Cyanidin 3-glucoside in diet-induced metabolic syndrome in rats

A dissertation submitted by Ryan du Preez, Bachelor of Health
(Biomedical Science)

For the award of Bachelor of Science (Honours)

2015

School of Health and Wellbeing

University of Southern Queensland

Toowoomba, Queensland 4350

Australia

TABLE OF CONTENTS

ABSTRACT

Metabolic syndrome combines the risk factors for type 2 diabetes and cardiovascular disease, including obesity, insulin resistance, impaired glucose tolerance, dyslipidaemia and hypertension. Obesity is now understood to be a chronic low-grade inflammatory state with sustained oxidative stress. There are few drug therapies for obesity and all may produce unacceptable adverse effects. Thus, functional foods may serve as a better alternative for treatment of metabolic syndrome. Anthocyanins are purple-coloured polyphenolic compounds found in dark-coloured fruits and vegetables, such as purple carrot, Queen Garnet plum, chokeberry and purple corn. As anthocyanins produce anti-oxidant and anti-inflammatory responses, this study investigated whether anthocyanin-rich black rice can reverse the signs of diet-induced metabolic syndrome. This study used high-carbohydrate, high-fat diet-fed male Wistar rats to mimic the symptoms of human metabolic syndrome. Rats developed hypertension, diabetes along with damage to cardiovascular and hepatic systems. However, rats supplemented with cyanidin 3-glucoside rich black rice extract mid-way through the protocol had noticeable overall health improvements. Black rice extract reversed many changes the rats developed while on the diet including reduced body weight (10%), abdominal obesity (6%) and normalising systolic blood pressure; improved hepatic structure (decreased inflammation) and function; (decreased liver enzyme activity) improved cardiovascular structure (decreased inflammation and fibrosis) and function along with reduced overall cholesterol and decreased liver enzyme activity.

Keywords: diet-induced metabolic syndrome, anthocyanin, cyanidin 3-glucoside, obesity

DECLARATION

I hereby certify that the experimental work, results, analyses, discussion and conclusions reported in this dissertation are entirely my own effort, except where otherwise acknowledged. I also certify this work is original and has not been previously submitted.

Candidate: Ryan du Preez

_____   Date:

Endorsement

Principal supervisor: Dr Sunil Panchal

_____   Date:

Associate supervisor: Professor Lindsay Brown

_____   Date:

ACKNOWLEDGEMENTS

LIST OF ABBREVIATIONS

ALP – alkaline phosphatase

ALT – alanine transaminase

AST – aspartate transaminase

BMI – body mass index

C – corn starch diet-fed rats

CBRE – corn starch diet-fed rats treated with black rice extract

CRP – C-reactive protein

CS – corn starch diet

DXA – dual-x-ray absorptiometry

EGIR – European group for the study of insulin resistance

FOXO1 – forkhead box protein O1

H – high-carbohydrate, high-fat diet-fed rats

HBRE – high-carbohydrate, high-fat diet-fed rats treated with black rice extract

HCHF – high-carbohydrate, high-fat diet

HDL – high-density lipoprotein

Hs-CRP – high-sensitivity- C-reactive protein

IDF – International Diabetes Foundation

IL-6 – interleukin-6

LDH – lactate dehydrogenase

LDL – low-density lipoprotein

NCEP ATP III – The National Cholesterol Education Program Adult Treatment Panel III

NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells

OGTT – oral glucose tolerance test

Ox-LDL – oxidised low-density lipoprotein

PPAR-α – peroxisome proliferator-activated receptor-alpha

SBP – systolic blood pressure

TNF-α – tumour necrosis factor-alpha

WHO – World Health Organisation

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

LITERATURE REVIEW AND INTRODUCTION

1.1 Metabolic syndrome

Metabolic syndrome refers to the co-occurrence of risk factors for cardiovascular disease and type 2 diabetes. These risk factors include hypertension, central obesity, insulin resistance, elevated fasting blood glucose concentrations, impaired glucose tolerance and dyslipidaemia (1, 2). Various definitions have been developed for the classification of metabolic syndrome. Four of these definitions given by World Health Organisation (WHO), European Group for the study of Insulin Resistance (EGIR), National Cholesterol Education Program Adult Treatment Plan III (NCEP ATP III) and International Diabetes Federation (IDF) are summarised in Table 1. The reported prevalence of metabolic syndrome varies according to the different parameters used to define this syndrome.

Obesity as the major symptom of metabolic syndrome has reached epidemic proportions (3). Obesity is characterised by low-grade inflammation with increased oxidative stress along with excessive fat deposition; obese individuals have a body mass index (BMI) of $\geq 30 \text{kg/m}^2$ (4). The prevalence of metabolic syndrome in adults from the United States of America during 1988-1994 was estimated at 18%, while during 1999-2006 it had increased to 34.1% (5). More than 35% of men and women in the United States of America are classified as having obesity with a similar proportion of the population overweight too (6). In Australia, during 1999-2000, the prevalence of metabolic syndrome in adults aged over 25 years was estimated to be between 22% (using IDF definition) and 31% (using NCEP ATP III and WHO definitions) (7). The Australian Diabetes, Obesity and Lifestyle study in 2000 found that between 19.3% (using IDF definition) and 29.1% (using NCEP ATP III definition) of adults

aged 25 and over met the criteria for a diagnosis of metabolic syndrome (8). In Australia during 2011-12, 64% of people aged 18 years or older were overweight or obese (BMI $\geq$ 25 kg/m^2) (9). The prevalence of obesity has increased over time from 56.3% in 1995, 61.2% in 2007-08 and 62.8% in 2011-12 (9).

Different definitions of metabolic syndrome provide a different prevalence rate due to the differences in definition. The IDF criteria for defining metabolic syndrome demonstrates the importance of treating risk factors in aged (64 years and older) individuals, especially higher fasting glucose and obesity, is important (10). A study compared the prevalence of metabolic syndrome and association with diabetes and cardiovascular disease using two definitions; it found prevalence of metabolic syndrome is higher using NCEP ATP III (odd-ratio 7.0, 95% confidence-interval 5.3–9.4) definition compared to the IDF definition (odd-ratio 4.7, 95% confidence-interval 3.4–6.3), which has a focus on prediabetes and type 2 diabetes, in a Bangladeshi population (11). In a study using the NCEP ATP III and IDF definitions it was found the prevalence of metabolic syndrome is high (between 38.9% using IDF definition and 46.1% using NCEP ATP III definition) in a Sri Lankan population with an emphasis on glycaemic control in the IDF definition (12). A study into the prevalence of metabolic syndrome in elderly women (60-83 years) using different criteria to define metabolic syndrome found that the NCEP ATP III criteria is the best indicator to predict a cardiovascular event in this age group (13). In 2002, the prevalence of metabolic syndrome ranged from 8.8% to 14.3% in Finnish men aged 42 to 60 years depending on the definition used (14). The American Heart Association reported in 2012, 35% (using a definition similar to NCEP ATP III) of the adults in the United States of America would be classified as having metabolic syndrome (15).

In Russia during 2010, 9.5% of men and 24% of women had metabolic syndrome (16). In Asian populations, the IDF definition will miss a sizable number of Asians if used in screening probably due to the inadequacy of current methods to identify central obesity (17). In South-East Asia, metabolic syndrome is estimated at 24% using the NCEP ATP III definition (18). A study reported the prevalence of metabolic syndrome to be high in Karachi, Pakistan with estimates between 34.8% and 49% using the IDF and modified NCEP ATP III definitions, respectively (19). This epidemiological evidence from Russia, Pakistan, Bangladesh, Sri-Lanka, South-East Asia, Finland, United States of America and Australia emphasises that metabolic syndrome is a global problem.

The alteration of diet composition is recognised as one of the major causes of increasing metabolic syndrome prevalence (20). Replacement of complex dietary carbohydrates (polysaccharides) with simple sugars (monosaccharides or disaccharides) in conjunction with an excessive consumption of animal products leads to changes in dietary metabolic responses (20). These dietary changes, specifically increases in sugars and lipids (long–chain saturated and *trans* fats) can induce metabolic disturbances that potentiate the development of inflammation and oxidative stress (21). Inflammation and oxidative stress underpin many of the pathologies associated with metabolic syndrome (22-24) including cardiovascular structure and function including cardiac hypertrophy, endothelial dysfunction, cardiac fibrosis and ventricular contractile dysfunction (25). Inhibition of inflammation and oxidative stress using synthetic and natural compounds can attenuate the development of metabolic syndrome (26-31).

Different diagnostic criteria for metabolic syndrome have been proposed by different organisations over the past decade (32). The most appropriate definition for metabolic syndrome must consider age and ethnic differences and vary each parameter threshold value

accordingly (32). A single set of threshold values should be decided upon for very specific age ranges and ethnic groups. These improvements should streamline diagnosis and expedite treatments.

Table 1. Definitions of metabolic syndrome Source: (1)

	WHO (1998)	EGIR (1999)	NCEP ATP III (2005 revision)	IDF (2005)
Compulsory	Insulin resistance* (IGT, IFG, T2D or other evidence of IR)	Hyperinsulinemia (plasma insulin >75th percentile)	None	Central obesity
Criteria	Insulin resistance or diabetes, plus two of the five criteria below	Hyperinsulinemia, plus two the four criteria below	Any three of the five criteria below	Obesity, plus two of the four criteria below
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 (>40 inches in males, >35 inches in females	Waist circumference (>94 cm in males, > 80 cm in females
Hyperglycaemia	Insulin resistance already required	Insulin resistance already required	Fasting glucose >100 mg/dl or Rx	Fasting glucose >100 mg/dl
Dyslipidaemia (Triglycerides)	TG >150 mg/dl or HDL-C (<35 mg/dl in males, <39 mg/dl in females	TG >177 mg/dl or HDL-C <39 mg/dl	TG >150 mg/dl or Rx	TG > 150 mg/dl or Rx
Dyslipidaemia (Cholesterol)			HDL cholesterol (<40 mg/dl in males, <50 mg/dl in females or Rx	HDL-C (<40 mg/dl in males, < 50 mg/dl in females or Rx
Hypertension	>140/90 mmHg	>140/90 mmHg or Rx	>130/85 mmHg or Rx	>130/85 mmHg or Rx
Other criteria	Microalbuminuria†			

*IGT, impaired glucose tolerance; IFG, impaired fasting glucose; T2D, type 2 diabetes; IR, insulin resistance; other evidence includes euglycemic clamp studies.

†Urinary albumin excretion of ≥ 20 μg/min or albumin-to-creatinine ratio of ≥ 30 mg/g.

‡Reliable only in patients without T2D.

Criteria for central obesity (waist circumference) are specific for each population; values given are for European men and women.

Rx, pharmacologic treatment.

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 Foundation; TG, triglycerides; HDL-C, high density lipoprotein cholesterol.

Cardiovascular disease, including coronary heart disease, ischaemic heart disease and stroke, remains the leading cause of death around the world. The Global Burden of Disease study conducted in 2010 estimated that 29.6% of all deaths worldwide were caused by cardiovascular disease (33). There were 52.8 million deaths globally in 2010 of which ischaemic heart disease and stroke accounted for 12.9 million (34). Deaths caused by strokes are expected to increase to 23.6 million people worldwide in 2030 (35).

Global obesity (BMI of $\geq 30\text{kg/m}^2$) prevalence has more than doubled since 1980. In 2008, more than 1.4 billion adults aged 20 or more years were overweight or obese with 500 million adult men and women defined as obese (36). Obesity is a complex condition which is associated with co-morbidities including cardiovascular disease, type 2 diabetes and non-alcoholic fatty liver disease (37). In 2003, global prevalence of type 2 diabetes was 194 million which is predicted to increase to 334 million in 2025 (38). Type 2 diabetes further increases the risk of cardiovascular disease. The increasing worldwide prevalence of metabolic syndrome increases the proportion of people who are suffering from type 2 diabetes and cardiovascular disease and hence increases the burden on the healthcare system (3). Cardiovascular disease represents a major economic burden with an estimated healthcare cost of USD 457.4 billion in 2006 (39) and this cost is expected to increase with the increase in prevalence of metabolic syndrome.

Recently, it has been identified that the gut microflora plays an important role in regulating health as altered gut microbiota can contribute to the induction of many disease states including obesity (40). The gut microbiota contains 10^{13} to 10^{14} microorganisms which equates to approximately 1.5 to 2 kilograms of bacteria. Gut microbiota interact with all consumed foods and therefore represent an important aspect of metabolism. This gut microbiota can modify the nutrient availability and uptake and consequently may define the

metabolism of the host. Similarly, a particular type of diet can selectively increase a particular population of gut microbiota (40). The selective increase of beneficial bacteria in the gut can be used as a strategy to improve metabolic syndrome.

However, the current body of knowledge is insufficient to evaluate the additional functions of foods. In order to identify foods that may provide health benefits beyond basic nutritional requirements, it is important to identify their physiological activities in chronic diseases (4). Many foods from plants contain polyphenols, a group of non-nutritive compounds that may produce the physiological responses to foods (41). Polyphenols are secondary plant metabolites synthesised from shikimate pathway products containing more than one phenolic ring and devoid of any nitrogen-based functional groups in their most basic structure (41). Flavonoids are a class of polyphenols that are found in fruits and vegetables which have potential beneficial health effects. Anthocyanins belong to the flavonoids class of polyphenols and they are water-soluble dark coloured natural pigments from fruits and vegetables (42).

1.2 Anthocyanins: structure and sources

Anthocyanins are water-soluble flavonoid pigments that, depending on pH, contribute a diverse range of colours including purple, red and dark blue seen in many fruits and vegetables (43). For example, chokeberry is extremely rich in the content of these pigments (44, 45). Anthocyanins are used as natural colouring agents because of the high content of anthocyanins in some fruits and vegetables (46). Anthocyanins have anti-oxidative and anti-inflammatory activities and these properties of anthocyanins can be helpful in providing health benefits against chronic diseases (47). Anthocyanins are susceptible to heat, light and pH changes as well as oxidation (48). Anthocyanidin is the aglycone (sugar-free) form of the anthocyanin compound. There are at least six main types of anthocyanidins: pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin. Cyanidins are the most widely

distributed species (49). Three of the major anthocyanidins found in nature are cyanidin, delphinidin and malvidin (48) and are shown below in Figure 1.
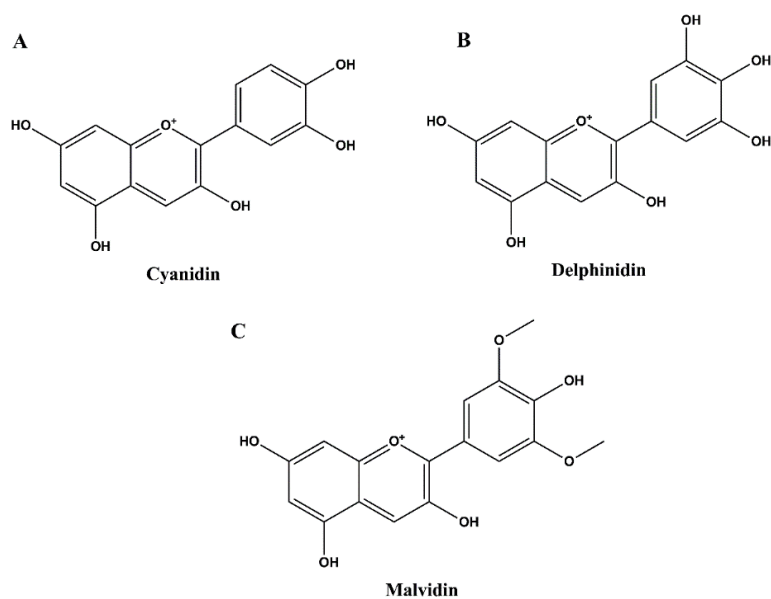
Figure 1. Structure of anthocyanidins

Chemically, anthocyanins are based on a single basic core structure, the flavylium ion. Anthocyanins have a C6-C3-C6 skeleton with a positive charge in their structure at acidic pH. The structure of common anthocyanins is presented in Figure 2 and the three side groups are presented in Table 2.
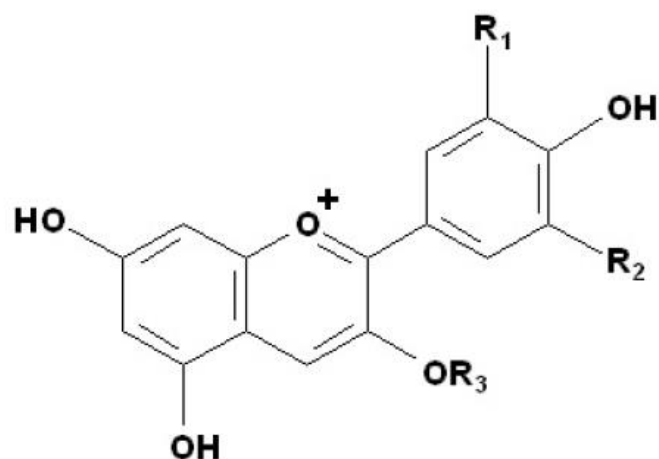
Figure 2. Structure of anthocyanins Source: (43)

Table 2. Structure of anthocyanins Source: (43)

Names	R1	R2	R3
Pelargonidin	H	H	H
Cyanidin	OH	H	H
Delphinidin	OH	OH	H
Peonidin	OCH_3	H	H
Petunidin	OCH_3	OH	H
Malvidin	OCH_3	OCH_3	H
Pelargonidin-3-0-glucoside	H	H	Glu
Cyanidin-3-0-glucoside	OH	H	Glu
Delphinidin-3-0-glucoside	OH	OH	Glu
Peonidin-3-0-glucoside	OCH_3	H	Glu
Petunidin-3-0-glucoside	OCH_3	OH	Glu
Malvidin-3-0-glucoside	OCH_3	OCH_3	Glu

Major sources of dietary anthocyanins include fruits and vegetables including berries (blueberry, billberry, Saskatoon berry and chokeberry), purple carrot, blackcurrant, plum, red radish, purple corn, purple cabbage and purple sweet potato (35, 50, 51). Anthocyanin concentrations in different foods are presented in Table 3.

