in RNA input amounts and reverse transcriptase efficiency, mRNA abundance of the genes of interest was normalised to housekeeping gene, β-actin, for heart, liver and skeletal muscle. Real-time PCR reactions were run for 50 cycles of 95°C for 15 sec and 60°C for 60 sec. Relative changes in mRNA abundance were quantified using the $2^{-\Delta\Delta CT}$ method (277) and reported in arbitrary units. CT values for β-actin were not altered by dietary intervention.

## 5.4. Results

### *5.4.1. Cardiovascular structure and function*

H rats showed increased left ventricular internal diameter in diastole (LVIDd) and left ventricular wet weight as signs of eccentric hypertrophy compared to C rats. This change in LVIDd was observed with no change in relative wall thickness in either of the groups (Table 5.2). H rats showed impaired systolic function seen as decreased fractional shortening, developed pressure and dP/dt with increased left ventricular diameter in systole (LVIDs), diastolic stiffness and systolic wall stress (Table 5.2). H rats showed increased diastolic,

systolic and stroke volumes, cardiac output and estimated left ventricular mass compared to C rats, with increases in heart rate.

**Table 5.1 Real-time PCR primer sequences.**

| Gene | Forward primer (5'-3') | Reverse primer (5'-3') |
|---|---|---|
| β-ACTIN | CTAAGGCCAACCGTGAAAAGA | CCAGAGGCATACAGGGACCAAC |
| CTGF | AAGGACCGCACAGTGGTT | AGAACAGGCGCTCCACTC |
| TGFβ | TTTAGGAAGGACCTGGCTTG | TGTTGGTTGTAGAGGGCAAG |
| MCP1 | GCCGTGTGTTCACAGTGCT | AGTTCTCCAGCCGACCATT |
| MMP2 | ACAGTGACACCACGTACAAGC | CATTCCCTGCGAAGAACACAG |
| AMPKα | ACTCTGCTGATGCACATGCT | AGGGGTCTTCAGGAAAGAGG |
| PPARα | TGTCGAATATGTGGGGACAA | ACTTGTCGTACGCCAGCTTT |

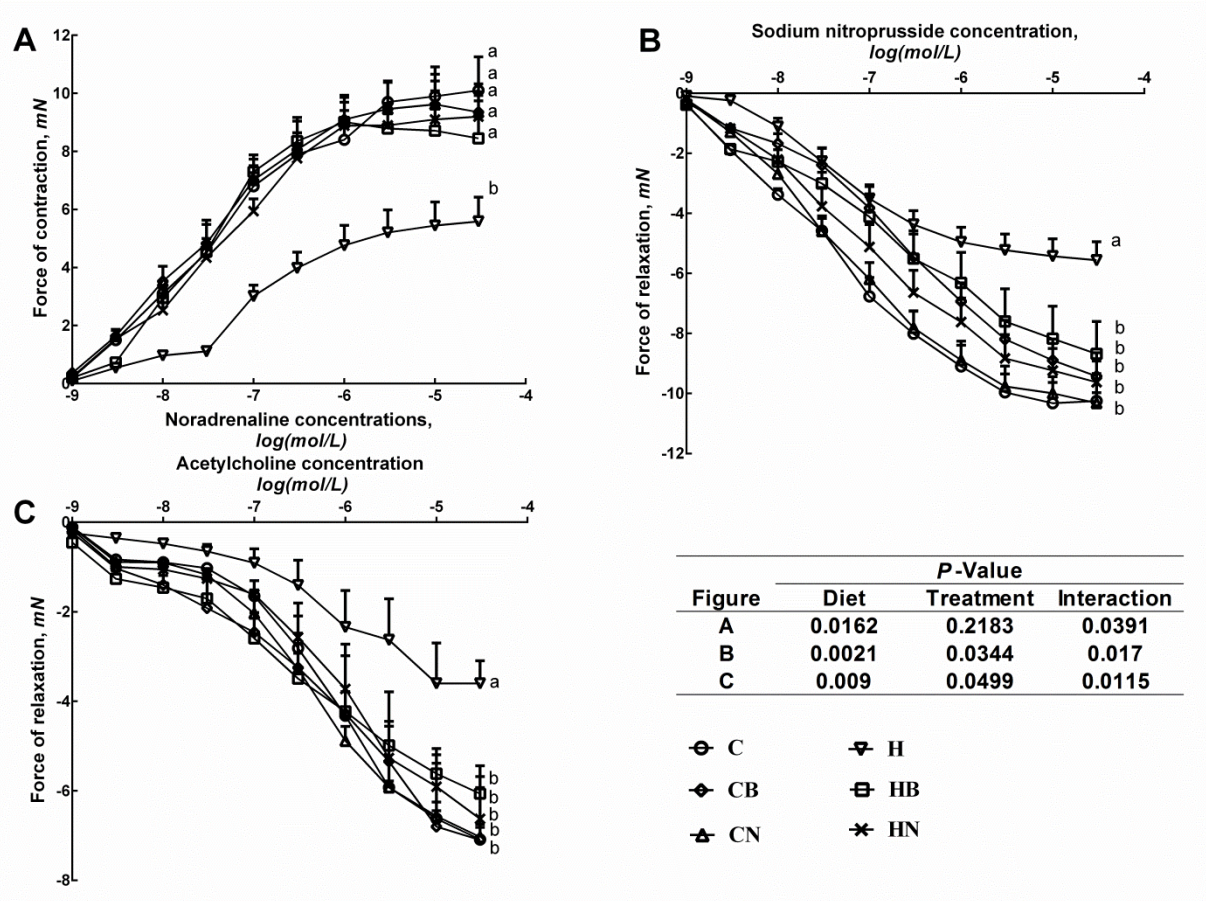
CTGF, connective tissue growth factor; TGFβ, transforming growth factor beta; MCP1, monocyte chemotactic protein; MMP2, matrix metalloproteinase-2; AMPKα, 5' adenosine monophosphate-activated protein kinase alpha; PPARα, peroxisome proliferator-activated receptor alpha.

Treatment of H rats with B and N decreased the LVIDd and LVIDs compared to H rats, with no change in left ventricular posterior wall diameter in systole and diastole. These effects were accompanied by increased fractional shortening with B and N (Table 5.2). Diastolic stiffness, systolic volumes, cardiac output, systolic wall stress and wet weight of left ventricle with septum were normalised with B and N, while heart rate was decreased with B and N treated rats. HB and HN rats increased diastolic volume and B and N treatment increased ejection time along with left-ventricular developed pressure compared to C and H rats (Table 5.2). Treatment of H rats with B and N normalised estimated left-ventricular mass and wet weight along with decreased systolic blood pressure (Table 5.2).

In isolated thoracic aortic rings, H rats showed decreased vascular contraction with noradrenaline (Figure 5.1A) and decreased vascular relaxation with sodium nitroprusside and acetylcholine compared to C rats (Figure 5.1B and C). B and N rats showed improved

contraction and relaxation in isolated thoracic aortic rings (Figure 5.1A, B and C), correlated with decreased systolic blood pressure in HB and HN rats.

Compared to C rats (Figure 5.2A and G), H rats showed increased infiltration of inflammatory cells (Figure 5.2D) and increased interstitial collagen deposition (Figure 5.2J). HB and HN showed decreased infiltration of inflammatory cells (Figure 5.2B, C, E and F) and reduced collagen deposition (Figure 5.2H, I, K and L), while no other changes were observed with tissue morphology appearing normal.




| Figure | P-Value | | |
|---|---|---|---|
| | Diet | Treatment | Interaction |
| A | 0.0162 | 0.2183 | 0.0391 |
| B | 0.0021 | 0.0344 | 0.017 |
| C | 0.009 | 0.0499 | 0.0115 |

Figure 5.1 Cumulative concentration-response curves for noradrenaline (A), sodium nitroprusside (B) and acetylcholine (C) in thoracic aortic rings from C, CB, CN, H, HB and HN rats. Data are shown as means ± SEM. End-point means without a common alphabet in each data set significantly differ, $P<0.05$ and n=10/group.

H diet-fed rats resulted in significant increases in CTGF, MCP1 and MMP2, mRNA expression (Figure 5.3A, C and D) compared to C rats. However, the H diet did not alter the TGFβ mRNA expression (Figure 5.3B) compared to C rats. H diet-fed rats increased AMPKα and decreased PPARα mRNA expression (Figure 5.4A and B) compared to C rats. HB and HN rats decreased CTGF, MCP1 and MMP2 mRNA expression (Figure 5.3A, C and D) and

no change in TGFβ mRNA expression (Figure 5.3B). HB and HN rats showed decreased AMPKα and increased PPARα mRNA expression (Figure 5.4A and B) compared to H rats.

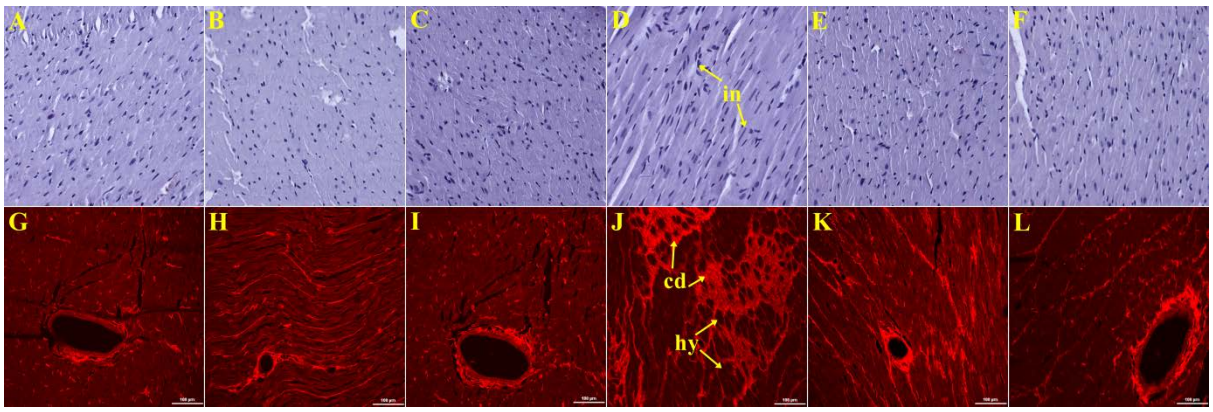



Figure 5.2 Haematoxylin and eosin staining of left ventricle (original magnification ×20) showing inflammatory cells (marked as "in") as dark spots outside the myocytes in C (A), CB (B), CN (C), H (D), HB (E) and HN (F) rats. Picrosirius red staining of left ventricular interstitial collagen deposition (original magnification ×20) in C (G), CB (H), CN (I), H (J), HB (K) and HN (L) rats. Collagen deposition is marked as "cd" and hypertrophied cardiomyocytes are marked as "hy".

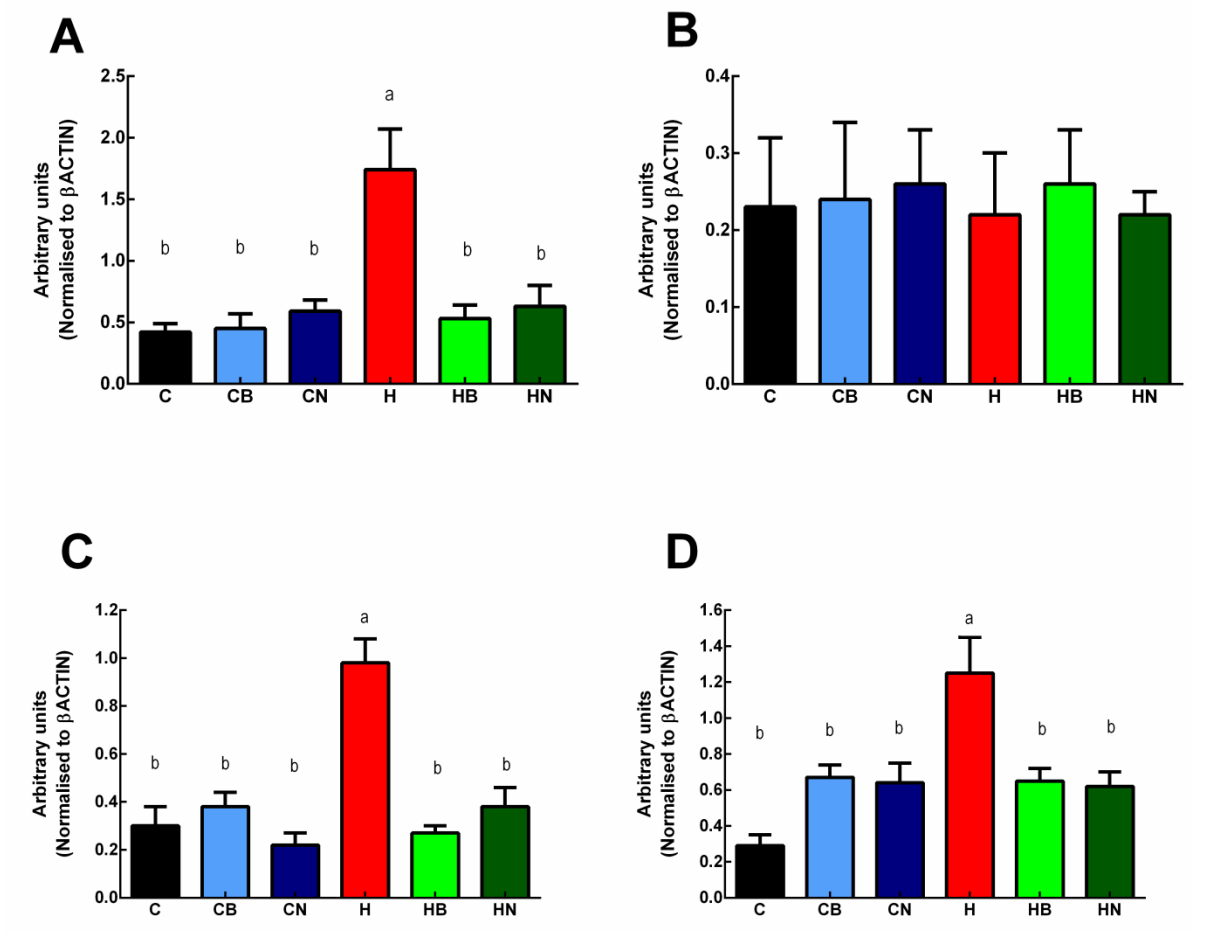



Figure 5.3 Left ventricular mRNA expressions of CTGF (A), TGFβ (B), MCP1 (C) and MMP2 (D) in C, CB, CN, H, HB and HN rats. Data expressed in arbitrary units normalised to

βACTIN. Means without a common alphabet in each data set significantly differ, P<0.05 and n=10/group.

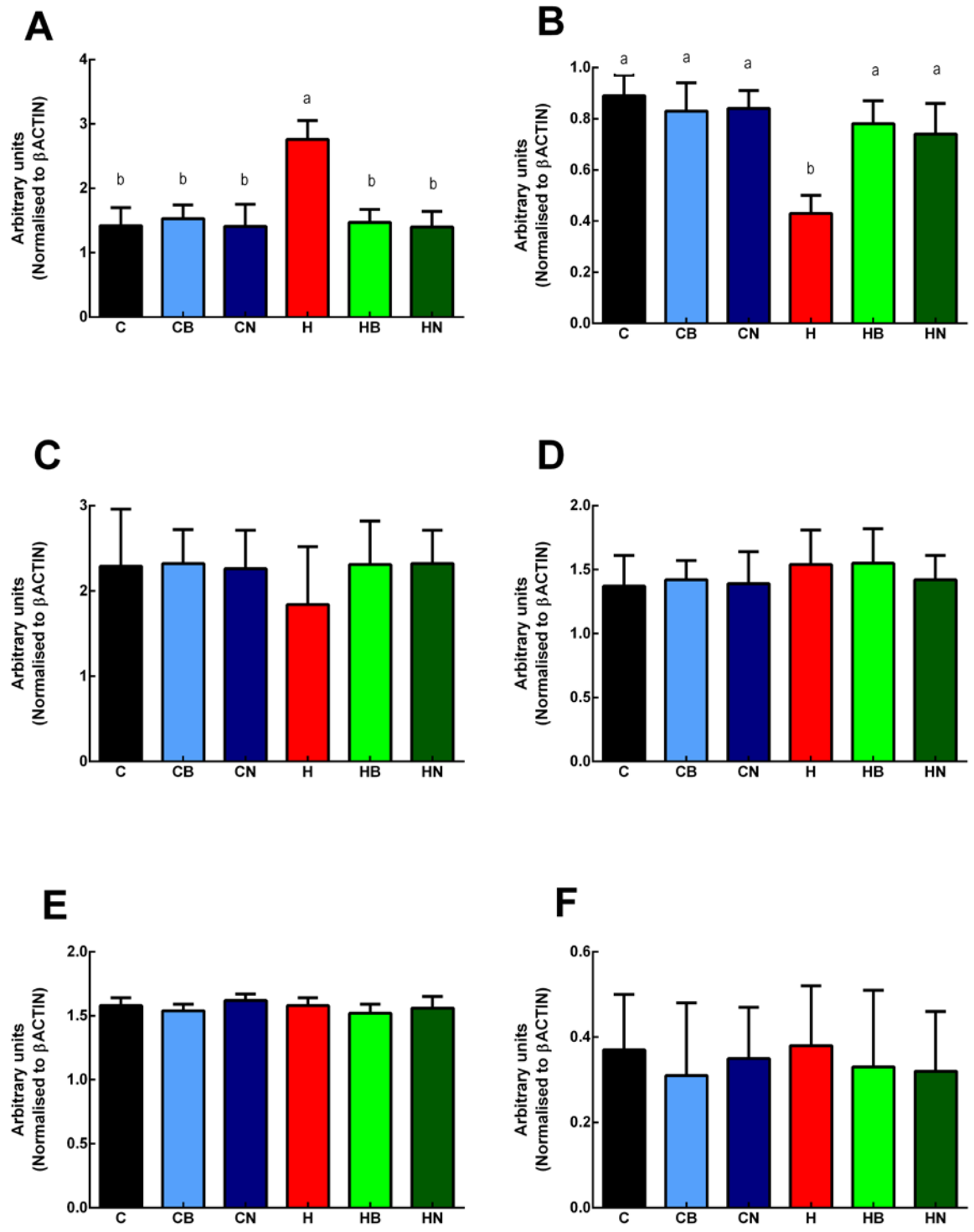



Figure 5.4 Left ventricular, hepatic and skeletal muscle mRNA expression of AMPKα and PPARα in C, CB, CN, H, HB and HN rats. Data expressed in arbitrary units normalised to β-actin. Means without a common alphabet in each data set significantly differ, P<0.05 and n=10/group.