Table 3. Concentrations of anthocyanins in various foods Source: (52)

Food source	Major type(s) of anthocyanidin	Concentration (mg/100g fresh weight)
Apple (Red Delicious)	Cyanidin	12.1
	Pelargonidin	0.2
Chokeberry	Cyanidin	1478
	Pelargonidin	2.3
Black bean	Delphinidin	18.5
	Petunidin	15.4
	Malvidin	10.6
Black plum	Cyanidin	124.5
Wild blueberry	Delphinidin	141.1
	Cyanidin	66.3
	Petunidin	87.6
	Peonidin	36.9
	Malvidin	154.6

1.3 Metabolism and pharmacokinetics of anthocyanins

The anthocyanin group is now known to have many beneficial health effects (53). However, information on pharmacokinetics in humans is scarce (54, 55). Studies have investigated the absorption of flavonoids after controlled oral administration of pharmacological doses rather than following regular dietary consumption of flavonoids (54). Anthocyanins are extensively metabolised with only 1.5 to 5.1% of anthocyanins recovered in urine within 12 hours after wine consumption (54). Depending on nutritional habits in humans, total daily flavonoid consumption is estimated to be a few hundred milligrams (56). Although the total anthocyanin consumption is substantially lower, consumption of the Queen Garnet plum containing of 272 mg/100 g fresh fruit, so 5-10 fold higher than other plums (57), should markedly increase anthocyanin intake. When taken in the diet, anthocyanins are absorbed from

the stomach (58) and intestinal cells (59) and rapidly detected in plasma *in vivo* (54). The major metabolites of cyanidin 3-glucoside are protocatechuic acid and 4-hydroxybenzoic acid (60). These two metabolites exert both anti-inflammatory and anti-oxidative effects and are therefore considered the active metabolites responsible for the systemic health benefits (60).

Cyanidin 3-glucoside is highly metabolised during the first pass effect (60). Anthocyanins, in general, are present in the circulation for less than 48 hours after ingestion (61). A tracer metabolite (^{13}C-labeled anthocyanin) was used to investigate the absorption, distribution, metabolism and elimination in humans (61). This study established a minimum relative bioavailability for cyanidin 3-glucoside of 12.38 ± 1.38% on the basis of the total elimination of the absorbed ^{13}C dose (49). The metabolites identified as degradation products included phenolic acid, hippuric acid, phenylacetic acid and phenylpropenoic acid (49). This study concluded that anthocyanins are more bioavailable than previously perceived, and their metabolites are present in the circulation for more than 48 hours after ingestion (49). Other pharmacokinetic studies have shown that plasma anthocyanins peak approximately one hour after oral consumption and then rapidly decrease (62).

Anthocyanin glycosides are hydrolysed into their anthocyanidin (aglycons) form by the intestinal microbiota in as little as 20 minutes to as long as 2 hours after incubation depending on the sugar moieties present (49). The Caco-2 cell line is a human epithelial cell model derived from a colon carcinoma, proven to be a good alternative to animal studies predicting intestinal absorption of anthocyanins (63). A study using this cell line found that although bioavailability of anthocyanins is very low, anthocyanins from several different food sources (including blackberry, blueberry, plum, black rice and grape) exert health benefits through anti-proliferative, anti-oxidant and anti-inflammatory actions (63). Although it is unknown whether anthocyanins accumulate in the cardiac or vascular tissues during long-term feeding, animal

studies have demonstrated that anthocyanins affect vascular reactivity (64). Relatively low-dose anthocyanin interventions with patients clinically diagnosed with vascular diseases have been associated with significant reductions in ischemia, blood pressure, lipid levels and inflammatory status (64).

1.4 Anthocyanin functions

Anthocyanins have anti-oxidative and anti-inflammatory properties (53, 65), decrease plasma concentrations of total cholesterol, LDL-cholesterol and triglycerides and inhibit enzymes responsible for fatty acid synthesis in rats (42). In previous studies, anthocyanins from purple carrots and Queen Garnet plums have shown beneficial effects against diet-induced metabolic syndrome (28, 57). In high-carbohydrate, high-fat diet-fed rats, purple carrot juice (5 % in food for 8 weeks; providing 15 mg/kg/day of cyanidin 3-glucoside) reduced systolic blood pressure, abdominal obesity, plasma lipids and body weight while improving glucose tolerance and structure and function of the heart and liver (28). In a recently published study, the Queen Garnet plum juice has shown very similar results with a lower concentration of cyanidin 3-glucoside (~8 mg/kg/day) being used for the treatment of obese rats (57). Based on these results, and the fact that the same active ingredient (cyanidin 3-glucoside) is being investigated from different sources, it can be suggested that many other food sources will have similar health effects in terms of physiological responses, e.g. reduction in systolic blood pressure. However, the variation in, types and concentrations of anthocyanins between dark-coloured food products may result in different responses which needs to be proven. This study has extended the evidence in supporting the responses provided by cyanidin 3-glucoside in attenuating metabolic syndrome in diet-induced rats.

1.5 Black Rice

Black rice (*Oryza sativa* L.) also known as purple rice has a seed coat containing anthocyanins, mainly cyanidin 3-glucoside (66). Black rice also contains phytosterols (24-methylene-ergosta-5-en-3β-ol, 24-methylene-ergosta-7-en-3β-ol, fucosterol, gramisterol, campesterol, stigmasterol and *β*-sitosterol) and triterpenoids (cycloeucalenol, lupenone, lupeol and 24-methylenecycloartanol) at much smaller concentrations (67). Some of these phytosterols and triterpenoids have shown their physiological responses (67). This study used a concentrated black rice extract provided by ChromaDex with ~36% of the extract as cyanidin 3-glucoside.

1.6 Cyanidin 3-glucoside

Cyanidin 3-glucoside (Figure 2, Table 2), a secondary metabolite produced by many plants, is an anthocyanin (68). It is a one of the major anthocyanins in many berries, purple carrots, plums and black rice (66, 69, 70). Below is the literature related to proven biological activities of cyanidin 3-glucoside.
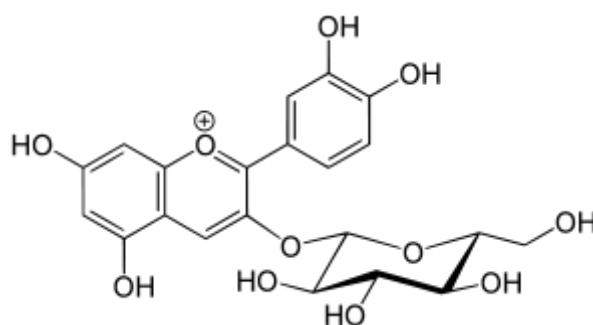
Figure 3. Structure of cyanidin 3-glucoside

1.7 *In vitro* studies

Anthocyanins from black soybean (72% was cyanidin 3-glucoside) at a dose of 10 μg/mL in a cell culture of human umbilical vein endothelial cells were used to investigate potential health benefits (71). In this study, black soybeans preferentially inhibited TNF-α-mediated induction of vascular cell adhesion protein 1 over intercellular adhesion molecule 1 (71). These results indicate the possible role that anthocyanins have for the molecular regulation of inflammatory pathways. The flavonol and anthocyanin subclasses have also been inversely associated with biomarkers of adiposity-associated inflammation, including increased TNF-α, IL-6, and hs-CRP (72). Delphinidin reduced the oxidised low-density lipoprotein (ox-LDL) levels in human umbilical vein endothelial cells (49, 73) and hence protected human umbilical vein endothelial cells against ox-LDL-induced injury (49).

Cyanidin 3-glucoside extracted from purple corn has shown health benefits in *in vitro* and animal studies especially as an anti-oxidant (35). These findings clearly indicate that cyanidin 3-glucoside has significant potency in anti-diabetic effects by modulating the c-Jun N-terminal kinase/FOXO1 signalling pathway and the related inflammatory adipocytokines (35).

Anthocyanins from black soybean seed coats (72% cyanidin 3-glucoside, 20% delphinidin 3-glucoside and 6% petunidin 3-glucoside) reduced TNF-α-mediated vascular cell adhesion protein 1 induction in a concentration-dependent manner (10, 50 and 100 μg/mL) in human umbilical vein endothelial cells (71). In the same cell line, delphinidin inhibited ox-LDL-induced cell viability loss primarily by up-regulating proteins involved in inhibiting apoptosis including Bcl-2 and Bax proteins (62). These results suggest that the responses to anthocyanins supplementation may be mediated by its anti-inflammatory and anti-apoptotic properties and up-regulation of the insulin-signalling cascade (62).

1.8 Animal studies

Chokeberry extract (106.8 mg cyanidin 3-glucoside/100 mL juice) reduced body weight gain and abdominal fat, reduced the cardiovascular risk factor related to metabolic syndrome (having an anti-hyperlipidaemic effect) in plasma and modulated multiple signalling pathways related to adipose dysfunction in a rat model (46). A study found that chokeberry fruit juice (5, 10, and 20 mL/kg body weight) for 30 days reduced total cholesterol, LDL-cholesterol and triglycerides in 4% cholesterol diet-fed rats (46). Chokeberries (100 and 200 mg/kg/day) also reduced visceral adiposity, blood glucose, serum triglycerides, total cholesterol and LDL-cholesterol in fructose-fed rats (44). In the same study, anthocyanin supplementation increased plasma adiponectin levels, inhibited the plasma levels of pro-inflammatory cytokines such as TNF-α and IL-6, down-regulated adipogenic markers (GSK3β, FABP4, FAS and LPL) with mRNA expression and up-regulated important intermediates of the insulin signalling cascade (IRS1, IRS2, PI3K, GLUT1, GLUT4 and GYS1) (44). In diabetes induced by high-fructose diet and simultaneous single injection of streptozotocin (20 mg/kg), dietary supplementation with chokeberry fruit extract (0.2% in food; ~400 mg/g of anthocyanin glycosides in the extract) decreased antioxidant status of vital organs, total plasma cholesterol and blood glucose concentrations (45). In Zucker rats fed a high-fat diet, supplementation with 2% dietary blueberry or 1% whole tart cherry powder reduced plasma triglycerides, fasting insulin, homeostatic model assessment insulin resistance. Furthermore, there was a reduction in abdominal fat mass and increased adipose and skeletal muscle peroxisome proliferator-activated receptor (PPAR)-α and PPAR-γ activity along with reductions in TNF-α, IL-6 and nuclear factor-κB (NF-κB) in the plasma and the adipose tissue and improved glucose tolerance (74). At the same dosage after 90 days, tart cherries reduced fasting blood glucose, hyperlipidaemia, hyperinsulinemia and fatty liver with increased hepatic PPAR-α expression in salt-sensitive Dahl rats (75).

Anthocyanin extract from black rice (5 g/kg diet) lowered body weight gain and serum triglyceride, raised hepatic carnitine palmitoyltransferase 1 expression and inhibited platelet hyperactivity suggested by decreased thromboxane A2, the thrombogenic ratio of thromboxane A_2 and prostacyclin, serum calmodulin and soluble P-selectin expression in high fat diet-fed rats (76). Studies in animal models have shown the flavonoid subclass, anthocyanins, improved glucose metabolism, insulin resistance and β-cell dysfunction through GLUT4 regulation.

Blackcurrants improved endothelial function by increasing nitric oxide synthesis which subsequently induces endothelium-dependent vasorelaxation via the H_1-recprotes on the endothelium in isolated rat aortic rings (77). These effects were abolished by nitric oxide-cGMP inhibitors suggesting the role this signalling pathway in anthocyanin-mediated vasodilation (78).

Cyanidin 3-glucoside reduced body weight gain and attenuated obesity-associated dyslipidaemia and insulin resistance in high-fat diet-fed rats by increasing serum adiponectin level (48, 79). Rats supplemented with cyanidin and its glucoside increased adiponectin and leptin secretion along with the stimulation of AMP-activated protein kinase (80). Cyanidin 3-glucoside ameliorated insulin sensitivity by down-regulating the retinol binding protein 4 expression in diabetic mice (41). Cyanidin 3-glucoside attenuated obesity-associated insulin resistance and hepatic steatosis in high-fat diet-fed and *db/db* mice potentially via the up-regulation of transcription FOXO1 (81). A study with bayberry fruit extract, containing cyanidin 3-glucoside, protected pancreatic β-cells and ameliorated oxidative stress-induced injury along with reduced blood glucose concentration in diabetic mice (69). Cyanidin 3-glucoside lowered fasting glucose levels and markedly improved insulin sensitivity in both high fat diet-fed and *db/db* mice compared with untreated controls (81). Cyanidin 3-glucoside

has shown the potential to improve insulin resistance and its consequences in 3T3-L1 adipocytes through the upregulation of GLUT4 gene expression (68).

White adipose tissue mRNA levels and serum concentrations of inflammatory cytokines (TNF-α, IL-6 and monocyte chemoattractant protein-1) were reduced by cyanidin 3-glucoside, as were macrophage infiltration in adipose tissue (81). Concomitantly, hepatic triglyceride content and steatosis were alleviated by cyanidin 3-glucoside (81). Moreover, cyanidin 3-glucoside treatment decreased c-Jun N-terminal kinase activation and promoted phosphorylation and nuclear exclusion of FOXO1 after refeeding (81).

From the research studies on animal (rats and mice), it is evident that anthocyanins have many health benefits, in summary, improved lipid profile, blood glucose, endothelial function, inhibited pro-inflammatory cytokines, attenuated inflammation, all while a high-fat diet was still fed the animals. Anthocyanins also up-regulated gene expression in glucose transporters leading to improvements of diabetes. These studies have shown many health benefits. Animal studies have not been able to translate to human clinical trials. This step is necessary to provide evidence-based research to support the right dosages and study technique to treat human diseases. The gap in knowledge occurs in human studies where anthocyanins are notoriously difficult to study. Details on the mechanisms of action for bioactivity, uptake, absorption, bioavailability, whole body distribution, and tissue localisation are still not fully understood (82).

1.9 Human studies

An epidemiological study of a large group (n=200,894 people) found that a higher consumption of anthocyanins and anthocyanin-rich fruit was associated with a lower risk of type 2 diabetes (83). Human studies aim to extend the therapeutic responses produced in animal

models to dietary anthocyanins. In patients with metabolic syndrome, chokeberry extract (3 x 100 mg/day) was given for two months. The results were decreased systolic and diastolic blood pressure, endothelin-1, total cholesterol, LDL-cholesterol, triglycerides, thiobarbituric acid reactive substance, catalase activity and induced superoxide dismutase activity (42). Clinical studies show little effect of pro-inflammatory markers on healthy human participants (64). However, one study showed significant improvements of plasma risk biomarkers after supplementation with anthocyanins (84). Consumption of anthocyanin-rich foods and beverages improved endothelium-dependent vasodilation in humans (78). In hypercholesterolaemic patients, 320 mg/day of purified anthocyanins isolated from bilberries and blackcurrants increased brachial artery flow-mediated dilatation and HDL-cholesterol concentrations and decreased the serum soluble vascular cell adhesion protein 1 and LDL-cholesterol concentrations (64).

Anthocyanins have health benefits evidenced in *in vitro*, animal and human studies. Based on a previous study in this laboratory and literature discussed for anthocyanins, this honours project characterised the effects of cyanidin 3-glucoside from black rice in diet-induced metabolic syndrome rats. Structural improvements in the rat heart were identified through histology and heart function through isolated perfusion heart studies (*ex vivo*). Systolic blood pressure was measured and isolated thoracic rings were used to measure vascular reactivity. Biochemical parameters (enzyme activities of alanine transaminase, aspartate transaminase and alkaline phosphatase) were assessed along with plasma lipid profile. Liver structural changes were defined through histopathological analysis. Small and large intestine structural changes were identified through histopathological analysis.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Black rice extract for this study was kindly provided by ChromaDex, Irvine, CA, USA. Beef tallow was purchased from Carey Brothers, Warwick, QLD, Australia. Condensed milk was purchased from Coles Kearney Springs, Toowoomba, QLD, Australia. Meat-free powdered rat food was purchased from Speciality Feeds, Glen Forrest, WA, Australia. Hubble, Mendel and Wakeman salt mixture was purchased from MP Biomedicals, Seven Hills, NSW, Australia. Fructose was purchased from Tate & Lyle, Wacol, QLD, Australia. Corn starch was purchased from Agri Food Ingredients, Kew East, VIC, Australia. All laboratory chemicals were purchased from Sigma-Aldrich Australia, Castle Hill, NSW, Australia, unless otherwise specified at their first appearance in text.