**Table 5.2 Changes in cardiovascular structure and function in C, CB, CN, H, HB and HN diet-fed rats (n=10 rats/group).**

| Variable | C | CB | CN | H | HB | HN | P values | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Diet | Treatment | Interaction |
| Heart rate (bpm) | 272±23[b] | 271±8[b] | 245±14[b] | 365±24[a] | 298 ±8[b] | 240 ±35[b] | 0.0308 | 0.0036 | 0.07 |
| IVSd (mm) | 1.9±0.1 | 1.8±0.0 | 1.9±0.0 | 2.0±0.1 | 1.8 ±0.0 | 2.0 ±0.0 | 0.16 | 0.017 | 0.61 |
| LVIDd (mm) | 6.9±0.2[b] | 6.8±0.1[b] | 6.4±0.2[b] | 8.7±0.4[a] | 6.4 ±0.2[b] | 6.5 ±0.2[b] | 0.001 | <0.0001 | <0.0001 |
| LVPWd (mm) | 1.7±0.1 | 1.8±0.0 | 1.8±0.0 | 1.8±0.0 | 1.8 ±0.1 | 1.8 ±0.0 | 0.57 | 0.72 | 0.72 |
| IVSs (mm) | 3.2±0.1 | 3.1±0.1 | 3.3±0.1 | 2.9±0.1 | 3.1 ±0.1 | 3.3 ±0.1 | 0.23 | 0.0393 | 0.23 |
| LVIDs (mm) | 3.8±0.2[b] | 3.7±0.2[b] | 3.8±0.2[b] | 4.8±0.4[a] | 3.8 ±0.2[b] | 3.4 ±0.3[b] | 0.24 | 0.0133 | 0.0178 |
| LVPWs (mm) | 2.9±0.1 | 2.8±0.1 | 2.8±0.1 | 2.9±0.1 | 3.0 ±0.1 | 3.1 ±0.1 | 0.0475 | 0.85 | 0.32 |
| Fractional shortening (%) | 48.5±2.1[b] | 58.8±0.6[a] | 54.3±1.2[a] | 42.3±2.7[b] | 55.5 ±1.0[a] | 58.5 ±0.8[a] | 0.14 | <0.0001 | 0.0027 |
| Ejection time (ms) | 80.1±2.5[b] | 93.2±2.1[a] | 95.6±2.4[a] | 72.9±3.9[b] | 85.9 ±2.1[a] | 88.4 ±2.0[a] | 0.0014 | <0.0001 | 1 |
| Ejection fraction (%) | 85.8±2.0 | 81.1±1.7 | 77.6±1.4 | 82.5±2.4 | 81.3 ±1.9 | 81.7 ±2.8 | 0.85 | 0.1 | 0.22 |
| Diastolic volume (μL) | 359±38[b] | 341±25[b] | 275±32[b] | 688±66[a] | 281 ±36[a] | 292±33[a] | 0.0061 | <0.0001 | <0.0001 |
| Systolic volume (μL) | 60±10[b] | 59±9[b] | 60±8[b] | 121±22[a] | 61 ±11[b] | 51 ±16[b] | 0.12 | 0.0282 | 0.0321 |
| Stroke volume (μL) | 309±33[b] | 282±21[b] | 215±28[b] | 567±38[a] | 220 ±31[b] | 242 ±22[b] | 0.0037 | <0.0001 | <0.0001 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Cardiac output (mL/min) | 88.7±13.6[b] | 77.1±6.4[b] | 62.8±9.6[b] | 184.4±19.0[a] | 65.5±10.0[b] | 62.4±12.1[b] | 0.0087 | <0.0001 | 0.0001 |
| LV developed pressure (mmHg) | 54.7±3.4[ab] | 62.4±5.1[a] | 68.2±4.7[a] | 44.7±3.6[b] | 64.2±4.2[a] | 66.8±3.8[a] | 0.37 | 0.0004 | 0.38 |
| (+)dP/dt (mmHg/S) | 1148±75[a] | 1229±67[a] | 1197±66[a] | 825±84[b] | 1210±64[a] | 1242±78[a] | 0.1 | 0.0022 | 0.0334 |
| (-)dP/dt (mmHg/S) | -705±52[a] | -785±56[a] | -758±47[a] | -473±47[b] | -767±50[a] | -783±55[a] | 0.08 | 0.0006 | 0.034 |
| Diastolic stiffness (k) | 22.9±0.6[b] | 23.1±0.4[b] | 22.6±0.7[b] | 28.6±0.7[a] | 24.3±0.6[b] | 23.8±0.9[b] | <0.0001 | 0.0008 | 0.0013 |
| Estimated LV mass, Litwin (g) | 0.8±0.1[b] | 0.7±0.0[b] | 0.8±0.0[b] | 1.1±0.1[a] | 0.8±0.0[b] | 0.8±0.0[b] | 0.0071 | 0.0035 | 0.0393 |
| LV+septum wet weight (mg/mm tibial length) | 17.8±0.5[b] | 17.1±0.4[b] | 15.3±0.5[c] | 20.1±0.6[a] | 17.6±0.4[b] | 16.2±0.4[bc] | 0.0023 | <0.0001 | 0.15 |
| Right ventricle wet weight (mg/mm tibial length) | 4.6±0.5[ab] | 4.1±0.1[b] | 3.7±0.2[c] | 5.6±0.4[a] | 4.9±0.3[ab] | 4.1±0.2[b] | 0.0059 | 0.0015 | 0.62 |
| Relative wall thickness | 0.5±0.03[b] | 0.5±0.02[b] | 0.5±0.02[b] | 0.6±0.03[a] | 0.5±0.02[b] | 0.5±0.01[b] | 0.08 | 0.0497 | 0.0497 |
| Systolic blood pressure (mmHg) | 129±3[b] | 126±1[b] | 123±2[b] | 151±2[a] | 134±2[b] | 132±2[b] | <0.0001 | <0.0001 | 0.0034 |
| Systolic wall stress (mmHg) | 87.7±7.0[b] | 85.8±6.6[b] | 83.4±6.5[b] | 111.2±7.4[a] | 82.4±7.3[b] | 74.9±5.8[b] | 0.49 | 0.0114 | 0.0483 |

Each value is a mean ± SEM. Means within a row with unlike superscripts differ, P<0.05

**Table 5.3 Dietary intakes, body composition and anthropometrics, organ wet weights, changes in glucose tolerance test, plasma insulin and plasma biochemistry in C, CB, CN, H, HB and HN diet-fed rats (n=10 rats/group).**

| Variable | C | CB | CN | H | HB | HN | P values | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Diet | Treatment | Interaction |
| Food intake (g/d) | 32.7±1.3$^{ab}$ | 35.1±0.6$^{a}$ | 36.0±0.8$^{a}$ | 26.9±0.9$^{c}$ | 25.5±0.5$^{c}$ | 25.2±0.6$^{c}$ | <0.0001 | 0.09 | 0.06 |
| Water intake (ml/d) | 25.5±1.6$^{a}$ | 24.9±1.2$^{ab}$ | 26.3±2.0$^{a}$ | 26.8±2.3$^{a}$ | 24.9±0.6$^{a}$ | 28.5±1.1$^{a}$ | 0.35 | 0.26 | 0.76 |
| Beetroot juice intake (ml/d) | 0.0±0.0 | 1.7±0.0$^{a}$ | 0.0±0.0 | 0.0±0.0 | 1.1±0.0$^{b}$ | 0.0±0.0 | <0.0001 | <0.0001 | <0.0001 |
| Nitrate intake (mg/d) | 0.0±0.0 | 7.3±0.0$^{a}$ | 7.9±0.0$^{a}$ | 0.0±0.0 | 5.3±0.0$^{b}$ | 5.4±0.0$^{b}$ | <0.0001 | <0.0001 | <0.0001 |
| Energy intake (kJ/d) | 393±15$^{b}$ | 395±6$^{b}$ | 399±9$^{b}$ | 565±14$^{a}$ | 554±8$^{a}$ | 585±13$^{a}$ | <0.0001 | 0.28 | 0.51 |
| Feed conversion efficiency (%) | 1.8±0.2$^{b}$ | 1.7±0.2$^{b}$ | 1.7±0.3$^{b}$ | 5.4±0.7$^{a}$ | 4.5±0.5$^{a}$ | 3.8±0.7$^{a}$ | <0.0001 | 0.22 | 0.31 |
| Body weight gained (8-16 weeks) (%) | 7.4±1.9$^{b}$ | 6.8±0.8$^{b}$ | 5.6±1.4$^{b}$ | 15.1±0.9$^{a}$ | 12.0±0.9$^{a}$ | 11.2±1.5$^{a}$ | <0.0001 | 0.13 | 0.4 |
| Visceral adiposity index (%) | 4.6±0.2$^{b}$ | 4.3±0.6$^{b}$ | 4.4±0.2$^{b}$ | 7.6±0.5$^{a}$ | 6.2±0.5$^{a}$ | 6.4±0.5$^{a}$ | <0.0001 | 0.14 | 0.4 |
| Abdominal circumference (cm) | 20.3±0.2$^{c}$ | 20.5±0.3$^{c}$ | 20.1±0.3$^{c}$ | 22.5±0.4$^{a}$ | 21.3±0.4$^{b}$ | 21.2±0.2$^{b}$ | <0.0001 | 0.06 | 0.07 |
| Body mass index (kg/m2) | 5.2±0.4$^{b}$ | 5.0±0.1$^{b}$ | 4.8±0.2$^{b}$ | 6.3±0.3$^{a}$ | 5.7±0.1$^{b}$ | 5.8±0.2$^{b}$ | <0.0001 | 0.13 | 0.69 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Bone mineral content (g) | 12.7±0.4$^{b}$ | 12.7±0.3$^{b}$ | 11.5±0.2$^{b}$ | 15.6±0.5$^{a}$ | 15.1±0.4$^{a}$ | 14.7±0.4$^{a}$ | <0.0001 | 0.0202 | 0.57 |
| Total body lean mass (g) | 298±7$^{a}$ | 272±7$^{ab}$ | 270±5$^{ab}$ | 302±9$^{a}$ | 283±7$^{a}$ | 285±8$^{a}$ | 0.11 | 0.0027 | 0.72 |
| Total body fat mass (g) | 101±8$^{b}$ | 104±9$^{b}$ | 96±5$^{b}$ | 187±20$^{a}$ | 181±13$^{a}$ | 189±11$^{a}$ | <0.0001 | 0.99 | 0.78 |
| **Tissue wet weight (mg/mm)** | | | | | | | | | |
| Retroperitoneal adipose tissue | 171.5±9.3$^{b}$ | 147.5±23.9$^{b}$ | 148.2±5.9$^{b}$ | 303.1±22.7$^{a}$ | 288.0±28.0$^{a}$ | 300.6±22.1$^{a}$ | <0.0001 | 0.62 | 0.88 |
| Epididymal adipose tissue | 118.0±6.8$^{b}$ | 90.3±10.6$^{b}$ | 86.6±4.5$^{b}$ | 173.9±20.4$^{a}$ | 150.5±9.2$^{a}$ | 159.2±15.1$^{a}$ | <0.0001 | 0.08 | 0.78 |
| Omental adipose tissue | 84.8±6.3$^{ab}$ | 97.2±13.8$^{ab}$ | 94.7±5.8$^{ab}$ | 132.1±17.1$^{a}$ | 152.0±16.2$^{a}$ | 131.7±18.2$^{a}$ | 0.0001 | 0.49 | 0.81 |
| Liver | 223.5±6.9$^{b}$ | 218.0±5.8$^{b}$ | 193.8±6.3$^{b}$ | 335.4±11.1$^{a}$ | 256.6±9.5$^{b}$ | 258.7±14.2$^{b}$ | <0.0001 | <0.0001 | 0.0011 |
| **Glucose metabolism and plasma biochemistry** | | | | | | | | | |
| OGTT-AUC (mmol/L min) | 751±12$^{c}$ | 670.1±7.5$^{de}$ | 639.3±6.2$^{e}$ | 827.4±12.9$^{a}$ | 797.1±18.5$^{b}$ | 695.3±12.2$^{d}$ | <0.0001 | <0.0001 | 0.0154 |
| Plasma insulin (μmol/L) | 1.9±0.4$^{b}$ | 2.0±0.5$^{b}$ | 1.7±0.3$^{b}$ | 4.1±0.5$^{a}$ | 2.3±0.4$^{b}$ | 2.1±0.4$^{b}$ | 0.0416 | 0.0091 | 0.0242 |
| Plamsa leptin (μmol/L) | 6.5±0.7$^{ab}$ | 6.3±1.1$^{ab}$ | 6.4±0.5$^{ab}$ | 10.3±1.2$^{a}$ | 8.1±0.9$^{ab}$ | 8.5±0.6$^{ab}$ | 0.0007 | 0.36 | 047 |
| ALP (U/L) | 115.3±8.3$^{c}$ | 136.9±6.6$^{c}$ | 135.8±12.3$^{c}$ | 325.8±27.6$^{a}$ | 235.8±18.8$^{b}$ | 228.3±17.1$^{b}$ | <0.0001 | 0.0488 | 0.001 |
| ALT (U/L) | 26.3±1.5$^{b}$ | 26.4±1.1$^{b}$ | 29.3±1.1$^{b}$ | 34.3±1.6$^{a}$ | 29.3±1.2$^{b}$ | 30.1±1.4$^{b}$ | 0.0007 | 0.17 | 0.0269 |
| AST (U/L) | 67.3±6.1$^{b}$ | 60.4±1.9$^{b}$ | 60.8±1.3$^{b}$ | 87.5±3.0$^{a}$ | 61.3±2.9$^{b}$ | 63.9±3.6$^{b}$ | 0.0064 | <0.0001 | 0.0144 |
| Total cholesterol (mmol/L) | 1.9±0.1$^{b}$ | 1.3±0.1$^{c}$ | 1.5±0.1$^{c}$ | 2.6±0.2$^{a}$ | 1.4±0.1$^{c}$ | 1.6±0.1$^{c}$ | 0.0013 | <0.0001 | 0.009 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Triglycerides (mmol/L) | 0.5±0.1[b] | 0.4±0.1[b] | 0.5±0.1[b] | 1.8±0.3[a] | 0.9±0.2[b] | 0.8±0.1[b] | <0.0001 | 0.0036 | 0.0083 |
| NEFA (mmol/L) | 1.3±0.2[b] | 1.3±0.1[b] | 1.4±0.1[b] | 5.4±0.6[a] | 2.4±0.3[b] | 2.5±0.3[b] | <0.0001 | <0.0001 | <0.0001 |

Each value is a mean ± SEM. Means within a row with unlike superscripts differ, P<0.05.

*In all groups body-weight gained calculated as percentage of body weight increase from 8 weeks to 16 weeks. OGTT-AUC, oral glucose tolerance test-area under the curve; ALP, alkaline phosphatase; ALT, aspartate transaminase; AST, aspartate transaminase; NEFA, non-esterified fatty acids.

### *5.4.2. Dietary intake, body composition and plasma biochemistry*

Food intakes were higher in C, CB and CN rats than in H, HB and HN rats, respectively (Table 5.3). Due to this difference, the average daily intake of nitrates was higher in CB and CN rats than in HB and HN rats, respectively (Table 5.3). Neither B nor N treatment altered food, water or energy intakes (Table 5.3). H rats increased feed conversion efficiency, body weight gain, abdominal circumference and body mass index compared to C rats, while these parameters were reduced in HB and HN rats compared to H rats (Table 5.3). Bone mineral content was higher in H rats than HB and HN rats, and all were higher than C, CB and CN rats (Table 5.3). HB and HN rats had unchanged total body fat mass compared to H rats and increased total body fat mass compared to C, CB and CN rats. These changes in total body fat are consistent with changes in omental and epididymal fat pads (Table 5.3). Total body lean mass was unchanged in HB and HN rats.

Plasma concentrations of total cholesterol, triglycerides and NEFA were increased in H rats compared to C, HB or HN rats, while C rats had higher total cholesterol concentrations than HB and HN rats (Table 5.3). Plasma leptin concentrations were increased in H rats compared to C rats and B and N-treatment did not change leptin concentrations. H rats also had higher fasting blood glucose concentration compared to C rats. B and N treatment decreased blood glucose concentrations. The plasma glucose response to oral glucose tolerance area under the curve was greater in H rats than C rats (Table 5.3). B and N-treatment improved glucose clearance compared to H rats. However, N treated rats showed greater clearance compared to B treated rats (Table 5.3). Plasma insulin concentrations almost doubled in H rats compared to C, HB and HN rats (Table 5.3).

### *5.4.3. Hepatic structure and function*

Compared to C rats, H rats had increased liver wet weight with increased plasma activities of ALT, ALP and AST as markers of liver damage; B and N treatment decreased these parameters compared to H rats. Liver wet weight and plasma activities were unchanged in CB and CN rats compared to C rats, except an increased plasma ALP in CB and CN rats compared to C rats (Table 5.3). H rats (Figure 5.5D) showed increased hepatic lipid deposition and inflammatory cell infiltration compared to C rats (Figure 5.5A) while HB and HN rats showed decreased inflammatory cell infiltration (Figure 5.5E and F) compared to H rats. CB and CN rats showed minimal macrovesicular steatosis and portal inflammation and tissue morphology appeared normal (Figure 5.5B and C). However, no changes in AMPKα

and PPARα mRNA expression were observed in hepatic tissue (Figure 5.4C and D) or skeletal muscle (Figure 5.4E and F).

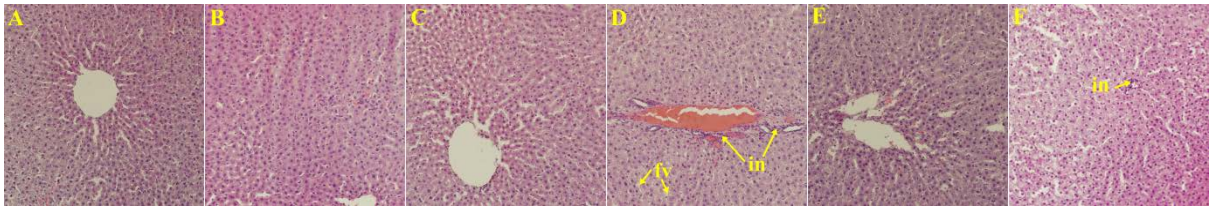



Figure 5.5 Haematoxylin and eosin staining of hepatocytes (original magnification ×20) showing inflammatory cells (marked as "in") and hepatocytes with fat vacuoles (marked as "fv") in C (A), CB (B), CN (C), H (D), HB (E) and HN (F) rats.

**5.5. Discussion**

Increases in dietary fruits and vegetables reduced blood pressure (275) but the cardioprotective effects of these diets remain contradictory and the mechanisms of action for these effects are unclear (278). Further, green leafy vegetables and beetroot with increased nitrates (265) improved cardiovascular health (268) hypothesised to be due to *in vivo* nitric oxide generation from nitrate. However, the daily dose of nitrates in these studies have exceeded the current recommended level of 3.7 mg/kg/day (275). In this study, we have used a lower dose of 12 mg/kg/day in rats which converts to ~2.5 mg/kg/day in a 70 kg human based on body surface area comparisons between rats and humans (167).

This study has measured the molecular and physiological effects in high-carbohydrate, high-fat diet-fed rats that mimic most of the signs of cardiometabolic disorders in humans. This diet produced increased blood pressure, left ventricular stiffness, vascular dysfunction, inflammatory cell infiltration in heart and liver, and collagen deposition in the left ventricle associated with increased CTGF, MCP1 and MMP2 mRNA expression. Decreased blood pressure and diastolic stiffness with both interventions correlated with decreased collagen deposition in left ventricle and normalised CTGF mRNA expression. Overexpression of MMP2 and CTGF increased collagen deposition in heart and aortic walls (279, 280) which was seen in H diet-fed rats. CTGF, activated by upstream TGFβ signal, stimulates the proliferation of connective tissue cells such as fibroblasts and extracellular matrix (281). In this study, decreased collagen deposition occurred with no change in the upstream activator TGFβ mRNA expression which is contradictory to previous studies (282). Our results also suggested that nitrate and beetroot interventions improved vascular function by improving endothelial function, perhaps by *in vivo* conversion of nitrate to nitrite increasing NO concentrations (275). NO inhibits endothelin-1 and release of noradrenaline

(283) so will increase cyclic guanosine monophosphate (cGMP) in smooth muscle (284) and improve vascular function (285). This improved vascular function is consistent with previous studies suggesting that NO normalised the MCP1 mRNA expression which inhibited the inflammatory cytokines such as TNF-α, induced signal transducer and activator of transcription (STAT3) phosphorylation, inducible nitric oxide synthase and cell adhesion molecules that attract inflammatory cells to the endothelial surface and facilitate their entrance into the vessel wall by inhibiting the activation of nuclear factor-κB (284, 286). Extending these effects could be the reason for reduced inflammatory cell infiltration in left ventricle and liver with both treatments.

In addition, this diet caused widespread metabolic changes including increased abdominal fat deposition, plasma lipids, plasma liver enzymes and liver weight, and impaired glucose tolerance when compared to rats fed a low-fat, corn starch-rich diet (146). Dysfunction of the left ventricle correlates with metabolic changes, oxidative stress and increase in inflammatory cell infiltration, and an increase in left ventricular fibrosis and stiffness (287). Intervention with either sodium nitrate or beetroot juice containing the same dose of sodium nitrate attenuated or reversed the changes in heart and liver structure and function. Further, these interventions reversed most of the metabolic changes but did not reduce total body weight.

Both nitrate- and beetroot-treated rats decreased fat vacuoles, hepatic inflammation and liver wet weight directly correlating to the reduction of plasma activity of liver enzymes and free fatty acids. NO activated AMPKα as an energy sensing enzyme which is activated in response to cellular stress by increasing intracellular cGMP (288). Increased AMPKα activation increases fatty acid oxidation and improves glucose homeostasis by increasing the peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α). In C2C12 murine monocytes, beetroot showed increased activation of PGC-1α with increased glucose uptake (289). In our study, both interventions improved the glucose homeostasis similar to previous study with nitrates in endothelial nitric oxide synthase-deficient mice model (194). However, neither treatment changed AMPKα mRNA expression in liver and skeletal muscle. In heart, the increased AMPKα expression in H diet rats was normalised in beetroot and nitrate-treated rats, suggesting that reduced blood pressure and improved cardiovascular function with either treatment reduced the cellular stress and workload, therefore normalising AMPKα expression. The results also suggest that increased AMPKα expression and

activation relates to tissue-specific conditions and could be an alternative mechanism for improving glucose homeostasis in disease states.

The activation of PPARα during energy deprivation regulated lipid metabolism in the liver and increased NO production (290). Few studies have explained how the improved relationship between PPARα and NO production benefits cardiovascular health (290). Activation of PPARα also increased mitochondrial fatty acid β-oxidation (291). Treatment with beetroot or nitrate did not change PPARα expression in liver or skeletal muscle but increased expression in the heart. Similarly, low dose treatment with nitrates showed no PPARα activation and expression in muscle and liver (292). However, plasma triglycerides and NEFA decreased with reduced fat vacuoles in liver. These results suggest that PPARα expression and activation is tissue-specific in beetroot and nitrate treatment. Additionally, endothelial nitric oxide synthase-deficient mice treated with sodium nitrate decreased body weight and visceral adiposity (194), where in our study, the same dose or nitrate had no effects on body weight and adiposity.

### 5.6. Conclusions

Our integrative study suggested that in the high-carbohydrate, high-fat diet fed rats, mimicking human metabolic syndrome conditions, beetroot and inorganic nitrate treatment reduced blood pressure, cardiovascular function and structure by normalising CTGF, MCP1 and MMP2 mRNA expression in heart along with decreased plasma total cholesterol, triglycerides and no-esterified fatty acids. Both interventions improved hepatic structure, glucose metabolism and decreased plasma insulin, and liver enzymes independent to AMPKα and PPARα mRNA expression. In addition, all these changes were seen with no change in body weight and total fat mass.

### 5.7. Study specific acknowledgements

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# CHAPTER 6

# 6. Effect of chokeberry and purple maize in reversing the signs of diet-induced metabolic syndrome in rats

## 6.1. Summary

Increased consumption of fruits and vegetables reduces signs of metabolic syndrome, leading to their definition as functional foods. This study measured cardiovascular, liver and metabolic parameters following chronic administration of either chokeberry juice (CB) or purple maize (PM) as sources of polyphenols, especially anthocyanins such as cyanidin 3-glucoside, in rats with diet-induced metabolic syndrome. Male Wistar rats (8-9 week old) were fed with corn starch diet (C) or high-carbohydrate, high-fat diet (H) diet and divided into six groups (n=12/group) for 16 week feeding with C, C with CB or PM for last 8 weeks (CCB or CPM), H, H with CB or PM for last 8 weeks (HCB or HPM). CB and PM-fed rats received approximately 8 mg cyanidin 3-glucoside/kg/day. H rats developed many of the signs of human metabolic syndrome such as abdominal obesity, hypertension, impaired glucose tolerance, changes in cardiovascular and hepatic structure and function and increased pro-inflammatory markers compared to rats fed C diet. Both CB and PM treatments were effective in reversing the cardiovascular, liver and metabolic signs in this rat model of human metabolic syndrome, suggesting that cyanidin 3-glucoside attenuated or reversed the signs of metabolic syndrome by preventing inflammation-induced damage to organs.

## 6.2. Introduction

Fruits and vegetables rich in antioxidant phytochemicals such as flavonoids and polyphenols are effective in attenuating the signs of metabolic syndrome (195, 293). An increased intake of fruits and vegetables intake has been associated with a decrease in cardiovascular diseases (294), and better control of type 2 diabetes (295) and non-alcoholic fatty liver disease (296). Anthocyanins are bioactive flavonoids that give red to purple colours to fruits and vegetables. Regular consumption of anthocyanins has been credited with reduced risk of chronic diseases such as obesity, non-alcoholic fatty liver, diabetes and cardiovascular diseases (196, 297, 298). It is estimated that ~1000 mg of polyphenols, up to 215 mg of anthocyanins are consumed daily by an average adult in the USA. (298, 299).

Black chokeberry (*Aronia melanocarpa*) is described as an attractive garden plant, native to eastern North America but now grown widely in northern Europe, primarily Poland, where the sour fruit is eaten raw or processed into a wide range of foods. Black chokeberries are rich in cyanidin anthocyanins, chlorogenic acids and proanthocyanidins, and also contain

quercetin flavonols (300). While these components indicate that black chokeberries may be an effective functional food, more rigorous studies are needed to support popular indications for heart disease, hypertension, hyperlipidaemia and urinary tract infections, as well as actions against bacteria and viruses, and to strengthen memory and digestion (301, 302). In metabolic syndrome subjects, chokeberry extract decreased blood pressure and plasma lipid concentrations with no change in body weight (211). In rats fed with fructose-rich diet, chokeberry attenuated body weight gain and insulin resistance (303).

Purple corn has been cultivated in the Andean region, especially Peru and Bolivia, for centuries where it is used as a food and a colourant in a drink believed to improve health (304, 305). Treatment with purple maize (*Zea mays*) decreased abdominal adiposity (306), improved glucose metabolism (307) and decreased blood pressure (308) in high-fat diet fed rats. However, these is no clear evidence that intervention with these anthocyanin-containing traditional functional foods will improve the range of organ dysfunction of the metabolic syndrome, despite improvements in individual signs.

We have previously shown that rats fed with a high-carbohydrate, high fat-diet developed the signs of metabolic syndrome such as abdominal adiposity, non-alcoholic fatty liver, elevated plasma lipid concentrations, impaired glucose and insulin tolerance, hypertension and cardiovascular remodelling mimicking the human condition (146). In this model, we have shown that anthocyanin-rich purple carrots or Queen Garnet plums improved glucose tolerance, reduced abdominal adiposity, reduced plasma lipid concentrations, normalised inflammation and fibrosis in the heart and liver, and improved cardiovascular structure and function (200, 309).

This study has compared the cardiovascular, liver and metabolic responses of two foods, chokeberry and purple maize, at the same daily dose of anthocyanins in a high-carbohydrate, high-fat diet-fed rat model of human metabolic syndrome (146). These measurements included systolic blood pressure, echocardiography, vascular reactivity, collagen deposition and stiffness of heart, plasma biochemistry and histology for structural changes on heart and liver. We suggest that an adequate intake of cyanidin-type anthocyanins can normalise the metabolic, cardiovascular and liver changes induced by a high-carbohydrate, high-fat diet, possibly by decreasing infiltration of inflammatory cells in the organs.

## 6.3. Materials & methods

### *6.3.1. Analysis of chokeberry juice and purple maize flour*

Chokeberry juice (CB) was supplied by Fasbay Pty Ltd, Sydney, Australia and purple maize flour (PM) was supplied by Spectrum Ingredients Pte Ltd, Singapore. The anthocyanin contents were determined by HPLC based on the method outlined in the British Pharmacopoeia 2014 (Eur. Pharm 2394) using an Agilent 100 series HPLC system. Briefly, samples were prepared by extraction by 2% v/v HCl in methanol, using sonication for 15 minutes in volumetric flasks, then made up to volume and diluted as required to be within the standard calibration. Analysis was performed using a gradient of mobile phases A (water and formic acid, 91.5:8.5) and B (acetonitrile, methanol, water and formic acid, 22.5:22.5:41.5:8.5) over 56 minutes. The gradient ran from 7 to 25% B in 35 minutes, to 65% at 45 minutes followed by 100% B to 50 minutes and return to 7%. The column used was a Phenomenex 250mm C18 5µm column with a flow rate of 1 mL per minute and temperature 30°C. Detection and quantification were performed using a diode array detector (DAD) at 535nm with cyanidin chloride (PhytoLab, CAS No. 528-58-5, B# 80022 5368) as the calibrating standard. Total anthocyanins were calculated as cyanidin chloride and cyanidin 3-glucoside by mass correction.

### *6.3.2. Rats and diets*

The experimental groups consisted of 72 male Wistar rats (8-9 weeks old; weighing 335 ± 3 g) were randomly divided into 6 groups (n = 12 each) and fed with maize starch (C), maize starch + chokeberry juice (CCB), maize starch + purple maize flour (CPM), high-carbohydrate, high-fat (H), high-carbohydrate, high-fat + chokeberry juice (HCB) and high-carbohydrate, high-fat + purple maize flour (HPM). C and H rats received their respective diets for 16 weeks and CCB, CPM, HCB and HPM rats received C and H diets respectively for first 8 weeks while both diets were supplemented with chokeberry juice 50 ml/kg or purple maize flour 50 g/kg by replacing equivalent amounts of water for a further 8 weeks.

Experimental procedures such as body composition, oral glucose tolerance, echocardiography, blood pressure, isolated heart preparation, organ bath, plasma analysis and histology were conducted as described in Chapter 3.1.

## 6.4. Results

### *6.4.1. Diet and body composition*

CB and PM contained similar concentrations of total anthocyanins (Table 6.11) with cyanidin 3-glucoside as the major anthocyanin. The average daily intake of anthocyanins was higher in CCB and CPM rats compared to HCB and HPM rats, as the food intake was higher in CCB and CPM rats (Table 6.2).

Compared to C rats, H rats consumed less food but a similar amount of water (Table 6.2). Despite the lower food intake, the mean energy intake, feed efficiency, and therefore the increases in body weight, were higher in H rats than in C rats (Table 6.2). Consequently, chronic H diet feeding for 16 weeks increased measures of abdominal circumference, body mass index, total body fat mass and the individual abdominal fat pads, and increased the visceral adiposity index (Table 6.2). No change in total body lean mass was measured (Table 6.2). The bone mineral content was higher in H rats compared to C rats (Table 6.2).

**Table 6.1 Chokeberry juice and purple maize flour analysis**

| Variables | Chokeberry juice/ 100 ml | Purple-maize flour/ 100 g |
|---|---|---|
| Total anthocyanins (mg) | 240 | 220 |
| Energy (KJ) | 279 | 1,592 |
| Protein (g) | 16.1 | 7.8 |
| Total fat (g) | 0.5 | 4.2 |
| Total carbohydrates (g) | 0.2 | 76.7 |

Values are represented as mean of duplicate assays.

Treatment with either CB or PM for 8 weeks, starting at 8 weeks of the feeding period, did not change food or water intake (Table 6.2). Compared to controls, CB treatment groups consumed same energy and PM treatment groups had higher energy intake. However, lower feed conversion efficiency and body weight gain were observed in HCB and HPM rats (Table 6.2). Both treatments decreased total body fat mass, except CPM rats had higher total body fat mass compared to C rats (Table 6.2). Abdominal fat (retroperitoneal, epididymal and omental fat pads), body mass index and visceral adiposity index decreased in both HCB and HPM rats (Table 6.2). Bone mineral content and total lean mass were unchanged in both

HCB and HPM rats, except HCB rats had decreased bone mineral content compared to H rats (Table 6.2).

### *6.4.2. Plasma biochemistry and glucose handling*

Plasma concentrations of total cholesterol, triglycerides and non-esterified fatty acids (NEFA) were increased in H rats compared to C rats (Table 6.2). HCB and HPM rats showed decreased plasma lipid concentrations, compared to H rats. However, HPM rats had higher concentrations of triglycerides and NEFA than HCB rats (Table 6.2). Plasma leptin concentrations were doubled in H rats compared to C rats. HCB and HPM showed normalised leptin concentrations, consistent with the changes in total fat mass and abdominal fat pads (Table 6.2).

H rats had increased fasting blood glucose concentrations compared to C rats; HCB and HPM rats showed similar concentrations to C rats (Figure 6.1). The plasma glucose response to oral glucose loading was greater in H rats than C rats (Figure 6.1). At 120 minutes, HCB and HPM rats along with C rats had lower plasma glucose concentrations compared to H rats (Figure 6.1). Plasma insulin concentrations almost doubled in H rats compared to C, HCB and HPM rats (Table 6.2). This change is consistent with glucose tolerance area under the curve (Table 6.2); similarly, HCB and HPM rats had improved glucose metabolism compared to H rats (Table 6.2).

**Table 6.2 Dietary intakes, body composition and anthropometrics, organ wet weights, changes in glucose tolerance test, plasma insulin and plasma biochemistry in C, CCB, CPM, H, HCB and HPM diet-fed rats (n=10 rats/group)**

| Variable | C | CCB | CPM | H | HCB | HPM | P values | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Diet | Treatment | Interaction |
| Food intake (g/d) | 32.9±1.1[a] | 33.2±0.6[a] | 34.1±0.4[a] | 27.1±0.8[b] | 26.4±0.8[b] | 28.5±0.7[b] | <0.0001 | 0.11 | 0.7 |
| Water intake (ml/d) | 27.4±2.1 | 24.3±1.5 | 27.2±1.8 | 26.6±1.4 | 26.5±1.6 | 29.7±1.5 | 0.34 | 0.2 | 0.55 |
| Chokeberry juice intake (ml/d) | 0.0±0.0 | 1.7±0.0[a] | 0.0±0.0 | 0.0±0.0 | 1.4±0.0[b] | 0.0±0.0 | <0.0001 | <0.0001 | <0.0001 |
| Purple maize powder intake (g/d) | 0.0±0.0 | 0.0±0.0 | 1.8±0.0[a] | 0.0±0.0 | 0.0±0.0 | 1.5±0.0[b] | <0.0001 | <0.0001 | <0.0001 |
| Anthocyanins intake (mg/d) | 0.0±0.0 | 4.2±0.0[a] | 4.0±0.0[a] | 0.0±0.0 | 3.8±0.0[b] | 3.4±0.0[b] | <0.0001 | <0.0001 | <0.0001 |
| Energy intake (kJ/d) | 369±12[d] | 378±7[d] | 409±5[c] | 580±17[b] | 570±19[b] | 640±17[a] | <0.0001 | 0.0005 | 0.39 |
| Feed conversion efficiency (%) | 2.4±0.3[b] | 2.1±0.3[b] | 2.9±0.3[b] | 7.1±0.9[a] | 4.4±1.3[b] | 5.5±0.9[ab] | <0.0001 | 0.16 | 0.25 |
| Body weight gained (8-16 weeks) (%) | 8.2±1.4[b] | 7.7±1.4[b] | 8.3±1.3[b] | 21.6±2.6[a] | 9.9±2.8[b] | 10.8±1.6[b] | 0.0004 | 0.0051 | 0.0075 |
| Visceral adiposity index (%) | 4.9±0.4[b] | 4.4±0.3[b] | 4.8±0.2[b] | 8.9±0.9[a] | 6.1±0.5[b] | 6.0±0.3[b] | <0.0001 | 0.0021 | 0.0137 |
| Abdominal circumference (cm) | 19.2±0.1[c] | 18.2±0.2[d] | 19.1±0.1[cd] | 22.3±0.3[a] | 19.7±0.4[c] | 20.7±0.2[b] | <0.0001 | <0.0001 | 0.0022 |
| Body mass index (kg/m$^2$) | 5.0±0.2[d] | 4.8±0.1[d] | 4.7±0.1[d] | 7.3±0.2[a] | 5.7±0.1[c] | 6.4±0.2[b] | <0.0001 | <0.0001 | 0.0002 |
| Bone mineral content (g) | 11.3±0.3[c] | 11.4±0.3[c] | 11.5±0.3[c] | 15.8±0.4[a] | 13.5±0.5[b] | 14.8±0.4[a] | <0.0001 | 0.0166 | 0.009 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Total body lean mass (g) | 309±12 | 303±12 | 307±11 | 311±14 | 305±11 | 304±8 | 0.98 | 0.87 | 0.98 |
| Total body fat mass (g) | 74.3±7.6$^{d}$ | 94.4±8.4$^{d}$ | 113.7±8.5$^{cd}$ | 224.6±12.6$^{a}$ | 144.4±17.2$^{c}$ | 177.3±10.4$^{b}$ | <0.0001 | 0.0203 | <0.0001 |
| **Tissue wet weight (mg/mm)** | | | | | | | | | |
| Retroperitoneal adipose tissue | 179.7±15.3$^{c}$ | 141.9±13.7$^{c}$ | 173.0±12.6$^{c}$ | 521.7±45.6$^{a}$ | 279.2±38.2$^{b}$ | 311.8±16.7$^{b}$ | <0.0001 | <0.0001 | 0.0003 |
| Epididymal adipose tissue | 93.4±7.9$^{c}$ | 83.3±6.9$^{c}$ | 95.4±5.2$^{c}$ | 278.5±22.5$^{a}$ | 155.4±15.2$^{b}$ | 156.8±11.7$^{b}$ | <0.0001 | <0.0001 | <0.0001 |
| Omental adipose tissue | 106.6±8.1$^{d}$ | 91.8±8.0$^{d}$ | 101.1±5.5$^{d}$ | 261.1±21.3$^{a}$ | 141.0±12.3$^{cd}$ | 193.1±9.9$^{b}$ | <0.0001 | <0.0001 | 0.0002 |
| Liver | 214.2±6.7$^{c}$ | 188.8±6.2$^{c}$ | 204.1±5.5$^{c}$ | 319.6±8.9$^{a}$ | 250.7±11.1$^{b}$ | 268.6±7.0$^{b}$ | <0.0001 | <0.0001 | 0.0111 |
| **Glucose metabolism and plasma biochemistry** | | | | | | | | | |
| OGTT-AUC (mmol/L min) | 591±12$^{d}$ | 645±9$^{c}$ | 613±6$^{d}$ | 786±18$^{a}$ | 658±7$^{c}$ | 694±6$^{b}$ | <0.0001 | 0.001 | <0.0001 |
| Plasma insulin (μmol/L) | 1.4±0.3$^{b}$ | 1.1±0.2$^{b}$ | 1.7±0.3$^{b}$ | 4.1±0.5$^{a}$ | 2.3±0.4$^{b}$ | 2.6±0.6$^{b}$ | <0.0001 | 0.042 | 0.07 |
| Plamsa leptin (μmol/L) | 5.3±0.7$^{b}$ | 4.9±0.6$^{b}$ | 6.1±0.5$^{b}$ | 11.1±0.9$^{a}$ | 7.0±1.5$^{b}$ | 7.9±1.0$^{b}$ | <0.0001 | 0.06 | 0.06 |
| ALP (U/L) | 181±12$^{c}$ | 214±16$^{c}$ | 166±12$^{c}$ | 312±18$^{a}$ | 252±20$^{bc}$ | 265±18$^{bc}$ | <0.0001 | 0.17 | 0.0224 |
| ALT (U/L) | 29.6±2.1$^{b}$ | 28.1±2.2$^{b}$ | 29.7±1.5$^{b}$ | 43.2±2.8$^{a}$ | 34.6±3.9$^{ab}$ | 38.0±3.1$^{ab}$ | <0.0001 | 0.19 | 0.4 |
| AST (U/L) | 60.4±1.9$^{b}$ | 60.9±2.7$^{b}$ | 64.0±1.6$^{b}$ | 83.5±3.1$^{a}$ | 63.1±7.9$^{b}$ | 64.6±2.9$^{b}$ | 0.0099 | 0.0383 | 0.0099 |
| Total cholesterol (mmol/L) | 1.5±0.2$^{b}$ | 1.5±0.1$^{b}$ | 1.6±0.1$^{b}$ | 2.2±0.0$^{a}$ | 1.6±0.1$^{b}$ | 1.6±0.0$^{b}$ | 0.0038 | 0.0166 | 0.0039 |
| Triglycerides (mmol/L) | 0.5±0.0$^{c}$ | 0.4±0.0$^{c}$ | 0.5±0.0$^{c}$ | 1.6±0.2$^{a}$ | 0.7±0.1$^{c}$ | 1.0±0.1$^{b}$ | <0.0001 | <0.0001 | 0.0005 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| NEFA (mmol/L) | 1.3 ±0.2[c] | 1.2 ±0.1[c] | 1.4 ±0.1[c] | 3.4 ±0.2[a] | 1.8 ±0.4[c] | 2.3 ±0.3[b] | <0.0001 | 0.0036 | 0.0073 |

Each value is a mean ± SEM. Means within a row with unlike superscripts differ, P<0.05.

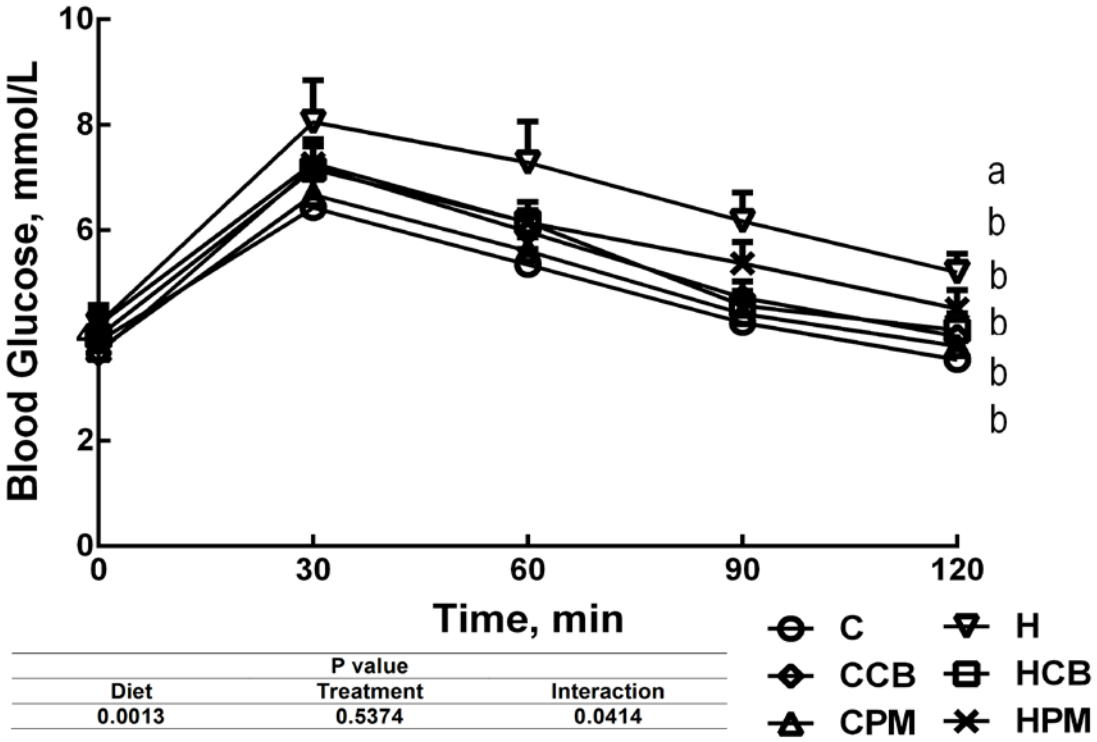
* In all groups body-weight gained calculated as percentage of body weight increase from 8 weeks to 16 weeks. OGTT-AUC, oral glucose tolerance test-area under the curve; ALP, alkaline phosphatase; ALT, aspartate transaminase; AST, aspartate transaminase; NEFA, non-esterified fatty acids.