2.2 Rats, experimental groups and housing

All experimental protocols were approved by the Animal Ethics Committee of the University of Southern Queensland (Project number – 13REA005) under the guidelines of the National Health and Medical Research Council of Australia. The experimental protocol consisted of 48 male Wistar rats (8-9 weeks old; 339 ± 2 g) purchased from the Animal Resource Centre, Murdoch, WA, Australia. Rats arrived a week before they were started on diets and these rats were acclimatised for one week on laboratory chow diet. Rats were randomly divided into following four experimental groups ($n = 12$ rats/group):

1. **C** – rats received corn starch (CS) diet for 16 weeks

2. **CBRE** – rats received CS diet for the initial 8 weeks and then CS diet was supplemented with 400 mg black rice extract/kg food for the last 8 weeks. 400 mg black rice extract containing 36% cyanidin 3-glucoside was added to each kilogram of CS food. This concentration was calculated based on the dose of cyanidin 3-glucoside used in the previous study with Queen Garnet plum and pure cyanidin 3-glucoside (57) of 8 mg/kg body weight/day.

3. **H** – rats received high-carbohydrate, high-fat (HCHF) diet for 16 weeks

4. **HBRE** – rats received HCHF diet for the initial 8 weeks and then HCHF diet was supplemented with 400 mg black rice extract/kg food for the last 8 weeks.

This protocol is represented in detail in Figure 4. All rats were housed in a temperature-controlled (21 ± 2ºC) room with an automated 12-hour light & dark cycle environment. All rats were housed in individual cages in the animal house at the University of Southern Queensland, Toowoomba campus and had *ad libitum* access to diet and water.
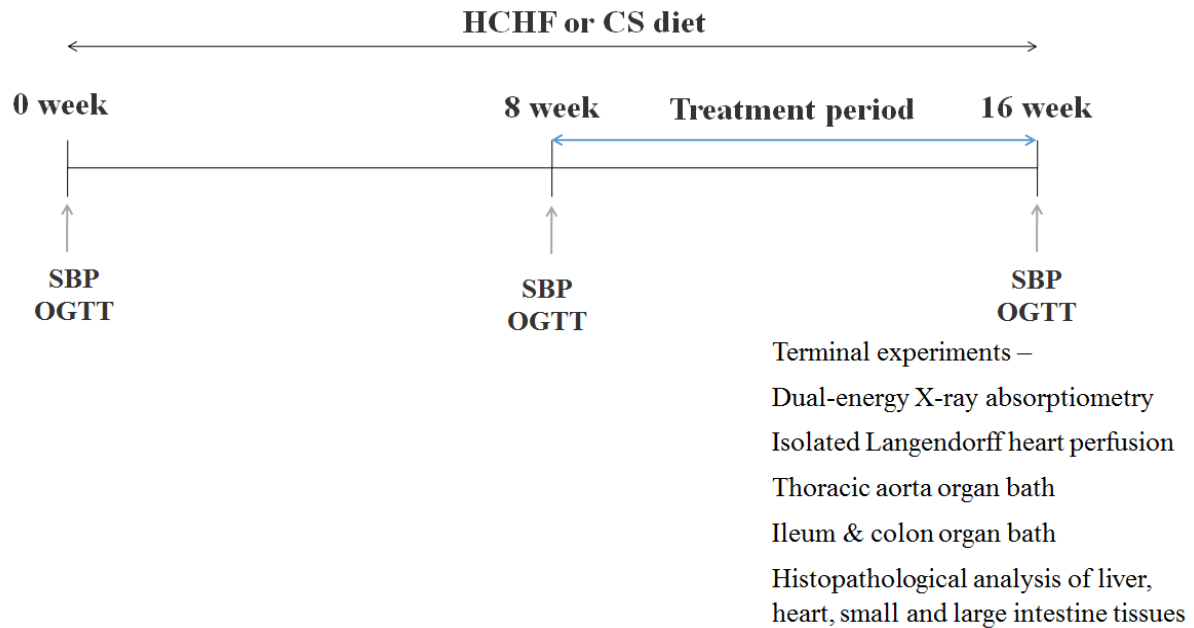
Figure 4. Timeline of the 16-week experimental protocol. SBP, systolic blood pressure; OGTT, oral glucose tolerance test; CS, corn starch diet; HCHF, high-carbohydrate, high-fat diet.

2.3 Diets

All group-specific diets were custom-prepared in the animal house food preparation room. The CS diet was prepared by thorough mixing of corn starch (570 g), powdered rat food (155 g), Hubble, Mendel and Wakeman salt mixture (25 g) and water (250 g) per kilogram of diet. The HCHF diet was prepared by thorough mixing of fructose (175 g), condensed milk (395 g), beef tallow (200 g), powdered rat food (155 g), Hubble, Mendel and Wakeman salt mixture (25 g) and water (50 g). C and CBRE rats were given tap water whereas H and HBRE rats were given 25% fructose (w/v) in drinking water. The energy densities of CS and HCHF diets were 11.23 kJ/g and 17.83 kJ/g, respectively, and an additional 3.85 kJ/mL in the drinking water for the HCHF diet–fed groups (H and HBRE rats). Energy content was calculated from the following values in kilojoules per gram: fructose, 15.40; corn starch, 15.94; condensed milk, 13.80; beef tallow, 37.70; and powdered rat food, 13.80.

2.4 Daily measurements

Rats were measured daily for their body weights and intakes of food and water. During these measurements, day-to-day health of rats was also monitored. Bedding of rats was changed regularly to provide cleaner housing for all rats. Energy intake of rats was calculated from the energy densities of CS and HCHF diets. Feed conversion efficiency was calculated using the following formula (26):

$$\text{Feed conversion efficiency} = \frac{\text{mean body weight gain (g)}}{\text{daily energy intake (kJ)}}$$

2.5 Systolic blood pressure measurement

Systolic blood pressure was measured at 0, 8 and 16 weeks under light sedation by intraperitoneal injection with Zoletil (tiletamine 10 mg/kg, zolazepam 10 mg/kg; Virbac, Peakhurst, NSW, Australia). Measurements were performed using an MLT1010 Piezo-Electric Pulse Transducer (ADInstruments, Bella Vista, NSW, Australia) and an inflatable tail-cuff connected to an MLT844 Physiological Pressure Transducer (ADInstruments) connected to a PowerLab data acquisition unit (ADInstruments) (26). The calibration of a transducer was performed on the day of the measurement using a pressure meter according to instructions provided by the manufacturer. Before the beginning of the sedation protocol, room temperature was set to 25ºC. After the rats were sedated, their tails were inserted through the tail cuff and the transducer was wrapped around the tail next to the cuff. A trace was detected for the blood pressure and five to six individual systolic blood pressure readings were taken for each rat and the average was calculated for each rat before using it for group calculations. After the blood pressure measurements, rats were given 5 mL of saline via sub-cutaneous injection on the dorsal side to prevent dehydration. Rats are then allowed to recover from sedation before returning to their individual cages. During the recovery period, rats were monitored every

fifteen minutes for the first hour with subsequent monitoring once every hour until the rat had completely recovered.

2.6 Abdominal circumference

Abdominal circumference was measured at 0, 8 and 16 weeks on sedated rats (immediately after systolic blood pressure measurement). A standard measuring tape was used to measure the circumference of the abdomen in the ventral recumbent position. The area measured is the soft tissue superior to the hind legs (26).

2.7 Oral glucose tolerance test

Oral glucose tolerance test was performed at 0, 8 and 16 weeks. For oral glucose tolerance test, rats were deprived of food overnight (6pm to 6am) in individual cages. For overnight food deprivation period, all types of diet were removed from their cages and fructose-supplemented drinking water in the H and HBRE groups was replaced with tap water. After the overnight food deprivation period, basal blood glucose concentrations were determined in tail vein blood using Medisense Precision Q.I.D. glucometer (Abbott Laboratories, Bedford, MA). Rat tails were pricked with a 30 gauge needle to obtain a drop of blood which was then transferred onto a glucose strip (Freestyle Optium Blood Glucose Test Strips, Abbott Diabetes Care Ltd., Witney, Oxon, UK) attached to the glucometer. After recording this initial 0 minute blood glucose concentration, all rats were given 40% aqueous glucose solution (2 g glucose/kg body weight) via oral gavage using a 5 mL syringe and a 18 gauge plastic gavage needle. Tail vein blood samples were taken at 30, 60, 90 and 120 minutes following glucose administration (26). Rats were returned to their group-specific diets after the last blood glucose concentration measurement. The area under the curve (AUC) was calculated from the graph showing blood glucose concentrations against time.

2.8 Body composition measurement

Body compositions of rats were measured using dual-energy X-ray absorptiometry (DXA) after 16 weeks of feeding protocol. These measurements were performed using a Norland XR36 DXA instrument (Norland Corp., Fort Atkinson, WI, USA). Rats were transported to The University of Queensland, St Lucia campus, Brisbane, in a University of Southern Queensland air-conditioned vehicle. For DXA scans, rats were anaesthetised using intraperitoneal injection of Zoletil (tiletamine 10 mg/kg and zolazepam 10 mg/kg) and Ilium Xylazil (xylazine 6 mg/kg; Troy Laboratories, Smithfield, NSW, Australia). These DXA scans recorded bone mineral density, bone mineral content, lean mass and fat mass and 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.). The precision error of lean mass for replicate measurements, with repositioning, was 3.2% (75). After the scans were completed, rats were given 5 mL of saline via sub-cutaneous injection on the dorsal side to prevent dehydration. Rats were maintained at 25°C to prevent hypothermia. Rats were monitored as described for systolic blood pressure measurements. Rats were then taken back to the University of Southern Queensland, Toowoomba campus and returned to their respective cages in the animal house facility with free access to their respective diets and water.

2.9 Terminal euthanasia and Langendorff heart preparation

Terminal euthanasia was induced via intraperitoneal injection of Lethabarb (pentobarbitone sodium, 100 mg/kg; Virbac, Peakhurst, NSW, Australia). Once euthanasia was induced in rats, heparin was administered (200 IU) into the right femoral vein. The abdomen was then opened and blood (~6 mL) was withdrawn from the abdominal aorta, collected into heparinised tubes and centrifuged at 5000 $\times$ g for 15 minutes within 30 minutes of collection to obtain plasma. Plasma from each rat was transferred to five Eppendorf tubes and stored at

-20°C before analysis. Hearts were removed and used in isolated Langendorff heart mode to assess left ventricular function of the rats. Hearts isolated from rats were perfused with a modified Krebs-Henseleit bicarbonate buffer containing (in mmol/L): 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. Buffer was bubbled with 95% O_2–5% CO_2 and maintained at 35°C. Isovolumetric ventricular function was measured by inserting a latex balloon catheter into the left ventricle 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 during pacing of the heart at 250 beats/minute using an electrical stimulator. End-diastolic pressures were obtained from 0 to 30 mmHg for calculation of diastolic stiffness constant (κ, dimensionless) (26).

2.10 Thoracic aortic organ bath

The thoracic aorta was isolated from the rat after blood collection and heart removal. The thoracic aorta was placed in a vessel filled with Tyrode's physiological salt solution bubbled with 95% O_2–5% CO_2 and maintained at 35°C. Tyrode's physiological salt solution contains (in mmol/L): NaCl, 136.9; KCl, 5.4; $CaCl_2$, 1.8; $MgCl_2.H_2O$, 1.05; $NaH_2PO_4.H_2O$, 0.42; $NaHCO_3$, 22.6; $C_6H_{12}O_6$, 1.8; ethylenediaminetetraacetic acid, 0.05; ascorbic acid (BDH laboratory supplies, Poole, UK), 0.3; glucose, 5.5. The fat around the aorta was carefully removed to expose the underlying tissue. Rings of approximately 4 mm in length were cut from each aorta. The rings were mounted onto the organ bath apparatus and allowed to stabilise at a resting tension of ~10 mN. Following stabilisation, submaximal (~70%) contraction was induced using noradrenaline to study the relaxation effect. One bath was kept intact to study the noradrenaline-induced contraction. After stabilisation, the cumulative concentration response curves (contraction) were obtained for noradrenaline and the cumulative

concentration-response curves (relaxation) were obtained for acetylcholine and sodium nitroprusside following submaximal (~70%) contraction (26).

2.11 Ileum and colon organ bath

Distal ileum and distal colon were isolated from rats. These pieces were cleaned using Tyrode solution and were then cut into ~1 cm long pieces. The ileum and colon tissues were then stitched using fine thread and suspended in an organ bath filled with Tyrode physiological salt solution bubbled with 95% O_2–5% CO_2 and maintained at 35ºC and allowed to stabilise at a resting tension of ~10 mN. Concentration-response curves were obtained for acetylcholine by measuring responses to individual concentrations of acetylcholine.

2.12 Organ weights

After performing Langendorff heart perfusion, hearts (n = 10) were separated into right ventricle and left ventricle with septum for weighing. Livers and abdominal fat pads (retroperitoneal, epididymal and omental) were isolated and weighed (n = 12). These organ weights were normalised relative to the tibial length at the time of their removal (in mg of tissue/mm of tibial length) (26).

Visceral adiposity index was calculated as follows:

$$\text{Visceral adiposity index (\%)} = \frac{\text{retroperitoneal fat (g)} + \text{omental fat (g)} + \text{epididymal fat (g)}}{\text{body weight (g)}} \times 100$$

2.13 Histology

Two rats were exclusively used for histopathological analysis from each group. Tissues were also collected from two other rats in each group. The heart, liver, small intestine and large intestine of rats were used for histopathological analysis. Two slides were prepared per tissue specimen and two random, non-overlapping fields per slide were taken to avoid biased analysis.

Approximately 5-7 minutes after euthanasia, heart, liver, small intestine and large intestine portions were collected and fixed in 10% neutral buffered formalin for 3 days. The samples were then dehydrated and embedded in paraffin wax. Thin sections (5 μm) of the samples were cut and stained with haematoxylin and eosin for determination of inflammatory cell infiltration (20×) and liver fat vacuole presence (40×). Collagen distribution was defined in the heart with picrosirius red stain. EVOS FLC microscope (Tokyo, Japan) was used to capture images for the above-mentioned slides to determine the extent of collagen deposition in selected tissue sections. Small intestine and large intestine sections were stained with haematoxylin and eosin stain to identify inflammatory cells (26).

2.13 Biochemical analyses

Plasma collected during terminal experiments were used to test for enzyme activities and plasma concentrations of biochemical markers. Plasma activities of alanine transaminase (ALT), aspartate transaminase (AST), and plasma concentrations of total cholesterol and triglycerides were determined at the School of Veterinary Sciences, The University of Queensland using kits and controls supplied by Olympus (Japan, Tokyo) using an AU 400 Olympus analyser (26). Non-esterified fatty acids were determined using a commercial kit (Wako Diagnostics, Osaka, Japan) (26) at the School of Veterinary Sciences, The University of Queensland Gatton campus.

2.14 Statistical analysis

All data are presented as mean ± standard error margin (SEM). Results were tested for variance using Bartlett's test and variables that are not normally distributed were transformed (using log10 function) prior to statistical analyses. Data from C, CBRE, H and HBRE groups were tested by two-way analysis of variance. When the interactions and/or the main effects

were significant, means were compared using the Newman-Keuls multiple comparison *post hoc* test. Where transformations did not result in normality or constant variance, a Kruskal-Wallis non-parametric test was performed. A *P*-value of <0.05 was considered as statistically significant. All statistical analyses were performed using Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

CHAPTER 3

RESULTS

3.1 Black rice extract composition and dosage

The main anthocyanin present in black rice extract was cyanidin 3-glucoside at 36% with 2% of other anthocyanins (not individually identified) information provided by ChromaDex (Irvine, CA, USA). 400 mg of black rice extract/kg food was administered to rats in CBRE and HBRE groups to obtain the dosage of cyanidin 3-glucoside 8 mg/kg of rat body weight, however the average daily intake of cyanidin 3-glucoside were higher in CBRE rats than in HBRE rats due to the higher food intake (Table 4).

3.2 Physiological variables

Rats were fed with their group-specific diet for 16 weeks. During 16 weeks of the protocol, C rats slowly increased their body weight (Figure 5A). CBRE rats were higher in body weight than the C rats at the end of 8 weeks. This difference was visible at the end of 16 weeks where body weight of CBRE rats was higher than C rats (Figure 5A). The increase in body weight of H rats was faster than C rats and they had higher body weight compared to C and CBRE rats at the end of 16 weeks. Supplementation of black rice extract for the last 8 weeks of the protocol suppressed the increase in body weight of HBRE rats compared to H rats (Figure 5A). To identify the effect of black rice extract with difference in body weight of C and CBRE, percentage body weight gain was calculated for the increase in body weight from week 8 to 16. The percentage body weight gain was unchanged by the black rice extract in CBRE rats compared to C rats while it was decreased in HBRE rats compared to H rats (Figure 5B).