**Table 6.3 Changes in cardiovascular structure and function in C, CCB, CPM, H, HCB and HPM diet-fed rats (n=10-8 rats/group)**

| | | | | | | | P values | | |
|---|---|---|---|---|---|---|---|---|---|
| **Variable** | **C** | **CCB** | **CPM** | **H** | **HCB** | **HPM** | **Diet** | **Treatment** | **Interaction** |
| Heart rate (bpm) | 277±18[b] | 246±9[b] | 256±15[b] | 335±16[a] | 243±9[b] | 265±11[b] | 0.06 | 0.0001 | 0.06 |
| IVSd (mm) | 1.9±0.1[ab] | 1.8±0.0[b] | 1.9±0.0[ab] | 2.1±0.1[a] | 1.9±0.1[ab] | 1.9±0.0[ab] | 0.09 | 0.11 | 0.38 |
| LVIDd (mm) | 6.4±0.2[b] | 6.7±0.3[b] | 7.0±0.1[b] | 7.9±0.3[a] | 7.1±0.2[b] | 7.2±0.2[b] | 0.0005 | 0.51 | 0.0139 |
| LVPWd (mm) | 1.8±0.1[b] | 1.7±0.0[b] | 1.8±0.0[b] | 2.1±0.0[a] | 1.9±0.1[b] | 1.9±0.0[b] | 0.0001 | 0.0393 | 0.23 |
| IVSs (mm) | 3.2±0.2[b] | 3.0±0.1[b] | 2.9±0.0[b] | 3.8±0.1[a] | 3.2±0.1[b] | 3.3±0.1[b] | <0.0001 | 0.0022 | 0.42 |
| LVIDs (mm) | 3.7±0.2 | 4.0±0.2 | 3.7±0.2 | 4.5±0.2 | 3.7±0.3 | 4.3±0.3 | 0.07 | 0.58 | 0.06 |
| LVPWs (mm) | 2.9±0.1[b] | 2.6±0.1[b] | 2.7±0.1[b] | 3.4±0.1[a] | 3.2±0.1[ab] | 3.0±0.1[ab] | <0.0001 | 0.0099 | 0.32 |
| Fractional shortening (%) | 50.9±2.1[a] | 53.7±0.8[a] | 53.4±0.9[a] | 47.5±2.1[ab] | 58.0±1.2[a] | 55.7±1.4[a] | 0.39 | 0.0002 | 0.0392 |
| Ejection time (ms) | 79.6±2.3 | 93.3±3.4[ab] | 86.0±2.7 | 92.8±2.9[ab] | 86.4±3.9 | 95.0±3.0[ab] | 0.0486 | 0.33 | 0.0053 |
| Ejection fraction (%) | 87.3±1.4 | 83.3±1.3 | 84.6±1.6 | 89.0±2.2 | 84.1±4.0 | 83.6±2.9 | 0.8 | 0.14 | 0.85 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Diastolic volume (µL) | 353±34[b] | 365±33[b] | 364±22[b] | 515±39[a] | 386±28[b] | 395±37[b] | 0.01 | 0.15 | 0.07 |
| Systolic volume (µL) | 45±10[b] | 57±8[a] | 56±8[a] | 90±10[a] | 65±23[a] | 63±12[a] | 0.0203 | 0.72 | 0.11 |
| Stroke volume (µL) | 268±18[b] | 298±20[b] | 307±18[b] | 425±29[a] | 306±26[b] | 322±19[b] | 0.0018 | 0.13 | 0.002 |
| Cardiac output (mL/min) | 92.3±11.2[b] | 70.4±13.6[b] | 78.6±6.6[b] | 144.8±21.7[a] | 93.0±7.7[b] | 100.3±10.7[b] | 0.0038 | 0.0165 | 0.41 |
| LV developed pressure (mmHg) | 69.6±3.5[a] | 71.4±4.1[a] | 64.8±5.6[a] | 43.7±3.6[b] | 63.7±5.7[a] | 60.2±3.8[a] | 0.001 | 0.06 | 0.0444 |
| (+)dP/dt (mmHg/S) | 1147±61[a] | 1285±53[a] | 1195±65[a] | 784±66[c] | 1089±63[a] | 1002±76[ab] | <0.0001 | 0.0044 | 0.33 |
| (-)dP/dt (mmHg/S) | -782±51[a] | -804±49[a] | -795±43[a] | -489±57[b] | -700±44[a] | -711±52[a] | 0.0002 | 0.0316 | 0.08 |
| Diastolic stiffness (k) | 22.9±0.8[b] | 22.3±0.4[b] | 23.1±0.6[b] | 28.6±0.6[a] | 23.9±0.7[b] | 24.2±0.5[b] | <0.0001 | 0.0002 | 0.0006 |
| Estimated LV mass, Litwin (g) | 0.93±0.06[b] | 0.79±0.08[b] | 0.88±0.02[b] | 1.14±0.05[a] | 1.01±0.06[ab] | 1.10±0.04[ab] | <0.0001 | 0.05 | 0.99 |
| LV+septum wet weight (mg/mm tibial length) | 16.1±0.5[c] | 15.3±0.5[c] | 15.0±0.5[c] | 19.5±0.8[a] | 16.9±0.8[c] | 18.5±0.5[b] | <0.0001 | 0.0268 | 0.23 |
| Right ventricle wet weight (mg/mm tibial length) | 3.8±0.2 | 3.8±0.2 | 4.0±0.2 | 4.4±0.2 | 4.3±0.3 | 5.0±0.4[a] | 0.0018 | 0.18 | 0.6 |
| Relative wall thickness | 0.50±0.03 | 0.57±0.09 | 0.53±0.02 | 0.56±0.03 | 0.51±0.01 | 0.48±0.01 | 0.63 | 0.69 | 0.29 |
| Systolic blood pressure (mmHg) | 130±2[b] | 130±3[b] | 132±2[b] | 152±2[a] | 120±1[b] | 134±3[b] | <0.0001 | <0.0001 | <0.0001 |
| Systolic wall stress (mmHg) | 83.0±4.0[b] | 79.1±4.3[b] | 75.3±11.8[b] | 119.6±7.6[a] | 76.8±7.5[b] | 89.5±9.7[b] | 0.0171 | 0.0129 | 0.06 |

Each value is a mean ± SEM. Means within a row with unlike superscripts differ, P<0.05.




| P value | | |
|---|---|---|
| Diet | Treatment | Interaction |
| 0.0013 | 0.5374 | 0.0414 |

Figure 6.1 Effect of CB and PM on oral glucose tolerance in C, CCB, CPM, H, HCB and HPM rats. Data are shown as mean ± SEM. End-point means without a common alphabet in each data set significantly differ, $P<0.05$ and n=10/group.

### *6.4.3. Cardiovascular structure and function*

Compared to C rats, H rats showed eccentric hypertrophy, characteristic of increased preload, measured as increased left ventricular internal diameter in diastole (LVIDd) without any changes in relative wall thickness or end systolic dimensions (Table 6.3). H rats showed impaired systolic function with decreased fractional shortening and increased wall stress compared to C rats (Table 6.3). However, ejection time and ejection fraction were not affected (Table 6.3). Diastolic and stroke volumes and consequently the cardiac output were increased in H rats compared to C rats. These effects were seen with increased heart rate in H rats compared to C rats (Table 6.3). These changes in H rats were accompanied by increased LV wet weight and elevated systolic blood pressure (Table 6.3).

Both CB and PM treatment improved LV function by decreasing LVIDd and normalising developed pressure (Table 6.3). Ejection time, ejection fraction, fractional shortening and LVIDs were unaffected in both treatment groups (Table 6.3). Systolic wall stress, cardiac output, diastolic and stroke volumes and heart rate were normalised with both CB and PM treatment. However, systolic volume was elevated in both HCB and HPM rats

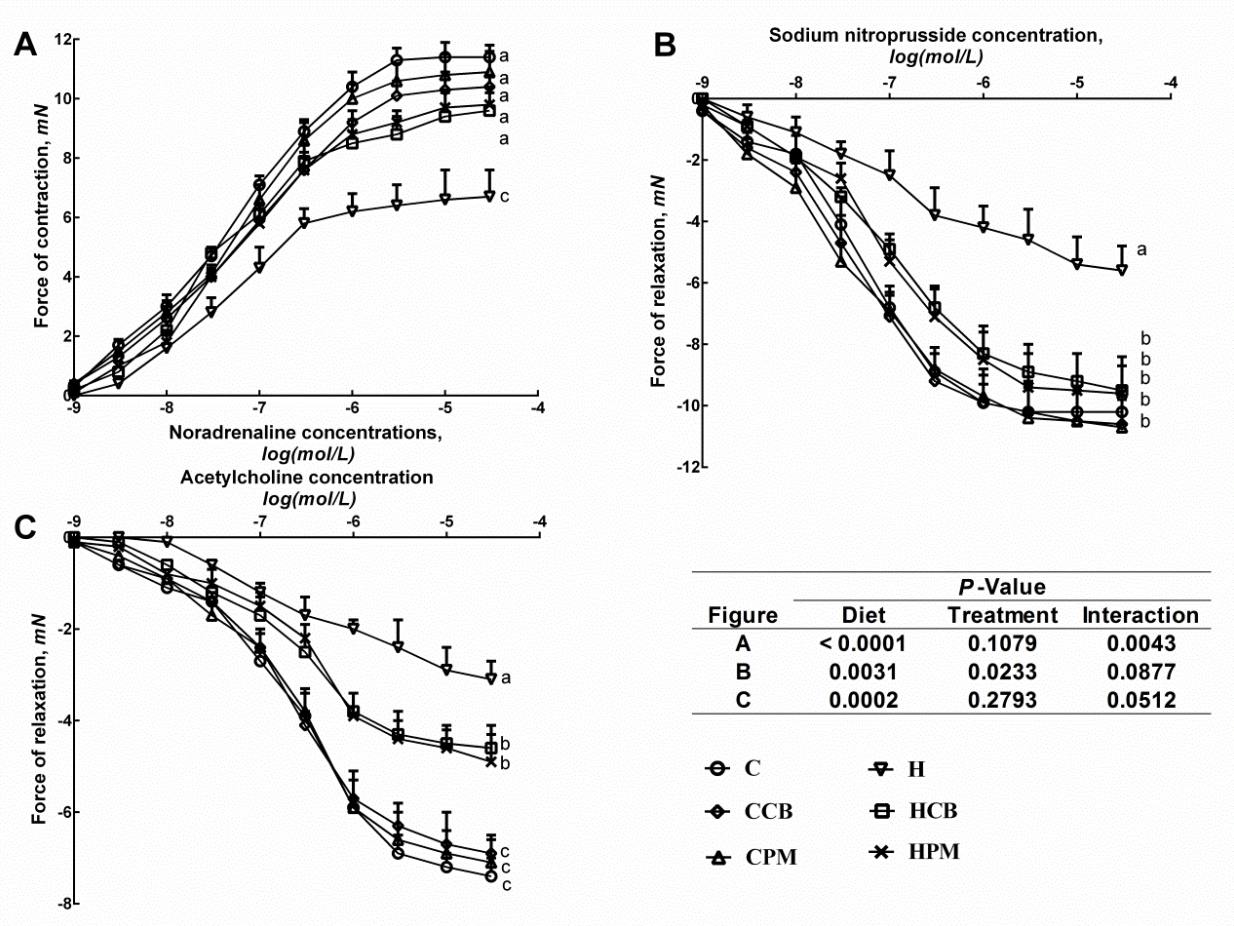
with no change in relative wall thickness (Table 6.3). In the isolated Langendorff heart, LV stiffness was increased while LV dP/dt was decreased in H rats; these changes were normalised in HCB and HPM. These effects were accompanied by decreased LV wet weight and systolic blood pressure in both HCB and HPM rats (Table 6.3).

Histological evaluation of the left ventricle after 16 weeks showed greater infiltration of inflammatory cells into the LV with H diet feeding (Figure 6.3D), as well as increased interstitial collagen deposition (Figure 6.3J) compared to C rats (Figure 6.3A and G). HCB and HPM rats showed normalised inflammatory cell numbers (Figure 6.3E and F) and markedly reduced ventricular collagen deposition (Figure 6.3K and L). The reduction in LV fibrosis and inflammation was consistent with the reduced diastolic stiffness in HCB and HPM rats (Table 6.3), while CCB (Figure 6.3B and H) and CPM (Figure 6.3C and I) rats showed minimal changes. No other changes were observed and tissue morphology appeared normal.

H diet feeding diminished α1-adrenoceptor-mediated vascular contraction to noradrenaline (Figure 6.2A), endothelium-independent relaxation to sodium nitroprusside (Figure 6.2B) and endothelium-dependent relaxation to acetylcholine (Figure 6.2C) in isolated thoracic aortic rings compared to C rats. HCB and HPM rats showed increased responses to noradrenaline (Figure 6.2A), sodium nitroprusside (Figure 6.2B) and acetylcholine (Figure 6.2C) in isolated thoracic aortic rings of HCB and HPM group (Table 6.3).

### *6.4.4. Hepatic structure and function*

Plasma alkaline phosphatase (ALP), alanine transaminase (ALT) and aspartate transaminase (AST) activities were increased in H rats compared to C rats, indicating liver damage (Table 6.2). HCB and HPM showed lowered but not normalised ALP, ALT and AST activities (Table 6.2). H rats showed increased inflammatory cell infiltration and lipid deposition as fat vacuoles in the liver (Figure 6.4D) compared to C rats (Figure 6.4A). In HCB and HPM rats, macrovesicular steatosis and portal inflammation were decreased compared to H rats (Figure 6.4E and F). Livers from CCB and CPM rats showed normal tissue architecture (Figure 6.4B and C).




| Figure | P-Value | | |
|---|---|---|---|
| | Diet | Treatment | Interaction |
| A | < 0.0001 | 0.1079 | 0.0043 |
| B | 0.0031 | 0.0233 | 0.0877 |
| C | 0.0002 | 0.2793 | 0.0512 |

Figure 6.2 Cumulative concentration-response curves for noradrenaline (A), sodium nitroprusside (B) and acetylcholine (C) in thoracic aortic rings from C, CCB, CPB, H, HCB and HPM rats. Data are shown as mean ± SEM. Significantly different end-point means and indicated by different letters, P<0.05 and n=10/group.

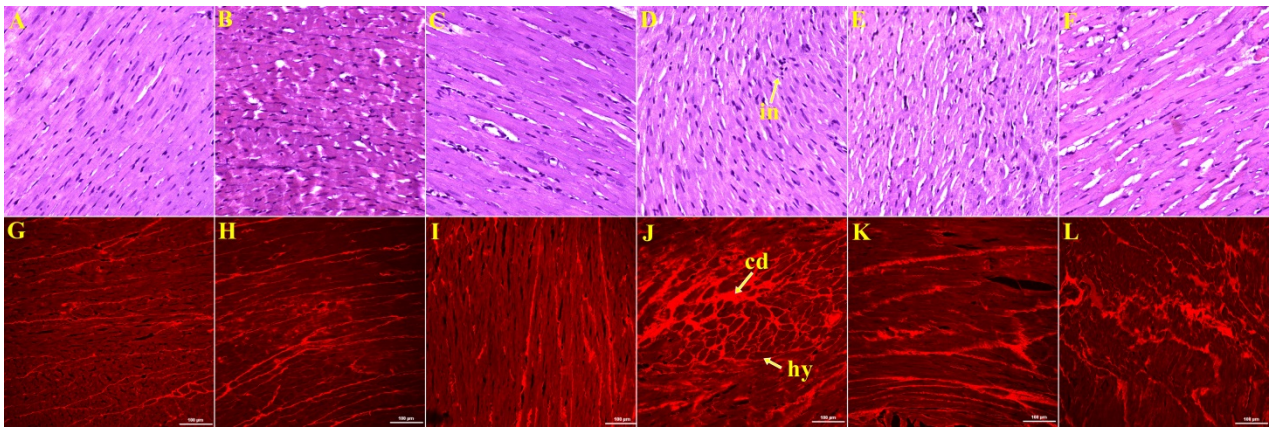



Figure 6.3 Hematoxylin and eosin staining of the lef- ventricle (original magnification ×20) showing inflammatory cells (marked as “in”) as dark spots outside the myocytes in C (A), CCB (B), CPM (C), H (D), HCB (E) and HPM (F) rats. Picrosirius red staining of left-ventricular interstitial collagen deposition (original magnification ×20) in rats fed the C (G), CCB (H), CPM (I), H (J), HCB (K) and HPM (L)diet. Collagen deposition is marked as “cd” and hypertrophied cardiomyocytes are marked as “hy”.

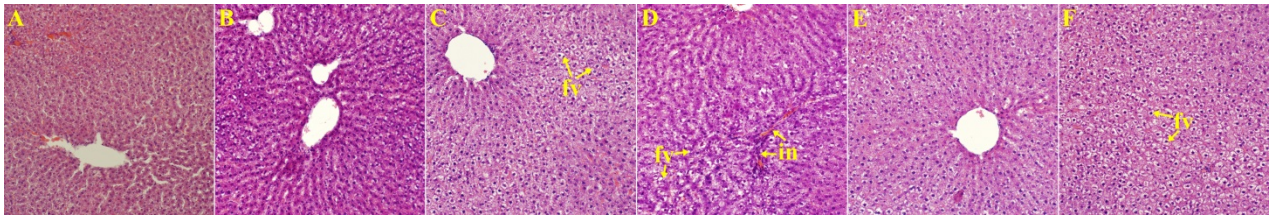



Figure 6.4 Hematoxylin and eosin staining of hepatocytes (original magnification ×20) showing inflammatory cells (marked as "in") and hepatocytes with fat vacuoles (marked as "fv") in C (A), CCB (B), CPM (C), H (D), HCB (E) and HPM (F) rats.

**6.5. Discussion**

Consumption of high-carbohydrate, high-fat diet in rats mimics the signs of the metabolic syndrome in humans (146). Rats fed with this diet developed abdominal obesity, hypertension with endothelial dysfunction, cardiac fibrosis together with increased left-ventricular stiffness, dyslipidemia, inflammation in heart and liver, increased plasma liver enzyme concentrations and impaired glucose tolerance. Excess fat deposition in the abdomen increases chronic low-grade inflammation, oxidative stress, dyslipidemia, non-alcoholic fatty liver disease, cardiovascular diseases, type 2 diabetes and insulin resistance (120, 310-314). In this study, we showed that both chokeberry and purple-maize attenuated the metabolic, cardiovascular and liver changes in rats fed a high-carbohydrate, high-fat diets in rats. We suggest that these improvements derive from decreased inflammatory cell infiltration as a consequence of decreased pro-inflammatory cytokines released from reduced abdominal fat pads as cyanidin 3-glucoside, a common anthocyanin present in both the interventions, showed anti-inflammatory activity (315).

Abdominal adipose tissue is a dynamic organ producing adipokines that have pro-inflammatory or anti-inflammatory responses. Dysregulated production of these adipokines leads to obesity and also induces low-grade inflammation and insulin resistance (316). In our diet-induced obese rats, plasma leptin concentrations were increased; treatment with either chokeberry or purple maize reduced both plasma leptin concentrations and abdominal fat mass. Similar results were shown with chokeberry extract expression (303) and in our previous studies with cyanidin 3-glucoside and Queen Garnet plums (309). All these interventions contain cyanidin 3-glucoside as the major anthocyanin and this compound is the likely bioactive component. Since leptin is pro-inflammatory, reduced leptin concentrations should reduce inflammation throughout the body, as we have shown in the heart and liver.

An increased abdominal fat mass in obesity leads to abnormal regulation of adipogenesis (314, 317). These results were consistent with the effects observed using an

anthocyanin rich purple maize extract in C57BL/6J mice, showed decrease in abdominal fat pads and reduced body weight (318). Decrease in adipose tissue decreases the production of pro-inflammatory cytokines and adipokines (38), where decrease in leptin concentration were observed in CB and PM treated groups. Similarly in fructose-rich diet-fed rats, CB and PM treatment also improved glucose metabolism which is consistent with the known effects of anthocyanin foods. It is also known that increased adipose tissue associated with obesity are linked with abnormal regulation of PPARγ plays a major role in glucose metabolism and energy homeostasis, and regulator of adipogenesis (319, 320). As excessive fructose feeding decreased PPARγ mRNA expression in white adipose tissue and treatment with chokeberry extract increased PPARγ mRNA expression (303).

Further, KK-*Ay* mice treated with anthocyanins also showed similar changes along with decrease in mean diameter of the visceral adipose cells and subcutaneous adipose cells, suggesting that anthocyanin supplementation inhibits lipid accumulation (321). Long-term fructose feeding induces the excess hepatic lipid storage in both humans and rodents (322-324). It is well known that the liver plays a major role in lipid metabolism and our current data suggests that CB and PM treatment decreased triglycerides and non-esterified free fatty acids which attenuated the liver steatosis. Cyanidin 3-glucoside showed increased phosphorylated AMP-activated protein kinase (pAMPK) and decreased lipoprotein lipase activity in skeletal muscle and adipocytes (321). In addition, purple sweet potato treatment in diet-induced obese mice and HepG2 hepatocytes showed similar phosphorylation of AMPK (325). AMP-activated protein kinase (AMPK) regulates and monitor cellular energy balance (326) and pAMPK stimulates free fatty acid oxidation via activation of acetyl coenzyme –A carboxylase in skeletal muscle (327) and regulates lipolysis and lipogenesis by converting adipocytes into lipid oxidizing cells (328). Anthocyanins also down regulated lipid-metabolism proteins, sterol regulatory element-binding protein-1c and fatty acid synthase via AMPK inhibitor compound C (325, 329). Additionally, in C57BL/KsJ db/db mice treated with purple maize extract also increased pAMPK and decreased phosphoenolpyruvate carboxykinase and glucose 6-phosphatase gene expression in liver and glucose transporter 4 expressions in skeletal muscle (330). Therefore, decrease in adipose storage leading to decrease in body weight gain and improved hepatic function and glucose metabolism.

CB and PM treatment also improved cardiovascular structure and function by decreasing plasma levels of non-esterified free fatty acids (NEFA). Increase in plasma NEFA inhibits aortic endothelia nitric oxide synthase via oxidative mechanism and causes hypertension (331). In subjects with metabolic syndrome, supplementation with CB extract

and PM extract powder decreased blood pressure and plasma lipids (211, 308). Similarly, many epidemiological studies suggest that increase in the dietary intake of anthocyanin rich strawberries, blueberries and moderate levels of red wine is associated with a reduction in cardiovascular health disease (298). However there is no direct evidence that anthocyanins are helpful in decreasing the cardiovascular diseases. Increase in expression of eNOS enhances nitric oxide release improves endothelial function (332). In cultured bovine artery endothelial cells, cyanidin 3-glucoside increased the expression of endothelial nitric oxide synthase (eNOS) in (333). Similarly, chokeberry juice treatment showed improved endothelial function in porcine coronary arteries by redox-sensitive activation (334). CB and PM treated groups also showed consistent results in improved endothelial function and decreased blood pressure.

In our previous study, rats treated with cyanidin 3-glucoisde, anthocyanin-rich purple carrots and Queen Garnet plum juice showed similar responses to CB and PM, such as decreased blood pressure, left ventricular stiffness and collagen depots in the LV (200, 309). In this study, we have treated rats either with 3.2 ml/kg BW of CB juice or 3.1 mg/kg BW of PM powder, corresponding to ~43 ml/day CB or ~45 g/day PM to achieve a dose of ~110 mg of anthocyanins in a 70 kg human, based on body surface area comparison between rats and humans (167). However, the dose of quercetin glycosides in this study was low at around 1 mg/kg/day, much lower than the quercetin dose of around 50 mg/kg/day used to reverse signs of metabolic syndrome in the same model (148). This indicates that anthocyanins are the major bioactive compound in CB and PM.

### 6.6. Conclusions

In conclusion, the effects of CB and PM in a diet-induced rat model of human metabolic syndrome are consistent with the reported effects of anthocyanins as it is the only common polyphenol group present in both the treatments. These findings suggest that chokeberry or purple maize might be beneficial in attenuating obesity and metabolic syndrome disease conditions. However, further investigation on CB and PC along with anthocyanins are necessary to understand the mechanism of action. Similar responses in CB and PM treated rats suggest a clinical trial corresponding to the same dose from this study to determine if these positive effects can be translated to humans with signs of metabolic syndrome.

### 6.7. Study specific acknowledgements

We thank Ms Lilly Slewo (Fasbay Pty Ltd, Australia) and Mr Kenneth C Davis (Spectrum Ingredients Pte Ltd, Singapore), for providing chokeberry juice and purple maize powder respectively. We thank Mr Jason Brightwell, The Prince Charles Hospital, Brisbane, Australia, for the acquisition of echocardiographic images.

# CHAPTER 7

# 7. Cyanidin 3-glucoside improves diet-induced metabolic syndrome in rats

## 7.1. Summary

Increased consumption of dark-coloured fruits and vegetables may mitigate metabolic syndrome. This study has determined the changes in metabolic parameters, and in cardiovascular and liver structure and function, following chronic administration of either cyanidin 3-glucoside (CG) or Queen Garnet plum juice (QG) containing cyanidin glycosides to rats fed either a corn starch (C) or a high-carbohydrate, high-fat (H) diet. Eight to nine-week-old male Wistar rats were randomly divided into six groups for 16-week feeding with C, C diet with CG or QG, or H or H diet with CG or QG. C or H diets were supplemented with 115 mg/kg food of CG either as a powder or in 50 ml of QG from week 8 to 16. H diet-fed rats developed signs of metabolic syndrome including visceral adiposity, impaired glucose tolerance, hypertension, cardiovascular remodelling, increased collagen depots in left ventricle, non-alcoholic fatty liver disease, increased plasma liver enzymes and increased inflammatory cell infiltration in heart and liver. Both CG and QG reversed these cardiovascular, liver and metabolic signs. However, no intact anthocyanins or common methylated/conjugated metabolites could be detected in the plasma samples and plasma hippuric acid concentrations were unchanged. Our results suggest CG is the most likely mediator of the responses to QG but that the pharmacokinetics of oral CG in rats require further investigation.

## 7.2. Introduction

The prevalence of obesity is increasing, now reaching epidemic proportions (260, 261). Obesity is accepted as a chronic, low-grade inflammatory state with increased oxidative stress (335, 336). Controlling inflammation is one mechanism to either reverse or attenuate obesity and associated tissue and organ changes (335). Eating fruits and vegetables can prevent chronic disease including cardiovascular disease and possibly prevent body weight gain (293). Increased consumption of polyphenol-containing fruits and vegetables provides anti-inflammatory responses that could reduce the risk factors for metabolic syndrome, producing cardiac and hepatic protection (297, 337-339). The most common polyphenols are flavonoids, and many flavonoids have been studied for their role in reducing obesity, probably by antioxidant or anti-inflammatory mechanisms (195, 340). Flavonoids are widespread in nature, including the anthocyanins commonly found in dark-coloured fruits and vegetables including Red Delicious apples, chokeberries, black beans, black plums and

wild blueberries (341); an example is cyanidin 3-glucoside (CG) (Supplementary Figure 1A) Anthocyanins are produced by plants as secondary metabolites to protect against environmental stress factors and fungal infections (342) and they also promote health in humans (298, 343). Their pharmacokinetics and metabolism have been reported (344, 345). It is estimated that the average daily oral intake is ~1000 mg of polyphenols in adults in the USA (298) and ~65 mg of anthocyanidins in Europe (346). As vegetables and fruits are rich in polyphenols, they may supply an adequate dietary intake of polyphenols including anthocyanins.

CG has shown responses in experimental models that indicate a potential role in reversing the signs of the metabolic syndrome. CG decreased obesity and circulating triglycerides in an *in vivo* study using KK-Ay mice (347). *In vitro*, CG decreased inflammation in isolated vascular endothelial cells and monocytes (348) and produced an insulin-like effect in human omental adipocytes and 3T3-L1 cells (349).

The Queen Garnet plum is a variety of the Japanese plum (*Prunus salicina* Lindl) with a high anthocyanin (mainly CG) content up to 272 mg/100 g fresh fruit, being 5-10 fold higher than other plums (350). Consumption of Queen Garnet plum juice (QG) decreased malondialdehyde concentrations in plasma and urine as a biomarker of oxidative stress (350, 351) and reduced platelet activation-related thrombogenesis in healthy volunteers (351). Other food sources of CG and other cyanidin glycosides also improved signs of metabolic syndrome. As examples, purple corn decreased body fat and hyperglycaemia in a high-fat diet-fed mice (306) and Moro orange supplementation decreased the high-fat diet-induced increases in lipid deposition in liver and wet weight in mice as symptoms of non-alcoholic fatty liver (352) and fat accumulation (353). Similarly, high-fat diet-fed mice given either blueberry or purple corn supplementation reduced central adiposity (354), abdominal fat pads and hyperglycaemia (306). Additionally, chokeberry juice decreased blood pressure in humans with metabolic syndrome (211). Purple carrot juice containing anthocyanins improved glucose tolerance, decreased body weight gain, and improved cardiovascular and liver structure and function in rats fed a high-carbohydrate, high-fat diet (200). However, there is no clear evidence that CG is the active anthocyanin in improving these signs of metabolic syndrome.

Thus, this study has compared CG and QG in rats fed a high-carbohydrate, high-fat diet for attenuation of signs of the human metabolic syndrome. Cardiovascular, hepatic and metabolic parameters were measured for this comparison. Further, plasma samples were

screened for intact anthocyanins and their common metabolites (methylated/conjugated forms and hippuric acid) as a measure of anthocyanin absorption and metabolism.

## 7.3. Materials and methods

### *7.3.1. Cyanidin 3-glucoside and Queen Garnet plum juice*

Pure CG was supplied by Biosynth AS, Sandnes, Norway. Fresh Queen Garnet plums were harvested in February 2013 and QG was prepared and analysed for anthocyanins and quercetin glycosides (350, 355). The QG was also analysed for protein, fat, total sugar, dietary fibre and energy value by a commercial laboratory (Symbio Alliance, Brisbane, QLD, Australia). QG was supplied by Nutrafruit Pty Ltd, Toowong, QLD, Australia.

### *7.3.2. Rats and diets*

The experimental group consisted of 72 male Wistar rats (8-9 weeks old) were randomly divided into 6 experimental diet groups (n=12 each) and fed with corn starch (C), C + cyanidin 3-glucoside (CCG), C + Queen Garnet plum juice (CQG), high-carbohydrate, high-fat (H), H + cyanidin 3-glucoside (HCG) or H + Queen Garnet plum juice (HQC) for 16 weeks. CG 115 mg/kg food was thoroughly mixed in the diet; 50 ml/kg food QG plum juice containing 2.3 mg/ml anthocyanins and 0.31 mg/ml quercetin glycosides replaced an equivalent volume of water.

Experimental procedures such as body composition, oral glucose tolerance, echocardiography, blood pressure, isolated heart preparation, organ bath, plasma analysis and histology were conducted as described in Chapter 3.1. Plasma was screened for intact anthocyanins, their common methylated and conjugated metabolites as well as hippuric acid by HPLC (350, 351, 356).

## 7.4.Results

### *7.4.1. Diet intake, body composition and plasma biochemistry*

The major flavonoids present in QG were anthocyanins, mainly cyanidin 3-glucoside and cyanidin 3-rutinoside (Table 7.1, Figure 7.1) and quercetin glycosides (Table 7.1). The nutritional composition of the QG used in this study is shown in Table 7.1. Food intakes were higher in C, CQG and CCG rats than in H, HQG and HCG rats, respectively (Table 7.2). Due to these differences, the average daily intake of anthocyanins was higher in CCG and CQG rats than in HCG and HQG rats, respectively (Table 7.2).

Neither CG nor QG treatment altered food, water or energy intakes (Table 7.2). H rats increased feed conversion efficiency, body weight gain, abdominal circumference and body mass index compared to C rats, while these parameters were reduced in HCG and HQG rats compared to H rats (Table 7.2). Bone mineral content was higher in H rats than HCG and HQG rats, and all were higher than C, CCG and CQG rats (Table 7.2). HCG and HQG rats had decreased total body fat mass compared to H rats and increased total body fat mass compared to C, CCG and CQG rats. These changes in total body fat are consistent with changes in omental and epididymal fat pads (Table 7.2). Total body lean mass was unchanged by CG or QG treatment.

**Table 7.1 Composition of the Queen Garnet plum juice by analysis**

| **Variables** | **Queen Garnet plum juice** |
|---|---|
| Cyanidin 3-glucoside (mg/100 ml)[#, a] | 200 |
| Cyanidin 3-rutinoside (mg/100 ml)[#, a] | 30 |
| Quercetin glycosides (mg/100 ml)[#, b] | 31 |
| Energy (KJ/100 ml)[*] | 374 |
| Protein (g/100 ml)[*] | 1.1 |
| Total fat (g/ 100 ml)[*] | < 1 |
| Total sugars (g/100 ml)[*] | 15.2 |
| Fibre (g/ 100 ml)[*] | < 1 |
| Sodium (g/100 ml)[*] | 0.073 |

Values are represented as mean of duplicate analysis.

[#] Analysed by authors.

[a] See Supplementary Figure 1 for chemical structures.

[b] Sum of quercetin 3-glucoside, quercetin 3-rutinoside and quercetin 3-galactoside; calculated as quercetin 3-glucoside equivalents.

[*] Analysed by a commercial laboratory (Symbio Alliance, Brisbane, QLD, Australia).

**Table 7.2 Dietary intakes, body composition and organ wet weights in C, CCG, CQG, H, HCG and HQG diet-fed rats (n=10 rats/group)**

| Variable | C | CCG | CQG | H | HCG | HQG | P values | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Diet | Treatment | Interaction |
| Food intake (g/d) | 33.1±1.8$^{a}$ | 33.6±0.8$^{a}$ | 33.4±0.5$^{a}$ | 26.5±0.9$^{b}$ | 25.8±0.7$^{b}$ | 25.5±0.5$^{b}$ | <0.0001 | 0.74 | 0.55 |
| Water intake (ml/d) | 30.6±2.1$^{a}$ | 30.5±2.3$^{a}$ | 29.5±1.3$^{a}$ | 25.7±1.1$^{b}$ | 23.6±1.0$^{b}$ | 22.0±0.7$^{b}$ | <0.0001 | 0.18 | 0.57 |
| Plums juice intake (ml/d) | 0.0±0.0 | 0.0±0.0 | 1.7±0.0$^{a}$ | 0.0±0.0 | 0.0±0.0 | 1.3±0.0$^{b}$ | <0.0001 | <0.0001 | <0.0001 |
| Anthocyanins intake (mg/kg/d) | 0.0±0.0 | 9.9±0.0$^{a}$ | 9.8±0.0$^{b}$ | 0.0±0.0 | 7.6±0.0$^{c}$ | 7.4±0.0$^{c}$ | <0.0001 | <0.0001 | <0.0001 |
| Quercetin glycoside intake (mg/kg/d) | 0.0±0.0 | 0.0±0.0 | 1.3±0.0$^{b}$ | 0.0±0.0 | 0.0±0.0 | 1.0±0.0$^{b}$ | <0.0001 | <0.0001 | <0.0001 |
| Energy intake (kJ/d) | 370±24$^{b}$ | 375±15$^{b}$ | 375±5$^{b}$ | 557±17$^{a}$ | 553±9$^{a}$ | 531±6$^{a}$ | <0.0001 | 0.5 | 0.33 |
| Feed conversion efficiency (%) | 2.1±0.4$^{b}$ | 2.2±0.4$^{b}$ | 1.3±0.2$^{b}$ | 7.9±1.8$^{a}$ | 4.1±0.6$^{ab}$ | 4.2±0.7$^{ab}$ | <0.0001 | 0.0293 | 0.15 |
| Body weight gain (8-16 weeks) (%) | 8.4±1.3$^{c}$ | 8.1±1.1$^{c}$ | 5.6±0.5$^{c}$ | 22.1±3.6$^{a}$ | 12.9±1.1$^{b}$ | 12.7±0.8$^{b}$ | <0.0001 | 0.0038 | 0.1 |
| Visceral adiposity index (%) | 4.8±0.4$^{b}$ | 4.8±0.2$^{b}$ | 4.8±0.3$^{b}$ | 9.2±1.1$^{a}$ | 6.7±0.5$^{b}$ | 6.6±0.4$^{b}$ | <0.0001 | 0.0485 | 0.0485 |
| Abdominal circumference (cm) | 20.7±0.2$^{b}$ | 20.4±0.2$^{b}$ | 20.6±0.3$^{b}$ | 22.8±0.2$^{a}$ | 21.1±0.2$^{b}$ | 21.3±0.2$^{b}$ | <0.0001 | 0.0013 | 0.0042 |
| Body mass index (kg/m$^{2}$) | 5.6±0.2$^{bc}$ | 5.1±0.1$^{c}$ | 4.6±0.1$^{c}$ | 6.9±0.3$^{a}$ | 5.9±0.2$^{b}$ | 5.5±0.1$^{bc}$ | <0.0001 | <0.0001 | 0.31 |
| Bone mineral content (g) | 11.3±0.3$^{c}$ | 11.5±0.4$^{c}$ | 11.4±0.2$^{c}$ | 16.9±0.7$^{a}$ | 15.5±0.5$^{b}$ | 14.4±0.2$^{b}$ | <0.0001 | 0.0055 | 0.0029 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Total body lean mass (g) | 318±11[a] | 302±7[a] | 279±3[ab] | 308±19[a] | 307±6[a] | 279±9[ab] | 0.66 | 0.0074 | 0.64 |
| Total body fat mass (g) | 72±8[c] | 86±8[c] | 94±5[c] | 217±18[a] | 162±6[b] | 160±6[b] | <0.0001 | 0.11 | 0.0008 |
| **Tissue wet weight (mg/mm)[#]** | | | | | | | | | |
| Retroperitoneal adipose tissue | 172.1±17.1[b] | 177.3±12.4[b] | 178.1±10.9[b] | 527.7±84.4[a] | 279.6±23.1[b] | 271.8±20.4[b] | <0.0001 | 0.0081 | 0.0057 |
| Epididymal adipose tissue | 98.6±8.3[b] | 94.3±7.8[b] | 97.7±7.5[b] | 236.2±31.9[a] | 150.5±10.4[b] | 149.7±9.4[b] | <0.0001 | 0.0175 | 0.0198 |
| Omental adipose tissue | 101.8±9.4[c] | 92.2±7.1[c] | 97.2±7.2[c] | 250.4±35.9[a] | 160.4±17.6[bc] | 163.6±10.4[bc] | <0.0001 | 0.0255 | 0.0431 |
| Total abdominal fat | 372.5±32.8[b] | 363.7±17.8[b] | 373.0±23.7[b] | 1014.3±151.1[a] | 590.5±43.7[b] | 585.1±36.1[b] | <0.0001 | 0.0111 | 0.0146 |
| Liver | 194.2±9.7[c] | 197.5±6.5[c] | 201.9±6.7[c] | 299.2±14.3[a] | 233.7±8.4[b] | 236.7±8.3[b] | <0.0001 | 0.0105 | 0.0014 |

Each value is a mean ± SEM. Means within a row with unlike superscripts differ, P<0.05.

* In all groups, the body weight gain (8-16 weeks) is calculated relative to body weight at 8 weeks.

[#] Normalized against tibial length at the time of removal.