Water intake was unchanged between all the groups (Table 4). The food intake was higher in C rats compared to H rats (Table 4). Black rice did not change the food intake in CBRE and HBRE rats when compared with C and H rats, respectively. Although the energy intake values were statistically not different between all the groups, H and HBRE rats had higher values compared to C and CBRE rats (Table 4). As a very small quantity (0.4 g/kg) of black rice extract was added to the food, the added energy density from the black rice was considered to be negligible. Feed efficiency was unchanged by the black rice extract in CBRE rats compared to C rats while it was decreased in HBRE rats compared to H rats Table (4).

3.3 Body composition measurements

Abdominal circumference in rats followed similar trend to the body weight. After 16 weeks of HCHF diet feeding, H rats had higher abdominal circumference compared to C rats (Figure 5C). Black rice extract did not change the abdominal circumference in CBRE rats compared to C rats while it decreased the abdominal circumference in HBRE rats compared to H rats (Figure 5C). The total abdominal fat deposition including retroperitoneal, epididymal, and omental fat was increased in H rats compared to C rats (Table 4). Black rice did not change the abdominal fat depositions in CBRE and HBRE rats when compared with C and H rats, respectively.

The dual X-ray absorptiometry scan provided data for lean mass, fat mass, bone mineral content and bone mineral density (Figure 6A-D). The fat mass was higher in H rats compared to C rats (Figure 6A). Black rice extract did not change the fat mass in CBRE and HBRE rats when compared with C and H rats, respectively (Figure 6A). There were no differences in lean mass between all the groups (Figure 6B). Visceral adiposity index was higher in H rats compared C rats and it was unaffected by the black rice extract in either of the treated groups (Table 4).

Bone mineral content was higher in H rats compared to C rats (Figure 6C). Black rice extract reduced bone mineral content in HBRE rats compared to H rats while not changing it in CBRE rats compared to C rats (Figure 6C). There were no differences in bone mineral density between all the groups (Figure 6D).

3.4 Metabolic variables

Basal blood glucose concentrations at 16 weeks were elevated in H rats compared to C rats (Figure 7A) and consequently the area under the curve was increased (Figure 7B). Black rice extract did not improve glucose sensitivity in CBRE rats or HBRE rats. HCHF diet induced dyslipidaemia in H rats where plasma concentrations of total cholesterol, triglycerides and Nonesterified fatty acids were higher compared to C rats (Table 4). These lipid parameters were decreased with black rice extract supplementation. Plasma activities of ALT and AST were measured as markers of liver damage. H rats had higher plasma activities of these enzymes compared to C rats while black rice supplementation normalised the activities of these enzymes in HBRE rats while not changing these activities in CBRE rats.

3.5 Cardiovascular structure and function

Systolic blood pressure in H rats was higher compared to HBRE rats. C rats did not develop hypertension and remained at ~120 mmHg which is within the normal range. H rats progressively increased to develop hypertension (Figure 8A). Black rice extract reduced the systolic blood pressure in CBRE and HBRE rats compared to C and H rats, respectively after 16 weeks on the protocol (Figure 8A).

At 16 weeks, the diastolic stiffness was increased in H rats compared to C rats (Figure 8B). There was no difference in diastolic stiffness between C rats and CBRE rats. Black rice extract decreased the diastolic stiffness in HBRE rats compared to H rats (Figure 8B). There

was no difference in left ventricle + septum wet weight across all the groups (Figure 8C). The right ventricle wet weight in H rats was greater than in HBRE rats (Figure 8D).

The vascular reactivity of the thoracic aorta to acetylcholine, noradrenaline and sodium nitroprusside was impaired in H rats compared to C rats shown by the decreased contraction and relaxation (Figure 9 A-C). The H rats had decreased force of noradrenaline-induced contraction, decreased sodium nitroprusside-induced relaxation and decreased acetylcholine-induced relaxation compared to C rats (Figure 9 A-C). Both CBRE and HBRE rats showed improved forces of contraction and relaxation. In H rats, the collagen deposition in the left ventricle was higher than in C rats (Figure 10). Black rice extract decreased the collagen deposition in the left ventricle in HBRE rats (Figure 10).

3.6 Hepatic structure and function

The H rats exhibited enlargement of the liver compared to C rats (Table 4). There were no differences in liver size between CBRE and C rats; HBRE and H rats, respectively. The H rat livers had increased infiltration of inflammatory cells and substantial fibrosis (Figure 12). The HBRE rats had reduced fibrosis and general inflammation (Figure 12). Liver enzymes profile, H rat has increased liver enzyme activity of ALT and AST compared to HBRE rats (Table 5). Livers from H rats showed fat depositions as a sign of non-alcoholic fatty liver disease. Whereas livers from HBRE rats showed decreased fat. The C rats had normal liver structure. HBRE rats showed similar fat deposition in the liver compared to H rats (Figure 12).

3.7 Gastrointestinal tract structure and function

In the ileum organ bath, the C rats had a greater acetylcholine-induced contraction compared to H rats (Figure 11A). The HBRE rats showed an improved response. The CBRE rats showed an improved response. In the colon organ bath, C rats had a greater acetylcholine-

induced contraction compared to H rats (Figure 11B). The HBRE rats showed an improved response. The CBRE rats showed an improved response. Haematoxylin and eosin staining of the ileum (Figure 13) and colon (Figure 14) from the H rats showed an increase in infiltration of inflammatory cells compared to C rats. In the CBRE and HBRE rats there was less inflammation.

Table 4. Effects of black rice extract on physiological variables and body composition

Variable	C	CBRE	H	HBRE	P-value Diet	P-value BRE	P-value Diet × BRE
Initial body weight, g	340 ± 1	341 ± 2	338 ± 1	337 ± 1	0.0283	1.0000	0.4537
Final body weight, g	379.9 ± 6.9^d	408.1 ± 5.8^c	555.0 ± 12.1^a	490.8 ± 9.8^b	<0.0001	0.0410	<0.0001
Water intake (0-8 weeks), mL/day	28.9 ± 2.9	24.8 ± 3.0	30.4 ± 2.3	30.5 ± 2.8	0.1994	0.4730	0.4513
Water intake (8-16 weeks), mL/day	29.8 ± 3.0	27.6 ± 3.8	29.9 ± 2.3	34.6 ± 4.1	0.2985	0.7128	0.3121
Food intake (0-8 weeks), g/day	38.2 ± 3.5^a	24.9 ± 1.7^b	43.4 ± 2.8^a	23.9 ± 1.6^b	0.4104	<0.0001	0.2264
Food intake (8-16 weeks), g/day	36.5 ± 3.6^a	26.4 ± 2.5^b	41.6 ± 3.0^a	23.3 ± 2.3^b	0.7443	<0.0001	0.1687
Energy intake, kJ/day	418.6 ± 40.5	475.5 ± 33.3	548.6 ± 41.8	529.4 ± 39.6	0.0227	0.6307	0.3338
Food conversion efficiency, g/kJ	0.09 ± 0.01^c	0.12 ± 0.01^c	0.40 ± 0.02^a	0.28 ± 0.01^b	<0.0001	0.0014	<0.0001
Cyanidin 3-glucoside intake, mg/kg body weight/day	N/A	13.7 ± 1.4^a	N/A	6.9 ± 0.7^b	<0.0001	<0.0001	<0.0001
Bone mineral content, g	11.4 ± 0.1^c	11.7 ± 0.2^c	16.2 ± 0.5^a	14.4 ± 0.4^b	<0.0001	0.0820	0.337
Bone mineral density, g/cm^2	0.172 ± 0.003	0.170 ± 0.002	0.181 ± 0.002	0.181 ± 0.002	0.9189	0.9894	0.9919
Total fat mass, g	73.0 ± 4.2^b	75.4 ± 7.1^b	203.5 ± 15.6^a	188.9 ± 11.8^a	<0.0001	0.6707	0.5072
Total lean mass, g	295.5 ± 9.9	309.3 ± 7.1	306.1 ± 10.9	287.1 ± 6.5	0.8365	0.6977	0.0936
Abdominal circumference at 16 weeks, cm	19.2 ± 0.2^c	19.5 ± 0.2^c	22.0 ± 0.3^a	20.8 ± 0.2^b	<0.0001	0.0639	0.0023
Visceral adiposity index, %	4.1 ± 0.1^b	4.1 ± 0.4^b	7.7 ± 0.6^a	8.5 ± 0.6^a	<0.0001	0.4010	0.4010
Body mass index, g/cm^2	0.61 ± 0.01^d	0.65 ± 0.01^c	0.82 ± 0.02^a	0.78 ± 0.01^b	<0.0001	1.0000	0.0046
Tissue wet weight, mg/mm tibial length							
Retroperitoneal	124.5 ± 11.2^b	164.7 ± 28.1^c	387.6 ± 41.1^a	381.7 ± 38.5^a	<0.0001	0.0844	0.1217
Epididymal	79.6 ± 6.7^c	83.4 ± 9.2^d	176.5 ± 12.9^b	215.0 ± 20.1^a	<0.0001	0.2437	0.0003
Omental	116.7 ± 9.39^b	90.72 ± 6.70^c	225.7 ± 18.4^a	252.5 ± 16.1^a	<0.0001	0.0089	<0.0001
Total abdominal fat	320.8 ± 13.3^d	338.8 ± 34.0^c	789.8 ± 65.9^a	849.2 ± 65.8^a	<0.0001	0.0377	0.0023

Values are mean $\pm$ SEM, n=10-12. Means in a row with superscripts without a common letter differ, $P<0.05$.

BRE, black rice extract; C, corn starch diet-fed rats; CBRE, corn starch diet-fed rats supplemented with black rice extract; H, high-carbohydrate high-fat diet-fed rats; HBRE, high-carbohydrate high-fat diet-fed rats supplemented with black rice extract.

Table 5. Effects of black rice extract on metabolic variables

Variable	C	CBRE	H	HBRE	P-value		
					Diet	BRE	Diet × BRE
Plasma lipid profile							
Total cholesterol, mmol/L	1.71 ± 0.07	1.60 ± 0.09	1.62 ± 0.07	1.62 ± 0.06	0.6360	0.4580	0.4580
Triglyceride, mmol/L	0.48 ± 0.08[c]	0.48 ± 0.05[c]	2.07 ± 0.31[a]	1.25 ± 0.14[b]	<0.0001	0.0259	0.0259
Non-esterified fatty acids, mmol/L	1.13 ± 0.24[c]	1.21 ± 0.14[c]	4.29 ± 0.30[a]	3.41 ± 0.32[b]	<0.0001	0.1321	0.0727
Liver enzymes							
Plasma ALT activity, U/L	32.1 ± 4.3[b]	26.7 ± 2.7[b]	48.0 ± 5.9[a]	30.8 ± 3.8[b]	0.0268	0.0131	0.1816
Plasma AST activity, U/L	96.5 ±13.6[b]	77.6 ± 3.5[b]	162.5 ± 14.0[a]	85.6 ± 11.5[b]	0.0027	0.0002	0.0159
Liver wet weight, mg/mm	220.1 ± 8.8[b]	250.8 ± 10.5[b]	325.7 ± 24.9[a]	341.1 ± 16.3[a]	<0.0001	0.1664	0.6428

Values are mean ± SEM, n = 10-12. Means in a row with superscripts without a common letter differ, $P<0.05$

ALT, alanine transaminase; AST, aspartate transferase; BRE, black rice extract; C, corn starch diet-fed rats; CBRE, corn starch diet-fed rats supplemented with black rice extract; H, high-carbohydrate high-fat diet-fed rats; HBRE, high-carbohydrate high-fat diet-fed rats supplemented with black rice extract.

A

B

C

	P-value		
Figure	**Diet**	**BRE**	**Diet *x* BRE**
A	**<0.0001**	**0.0410**	**<0.0001**
B	**0.0002**	**0.1269**	**0.0011**
C	**<0.0001**	**0.0639**	**0.0023**

Figure 5. Effects of black rice extract on body weight (A), body weight gain from 8-16 weeks (B) and abdominal circumference (C) in C, CBRE, H and HBRE rats. Values are mean ± SEM, *n* = 10-12. End-point means or overall mean values without a common letter differ, *P* < 0.05. BRE, black rice extract; C, corn starch diet-fed rats; CBRE, corn starch diet-fed rats supplemented with black rice extract; H, high-carbohydrate, high-fat diet-fed rats; HBRE, high-carbohydrate, high-fat diet-fed rats supplemented with black rice extract.

A

B

C

D

	P-value		
Figure	**Diet**	**BRE**	**Diet *x* BRE**
A	<0.0001	0.6707	0.5072
B	0.8365	0.6977	0.0936
C	<0.0001	0.0820	0.0337
D	0.0012	0.8994	0.8005

Figure 6. Effects of black rice extract on lean mass (A), fat mass (B), bone mineral content (C) and bone mineral density (D) in C, CBRE, H and HBRE rats. Values are mean ± SEM, *n* = 10. Means without a common letter differ, *P* < 0.05. BRE, black rice extract; C, corn starch diet-fed rats; CBRE, corn starch diet-fed rats supplemented with black rice extract; H, high-carbohydrate, high-fat diet-fed rats; HBRE, high-carbohydrate, high-fat diet-fed rats supplemented with black rice extract.
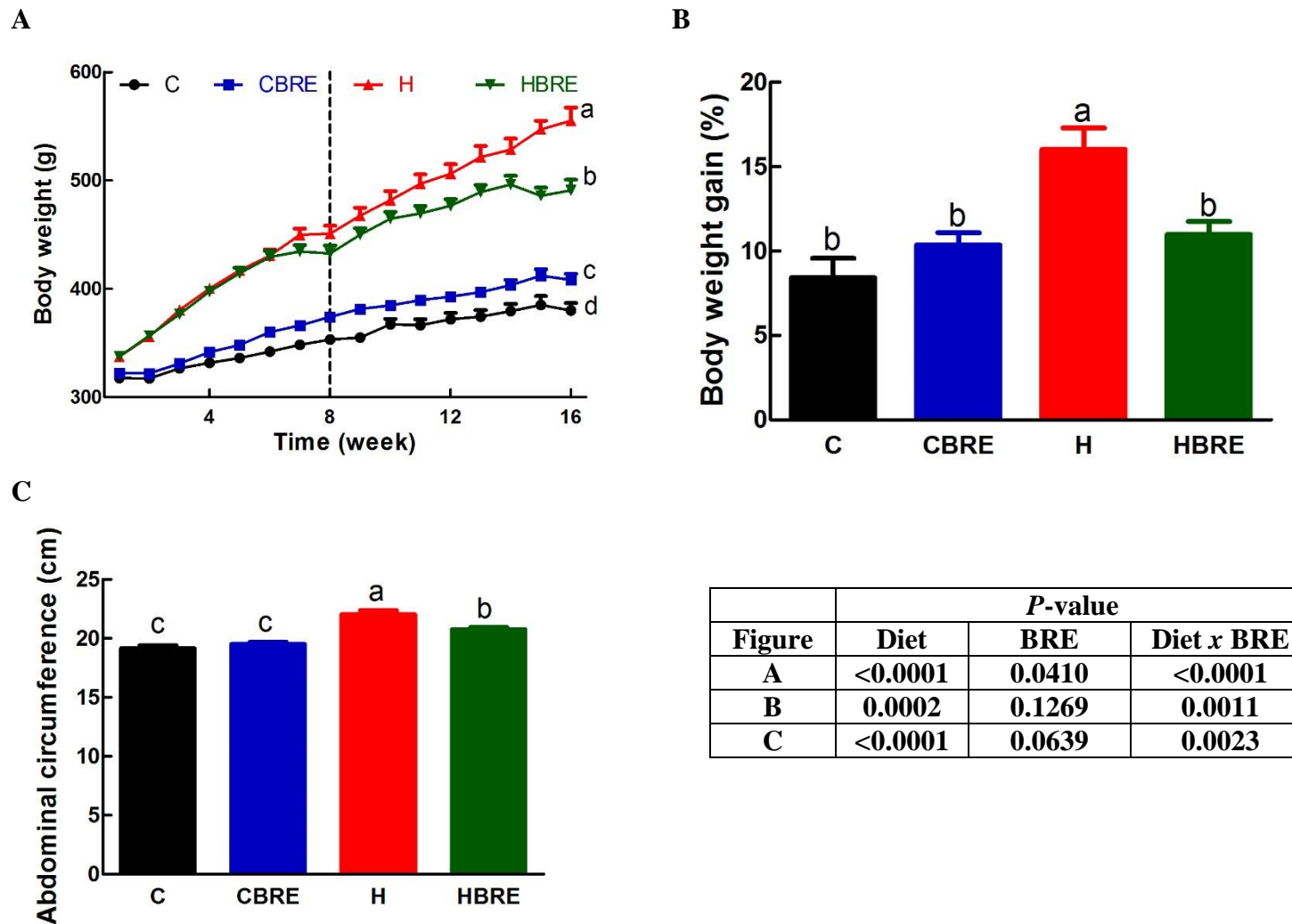
A