**Table 7.3 Changes in glucose tolerance test, plasma hormones, plasma metabolites and plasma biochemistry in C, CCG, CQG, H, HCG and HQG diet-fed rats (n=10 rats/group)**

| | | | | | | | P values | | |
|---|---|---|---|---|---|---|---|---|---|
| **Variable** | **C** | **CCG** | **CQG** | **H** | **HCG** | **HQG** | **Diet** | **Treat-ment** | **Interaction** |
| OGTT-AUC (mmol/L min) | 691±22[b] | 666±17[b] | 646±38[b] | 843±27[a] | 709±12[b] | 727±37[b] | 0.0008 | 0.0164 | 0.27 |
| Plasma insulin (μmol/L) | 1.8±0.5[b] | 1.8±0.7[b] | 1.7±0.2[b] | 3.9±0.3[a] | 2.1±0.4[b] | 1.9±0.3[b] | 0.0019 | 0.0041 | 0.0088 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Plamsa leptin (μmol/L) | 7.3±0.7$^{b}$ | 7.2±0.9$^{b}$ | 7.1±0.9$^{b}$ | 11.8±0.9$^{a}$ | 8.6±0.8$^{b}$ | 8.9±1.0$^{b}$ | 0.001 | 0.09 | 0.13 |
| Plasma total cholesterol (mmol/L) | 1.6±0.1$^{b}$ | 1.6±0.1$^{b}$ | 1.4±0.1$^{b}$ | 2.4±0.1$^{a}$ | 1.7±0.0$^{b}$ | 1.6±0.1$^{b}$ | <0.0001 | <0.0001 | 0.0049 |
| Plasma triglycerides (mmol/L) | 0.4±0.0$^{c}$ | 0.4±0.0$^{c}$ | 0.4±0.1$^{c}$ | 1.9±0.2$^{a}$ | 0.9±0.2$^{b}$ | 0.6±0.1$^{b}$ | <0.0001 | <0.0001 | <0.0001 |
| Plasma NEFA (mmol/L) | 1.5±0.2$^{c}$ | 1.5±0.1$^{c}$ | 1.1±0.1$^{c}$ | 4.9±0.3$^{a}$ | 1.6±0.2$^{c}$ | 2.4±0.4$^{bc}$ | <0.0001 | <0.0001 | 0.0005 |
| Plasma hippuric acid (ng/ml) | 85±60 | 86±45 | 59±82 | 139±69 | 91±69 | 80±49 | 0.63 | 0.70 | 0.69 |
| Plasma ALP (U/L) | 123.7±5.2$^{c}$ | 116.5±11.6$^{c}$ | 123.8±6.5$^{c}$ | 315.8±17.2$^{a}$ | 192.2±14.5$^{b}$ | 212.8±12.0$^{b}$ | <0.0001 | <0.0001 | <0.0001 |
| Plasma ALT (U/L) | 25.9±1.9$^{c}$ | 23.0±1.5$^{c}$ | 20.4±1.5$^{c}$ | 45.9±2.9$^{a}$ | 27.0±2.0$^{c}$ | 31.9±1.3$^{bc}$ | <0.0001 | <0.0001 | 0.0403 |
| Plasma AST (U/L) | 61.1±1.9$^{b}$ | 61.8±1.4$^{b}$ | 58.2±3.8$^{b}$ | 86.6±2.1$^{a}$ | 62.0±2.0$^{b}$ | 62.4±2.1$^{b}$ | <0.0001 | <0.0001 | 0.0002 |

Each value is a mean ± SEM. Means within a row with unlike superscripts differ, P<0.05.

**Table 7.4 Changes in cardiovascular structure and function in C, CCG, CQG, H, HCG and HQG diet-fed rats (n=10-8 rats/group)**

| | | | | | | | P values | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Diet | Treatment | Interaction |
| **Variable** | **C** | **CCG** | **CQG** | **H** | **HCG** | **HQG** | | | |
| Heart rate (bpm) | 307±16$^{a}$ | 264±13$^{b}$ | 253±11$^{b}$ | 339±18$^{a}$ | 249±8$^{b}$ | 269±8$^{b}$ | 0.09 | 0.0001 | 0.56 |
| IVSd (mm) | 1.9±0.1 | 1.9±0.1 | 1.8±0.0 | 2.0±0.1 | 1.8±0.1 | 1.8±0.1 | 0.49 | 0.0429 | 0.49 |
| LVIDd (mm) | 6.3±0.2$^{c}$ | 6.9±0.1$^{b}$ | 7.0±0.2$^{b}$ | 7.9±0.1$^{a}$ | 6.9±0.2$^{b}$ | 7.0±0.1$^{b}$ | <0.0001 | 0.53 | <0.0001 |
| LVPWd (mm) | 1.6±0.0$^{b}$ | 1.7±0.0$^{b}$ | 1.7±0.0$^{b}$ | 1.9±0.0$^{a}$ | 1.8±0.1$^{b}$ | 1.7±0.1$^{b}$ | 0.0056 | 0.33 | 0.0056 |
| IVSs (mm) | 2.8±0.2 | 2.9±0.1 | 3.0±0.1 | 3.1±0.1 | 3.0±0.1 | 3.0±0.1 | 0.27 | 0.71 | 0.27 |
| LVIDs (mm) | 3.2±0.3$^{b}$ | 3.5±0.1$^{b}$ | 3.4±0.1$^{b}$ | 4.1±0.2$^{a}$ | 3.7±0.2$^{b}$ | 3.5±0.1$^{b}$ | 0.0153 | 0.31 | 0.0482 |
| LVPWs (mm) | 2.5±0.1 | 2.9±0.0 | 2.8±0.1 | 2.8±0.1 | 3.0±0.2$^{ab}$ | 3.0±0.1$^{ab}$ | 0.0186 | 0.0186 | 0.62 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Fractional shortening (%) | 53.9±2.5[a] | 58.4±1.7[a] | 59.8±2.3[a] | 45.8±2.1[b] | 57.3±1.5[a] | 57.1±1.3[a] | 0.0157 | 0.0003 | 0.21 |
| Ejection time (ms) | 70.4±2.4[c] | 77.6±1.4[bc] | 75.3±2.1[bc] | 90.6±2.4[a] | 83.4±4.1[bc] | 84.3±2.1[bc] | <0.0001 | 0.76 | 0.0193 |
| Ejection fraction (%) | 80.2±1.9 | 81.6±1.3 | 82.6±1.9 | 83.6±1.8 | 78.6±4.4 | 82.3±1.8 | 0.41 | 0.77 | 0.33 |
| LV developed pressure (mmHg) | 70.2±4.7[a] | 70.8±3.7[a] | 72.7±5.4[a] | 49.4±5.1[b] | 67.2±4.1[a] | 68.6±3.9[a] | 0.0138 | 0.0302 | 0.09 |
| (+)dP/dt (mmHg/S) | 1096±64[a] | 1046±73[a] | 1077±60[a] | 809±74[b] | 1006±59[ab] | 986±61[ab] | 0.0065 | 0.23 | 0.14 |
| (-)dP/dt (mmHg/S) | -714±48[a] | -756±36[a] | -734±50[a] | -506±44[b] | -669±39[a] | -686±39[a] | 0.0085 | 0.0363 | 0.09 |
| Diastolic stiffness (k) | 22.9±0.8[b] | 22.5±0.6[b] | 23.4±0.8[b] | 28.6±0.6[a] | 23.8±0.8[b] | 24.8±0.9[b] | <0.0001 | 0.042 | 0.0093 |
| Diastolic volume (μL) | 356±24[b] | 344±18[b] | 357±24[b] | 533±49[a] | 360±33[b] | 361±18[b] | 0.0069 | 0.0096 | 0.0093 |
| Systolic volume (μL) | 48±7[b] | 45±3[b] | 42.3±3[b] | 85±11[a] | 71±10[ab] | 48±5[b] | 0.0054 | 0.0044 | 0.033 |
| Stroke volume (μL) | 308±27 [ab] | 298±19 [ab] | 314±23 [ab] | 397±18[a] | 289±40 [ab] | 313±20 [ab] | 0.06 | 0.1 | 0.05 |
| Cardiac output (mL) | 88.1±7.5[b] | 78.9±6.7[b] | 81±8.8[b] | 127.5±8.7[a] | 71.7±10.0[b] | 84.7±6.7[b] | 0.0115 | 0.0041 | 0.0333 |
| Estimated LV mass, Litwin (g) | 0.73±0.00[b] | 0.84±0.03[b] | 0.80±0.03[b] | 0.99±0.05[a] | 0.82±0.07[b] | 0.83±0.03[b] | 0.0001 | 0.18 | 0.0015 |
| LV+septum wet weight (mg/mm tibial length) | 16.1±0.5[b] | 14.6±0.4[b] | 16.5±0.5[b] | 19.5±0.8[a] | 16.6±0.5[b] | 16.1±0.5[b] | 0.0154 | 0.0154 | 0.0027 |
| Right ventricle wet weight (mg/mm tibial length) | 3.8±0.2 [ab] | 4.2±0.2 [ab] | 3.9±0.1 [ab] | 4.4±0.2 [ab] | 4.9±0.2[a] | 4.0±0.2 [ab] | 0.06 | 0.41 | 0.17 |
| Relative wall thickness | 0.52±0.01 | 0.48±0.01 | 0.50±0.01 | 0.50±0.02 | 0.48±0.03 | 0.50±0.01 | 0.46 | 0.46 | 0.46 |
| Systolic blood pressure (mmHg) | 123±3[b] | 123±1[b] | 126±3[b] | 157±4[a] | 133±1[b] | 129±2[b] | <0.0001 | 0.0001 | <0.0001 |
| Systolic wall stress (mmHG) | 91.3±5.2[b] | 81.2±2.5[b] | 78.3±4.7[b] | 118.0±8.7[a] | 86.9±8.6[b] | 85.1±3.5[b] | 0.007 | 0.0004 | 0.1 |

Each value is a mean±S.E.M. Number of repetitive experiments indicated within parenthesis. Means within a row with unlike superscripts differ, P<0.05.

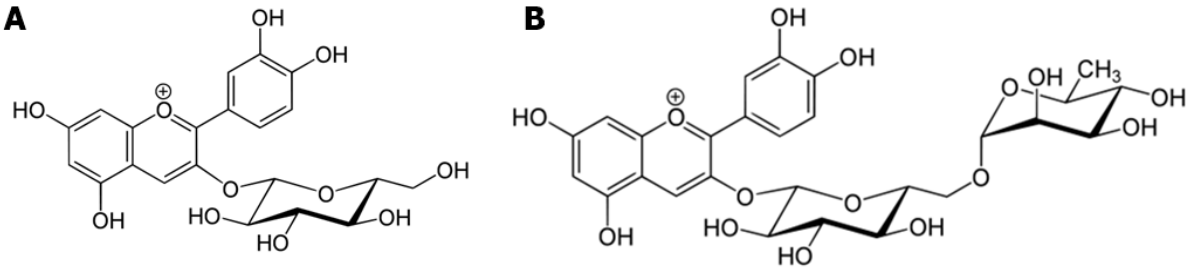
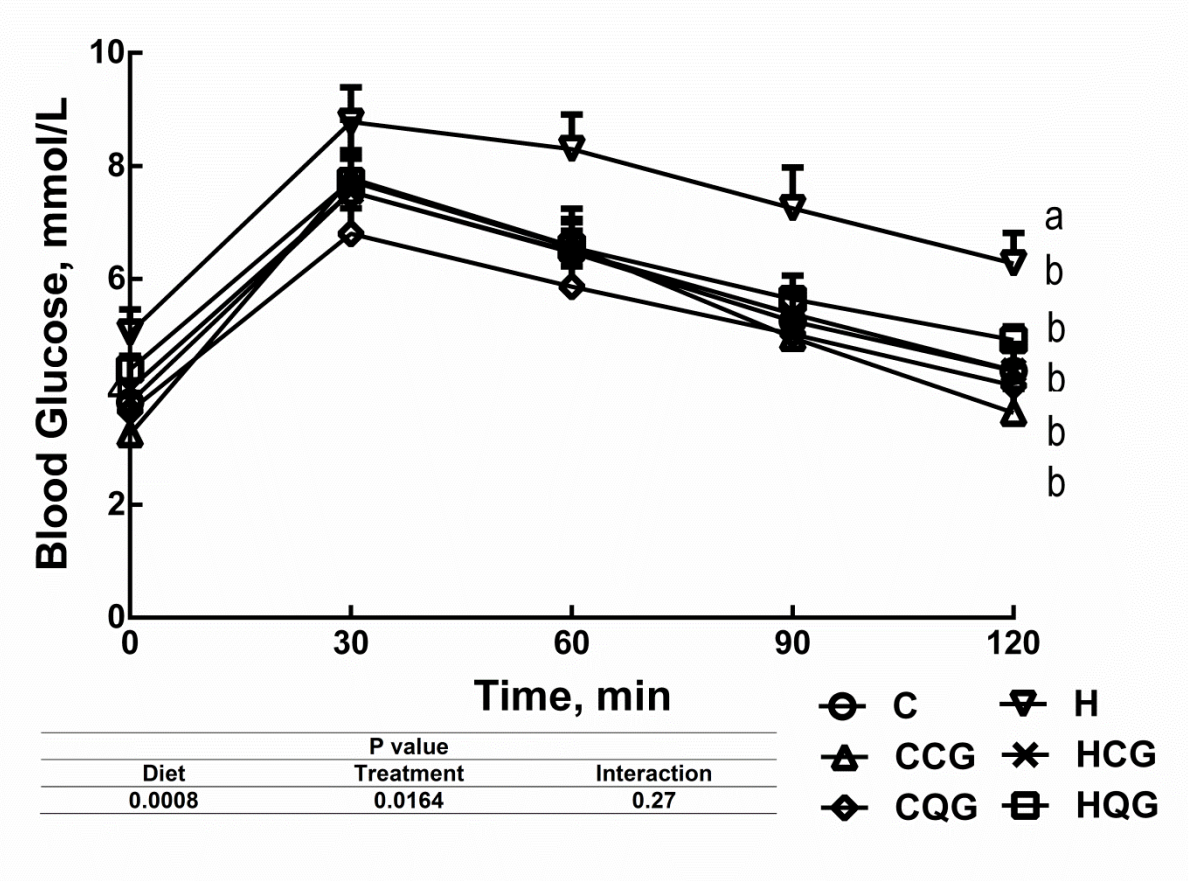



Figure 7.1 Chemical structures of cyanidin 3-rutinoside (A) and cyanidin 3-glucoside (B) as the characteristic anthocyanins in QG.




| P value | | |
|---|---|---|
| Diet | Treatment | Interaction |
| 0.0008 | 0.0164 | 0.27 |

Figure 7.2 Effect of cyanidin 3-glucoside and Queen Garnet plum juice on oral glucose tolerance in C, CCG, CQG, H, HCG and HQG rats. Data are shown as mean ± S.E.M. End-point means without a common alphabet in each data set significantly differ, $P<0.05$ and n=10/group.

### *7.4.2. Plasma biochemistry, oral glucose tolerance and plasma metabolites*

Plasma concentrations of total cholesterol, triglycerides and NEFA were increased in H rats compared to C or to CG and QG-treated rats, while HQG rats had higher NEFA concentrations than HCG rats; HCG and HQG rats had higher concentrations of triglycerides than CCG and CQG rats (Table 7.3). Plasma leptin concentrations were increased in H rats compared to C rats; leptin concentrations were normalised in HCG and HQG rats (Table 7.3).

H rats also had higher fasting blood glucose concentration compared to C rats. CG and QG treatment decreased blood glucose concentrations. The plasma glucose response to oral glucose loading was greater in H rats than C rats (Figure 7.2). At 120 minutes, HCG and HQG rats had lower plasma glucose concentrations than H rats (Figure 7.2). Plasma insulin concentrations almost doubled in H rats compared to C, and CG and QG-treated rats. This change is consistent with glucose tolerance curves (Table 7.3).

Neither cyanidin 3-glucoside nor cyanidin 3-rutinoside, the main QG anthocyanins, could be detected in the plasma of CG or QG-treated rats. Cyanidin glucuronide, the most common conjugated metabolite of cyanidin-based anthocyanins, was tentatively identified in some plasma samples after QG treatment. However, further evaluation was not undertaken since the concentration of this metabolite was below the limit of quantification. No other conjugated or methylated anthocyanin forms could be detected. Plasma hippuric acid concentrations were not different in CG and QG-treated rats (Table 7.3).

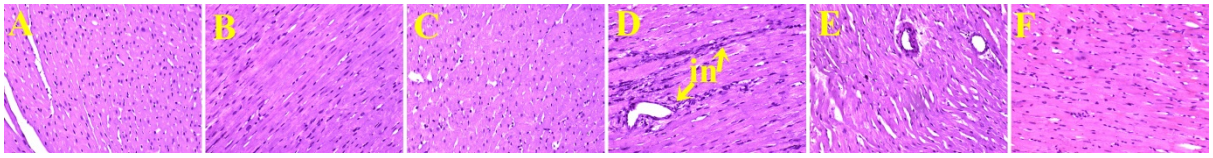



Figure 7.3 Haematoxylin and eosin staining of left ventricle (original magnification ×20) showing inflammatory cells (marked as “in”) as dark spots outside the myocytes in C (A), CCG (B), CCG (C), H (D), HCG (E) and HCG (F) rats.

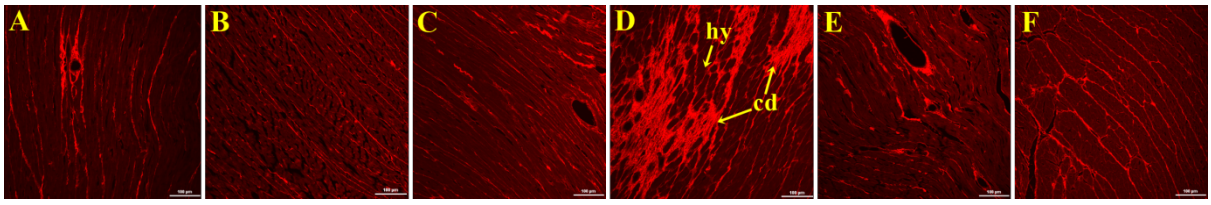



Figure 7.4 Picrosirius red staining of left ventricular interstitial collagen deposition (original magnification ×20) in C (A), CCG (B), CQG (C), H (D), HCG (E) and HQG (F) rats.; Collagen deposition is marked as “cd” and hypertrophied cardiomyocytes are marked as “hy”.

### *7.4.3. Cardiovascular structure and function*

H rats showed increased left ventricular internal diameter in diastole (LVIDd) and left ventricular wet weight as signs of eccentric hypertrophy compared to C rats. This change in LVIDd was observed with no change in relative wall thickness in either of the groups (Table 8.4). H rats showed impaired systolic function seen as decreased fractional shortening, developed pressure and dP/dt, increased left ventricular diameter in systole (LVIDs), diastolic

stiffness and systolic wall stress (Table 7.4). H rats also showed increased diastolic, systolic and stroke volumes, cardiac output and estimated left ventricular mass compared to C rats, without any change in heart rate.

Treatment of H rats with CG and QG decreased LVIDd and LVIDs compared to H rats, with increased left ventricular posterior wall diameter in systole in HCG and HQG rats but diastolic diameter remained constant. These responses were accompanied by increased fractional shortening with CG and QG (Table 7.4). Diastolic stiffness, diastolic and systolic volumes, cardiac output, systolic wall stress and wet weight of left ventricle with septum were normalised with CG and QG, while heart rate was decreased with CG and QG normalised ejection time compared to H rats (Table 7.4).

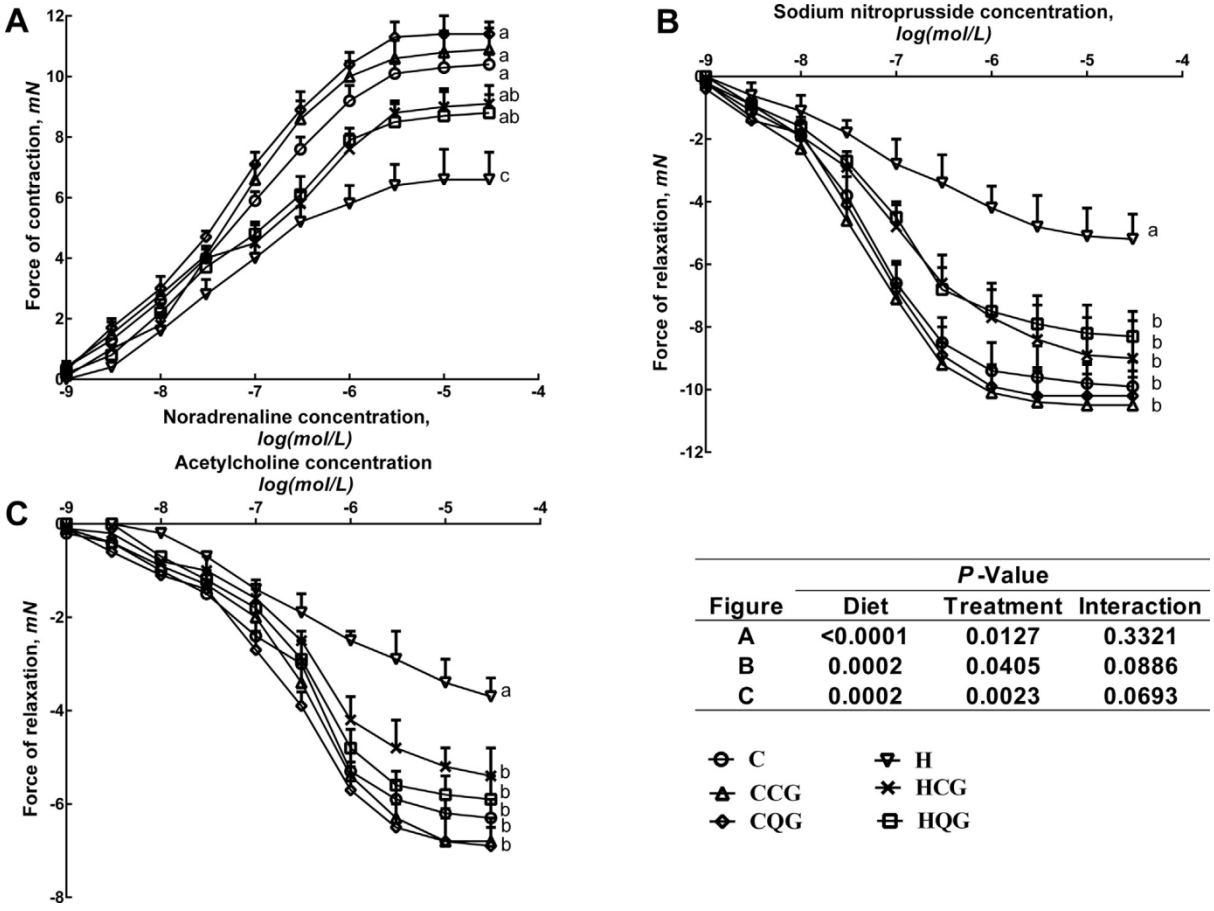



Figure 7.5 Cumulative concentration-response curves for noradrenaline (A), sodium nitroprusside (B) and acetylcholine (C) in thoracic aortic rings from C, CCG, CQG, H, HCG and HQG rats. Data are shown as means±S.E.M. End-point means without a common alphabet in each data set significantly differ, P<0.05 and n=10/group.