B
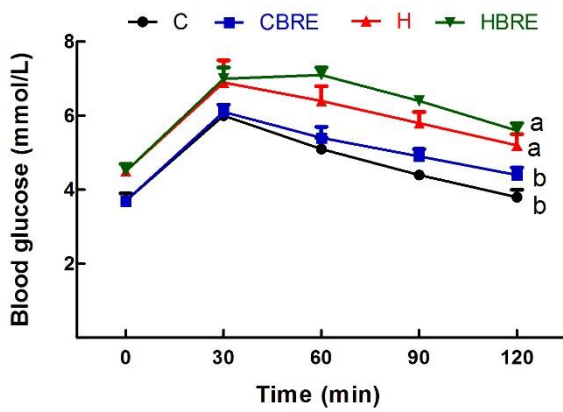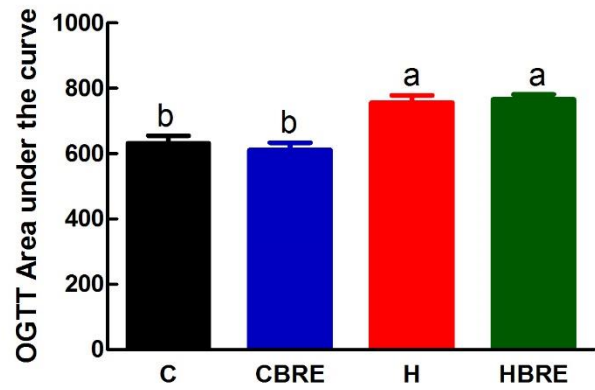
	P-value		
Figure	**Diet**	**BRE**	**Diet *x* BRE**
A	<0.0001	0.0799	0.7199
B	<0.0001	0.8170	0.4887

Figure 7. Effects of black rice extract on blood glucose concentration (A) and area under the curve (B) in C, CBRE, H and HBRE rats. Data presented as intakes from week 0-8 and 8-16. Values are mean ± SEM, *n* = 10-12. End-point means and overall means without a common letter differ, *P* < 0.05. BRE, black rice extract; C, corn starch diet-fed rats; CBRE, corn starch diet-fed rats supplemented with black rice extract; H, high-carbohydrate, high-fat diet-fed rats; HBRE, high-carbohydrate, high-fat diet-fed rats supplemented with black rice extract.
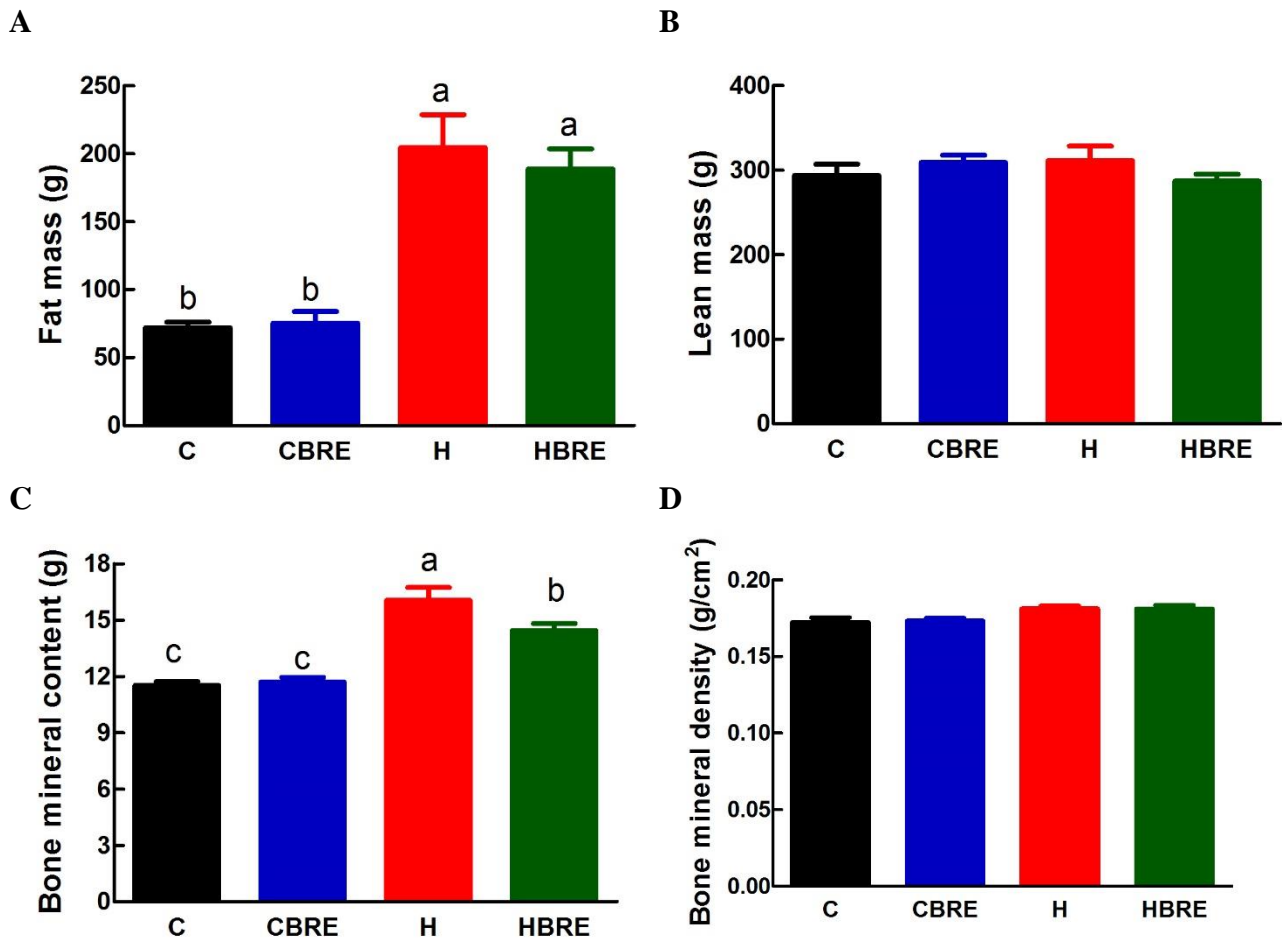
	P-value		
Figure	**Diet**	**BRE**	**Diet *x* BRE**
A	0.6697	0.1808	0.0006
B	0.5998	0.8437	0.3402
C	0.5583	0.5050	0.3750
D	0.2578	0.5821	0.4461

Figure 8. Effects of black rice extract on systolic blood pressure (A), diastolic stiffness constant (B), left ventricle + septum weight (C) and right ventricle weight (D) in C, CBRE, H and HBRE rats. Values are mean ± SEM, n = 10-12. Means without a common letter differ, P < 0.05. BRE, black rice extract; C, corn starch diet-fed rats; CBRE, corn starch diet-fed rats supplemented with black rice extract; H, high-carbohydrate, high-fat diet-fed rats; HBRE, high-carbohydrate, high-fat diet-fed rats supplemented with black rice extract.

A

B

C

Figure	*P*-value		
	Diet	BRE	Diet *x* BRE
A	0.4931	0.0261	0.1685
B	0.4201	0.4525	0.2398
C	0.7054	0.8117	0.4115

Figure 9. Effects of black rice extract on acetylcholine-induced relaxation (A), noradrenaline-induced contraction (B) and sodium nitroprusside-induced relaxation (C) in thoracic aortic preparations from C, CBRE, H and HBRE rats. Values are mean ± SEM, *n* = 10-12. End-point means without a common letter differ, *P* < 0.05. BRE, black rice extract; C, corn starch diet-fed rats; CBRE, corn starch diet-fed rats supplemented with black rice extract; H, high-carbohydrate, high-fat diet-fed rats; HBRE, high-carbohydrate, high-fat diet-fed rats supplemented with black rice extract.
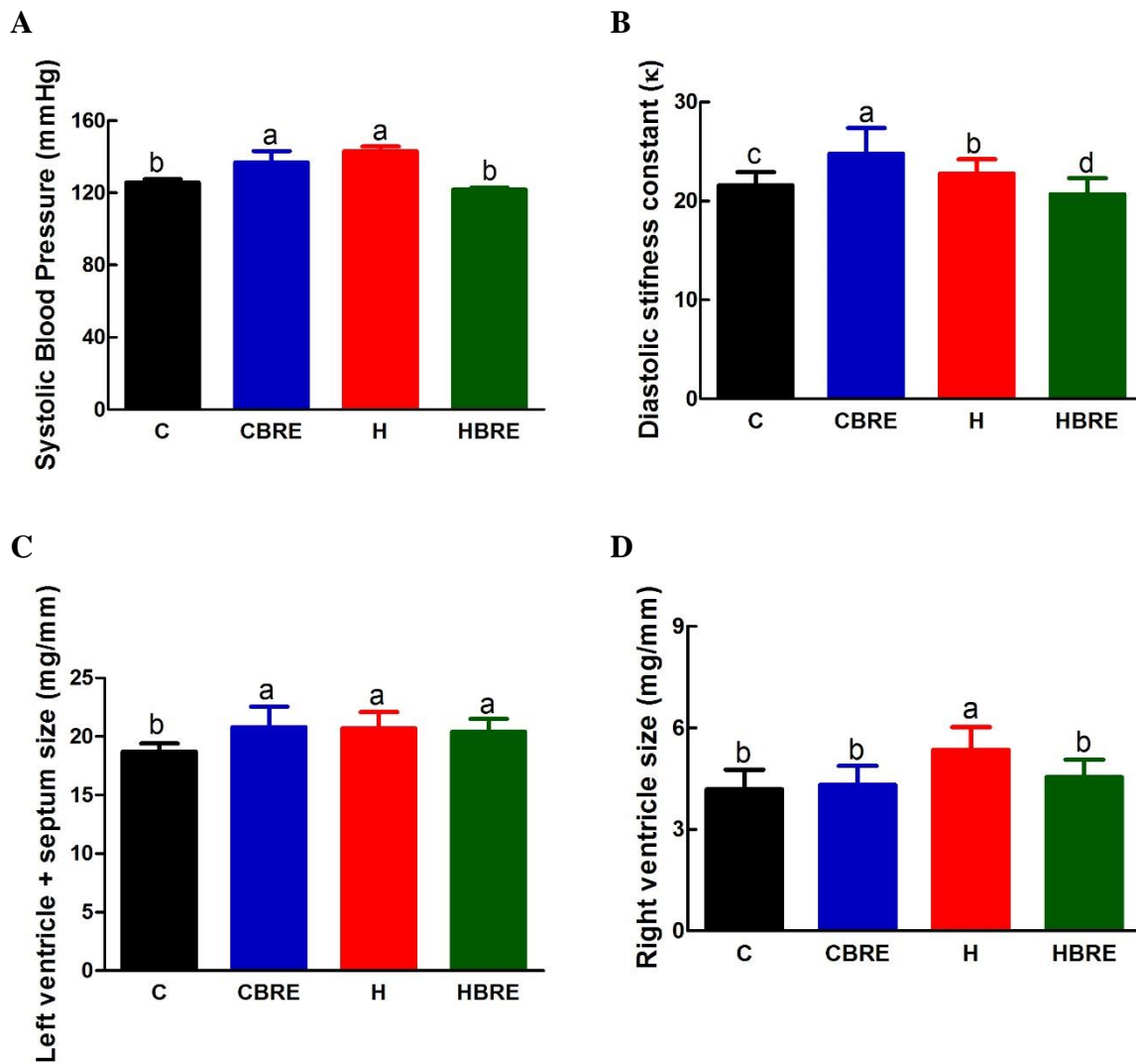
Figure 10. Effects of black rice extract on collagen (marked as 'cg') deposition in hearts using picrosirius red stain at 20x in C, corn starch diet-fed rats (A); CBRE, corn starch diet-fed rats supplemented with black rice extract (B) H, high-carbohydrate, high-fat diet-fed rats (C); HBRE, high-carbohydrate, high-fat diet-fed rats supplemented with black rice extract (D).

A

B

	P-value		
Figure	**Diet**	**BRE**	**Diet x BRE**
A	0.1148	0.9739	0.1666
B	0.3250	0.6063	0.2447

Figure 11. Effects of black rice extract on acetylcholine-induced relaxation in distal ileum preparation (A) and acetylcholine-induced relaxation in distal colon preparation (B) in C, CBRE, H and HBRE rats. Values are mean ± SEM, n = 6-8. End-point means without a common letter differ, $P < 0.05$. BRE, black rice extract; C, corn starch diet-fed rats; CBRE, corn starch diet-fed rats supplemented with black rice extract; H, high-carbohydrate, high-fat diet-fed rats; HBRE, high-carbohydrate, high-fat diet-fed rats supplemented with black rice extract.
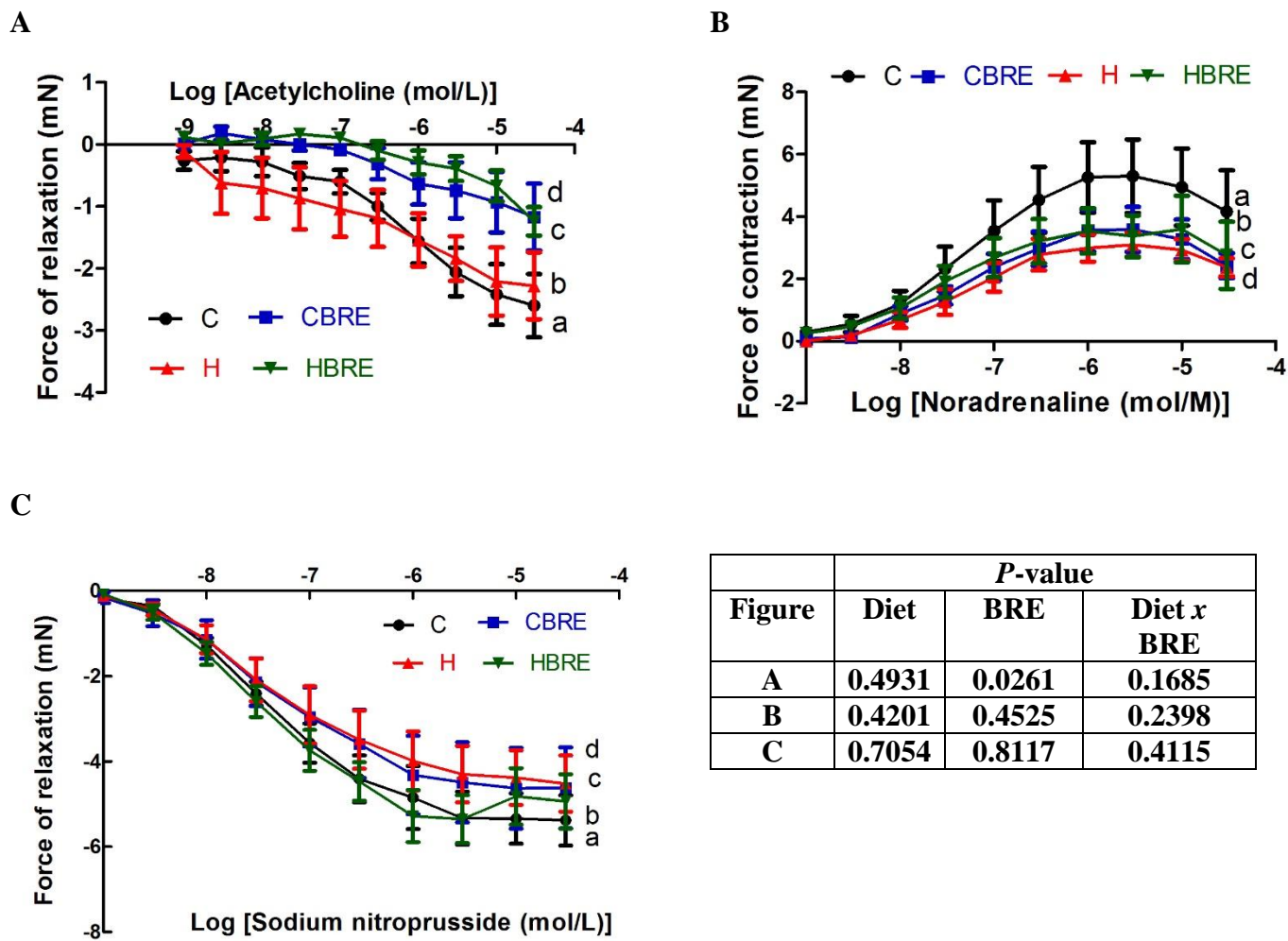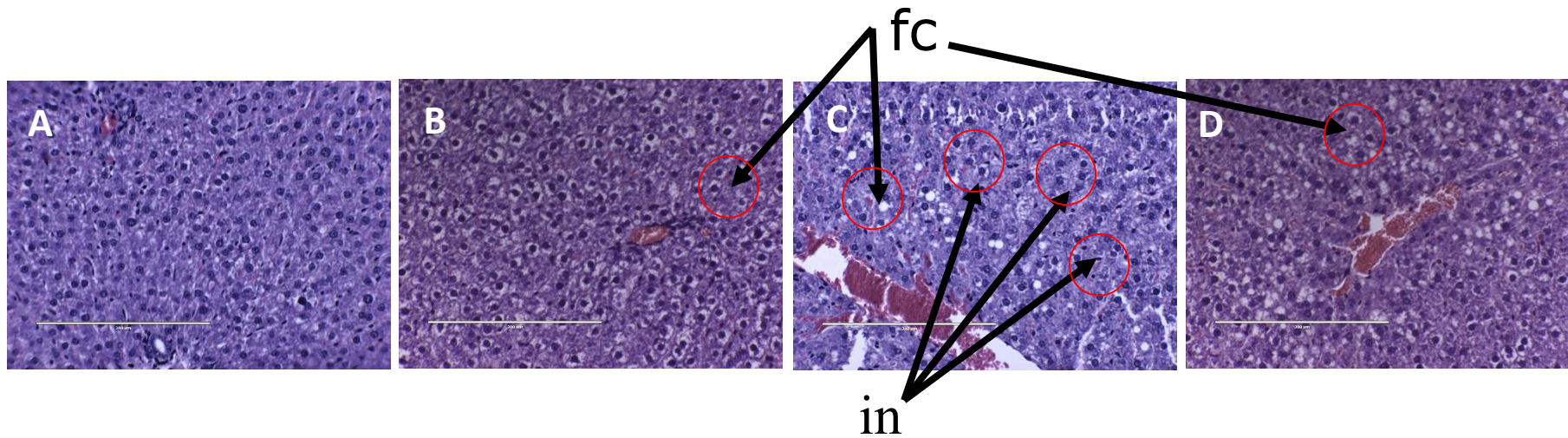
Figure 12. Effects of black rice extract on fat deposition and inflammation in the liver. A–D represent hematoxylin and eosin staining of the liver showing fat deposition (marked as 'vc') and inflammatory cells (marked as 'in) (20x) in C, corn starch diet-fed rats; CBRE, corn starch diet-fed rats supplemented with black rice extract; H, high-carbohydrate, high-fat diet-fed rats; HBRE, high-carbohydrate, high-fat diet-fed rats supplemented with black rice extract.
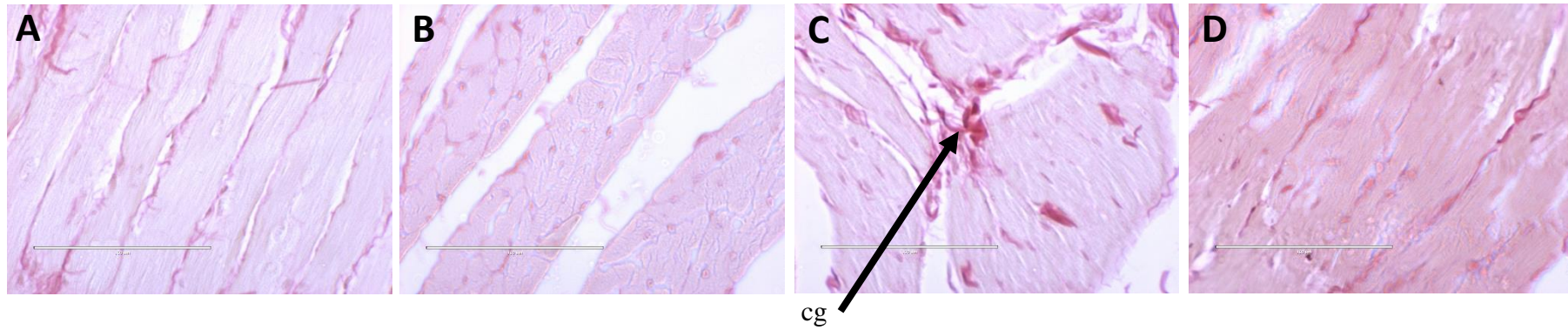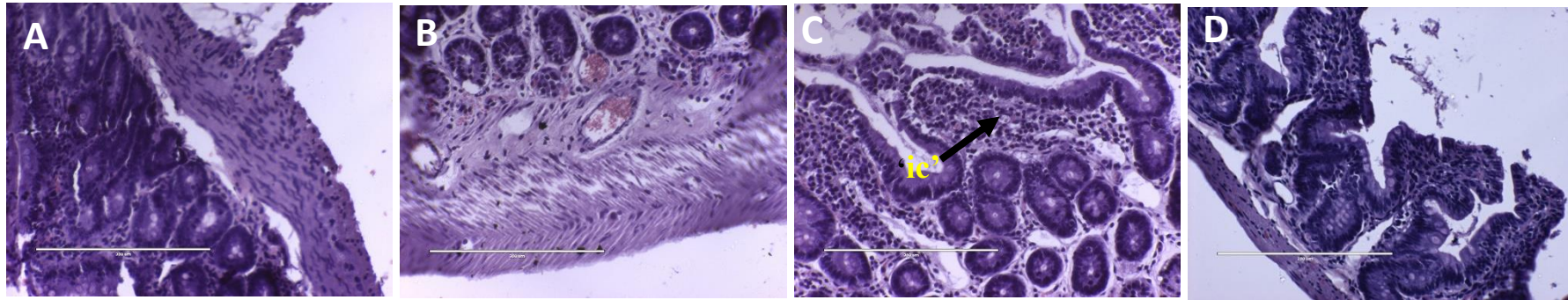
Figure 13. Effects of black rice extract on inflammation in small intestine using haematoxylin and eosin stain (20x) showing inflammatory cells (marked as "ic") in C, corn starch diet-fed rats (A); CBRE, corn starch diet-fed rats supplemented with black rice extract (B) H, high-carbohydrate, high-fat diet-fed rats (C); HBRE, high-carbohydrate, high-fat diet-fed rats supplemented with black rice extract (D).
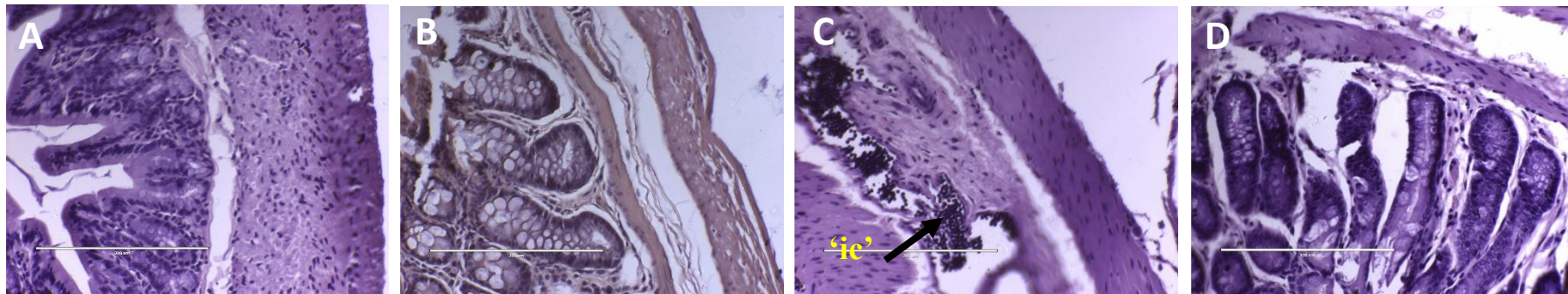
Figure 14. Effects of black rice extract on inflammation in large intestine using haematoxylin and eosin stain (20x) showing inflammatory cells (marked as "ic") in C, corn starch diet-fed rats (A); CBRE, corn starch diet-fed rats supplemented with black rice extract (B) H, high-carbohydrate, high-fat diet-fed rats (C); HBRE, high-carbohydrate, high-fat diet-fed rats supplemented with black rice extract (D).
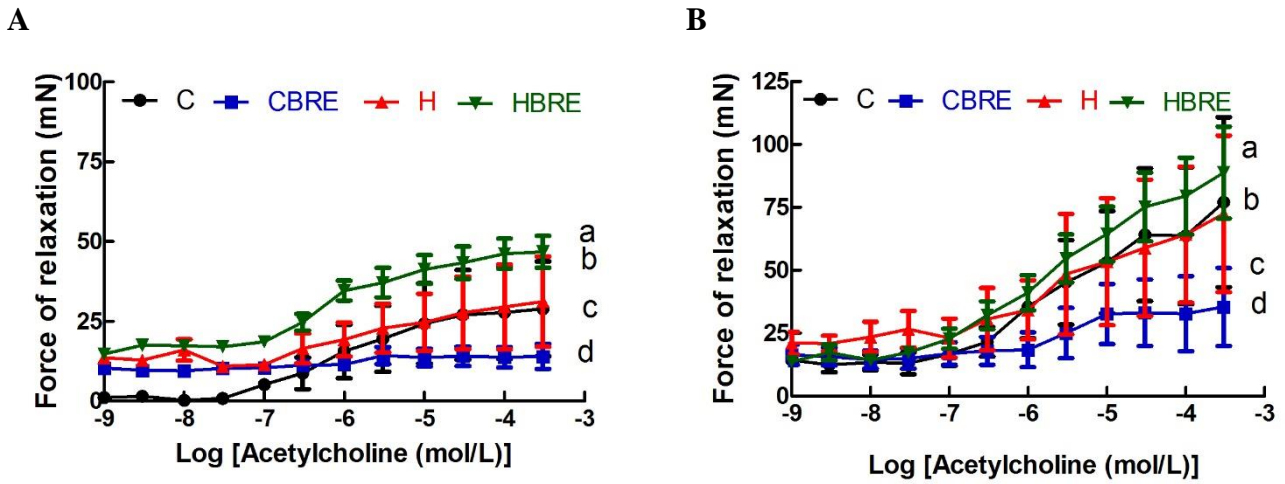
CHAPTER 4

DISCUSSION

This study identified health benefits of cyanidin 3-glucoside from black rice extract in a rat model of diet-induced metabolic syndrome. This study has extended the results obtained from previous studies at the University of Southern Queensland using the functional foods containing anthocyanins. Previous studies with purple carrots and Queen Garnet plum containing cyanidin 3-glucoside showed attenuation of diet-induced metabolic syndrome. This study used the same dose of cyanidin 3-glucoside as in the Queen Garnet plum study (28). Purple carrot study had used a higher dose of cyanidin 3-glucoside to obtain similar results as with Queen Garnet plums (28, 57), thus indicating the 8 mg/kg body weight/day as the effective dose of cyanidin 3-glucoside against diet-induced metabolic syndrome and obesity.

4.1 Diet-induced metabolic syndrome

The combination of feeding a high-carbohydrate, high-fat diet to rats for 16 weeks was used to mimic the human metabolic syndrome as in the previous studies (26, 28, 57). The high-carbohydrate, high-fat diet induced metabolic syndrome in rats including central obesity, dyslipidaemia, impaired glucose tolerance and hypertension. These changes were also accompanied with the alterations in structure and function of the cardiovascular, hepatic and gastrointestinal systems. The changes in the cardiovascular system included hypertension, increased diastolic stiffness, inflammation, fibrosis and endothelial dysfunction whereas the liver changes included hypertrophy, inflammation and steatosis along with increased plasma activity of liver enzymes (ALT and AST). This rat model is validated and has been used to identify potential benefits of various functional foods against diet-induced metabolic syndrome (27, 28, 57, 85, 86).

CS diet was used as control healthy diet for identifying the metabolic syndrome-associated changes in HCHF diet-fed rats. CS diet was rich in complex polysaccharide and hence it was able to provide balanced metabolism for the rats whereas the HCHF diet contained fructose and sucrose (26). Excess fructose (and sucrose as it breaks down to glucose and fructose) consumption has been linked to the development of metabolic complications (87, 88) and it has been shown to be the major contributing factor for the development of obesity (88). Unlike fructose, corn starch does not increase systolic blood pressure, abdominal fat deposition and concentration of blood glucose and plasma lipids (26). Moreover, the HCHF diet contained long-chain saturated and *trans* fatty acids. There are clear evidence that suggest that these fatty acids can initiate metabolic impairments and hence contribute towards the development of metabolic syndrome (89).

4.2 Significance

Obesity is a major medical problem exacerbating several health conditions such as type 2 diabetes, hypertension and dyslipidaemia (90). Although the prevalence of obesity has increased throughout the world, there are no suitable treatments available for it (4) especially the pharmacological approaches being associated with various complications (4, 91). Therefore, it has been proposed that functional foods, foods with defined health benefits, may be the alternative to be used as a treatment for obesity (4). Foods containing polyphenols, especially the anthocyanins, have many beneficial health effects associated with them (92), although they have a low bioavailability (93).

4.3 Black rice

Rice is an important crop and a staple food for more than half the world's population (94). Black rice (O*ryza sativa L.*) originated from South-Eastern Asia in the early 1200s (94). It has high levels of cyanidin 3-glucoside and hence the colour, along with high levels of iron

and vitamin E. The bran hull (outermost layer) contains one of the highest levels of anthocyanins found in food (higher by weight than any other coloured grains). Black rice also contains small quantities of amylose and amylopectin, the two components of starch. The black rice extract used in this study contained 36% cyanidin 3-glucoside with ~2% other anthocyanins.

In this study, black rice extract reduced body weight at the end of the protocol, however, it did not change the abdominal fat or total body fat content. In a recently published study, the body weight, abdominal fat and total body fat were reduced with a similar dose of cyanidin 3-glucoside as a pure compound as well as from Queen Garnet plum juice (57). In the purple carrot study, body weight and abdominal fat were reduced with the purple carrot juice with total body fat data unavailable (28). A recent report analysing the effects of functional foods including foods containing anthocyanins supports the reduction in body weight and abdominal fat reduction but black rice extract was unable to reduce the abdominal fat (4). Abdominal obesity has been linked with many pathophysiological complications including dyslipidaemia, cardiovascular disease and insulin resistance (95-97). So, with no change in abdominal fat, it was expected that the complications of metabolic syndrome would remain in the treated animals. According to a previous study, coffee extract did not reduce body weight and abdominal fat but it was still able to produce improvements in other complications of metabolic syndrome (98).

Anthocyanin bioavailability is lower than other polyphenols with only 1% being absorbed (99). In animal models, anthocyanins administered at relatively high dosages (1-2 mg/kg diet) are protective against oxidative stress (100). Dosage for this study was 8 mg/kg diet of total anthocyanins of which approximately 36% was in the form of cyanidin 3-glucoside (therefore, 2.8 mg/kg diet of cyanidin 3-glucoside was used). This cyanidin 3-glucoside dose

was previously investigated using purple carrot juice (dose of 5% in food for 8 weeks) using the 16-week high-carbohydrate, high-fat model (28). The results in this study supported the evidence that anthocyanins have beneficial health effects. Dietary phenols are divided into one third phenolic acids and two thirds flavonoids (101). The CBRE and HBRE rats had significantly reduced systolic blood pressure, abdominal obesity, body weight gain, diastolic stiffness along with reductions in inflammatory cells in the liver and heart. There was also reduced collagen deposition in the left ventricle of the heart. In a study (n= 920) conducted in Germany, investigated the consumption of young people (age 4-14 years) and found that anthocyanidin intake among the group was 6 mg/day, with strawberries representing the main source (102). Anthocyanins are widely ingested with median values of 180 mg/day, many derived from fruits and wines (103). It is evident that anthocyanins are consumed more widely than their sugar-free form (anthocyanidins). The HBRE rats showed 10% body weight reduction compared to H rats. Additionally, the HBRE line (Figure 6A) was beginning to plateau demonstrating body weight was maintained, which is the first stage to weight loss. The reduction in the overall body weight of HBRE rats is correlated to the reduction in abdominal fat deposition and is possibly due to the up-regulation of adiponectin. Adiponectin is an adipokine that has a role in the regulation of glucose concentrations and fatty acid catabolism.

4.4 Cardiovascular effects

4.4.1 Diastolic stiffness

Myocardial fibrosis, the major mechanism in the development of left ventricular dysfunction, results from disproportionate collagen deposition accompanied by reduced degradation of extracellular matrix leading to an increased tissue stiffness (26).

The left ventricle diastolic stiffness was higher in H rats compared to C rats (Figure 7B). Black rice extract decreased the diastolic stiffness in CBRE and HBRE rats. An increased

left ventricle stiffness correlates to a reduced heart function, decreasing the efficiency with which the oxygenated blood is pumped around the systemic circulation. This reduces the perfusion to tissues in the body. Hence, there is reduced oxygen and nutrient are transportation to tissues with decreased waste removal occurring. This is detrimental to the overall health and wellbeing of the individual.

4.4.2 Endothelial function

The cyclic guanosine monophosphate (cGMP) secondary messenger pathway for nitric oxide and endothelial function carried out in the cytoplasm. Guanylyl cyclase (converts GTP to cGMP). Protein kinase G to have vasodilatory effects. Nitric oxide is a metabolite of sodium nitroprusside. Build of cGMP stimulates protein kinase G which adds a phosphate group to the molecule. Phosphorylated protein activates the function which is vasodilation. Vasodilatory effects lower the pre-load on the heart, which decreases the volume of blood in the heart at the end of diastole (104).

Fatty acid oxidation (β-oxidation) occurs in the mitochondria to generate acetyl-CoA, NADH and $FADH_2$. Obesity occurs when triglycerides are stored as fat tissue instead of being utilised for energy production. Fatty acid degradation involves three stages. Stage one is β-oxidation cleaves two carbons at a time. Electrons carriers are NADH and $FADH_2$. Stage two is Krebs cycle where acetyl-CoA is consumed. Stage three is the electron transport chain (similar to aerobic respiration) to generate energy in the form of adenosine tri-phosphate (105).