Compared to C rats (Figure 7.3A and 7.4A), H rats showed increased infiltration of inflammatory cells in the left ventricle (Figure 7.3D) and increased interstitial collagen

deposition (Figure 7.4D). CG and QG suppressed the infiltration of inflammatory cells (Figure 7.3B, C, E and F) and reduced collagen deposition (Figure 7.4B, C, E and F), while no other changes were observed and tissue morphology appeared normal.

In isolated thoracic aortic rings, H rats showed decreased vascular contraction with noradrenaline (Figure 7.5A) and decreased vascular relaxation with sodium nitroprusside and acetylcholine compared to C rats (Figure 7.5B and C). CG and QG rats showed improved contraction and relaxation in isolated thoracic aortic rings (Figure 7.5A, B and C).

### *7.4.4. Hepatic structure and function*

Compared to C rats, H rats had increased liver wet weight with increased plasma activities of ALT, ALP and AST as markers of liver damage. HCG and HQG rats had decreased liver wet weight and plasma ALP, ALT and AST activities compared to H rats. Liver wet weight and plasma activities were unchanged in CCG and CQG rats compared to C rats (Table 7.3). H rats (Figure 7.6D) showed increased hepatic lipid deposition and inflammatory cell infiltration compared to C rats (Figure 7.6A) while HCG and HQG rats showed decreased inflammatory cell infiltration (Figure 7.6E and F) compared to H rats. CCG and CQG rats showed minimal macrovesicular steatosis and portal inflammation and tissue morphology appeared normal (Figure 7.6B and C).

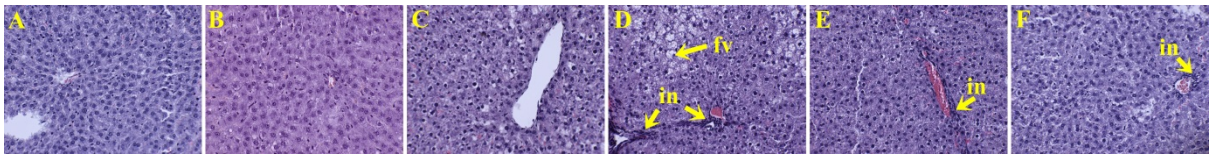



Figure 7.6 Haematoxylin and eosin staining of hepatocytes (original magnification ×20) showing inflammatory cells (marked as “in”) and hepatocytes with fat vacuoles (marked as “fv”) in C (A), CCG (B), CQG (C), H (D), HCG (E) and HQG (F) rats.

## 7.5. Discussion

Cyanidin-based anthocyanins, the characteristic polyphenols in QG, are one of the most abundant pigments in nature, being responsible for the dark red colours of many fruits and vegetables (357). Since continued human health has been associated with an increased intake of fresh fruits and vegetables, it is important to determine the anti-diabetic, anti-obesity and anti-inflammatory activities of purified cyanidin-based anthocyanins, especially cyaniding 3-glucoside, as well as foods containing these compounds in relevant rodent models of human disease. QG contain both anthocyanins and quercetin glycosides, suggesting that either could produce the physiological responses. However, the dose of

quercetin glycosides in this study was low at around 1 mg/kg/day, much lower than the quercetin dose of around 50 mg/kg/day or the rutin dose of around 100 mg/kg/day used to reverse signs of the metabolic syndrome in the same model (148, 358). This indicates that cyanidin 3-glucoside is the major bioactive compound in QG.

The high-carbohydrate, high-fat diet-fed rat mimics most of the signs of metabolic syndrome in humans. This diet increased abdominal fat deposition, plasma lipids, liver enzymes, liver weight, infiltration of inflammatory cells in heart and liver, blood pressure and collagen deposition, and impaired glucose tolerance. In addition, increased left ventricular stiffness and diminished aortic responses were observed when compared to rats fed a low-fat, corn starch-rich diet (146).

Both CG and QG improved cardiovascular and hepatic structure and function and reduced metabolic parameters such as body weight gain, visceral adiposity index and total body fat mass induced by the H diet, consistent with the metabolic responses to purple carrots (200) and purple corn (306), both dietary sources of cyanidin glycosides. CG increased fatty acid oxidation via AMP-activated protein kinase (AMPK) (359). AMPK activation leads to acetyl-CoA carboxylase phosphorylation and inactivation, which stimulates CPT1 expression, thereby increasing fatty acid oxidation, leading to decreased abdominal fat and improved glucose metabolism (359). Decreases in total body fat mass correlated with decreases in visceral adiposity as CG and QG reduced the weight of all abdominal fat pads. Leptin is a hormone secreted from adipose tissue (360) and the decrease in adipose tissue with CG and QG correlated with decreased plasma leptin concentrations. Similarly, CG and QG supplementation improved plasma lipid profiles by reducing plasma concentrations of triglycerides, total cholesterol and NEFA.

Dysfunction of the left ventricle correlates with metabolic changes, oxidative stress and increased inflammatory cell infiltration, with an increase in left ventricular fibrosis and stiffness (287). This study also shows that CG and QG improved cardiovascular function. The decrease in left ventricular weight suggests that CG and QG remodelled the structural damage caused by the H diet. It is clear from echocardiographic assessment that CG and QG improved systolic function and decreased left ventricular dimension, probably by adapting to the reduced wall stress. Additionally, CG and QG rats decreased the ejection time with no change in ejection fraction suggesting that a decreased rate of contraction was required to eject the smaller diastolic volume, correlating with reduced blood pressure and reduced left ventricular internal size during systole and diastole. Decreased blood pressure and diastolic

stiffness with CG and QG supplementation also correlated with decreased collagen deposition in left ventricle. Our results also suggested that CG and QG supplementation improved vascular function by improving endothelial function, perhaps by increasing NO concentrations as shown in similar studies with anthocyanin-containing chokeberry and bilberry extracts (361). This improved vascular function by CG and QG is consistent with the findings that anthocyanins inhibited inflammatory cytokines such as TNF-α, induced signal transducer and activator of transcription (STAT3) phosphorylation, inducible NO synthase, IL-1β and IL-6 by inhibiting the activation of NF-κB (348, 362, 363). Extending these effects could be the reason for reduced inflammatory cell infiltration in left ventricle and liver with supplementation of CG and QG. Both CG and QG supplemented rats showed decreased liver weight, with decreased fat vacuoles and decreased hepatic inflammation. Decreases in liver weight, adipose tissue and inflammation directly correlated with reduction of plasma activity of liver enzymes.

The absence of intact anthocyanins and their common methylated and conjugated metabolites in plasma was similar to the results of previous studies in which these compounds were not detected in plasma of rats and pigs following feeding with anthocyanin-containing foods (blackberries or blueberries) (364-366). This was presumed to be due to the rapid absorption and metabolism of the anthocyanins. Similar findings of observed biological effects but no detected anthocyanins were also reported with pre- and mildly hypertensive human subjects (367). In these subjects, blood pressure was lowered with tea containing delphinidin and cyanidin glycosides, but the anthocyanins were not detected in the collected plasma and urine samples, possibly because of insufficient sensitivity of the HPLC method to detect the anthocyanins (367). Further, hippuric acid, a colon microbial/liver-derived metabolite of dietary polyphenols and anthocyanins, may represent the final product of the *in vivo* biotransformation of these plant compounds. However, it can also be generated by the metabolic degradation and transformation of amino acids and fibre (364). Similar plasma hippuric acid concentrations between treatments, following 8 weeks of CG or QG diet, is also consistent with the findings of a study in which rats received either a control diet or a blueberry powder-supplemented diet (364). Plasma hippuric acid concentrations in rats can therefore not be considered as a reliable biomarker to assess anthocyanin absorption and metabolism. However, a relationship between the urinary excretion rate of hippuric acid and the ingested amount of blueberry anthocyanins has been demonstrated (364), suggesting that urinary hippuric acid, in contrast to plasma, could represent a potential biomarker for

anthocyanin absorption and metabolism. Due to technical reasons, no urine samples were available for biochemical analysis.

Further, the metabolism of the anthocyanins, especially cyanidin glycosides, by gut microbiota to protocatechuic acid (21, 368), one of a group of hydroxybenzoic acids that reduce blood pressure and improve lipid profiles (369), could be another reason for the improvements in CG and QG rats and could also explain the low or absent anthocyanin concentrations in plasma. Mice fed with a high-fat, high-sucrose diet became obese with increased plasma concentrations of cholesterol and triglycerides; treatment with anthocyanin-containing cranberry juice reversed these features of metabolic syndrome. These effects were associated with decreases in intestinal inflammation and increases in gut bacteria especially *Akkermansia* spp. (370). In mice, *Akkermansia muciniphila* increased with increased dietary polyphenol intake and attenuated the high-fat diet induced metabolic syndrome disorders (371). Increased *Bifidobacteria* in faeces together with increased urinary concentrations of anthocyanin metabolites including syringic acid, *p*-coumaric acid, 4-hydroxybenzoic acid and homovanillic acid confirm the important role of anthocyanins/polyphenols as bacterial substrates (372). The colonic metabolites of anthocyanins such as phenolic acids produced by gut bacteria may act as potential systemic bioactive compounds to produce the positive responses to anthocyanins (373, 374). Further, anthocyanins may act as prebiotics to increase the growth of beneficial gut bacteria (373). The current study is limited as gut microbiota was not analysed.

### 7.6. Conclusion

Both CG and QG containing cyanidin glycosides dosed at ~8 mg/kg body weight showed similar responses in reversing the signs of metabolic syndrome in rats fed a high-carbohydrate, high-fat diet. Reduction of body weight gain with decreased abdominal fat pads and improved lipid profile and glucose metabolism along with improved cardiovascular and hepatic structure and function suggests that both CG and QG can be possible treatments for reversing or attenuating the complications of metabolic syndrome. However, further investigation on CG and QG will be necessary to understand the mechanisms underlying their improvement of the signs of metabolic syndrome. Similar responses were observed in CG and QG rats, indicating that further investigations with QG are warranted to determine if these positive effects can be translated to obese or overweight humans.

### 7.7. Study specific acknowledgements

I thank Mr Kristian Larsen (Biosynth AS, Sandnes, Norway) and Mr Hugh Macintosh (Nutrafruit Pty Ltd, Toowong, QLD, Australia) for providing CG and QG, respectively. I thank Mr Jason Brightwell, The Prince Charles Hospital, Brisbane, Australia, for the acquisition of echocardiographic images. I thank Dr Kate Kauter, School of Health and Wellbeing, University of Southern Queensland, for her assistance and expert advice on ELISA.

# CHAPTER 8

# 8. Effect of Queen Garnet plum juice in mildly hypertensive obese or over-weight subjects: a randomised, double-blind, placebo-controlled study

## 8.1. Summary

The studies from Chapters 6 & 7 have shown that anthocyanins and anthocyanin-rich food attenuated metabolic syndrome risk factors in a validated rat model of human metabolic syndrome. The aim of this study was to determine whether Queen Garnet plum juice (QG) reduced blood pressure, adiposity and other metabolic syndrome risk factors in obese or over-weight adult humans. This study comprised 29 obese or over-weight subjects treated with QG or placebo drinks (250 ml/day) for 12 weeks. QG supplementation for 12 weeks decreased blood pressure, fasting plasma glucose and LDL, and increased fasting plasma HDL, whereas no changes in total body fat mass or body weight were observed. Our findings show that QG but not placebo attenuated some of the risk factors of metabolic syndrome. We assume that the major anthocyanin in QG, cyanidin 3-glucoside, is the most active component, with possible additive effects of flavonoids such as quercetin glycosides.

## 8.2. Introduction

Dietary habits and lifestyle in general are major risk factors for the development and progression of metabolic syndrome risk factors (375). The prevalence of the metabolic syndrome and incidence of cardiovascular diseases is increasing worldwide (376). As a consequence, costs for health care are rising and there is a strong demand for preventive strategies that can be easily implemented by the majority of the population. Diets rich in fruits and vegetables are among the recommended lifestyle modifications to decrease the risk of cardiovascular diseases, but they can also reduce the complications associated with disturbed metabolic states or already established disorders (337, 377). There is increasing evidence that anthocyanin-rich products may be beneficial in patients with cardiovascular diseases or metabolic disorders. In humans, cranberries rich in anthocyanins, flavonols and proanthocyanidins showed improved lipid profiles and lowered plasma insulin concentrations (378, 379). Similarly, pomegranate juice rich in anthocyanins and tannins decreased blood pressure in patients with carotid artery stenosis (380). Further, cherry juice reduced body weight blood pressure and LDL in diabetic subjects (381). Queen Garnet plums are a rich source of anti-oxidant anthocyanins that attenuated thrombosis in humans (351) and metabolic syndrome risk factors in rats (Chapter 7).

The aim of the present intervention study was to investigate the hypothesis that regular intake of QG for 12 weeks could improve cardiometabolic risk factors in obese or over-weight humans.

## 8.3. Materials and methods

### *8.3.1. Queen Garnet plum juice and Raspberry cordial*

Queen Garnet plums were harvested in February 2015 and plum juice (QG) was prepared as described (350). Placebo was prepared by diluting a commercial raspberry flavoured cordial (ingredients: water, sugar, acidity regulator [E330], flavour, natural colour [E163], preservatives [E211, E223] Coles Smart Buy – Raspberry flavoured cordial (Coles, Brisbane, QLD, Australia) 1:4 with water. All study beverages were heated to 72 °C, held for 5 minutes prior to packing into identical 2 L plastic bottles and stored at 4 °C prior to distribution to participants. The drinks were analysed for anthocyanins and quercetin glycosides, energy, protein, fat, total sugar, fibre, vitamin C and several B-vitamins (Table 8.1).

### *8.3.2. Experimental design, participant recruitment and randomisation*

This study was a randomised, double-blind, placebo-controlled trial involving 29 human volunteers residing in Melbourne between the ages of 20 and 60 years, with a mean age of 45. Participant recruitment and randomisation were performed as shown in Figure 3.2.

### *8.3.3. Administration of juice and fortnightly visits*

All participants received 2 x 2 L treatment or placebo drink packed in opaque brown bags with blinded label every fortnight for 12 weeks. Participants were requested to drink 250 ml every morning. Anthropometric and blood pressure measures were taken during fortnightly consultations using standard equipment and techniques as described in Chapter 3.2

**Table 8.1 Composition of the study drinks by analysis**

| Variables | Placebo drink | QG drink |
|---|---|---|
| Cyanidin 3-glucoside (mg/100 mL) | 0.1 | 76 |
| Cyanidin 3-rutinoside (mg/100 mL) | 0.1 | 25 |
| Quercetin glycosides (mg QGE/100 mL)* | 0.4 | 31 |
| Energy (kJ/100 g) | 186 | 213 |
| Protein (g/100 mL) | 1 | 1.1 |
| Fat (g/100 mL) | 0.1 | 0.1 |
| Total sugar (g/100 mL) | 7.8 | 8.5 |
| Fibre (g/100 mL) | <0.1 | <0.1 |

*Sum of quercetin 3-glucoside, quercetin 3-rutinoside and quercetin 3-galactoside; calculated as quercetin 3-glucoside equivalents

**Table 8.2 Baseline measurement stats**

| Variables | Placebo group (n=14) | Treatment group (n=15) | p Value |
|---|---|---|---|
| Body weight (kg) | 91±15 | 86±19 | 0.89 |
| Body mass index (kg/m$^2$) | 32±5 | 31±5 | 0.46 |
| Age (years) | 38.4±14.2 | 47±11 | 0.37 |
| Systolic blood pressure (mmHg) | 139±5 | 142±7 | 0.27 |
| Diastolic blood pressure (mmHg) | 91±3 | 92±4 | 0.37 |

Values are expressed as mean ± standard deviation (SD), n = number of subjects in group.

## 8.4. Results

### *8.4.1. Study Participants*

There were 94 volunteers screened for enrolment into the study, with 62 volunteers excluded from study bases on exclusion criteria, obese or overweight but not hypertensive or taking blood pressure medication (Figure 3.2). A total of 32 volunteers were randomised into two groups to receive QG or placebo. The data analysis was completed for 14 participants in

placebo group and 15 participants in QG group (Figure 3.2). In addition, there was no breach of the blinding process identified throughout the intervention period.

### *8.4.2. Participant characteristics*

Baseline general characteristics of participants who completed the study are shown in Table 8.2. There were no significant differences recorded in the baseline characteristics such as age, body weight, body mass index, systolic blood pressure and diastolic blood pressure between QG and placebo groups

### *8.4.3. Anthropometric parameters*

Baseline and post-intervention anthropometric parameters such as body weight, BMI, waist circumference, hip circumference, waist to hip ratio and percentage body fat (Table 8.3) had no significant changes between groups for all post-intervention anthropometric variables. No significant changes were observed in energy intake, physical activity (Table 8.5) and resting respiratory exchange ratio (Table 8.3).

### *8.4.4. Cardiovascular parameters*

The cardiovascular parameters including heart rate, systolic and diastolic blood pressure are presented in Table 8.3. There were significant differences between groups and change over time in systolic and diastolic blood pressure in treatment group (Figure 8.1). A significant decrease in heart rate over time was recorded in both the groups.

### *8.4.5. Fasting plasma lipids, liver enzymes and glucose*

QG treatment for 12 weeks decreased fasting plasma LDL and placebo drink decreased HDL. QG treatment for 12 weeks decreased fasting plasma glucose and placebo drink decreased gamma-glutamyl transpeptidase (Table 8.4). However, no changes were observed in plasma total cholesterol, triglycerides, alanine transaminase, aspartate transaminase and creatinine for either group.

**Table 8.3 Anthropometric & cardiovascular parameters baseline and post-intervention**

| Variable | Group | Baseline | Week 12 | Group | Time | Interaction |
|---|---|---|---|---|---|---|
| **Anthropometry and body composition measurements** | | | | | | |
| Body weight (kg) | Placebo | 92.5±14.7 | 92.7±14.0 | 0.3 | 0.8 | 0.4 |
| | Treatment | 86.6±20.7 | 86.2±21.9 | | | |
| Waist (cm) | Placebo | 107.5±14.0 | 107.5±13.3 | 0.5 | 0.3 | 0.4 |
| | Treatment | 103.7±14.9 | 104.3±14.9 | | | |
| Hip (cm) | Placebo | 115.2±12.2 | 116.2±12.1 | 0.1 | 0.2 | 0.2 |
| | Treatment | 109.2±9.7 | 109.2±9.8 | | | |
| Waist to hip ratio | Placebo | 0.93±0.06 | 0.92±0.05 | 0.3 | 0.8 | 0.08 |
| | Treatment | 0.94±0.06 | 0.95±0.06 | | | |
| Respiratory exchange ratio | Placebo | 0.86±0.06 | 0.84±0.07 | 0.8 | 0.5 | 0.5 |
| | Treatment | 0.85±0.7 | 0.85±0.5 | | | |
| Total body lean mass (kg) | Placebo | 50.1±9 | 50.1±9 | 0.7 | 0.8 | 0.3 |
| | Treatment | 51.1±11 | 51.3±11 | | | |
| Total body fat mass (kg) | Placebo | 37.3±13 | 37.4±13 | 0.2 | 0.9 | 0.6 |
| | Treatment | 32.4±14 | 32.4±14 | | | |
| Total fat (%) | Placebo | 42.2±9.8 | 42.25±9.9 | 0.5 | 0.3 | 0.4 |
| | Treatment | 62.1±96.2 | 37.83±9.9 | | | |
| Android (% fat) | Placebo | 48.8±10.7 | 48.8±11.2 | 0.3 | 0.9 | 1.0 |
| | Treatment | 45.1±11.4 | 45.1±10.3 | | | |
| Gynoid (% fat) | Placebo | 43.3±10.1 | 43.4±10.1 | 0.1 | 0.3 | 0.5 |
| | Treatment | 37.5±11.1 | 37.4±10.9 | | | |
| Android to gynoid ratio (% fat) | Placebo | 1.1±0.2 | 1.14±0.2 | 0.1 | 0.4 | 0.3 |
| | Treatment | 1.2±0.2 | 1.42±0.8 | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Bone mineral content (g) | Placebo | 2799±552 | 2807±531 | 0.4 | 0.8 | 0.4 |
| | Treatment | 2965±598 | 2951±580 | | | |
| **Cardiovascular measurements** | | | | | | |
| Systolic blood pressure (mmHg) | Placebo | 140±3 | 138±2 | 0.05 | <0.001 | <0.0001 |
| | Treatment | 142±6 | 130±4$^{*\#}$ | | | |
| Diastolic blood pressure (mmHg) | Placebo | 92±5 | 91±4 | 0.006 | <0.0001 | <0.0001 |
| | Treatment | 92±4 | 83±2$^{*\#}$ | | | |
| Heart rate (bpm) | Placebo | 77±8 | 73±7$^{\#}$ | 0.4 | 0.013 | 0.6 |
| | Treatment | 76±8 | 71±7$^{\#}$ | | | |

Values are expressed in mean ± SD. $^{*}$ p<0.05, compared to other group, $^{\#}$ p<0.05, compared to baseline of each intervention.