Cholesteryl ester transfer protein facilities the transfer of triglycerides between lipoproteins. This protein is involved in transfer of cholesteryl ester from HDL to other lipoproteins. Black rice extract can modulate this pathway causing decreased atherosclerotic plaque build-up. Vasodilation is the widening of blood vessels. Impaired vasodilation increases

the cardiovascular risk. Endothelium-dependent vasodilation is lost with increasing age. Cholesteryl ester transfer proteins are a novel target for increasing HDL levels (106).

4.4.3 Systolic blood pressure

Cyanidin 3-glucoside reduced the systolic blood pressure possibly through inhibition of angiotensin converting enzyme (ACE) (107). ACE is produced in the lung and functions in converting angiotensin I to angiotensin II (a potent vasoconstrictor). ACE is important in homeostasis and regulation of blood volume and thus blood pressure (108). ACE acts to decrease peripheral vascular resistance which leads to a decrease in blood pressure (109). The improvement of approximately 20 mmHg is substantial because it demonstrates the positive effect black rice extract had on normalising the systolic blood pressure thus decreasing the risk of developing cardiovascular diseases. The normal systolic blood pressure for all rats at 0 weeks was approximately 120 mmHg. The vascular reactivity seen in thoracic aorta rings organ bath showed an improvement in endothelial function shown by improved contraction and relaxation respective to the drug used. Improved endothelial function is seen by an increased production of nitric oxide synthase (NOS), consequently causing vasodilation of the blood vessels *in vivo*. Nitric oxide has three isoforms: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS). The eNOS was possibly increased. In rats, the absorption rate of anthocyanins is less predictable than other flavonoids such as tea catechin (110). In humans, the absorption rate of anthocyanins is less than tea catechins (110).

Black rice suppresses the nitric oxide and reactive chemical species in a biological model (94). In a study conducted in China, also using black rice extract it was found that the formation of nitric oxide through suppression of iNOS in macrophages from murine cells was significantly reduced ($P<0.05$) (94). Black rice decreases atherosclerotic plaque formation and increases the antioxidant status in rabbits (111). Cyanidin 3-glucopyransoide is metabolised in

50

the liver by catechol-O-methyltransferase into two metabolites, Pn3G and 4'-O-methyl-Cy3G (112). A study found that the structure of the glucoside affects its absorption and overall bioavailability. The increase in number of hydroxyl groups in the molecule decreases its availability (113).

4.5 Oxidative stress

Oxidative stress is caused by an imbalance between the production of reactive oxygen species (ROS) and their detoxification in biological systems (114). Oxidative stress potentiates ageing and chronic degenerative diseases (47). The consumption of dietary antioxidants reduces this effect. Obesity is associated with systemic oxidative stress, adipokine imbalance and reduced antioxidant defences, leading to dyslipidemia, vascular disease and hepatic steatosis (115). Cyanidin 3-glucoside functions as a free radical scavenger, i.e. removing toxic ROS from cells (107). The role of anthocyanins in cardiovascular disease protection is strongly linked to decreased oxidative stress production (82). Anthocyanins have diverse pharmacological actions including their antioxidant activity and protective effects against DNA damage, induced hepatic toxicity in rats and peroxidase activity in human's blood (116). ROS generation occurs when the unstable unpaired electrons, generated from metabolism by-products, cause damage to the cells and tissues (117). When blood flow is interrupted (hypoxia) to a tissue, ischemia occurs. The absence of blood and nutrients creates a problem when blood flow is returned and the tissue is perfused once again. The ROS species cause inflammation and oxidative damage to the tissue, causing a reperfusion injury. Orally administered cyanidin 3-glucoside attenuates hepatic ischemia reperfusion injury (118). Prolonged oxidative stress results in the uptake of glucose in both muscle and adipose tissue and decreases insulin secretion from pancreatic β-cells, thereby accelerating the pathological development of type 2 diabetes (80).

A study showed that anti-oxidative effects exerted by black rice extract are mediated through decreases in free-radical generation as well as increases in superoxide dismutase and catalase activities both *in vitro* and *in vivo* (119). Anthocyanins have direct protective effects on the heart. Hearts from male Wistar rats fed on a diet based on anthocyanins containing maize kernels for eight weeks were more resistant to regional ischemia and reperfusion insult induced in an isolated heart preparation (62). This diet also reduced the infarct size in a coronary occlusion and reperfusion model (62).

4.6 Diabetes and non-alcoholic fatty liver disease

Diabetes is a glucose regulation disorder and of growing health concern with approximately 2.8% of the world population diagnosed with diabetes in 2000 (38). There are two types of diabetes; type 1 affects 10% of the diabetics and is insulin independent with juvenile onset (strong genetic role); type 2 affects 90% of diabetics and is insulin dependent. The onset of type 2 diabetes is lifestyle, mainlydiet problems. Diabetes is set to increase due to the increasing proportion of the population who are 65 years and older. This increasing age will have greater impact in contributing to increased worldwide prevalence (38). Diabetic complications include liver problems, ranging from non-progressive steatosis to non-alcoholic fatty liver disease (NAFLD) (120). NAFLD is characterised by excessive lipid accumulation in the liver, manifesting the signs of metabolic syndrome and insulin resistance (99). NAFLD is the most common hepatic manifestation among metabolic disease, diabetes and obesity (121). In studies of type 2 diabetes and metabolic syndrome, the related risk factors including the consumption of energy-rich food has been linked to increased risk of developing these disease states (122). Cyanidin 3-glucoside from black berry extract inhibits nitric oxide biosynthesis (123). Diabetes is associated with oxidative stress caused from hyperglycaemia and hyperlipidaemia (122). The increased carbonyl stress (includes both oxidative and non-

oxidative stress) is possibly due to substrate overload and or deficiencies in detoxification pathways (124). The oral glucose tolerance test showed H rats to have an increased fasting blood glucose level. This correlates to a lack of insulin sensitivity and possibly in extremes cases such as diabetes and insulin resistance. There was a decreased sensitivity to glucose in H rats compared to C rats. This means higher basal blood glucose levels with an increased area under the curve for the H rats. Black rice extract did not improve blood glucose sensitivity. Blood glucose concentrations are controlled by the pancreas. In healthy individuals, the pancreatic β-cells located in the islets of Langerhans secrete insulin. After food consumption, insulin is released from the pancreatic β-cells to assist in glucose-uptake into the cell. Incretins are a useful group of hormones that increase insulin production, used as treatment for type 2 diabetics (125). The two most important incretins are glucagon like pepide-1 and glucose-dependent insulinotropic polypeptide (125). Obesity is a complex energy imbalance disorder with complications leading to the development of metabolic syndrome. In healthy individuals the endocrine system regulates several hormones, e.g. ghrelin, leptin and adiponectin in conjunction to maintain homeostatic, however in disease states such as metabolic syndrome the regulation is disrupted and obesity occurs.

4.7 Inflammation

Inflammation occurs in every organ in the body and is the typical response to cell damage and vascularised tissue injury (126). Inflammation has a protective function to foreign antigens; however over inflammation is a problem. Enzymes and lipid mediators are released after cell damage occurs. Leukotrienes are a group of G-coupled receptors originally from isolated leukocytes. They are formed by oxidation of arachidonic acid and eicosanoid inflammatory mediators. Their function is to sustain inflammatory reactions.

4.8 Black rice extract health claims

The black rice extract (ProC3G) provided by ChromaDex claims the following health benefits: improved cholesterol levels and vasodilation (decreases CVD), glucose metabolism (promotion of superoxide dismutase (SOD) activity), weight loss – anthocyanins influence the gene expression for fat metabolism, signalling body to burn fat and not store it, Anti-aging (contains antioxidants to defend against free radicals generated by oxidative stress). Black rice is claimed to include benefits for multiple organs specifically the kidney, liver and stomach. This study provided evidence to support that black rice extract decreases systolic blood pressure, reduces inflammation in the liver and reduces the overall body weight.

4.9 Anthocyanin mechanism of action

Mechanistic studies support the beneficial health effects of anthocyanins on the established biomarkers of cardiovascular disease which are nitric oxide, inflammation and endothelial dysfunction (64). The role of anthocyanins in cardiovascular disease prevention is strongly linked to protection against oxidative stress (64). Several mechanisms of action have been proposed to explain the anti-inflammatory actions of anthocyanins. Specifically these compounds may provide protection from DNA cleavage, enzyme inhibition, increased anti-inflammatory cytokine production, decreased lipid peroxidation, decreased capillary permeability, fragility and membrane strengthening (64). Commercial grape juice (10 mL/kg) has been shown to significantly inhibit platelet activity and experimental coronary thrombosis *in vivo* (127). Corn-derived anthocyanins made the myocardium less susceptible to ischemia reperfusion injury *ex vivo* and *in vivo* compared with the anthocyanin-free control (64).

NF-κB is a transcription factor activated by oxidative stress and pro-inflammatory stimuli and controls the expression of numerous genes involved in inflammation. Anthocyanins exert their effects through blocking NF-κB. Dampening NF-κB expression will thereby limit

54

the inflammatory response (84). The extent to which anthocyanins exert their effects can be measured through decreased plasma concentrations of pro-inflammatory chemokines, cytokines and inflammatory mediators (84).

4.10 Mice studies

Mice studies can also be used to study different aspects where anthocyanins have action. The benefit of mice studies include genetic modification, to 'knock out' or 'knock in' genes, e.g. knockout mice. The alteration of existing genes with modified genes focuses the study area. Heart disease uses *Apoe* knockout mouse and C57BL/6J. Type 2 diabetes The Jackson Laboratory: *Cpe^{fat}, Lep^{ob}, Lepr^{db}* and *tub (128).* In type 2 diabetic mice cyanidin 3-glucoside ameliorates hyperglycaemia and insulin insensitivity due to the reduction of retinol binding protein 4 expression (129). Orally administered cyanidin 3-glucoside is metabolised to protocatechuic acid via cyanidin by intestinal microbiota (130). Anti-scratching effects of black coloured rice for diseases such as dermatitis, rhinitis, and psoriasis (130). Cyanidin 3-glucoside metabolites also exhibited inhibition the expression of allergic cytokines such as IL-4, TNF-α and activation of their transcription factor, NF-κB in RBL-2H3 cells stimulated with IgE-antigen. Anthocyanin fraction from purple sweet potato decreases hepatic triglyceride accumulation through AMPK-mediated modulation of fatty acid metabolism, potentially ameliorating hyperkaliemia (131). Black rice extract attenuates hepatic steatosis in mice fed a high fat diet via fatty acid oxidation (132).

4.11 Limitations

Due to the time constraints and unforeseen delays, plasma analyses, gastrointestinal tract microbiota characterisation and Western Blot analyses were not completed. However, plasma was taken, faecal samples were collected for gut microbiota studies and tissue samples (liver, heart, pancreas, small and large and intestines) were collected for Western Blot studies

to be completed in the future. Pancreas tissue samples for histopathological analysis was collected but not used. The limitation in using a rodent model to study disease is the difficulty in translating positive results into higher order organisms such as humans. This requires human testing to provide further evidence-based validation before wide scale health claims can be made.

CHAPTER 5

5.1 SUMMARY AND CONCLUSION

The major finding from this study was that black rice extract attenuated or normalised signs of metabolic syndrome in high-carbohydrate, high-fat fed rats. Rats supplemented with black rice extract rats while still fed a high-carbohydrate, high-fat diet showed reduced abdominal obesity and overall body weight. Cardiovascular structure and function was improved with marked reductions in systolic blood pressure, inflammatory cell infiltration and fibrosis. Liver structure and function was improved as demonstrated by reduced inflammation. The gastrointestinal tract was improved with a decreased amount of general inflammation in the small and large intestines. Hence, black rice extract can provide cardiovascular, hepatic and gastrointestinal protection along with cholesterol modulation. A small dietary change in swapping brown rice to black rice or supplementing black rice into the human diet may offer health benefits. This study further extended the body of evidence that anthocyanins have functional food qualities. In particular, this study supports health claims for foods containing cyanidin 3-glucoside to improve the signs associated with metabolic syndrome. Further investigations are required to determine if these health benefits can translate to overweight or obese humans.

5.2 FUTURE DIRECTIONS

A 16-week high-carbohydrate, high-fat diet was used to model most of the systemic effects seen in the metabolic syndrome. Metabolic syndrome affects multiple organ systems including cardiovascular, hepatic and endocrine. The human body is a highly complex organism which cannot be ethically studied *in vivo* without validated evidence conducted on an animal model. The animal model must effectively mimic most of metabolic syndrome effects on the body. The translation of the results from an animal model (pre-clinical) to the gold standard of human clinical trials is important. Eventually, provided positive effects are seen, this black rice extract treatment can be tested in the human system. Insulin activity measurements can be used to determine if the insulin sensitivity to glucose.. Gene expression for weight loss would be useful in order to determine the expression of genes, proteins and hormones including such adiponectin and leptin in the treatment rats.

5.2.1 Gut microbiota (meta-genomic) analysis method

Faecal samples from rats will be collected immediately following euthanasia and stored at -80°C for analysis of the gut microbiota. Frozen samples will be delivered to the Australian Genome Research Facility (AGRF), Brisbane, Queensland, Australia. DNA from rat stool samples will be isolated by staff at AGRF and then sequenced on 1 x lane of MiSeq with 300bp paired-end reads using Illumina SBS (provides high speed, multiplex 16S amplicon sequencing on the MiSeq® system) to identify the various microbial organisms in the provided samples. Bioinformatics reports for Operational Taxonomic Unit (OTU) chart up to phylum/family level and reporting of taxonomic distribution will be provided by AGRF using their in-house analytical techniques.

5.2.2 Gene sequencing method

16S rRNA useful for identification of bacteria and species profiling (133). An investigation into the different regions of the gut (proximal, mid, distal) may determine the effect diet has on the variance of bacterial species. The gut microbiota impacts the metabolism and overall health of the host (134).

5.2.3 Polymerase chain reaction method

Polymerase chain reaction (PCR) is used to amplify DNA to several orders of magnitude which is good for DNA sequencing. PCR involves a three step process. Step one – denaturation of the DNA strands, separate into separate strands. *Taq* polymerase to synthesis more strands. Step two – anneal the strands. Step three – amplification of strands.

5.2.4 Western Blot method

Separate and identify specific amino acid sequence from protein mixture. Cell lysis to extract proteins, gel preparation, transfer, gel electrophoresis, electro transfer, blocking and antibody incubation (135).

5.2.5 Biochemical measurement methods

For oxidative stress the plasma concentrations of malondialdehyde may be measured. As obesity is a chronic low-grade inflammatory condition it would be useful to measure the CRP levels. The following cytokines would be useful to measure the concentrations of leptin, ghrelin and adiponectin.

5.2.6mRNA methods

mRNA studies indicate obesity has a strong genetic predisposition (136). Investigating mRNA expression and distribution in the adipose tissue of rats may lead to a better understanding of the physiology and hence clinical implications of a variety of genes.

REFERENCES

1.   Huang PL. A comprehensive definition for metabolic syndrome. Disease Models and Mechanisms. 2009;2(5-6):231-7.

2.   Kassi E, Pervanidou P, Kaltsas G, Chrousos G. Metabolic syndrome: definitions and controversies. BMC Medicine. 2011;9:48.

3.   O'Neill S, O'Driscoll L. Metabolic syndrome: a closer look at the growing epidemic and its associated pathologies. Obesity Reviews 2015;16(1):1-12.

4.   Brown L, Poudyal H, Panchal SK. Functional foods as potential therapeutic options for metabolic syndrome. Obesity Reviews. 2015;16(11):914-41.

5.   Mozumdar A, Liguori G. Persistent increase of prevalence of metabolic syndrome among U.S. adults: NHANES III to NHANES 1999-2006. Diabetes Care. 2011;34(1):216-9.

6.   Yang L, Colditz GA. Prevalence of overweight and obesity in the United States, 2007-2012. JAMA Internal Medicine. 2015;175(8):1412-3.

7.   Cameron AJ, Magliano DJ, Zimmet PZ, Welborn T, Shaw JE. The metabolic syndrome in Australia: prevalence using four definitions. Diabetes Research and Clinical Practice. 2007;77(3):471-8.

8.   Zimmet PZ, Alberti KG, Shaw JE. Mainstreaming the metabolic syndrome: a definitive definition. Medical Journal of Australia. 2005;183(4):175-6.

9.   Statistics ABo. Australian Health Survey: Updated Results, 2011-2012 2013.

10.   Salminen M, Kuoppamaki M, Vahlberg T, Raiha I, Irjala K, Kivela SL. Metabolic syndrome defined by modified International Diabetes Federation criteria and type 2 diabetes mellitus risk: a 9-year follow-up among the aged in Finland. Diabetes and Vascular Disease Research. 2013;10(1):11-6.