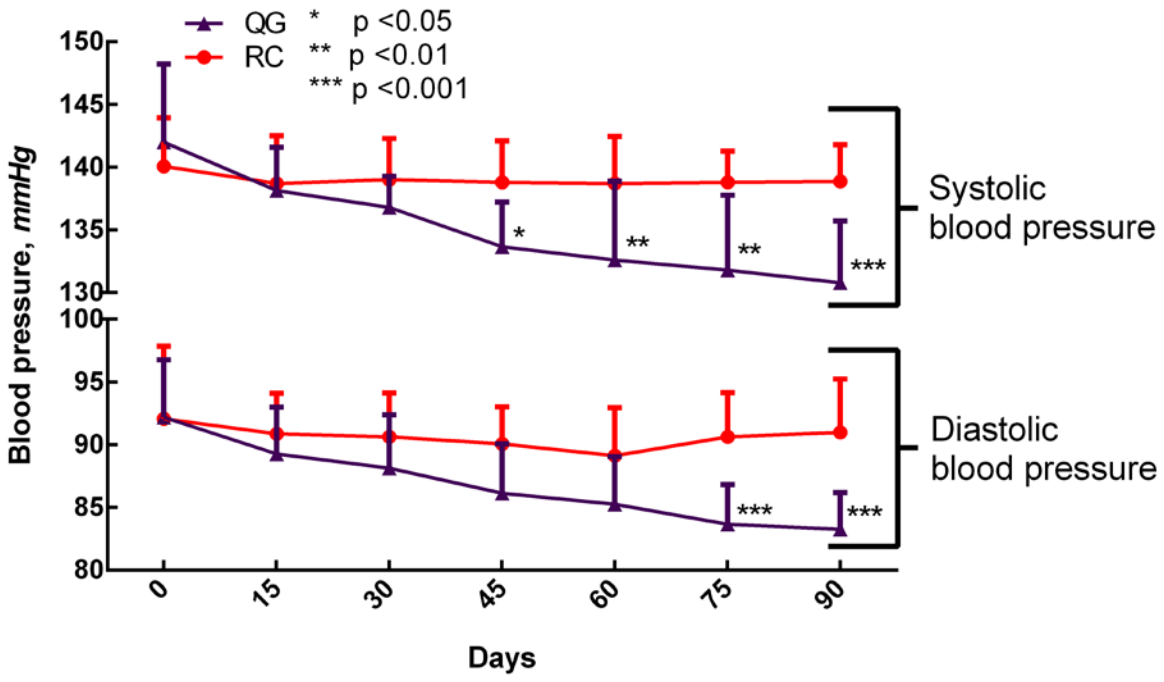



Figure 8.1 Effect of QG and placebo drinks on systolic and diastolic blood pressure. Data are shown as mean ± SD.

**Table 8.4 Fasting plasma analyses at baseline and post-intervention**

| Variable | Group | Baseline | Week 12 | Group | Time | Interaction |
|---|---|---|---|---|---|---|
| Glucose (mmol/L) | Placebo | 5.5±0.3 | 5.6±0.3 | 0.7 | 0.016 | 0.0005 |
| | Treatment | 5.8±0.7 | 5.2±0.4$^{*\#}$ | | | |
| Gamma-glutamyl transpeptidase (U/L) | Placebo | 21.9±15.4 | 14.6±5.7$^{*}$ | 0.2 | 0.3 | 0.031 |
| | Treatment | 26.2±28.2 | 29.2±25.1 | | | |
| Alanine transaminase (U/L) | Placebo | 34.6±11.3 | 35.6±13.8 | 0.9 | 0.5 | 0.7 |
| | Treatment | 33.6±20.4 | 36.6±24.2 | | | |
| Aspartate transaminase (U/L) | Placebo | 18.7±5.8 | 21.3±10.1 | 0.4 | 0.5 | 0.6 |
| | Treatment | 22.1±13.5 | 22.4±8.8 | | | |
| Creatinine (μmol/L) | Placebo | 65.9±22.9 | 76.8±17 | 0.5 | 0.1 | 0.2 |
| | Treatment | 66.9±22.3 | 67.5±24.4 | | | |
| Cholesterol (mmol/L) | Placebo | 5.1±1.1 | 5.1±0.8 | 0.7 | 0.2 | 0.3 |
| | Treatment | 5.2±0.9 | 4.8±0.8 | | | |
| Triglycerides (mmol/L) | Placebo | 1.5±0.6 | 1.5±0.8 | 0.8 | 0.9 | 0.9 |
| | Treatment | 1.4±0.7 | 1.4±0.5 | | | |
| High-density lipoprotein (mmol/L) | Placebo | 1.1±0.3 | 0.9±0.2$^{*}$ | 0.1 | 0.8 | 0.001 |
| | Treatment | 1.1±0.2 | 1.2±0.2 | | | |
| Low-density lipoprotein (mmol/L) | Placebo | 3.3±0.9 | 3.3±0.6 | 0.5 | 0.1 | 0.034 |
| | Treatment | 3.4±0.9 | 2.9±0.6$^{*}$ | | | |

Values are expressed in mean ± SD. $^{*}$ $p<0.05$, compared to other group, $^{\#}$ $p<0.05$, compared to baseline of each intervention.

### 8.5. Discussion

This human trial was conducted to evaluate the effect of Queen Garnet plum juice on weight management and metabolic syndrome risk factors in over-weight or obese men or women. QG plums contain both anthocyanins and quercetin glycosides, suggesting that either could produce the physiological responses. The anthocyanin dose in the current study was

~250 mg/day, which is higher than other human studies and the dose translated from animal studies (211, 309, 380, 382) and the quercetin glycoside dose in this study was around ~77 mg/day. It has been previously shown that a quercetin dose of 150 mg/day (double the dose from 250ml QG juice) is required to produce positive effects such as reduced blood pressure, LDL and plasma inflammatory markers in humans with metabolic syndrome (162, 383). This suggests that the positive effects seen with QG juice could be either primarily mediated by anthocyanins or a synergistic effect in combination with quercetin glycosides. Further investigation is required to determine the possible interactions of these compounds to produce the beneficial effects.

**Table 8.5 Self-reported dietary intake and physical activity**

| Variable | Group | Baseline | Week 12 | Group | Time | Interaction |
|---|---|---|---|---|---|---|
| Total energy intake (kJ/day) | Placebo | 7215±1624 | 7819±2473 | 0.8 | 0.9 | 0.7 |
| | Treatment | 7068±2403 | 7716±1773 | | | |
| Protein (g/day) | Placebo | 88±31 | 97±28 | 0.7 | 0.6 | 1 |
| | Treatment | 84±40 | 95±37 | | | |
| Fat (g/day) | Placebo | 70±43 | 68±39 | 0.4 | 0.8 | 0.9 |
| | Treatment | 65±29 | 67±33 | | | |
| Saturated fat (g day) | Placebo | 27±11 | 28±9 | 0.9 | 0.4 | 0.6 |
| | Treatment | 24±14 | 25±13 | | | |
| Carbohydrates (g/day) | Placebo | 171±62 | 203±57 | 0.5 | 0.7 | 0.9 |
| | Treatment | 163±83 | 199±70 | | | |
| Sugar (g/day) | Placebo | 77±21 | 86±18 | 0.9 | 0.9 | 0.5 |
| | Treatment | 76±23 | 81±21 | | | |
| Fibre (g/day) | Placebo | 22±6 | 23±8 | 0.7 | 0.5 | 0.3 |
| | Treatment | 21±8 | 24±8 | | | |
| Salt (mg/day) | Placebo | 2361±827 | 2404±801 | 0.6 | 0.5 | 0.8 |
| | Treatment | 2167±716 | 2044±624 | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Total work (MET min/week) | Placebo | 3404±2054 | 3089±1983 | 0.8 | 0.4 | 0.1 |
| | Treatment | 2879±1978 | 2994±1865 | | | |

Values are expressed in mean ± SD. MET, metabolic equivalent task.

The major results of the study were the decreases in blood pressure and fasting plasma glucose concentrations in the QG treatment group. In a controlled randomised study, the antihypertensive activity of an extract of *Hibiscus sabdariffa* (9.6 mg of anthocyanins/day) was compared with that of captopril (50 mg/d) in 75 patients with mild to moderate hypertension for a period of four weeks; both showed decreased systolic and diastolic blood pressure with no significant differences related to antihypertensive effectiveness and tolerability between the groups (384). Similarly, a double-blind placebo-controlled parallel study with *Aronia melanocarpa* (100 mg of anthocyanins/day) for six weeks in subjects with myocardial infarction showed decreased systolic and diastolic blood pressure (385). Three years of pomegranate juice (20 mg of anthocyanins/day) reduced carotid intima-media thickness and decreased systolic blood pressure (380). These beneficial effects were related to antioxidant mechanisms because an increase in serum paraoxonase-1 activity, decreases in the levels of antibodies against oxidised LDL, and an increase in total antioxidant status were observed in the patients supplemented with pomegranate juice (380). Additionally, anthocyanins showed an inhibitory effect on both protein and mRNA concentrations for endothelin-1 and increased the protein concentrations for endothelial nitric oxide synthase (386). Similar to the current study, *Aronia melanocarpa* juice supplementation in metabolic syndrome subjects decreased blood pressure and endothelin-1 concentrations suggesting that the blood pressure lowering effects of anthocyanins are related to regulation of the production of ET-1 and NO in the vascular wall (211). In addition, dietary administration of anthocyanin-rich foods such as purple maize, purple sweet potato and red radish showed decreased systolic and mean blood pressures in Spontaneously Hypertensive Rats by preservation of endothelial nitric oxide and prevention of serum lipid oxidation, but inhibition of angiotensin converting enzyme (ACE) activity was not found (387). In contrast, some of the anthocyanin-containing foods and the anthocyanin, delphinidin, possess ACE-inhibitory activity (388-390). Therefore, it is important to investigate further to understand the possible mechanisms of QG juice.

Beyond the blood pressure lowering effects, QG treatment decreased fasting plasma LDL concentrations with no change in HDL, total cholesterol or triglycerides. Similarly,

*Aronia melanocarpa* extract administration decreased total cholesterol, LDL and triglycerides concentrations with no change in HDL (211). Further, triglycerides, total cholesterol and LDL concentrations were reduced with grape powder and grape seed proanthocyanidin extract (~5 mg anthocyanins/day) (391, 392). Further, experiments on rats also showed that QG administration (~8 mg/kg/day) improved glucose metabolism and decreased fasting blood glucose concentrations (Chapter 7). Similarly, *Viburnum dilatum* extract containing ~25 mg/g cyanidin glycosides exerted hypoglycaemic effect in diabetic rats through inhibition of α-glucosidase (382).

Additionally, in diabetic patients, *Aronia melanocarpa* juice consumption decreased mean fasting blood glucose concentrations (393). Similar observations of decreased fasting glucose concentrations were seen in this study with QG supplementation. *In vitro* studies using anthocyanin-rich foods showed beneficial effects for regulating glucose are associated with insulin-releasing stimulatory properties and protective effects on pancreatic β-cells (394, 395). It may be of importance that the volunteers in our study were free of a history of type 2 diabetes and thus, a separate investigation on the glucose reducting properties of QG in diabetic subjects is justified.

The dietary intake and physical activity level was monitored throughout the intervention period and participants were requested to maintain usual dietary habits and physical activity during the intervention time, and there was no significant change reported in either treatment or placebo groups for all domains. A previous human trial evaluating the efficacy of anthocyanin ingestion only asked participants to maintain habitual diet and lifestyle during the intervention period, with no significant change observed in mean daily intakes of nutrients reported over the 12-week treatment period (396).

### 8.6. Conclusion

Not all of the benefits shown in the animal studies were translated to the current study in humans. However, the results from these studies show that anthocyanins from QG may be of benefit to human subjects with cardiometabolic risk factors as far as reduction of blood pressure and fasting plasma glucose and LDL concentration are concerned. Further investigations on QG are important to determine the beneficial effects in improving glucose metabolism in type 2 diabetic humans. In rats, QG treatment for 8 weeks reversed most of the signs of metabolic syndrome, it is important to understand that 8 weeks in rats is equivalent to four years in humans (397), the duration of the current study could be a possible limitation

in reversing the signs of metabolic syndrome. Hence, it would be important to study the potential benefits of long-term supplementation of QG, which may lead to reduced body weight and fat mass.

### 8.7. Study specific acknowledgements

I thank Mr Hugh Macintosh, Nutrafruit Pty, Ltd, Queensland, Australia, for providing QG. I thank Dr Kent Fanning, Department of Agriculture and Fisheries, Queensland, for helping in making and bottling QG and placebo drinks. I thank Ms Hedy Sung, Victoria University, for assisting with blood collection. I thank Ms Katie Astell, Victoria University, for providing her expert advice in statistics.

# CHAPTER 9

# 9. General conclusions and future directions

## 9.1. Conclusions

It is well established that human metabolic syndrome is produced by a combination of genetic and environmental factors. Sedentary lifestyles with less physical or recreational activities and diets rich in fat and sugars giving a decreased quantity and quality of nutrients are the most important environmental risk factors (375). Rapid urbanisation has led to cultivation of unhealthy foods and sedentary lifestyles, escalating a constellation of adverse health consequences of metabolic syndrome risk factors namely obesity, diabetes, cardiovascular and liver diseases (as discussed in Chapter 2). These unhealthy events consist of a complex cascade of molecular and cellular changes that alters hormonal control such as insulin, and increases expression of pro-inflammatory cytokines that result in inflammation (as discussed in Chapter 2). Metabolic syndrome is prevalent throughout the world (as described in Chapter 1), hence understanding and controlling these pathological changes is of great clinical importance.

Research in metabolic syndrome, as in other human diseases, relies on appropriate animal models to test possible interventions in humans and animals. These studies have contributed enormously to the understanding of human biochemistry, physiology, pathophysiology and pharmacology (122). Different models have been developed, characterised, and then successfully used for the development of preventive measures or cures for human diseases (122). One of the important human conditions is metabolic syndrome, which has been successfully mimicked in rodents (as described in Chapter1). The high-carbohydrate, high-fat diet contained 17.5% fructose, 20% beef tallow with predominantly saturated and *trans* fats and an additional 25% fructose in drinking water. Sixteen weeks of feeding this diet to young male Wistar rats induced abdominal adiposity, impaired glucose tolerance, dyslipidaemia, hyperinsulinaemia, increased plasma leptin and malondialdehyde concentrations, increased systolic blood pressure, endothelial dysfunction together with inflammation, fibrosis, hypertrophy, increased stiffness and delayed repolarisation in the left ventricle of the heart (146). Additionally, this diet induced hepatic steatosis with increased plasma activity of liver enzymes, renal inflammation and fibrosis and increased pancreatic islet size, and so this state appropriately mimics the human metabolic syndrome. Initially, I used this diet-induced rat model of metabolic syndrome to study the responses to interventions with green and black cardamom, beetroot and inorganic nitrates, different food sources of anthocyanins including chokeberry, purple maize and Queen Garnet

plums and the purified anthocyanin, cyanidin 3-glucoside, for the last 8 weeks of the 16 week protocol. Subsequently, the most successful intervention from rat studies has been chosen to examine the translational effects in mild-hypertensive obese or over-weight humans.

Initially, in Chapter 5, green and black cardamom were studied for their effects in this diet-induced metabolic syndrome model in rats. Green and black cardamom had volatile components which are the closely related terpenes, α-terpinyl acetate and 1,8-cineole respectively (Table 5.1). Black cardamom improved cardiovascular and hepatic function, decreased body weight gain and total body fat mass whereas green cardamom exacerbated adiposity, decreased liver function and worsened cardiovascular structure and function. These differences might arise from differences in the absorption and metabolism of these volatile oils or other components such as phenolics and flavonoids. From this chapter, it is clear that the definition of cardamom as a spice does not adequately describe the different biological characteristics. However, this study should be extended to determine the effects of two major volatile components, α-terpinyl acetate and 1,8-cineole, at the same dose as pure compounds.

Later, I studied beetroot containing inorganic nitrates and the same dose of sodium nitrate (Chapter 6), which showed similar responses in improving cardiovascular and hepatic function and glucose metabolism, and reduced inflammatory cell infiltration in heart and liver. The changes in cardiovascular function related to improved endothelial function by nitric oxide derived from nitrates. Both interventions also decreased blood pressure which then reduced the preload and afterload of the heart and cardiac energy expenditure, and altered the fibrotic and metabolic gene expression in left ventricle. However, no changes were observed in body weight, total body fat and metabolic gene expression in liver and skeletal muscle with either of the treatments.

Further, chokeberry and purple maize containing polyphenols mainly anthocyanins (studied in Chapter 7) showed improved glucose metabolism, endothelial function, cardiac and hepatic structure, reduced inflammatory cell infiltration in heart and liver, and total body fat mass. In addition, these interventions reduced blood pressure and cardiac fibrosis, and improved cardiovascular function. The remarkably similar responses to chokeberry and purple maize when the anthocyanin dose was similar strongly supports cyanidin glycosides as an excellent dietary supplement for the management of metabolic syndrome related risk factors.

In Chapter 8, I studied the responses to cyanidin 3-glucoside for comparison with the same dose of this anthocyanin from a new variety of Japanese plums, the Queen Garnet plum, in young male rats fed with high-carbohydrate, high-fat diet. Both treatments showed similar responses in improving glucose metabolism, cardiovascular and hepatic structure and function and plasma lipid profile along with decreased total body fat mass, body weight gain and inflammatory cell infiltration in heart and liver. These results clearly suggest that anthocyanin-containing foods produce important health benefits in attenuating metabolic syndrome risk factors and so will be a suitable supplementation to translate into humans. Further, in Chapter 9, I have chosen Queen Garnet plum juice to study these translational effects in mild-hypertensive obese or over-weight humans. Results from this randomised double-blind placebo-controlled study for 12 weeks showed decreased blood pressure, fasting plasma blood glucose and LDL concentrations and increased HDL concentrations, with no change in body weight and fat mass, suggesting that all the effects from Queen Garnet plum juice rat study have not been translated into humans with a short-term intervention.

In summary, phytonutrients present in foods can potentially be used to reduce the symptoms of metabolic syndrome, cardiovascular disease and fatty liver disease. This thesis has shown that increasing these foods in the diet or selected components of these foods can improve many or even all of the symptoms of the metabolic syndrome and associated complications. It is also evident that all the physiological or morphological changes seen in animals were not translated into humans in a short-term trial possibly since metabolic conditions vary from individual to individual. This thesis also suggests that the combination of two or more natural food sources or bioactive molecules in addition to nutritional advice can be used to improve translational effects in reducing or attenuating the risk factors of metabolic syndrome. Overall, food can be used as a medicine if taken in the proper amount and in the proper way.

### 9.2. Observed limitations and future directions

It is now generally accepted that an increased intake of fruits and vegetables decreases the risk factors of metabolic syndrome. However, the mechanism of action of these foods and their bioactive molecules still remains unclear, which is a major limitation for these studies. From this thesis, it is evident that different food sources have different beneficial effects in decreasing metabolic risk factors. The investigations in this thesis raised several questions which are possible limitations and need to be addressed by further investigations. I have shown reduced inflammatory infiltrates in the heart and liver of obese rats with most of the interventions, but the anti-inflammatory mechanisms in these studies remain unanswered. It

is known that adipose tissue functions as an endocrine organ and produces pro-inflammatory cytokines and adipokines which cause inflammation and insulin resistance and affect cardiovascular and hepatic function. Hence, further studies are warranted to understand the mechanisms involved in improving the health benefits of humans. These studies could include:

- α-terpinyl acetate and 1,8-cineole investigation to understand the beneficial effects of black cardamom.
- Nitric oxide molecular mechanism in improving the glucose metabolism and reducing inflammation.
- Investigating NF-κB mediated inflammation such as TNF-α, IL-1, IL-6, IL-8 and COX-2.
- Effects on diversity of gut microbiota population and metabolism with anthocyanins.
- Combination of beetroot juice and anthocyanins in preclinical studies and proceeding with larger clinical trials to study the synergistic effects.
- Understanding the mechanism of lipolysis and steatorrhea using these bioactive molecules (either 1,8-cineole or nitrates or anthocyanins) in the reduction of visceral adiposity.
- Long-term effects of QG supplementation in a diabetic cohort for understanding the mechanisms of the improvement in glucose utilization.

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