11.   Bhowmik B, Afsana F, Siddiquee T, Munir SB, Sheikh F, Wright E, et al. Comparison of the prevalence of metabolic syndrome and its association with diabetes and cardiovascular disease in the rural population of Bangladesh using the modified National Cholesterol Education Program Expert Panel Adult Treatment Panel III and International Diabetes Federation definitions. Journal of Diabetes Investigation. 2015;6(3):280-8.

12.   Chackrewarthy S, Gunasekera D, Pathmeswaren A, Wijekoon CN, Ranawaka UK, Kato N, et al. A comparison between revised NCEP ATP III and IDF definitions in diagnosing metabolic syndrome in an urban Sri Lankan population: The Ragama Health Study. International Scholarly Research Notices: Endocrinology. 2013;2013:320176.

13.   Paula HA, Ribeiro Rde C, Rosado LE, Pereira RS, Franceschini Sdo C. Comparison of the different definition criteria for the diagnosis of the metabolic syndrome in elderly women. Arquivos Brasileiros de Cardiologia. 2010;95(3):346-53.

14.   Lakka HM, Laaksonen DE, Lakka TA, Niskanen LK, Kumpusalo E, Tuomilehto J, et al. The metabolic syndrome and total and cardiovascular disease mortality in middle-aged men. JAMA. 2002;288(21):2709-16.

15.   Esposito K, Chiodini P, Colao A, Lenzi A, Giugliano D. Metabolic syndrome and risk of cancer: a systematic review and meta-analysis. Diabetes Care. 2012;35(11):2402-11.

16.   Sidorenkov O, Nilssen O, Grjibovski AM. Metabolic syndrome in Russian adults: associated factors and mortality from cardiovascular diseases and all causes. BMC Public Health. 2010;10:582.

17.   Pan WH, Yeh WT, Weng LC. Epidemiology of metabolic syndrome in Asia. Asia Pacific Journal of Clinical Nutrition. 2008;17 Suppl 1:37-42.

18.   Pandit K, Goswami S, Ghosh S, Mukhopadhyay P, Chowdhury S. Metabolic syndrome in South Asians. Indian Journal of Endocrinology and Metabolism. 2012;16(1):44-55.

19.   Hydrie MZ, Shera AS, Fawwad A, Basit A, Hussain A. Prevalence of metabolic syndrome in urban Pakistan (Karachi): comparison of newly proposed International Diabetes Federation and modified Adult Treatment Panel III criteria. Metabolic Syndrome and Related Disorders. 2009;7(2):119-24.

20.   Ilich JZ, Kelly OJ, Kim Y, Spicer MT. Low-grade chronic inflammation perpetuated by modern diet as a promoter of obesity and osteoporosis. Archives of Industrial Hygiene and Toxicology. 2014;65(2):139-48.

21.   Lee CY. The effect of high-fat diet-induced pathophysiological changes in the gut on obesity: What should be the ideal treatment? Clinical and Translational Gastroenterology. 2013;4:e39.

22.   Esser N, Legrand-Poels S, Piette J, Scheen AJ, Paquot N. Inflammation as a link between obesity, metabolic syndrome and type 2 diabetes. Diabetes Research and Clinical Practice. 2014;105(2):141-50.

23.   Monteiro R, Azevedo I. Chronic inflammation in obesity and the metabolic syndrome. Mediators of Inflammation. 2010;2010.

24.   Yubero-Serrano EM, Delgado-Lista J, Pena-Orihuela P, Perez-Martinez P, Fuentes F, Marin C, et al. Oxidative stress is associated with the number of components of metabolic syndrome: LIPGENE study. Experimental & Molecular Medicine 2013;45:e28.

25.   Dandona P, Aljada A, Chaudhuri A, Mohanty P, Garg R. Metabolic syndrome: a comprehensive perspective based on interactions between obesity, diabetes, and inflammation. Circulation. 2005;111(11):1448-54.

26.   Panchal SK, Poudyal H, Iyer A, Nazer R, Alam A, Diwan V, et al. High-carbohydrate high-fat diet-induced metabolic syndrome and cardiovascular remodeling in rats. Journal of Cardiovascular Pharmacology. 2011;57(1):51-64.

27.   Panchal SK, Poudyal H, Arumugam TV, Brown L. Rutin attenuates metabolic changes, nonalcoholic steatohepatitis, and cardiovascular remodeling in high-carbohydrate, high-fat diet-fed rats. Journal of Nutrition. 2011;141(6):1062-9.

28.   Poudyal H, Panchal S, Brown L. Comparison of purple carrot juice and beta-carotene in a high-carbohydrate, high-fat diet-fed rat model of the metabolic syndrome. British Journal of Nutrition. 2010;104(9):1322-32.

29.   Iyer A, Lim J, Poudyal H, Reid RC, Suen JY, Webster J, et al. An inhibitor of phospholipase A2 group IIA modulates adipocyte signaling and protects against diet-induced metabolic syndrome in rats. Diabetes. 2012;61(9):2320-9.

30.   Lim J, Iyer A, Liu L, Suen JY, Lohman RJ, Seow V, et al. Diet-induced obesity, adipose inflammation, and metabolic dysfunction correlating with PAR2 expression are attenuated by PAR2 antagonism. Federation of American Societies for Experimental Biology Journal. 2013;27(12):4757-67.

31.   Lim J, Iyer A, Suen JY, Seow V, Reid RC, Brown L, et al. C5aR and C3aR antagonists each inhibit diet-induced obesity, metabolic dysfunction, and adipocyte and macrophage signaling. Federation of American Societies for Experimental Biology Journal. 2013;27(2):822-31.

32.   Alberti KG, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, et al. Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. Circulation. 2009;120(16):1640-5.

33.   Nichols M, Townsend N, Scarborough P, Rayner M. Cardiovascular disease in Europe 2014: epidemiological update. European Heart Journal. 2014;35(42):2950-9.

34.   Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic

analysis for the Global Burden of Disease Study 2010. The Lancet. 2012;380(9859):2095-128.

35.   Lieberman S. The antioxidant power of purple corn: A research review. Alternative and Complementary Therapies. 2007;13(2):107-10.

36.   Shukla A, Kumar K, Singh A. Association between obesity and selected morbidities: a study of BRICS countries. PLoS One. 2014;9(4):e94433.

37.   Butsch WS. Obesity medications: what does the future look like? Current Opinion in Endocrinology, Diabetes and Obesity. 2015;22(5):360-6.

38.   Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care. 2004;27(5):1047-53.

39.   Tarride JE, Lim M, DesMeules M, Luo W, Burke N, O'Reilly D, et al. A review of the cost of cardiovascular disease. Canadian Journal of Cardiology. 2009;25(6):e195-202.

40.   Musso G, Gambino R, Cassader M. Interactions between gut microbiota and host metabolism predisposing to obesity and diabetes. Annual Review of Medicine. 2011;62:361-80.

41.   Quideau S, Deffieux D, Douat-Casassus C, Pouysegu L. Plant polyphenols: chemical properties, biological activities, and synthesis. Angewandte Chemie International Edition. 2011;50(3):586-621.

42.   Broncel M, Kozirog M, Duchnowicz P, Koter-Michalak M, Sikora J, Chojnowska-Jezierska J. *Aronia melanocarpa* extract reduces blood pressure, serum endothelin, lipid, and oxidative stress marker levels in patients with metabolic syndrome. Medical Science Monitor. 2010;16(1):CR28-34.

43.   He F, Liang NN, Mu L, Pan QH, Wang J, Reeves MJ, et al. Anthocyanins and their variation in red wines I. Monomeric anthocyanins and their color expression. Molecules. 2012;17(2):1571-601.

44.   Qin B, Anderson RA. An extract of chokeberry attenuates weight gain and modulates insulin, adipogenic and inflammatory signalling pathways in epididymal adipose tissue of rats fed a fructose-rich diet. British Journal of Nutrition. 2012;108(4):581-7.

45.   Jurgonski A, Juskiewicz J, Zdunczyk Z. Ingestion of black chokeberry fruit extract leads to intestinal and systemic changes in a rat model of prediabetes and hyperlipidemia. Plant Foods for Human Nutrition. 2008;63(4):176-82.

46.   Valcheva-Kuzmanova S, Kuzmanov K, Mihova V, Krasnaliev I, Borisova P, Belcheva A. Antihyperlipidemic effect of *Aronia melanocarpa* fruit juice in rats fed a high-cholesterol diet. Plant Foods for Human Nutrition. 2007;62(1):19-24.

47.   He J, Giusti MM. Anthocyanins: natural colorants with health-promoting properties. Annual Review of Food Science and Technology. 2010;1:163-87.

48.   Park S, Kang S, Jeong DY, Jeong SY, Park JJ, Yun HS. Cyanidin and malvidin in aqueous extracts of black carrots fermented with *Aspergillus oryzae* prevent the impairment of energy, lipid and glucose metabolism in estrogen-deficient rats by AMPK activation. Genes and Nutrition. 2015;10(2):455.

49.   Tsuda T. Dietary anthocyanin-rich plants: biochemical basis and recent progress in health benefits studies. Molecular Nutrition and Food Research. 2012;56(1):159-70.

50.   Li CY, Kim HW, Won SR, Min HK, Park KJ, Park JY, et al. Corn husk as a potential source of anthocyanins. J Agric Food Chem. 2008;56(23):11413-6.

51.   Hosseinian FS, Beta T. Saskatoon and wild blueberries have higher anthocyanin contents than other Manitoba berries. Journal of Agricultural and Food Chemistry. 2007;55(26):10832-8.

52.   Wu X, Beecher GR, Holden JM, Haytowitz DB, Gebhardt SE, Prior RL. Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption. Journal of Agricultural and Food Chemistry. 2006;54(11):4069-75.

53.   Ge Q, Ma X. Composition and antioxidant activity of anthocyanins isolated from Yunnan edible rose (*An ning*). Food Science and Human Wellness. 2013;2(2):68-74.

54.   Mazza G, Kay CD, Cottrell T, Holub BJ. Absorption of anthocyanins from blueberries and serum antioxidant status in human subjects. Journal of Agricultural and Food Chemistry. 2002;50(26):7731-7.

55.   Cook NC, Sammam S. Flavonoids-Chemistry, metabolism, cardioprotective effects, and dietary sources. Journal of Nutritional Biochemistry. 1996;7:66-76.

56.   Hollman PC, Katan MB. Dietary flavonoids: intake, health effects and bioavailability. Food and Chemical Toxicology. 1999;37(9-10):937-42.

57.   Bhaswant M, Fanning K, Netzel M, Mathai ML, Panchal SK, Brown L. Cyanidin 3-glucoside improves diet-induced metabolic syndrome in rats. Pharmacological Research. 2015;102:208-17.

58.   Passamonti S, Vrhovsek U, Vanzo A, Mattivi F. The stomach as a site for anthocyanins absorption from food. FEBS Letters. 2003;544(1-3):210-3.

59.   Talavera S, Felgines C, Texier O, Besson C, Manach C, Lamaison JL, et al. Anthocyanins are efficiently absorbed from the small intestine in rats. Journal of Nutrition. 2004;134(9):2275-9.

60.   Fang J. Some anthocyanins could be efficiently absorbed across the gastrointestinal mucosa: extensive presystemic metabolism reduces apparent bioavailability. Journal of Agricultural and Food Chemistry. 2014;62(18):3904-11.

61.   Czank C, Cassidy A, Zhang Q, Morrison DJ, Preston T, Kroon PA, et al. Human metabolism and elimination of the anthocyanin, cyanidin-3-glucoside: a (13)C-tracer study. The American Journal of Clinical Nutrition. 2013;97(5):995-1003.

62.   Toufektsian MC, de Lorgeril M, Nagy N, Salen P, Donati MB, Giordano L, et al. Chronic dietary intake of plant-derived anthocyanins protects the rat heart against ischemia-reperfusion injury. Journal of Nutrition. 2008;138(4):747-52.

63.   Kamiloglu S, Capanoglu E, Grootaert C, Van Camp J. Anthocyanin absorption and metabolism by human intestinal Caco-2 cells-A Review. International Journal of Molecular Sciences. 2015;16(9):21555-74.

64.   Wallace TC. Anthocyanins in cardiovascular disease. Advances in Nutrition. 2011;2(1):1-7.

65.   Bowen-Forbes CS, Zhang Y, Nair MG. Anthocyanin content, antioxidant, anti-inflammatory and anticancer properties of blackberry and raspberry fruits. Journal of Food Composition and Analysis. 2010;23(6):554-60.

66.   Fan MJ, Wang IC, Hsiao YT, Lin HY, Tang NY, Hung TC, et al. Anthocyanins from black rice (*Oryza sativa* L.) demonstrate antimetastatic properties by reducing MMPs and NF-κB expressions in human oral cancer CAL 27 cells. Nutrition and Cancer. 2015;67(2):327-38.

67.   Suttiarporn P, Chumpolsri W, Mahatheeranont S, Luangkamin S, Teepsawang S, Leardkamolkarn V. Structures of phytosterols and triterpenoids with potential anti-cancer activity in bran of black non-glutinous rice. Nutrients. 2015;7(3):1672-87.

68.   Inaguma T, Han J, Isoda H. Improvement of insulin resistance by cyanidin 3-glucoside, anthocyanin from black beans through the up-regulation of GLUT4 gene expression BMC Proceedings. 2011;5(8):21.

69.   Sun CD, Zhang B, Zhang JK, Xu CJ, Wu YL, Li X, et al. Cyanidin-3-glucoside-rich extract from Chinese bayberry fruit protects pancreatic beta cells and ameliorates hyperglycemia in streptozotocin-induced diabetic mice. Journal of Medicinal Food. 2012;15(3):288-98.

70.   Sri Harsha PSC, Khan MI, Prabhakar P, Giridhar P. Cyanidin-3-glucoside, nutritionally important constituents and in vitro antioxidant activities of *Santalum album* L. berries. Food Research International. 2013;50(1):275-81.

71.   Nizamutdinova IT, Kim YM, Chung JI, Shin SC, Jeong YK, Seo HG, et al. Anthocyanins from black soybean seed coats preferentially inhibit TNF-alpha-mediated induction of VCAM-1 over ICAM-1 through the regulation of GATAs and IRF-1. Journal of Agricultural and Food Chemistry. 2009;57(16):7324-30.

72.   Jennings A, Welch AA, Spector T, Macgregor A, Cassidy A. Intakes of anthocyanins and flavones are associated with biomarkers of insulin resistance and inflammation in women. Journal of Nutrition. 2014;144(2):202-8.

73.   Chen CY, Yi L, Jin X, Mi MT, Zhang T, Ling WH, et al. Delphinidin attenuates stress injury induced by oxidized low-density lipoprotein in human umbilical vein endothelial cells. Chemico-Biological Interactions. 2010;183(1):105-12.

74.   Seymour EM, Tanone, II, Urcuyo-Llanes DE, Lewis SK, Kirakosyan A, Kondoleon MG, et al. Blueberry intake alters skeletal muscle and adipose tissue peroxisome proliferator-activated receptor activity and reduces insulin resistance in obese rats. Journal of Medicinal Food. 2011;14(12):1511-8.

75.   Seymour EM, Singer AAM, Kirakosyan A, Urcuyo-Llanes DE, Kaufman PB, Bolling SF. Altered hyperlipidemia, hepatic steatosis, and hepatic peroxisome proliferator-activated receptors in rats with intake of tart cherry. Journal of Medicinal Food. 2008;11(2):252-9.

76.   Yang Y, Andrews MC, Hu Y, Wang D, Qin Y, Zhu Y, et al. Anthocyanin extract from black rice significantly ameliorates platelet hyperactivity and hypertriglyceridemia in dyslipidemic rats induced by high fat diets. Journal of Agricultural and Food Chemistry. 2011;59(12):6759-64.

77.   Nakamura Y, Matsumoto H, Todoki K. Endothelium-dependent vasorelaxation induced by black currant concentrate in rat thoracic aorta. The Japanese Journal of Pharmacology. 2002;89(1):29-35.

78.   Zhu Y, Xia M, Yang Y, Liu F, Li Z, Hao Y, et al. Purified anthocyanin supplementation improves endothelial function via NO-cGMP activation in hypercholesterolemic individuals. Clinical Chemistry. 2011;57(11):1524-33.

79.   Yu RQ, Wu XY, Zhou X, Zhu J, Ma LY. Cyanidin-3-glucoside attenuates body weight gain, serum lipid concentrations and insulin resistance in high-fat diet-induced obese rats. Chinese Journal of Contemporary Pediatrics. 2014;16(5):534-8.

80.   Guo H, Ling W. The update of anthocyanins on obesity and type 2 diabetes: experimental evidence and clinical perspectives. Reviews in Endocrine and Metabolic Disorders. 2015;16(1):1-13.

81.   Guo H, Xia M, Zou T, Ling W, Zhong R, Zhang W. Cyanidin 3-glucoside attenuates obesity-associated insulin resistance and hepatic steatosis in high-fat diet-fed and db/db mice via the transcription factor FoxO1. The Journal of Nutritional Biochemistry. 2012;23(4):349-60.

82.   Lila MA. Anthocyanins and human health: an *in vitro* investigative approach. Journal of Biomedicine and Biotechnology. 2004;2004(5):306-13.

83.   Wedick NM, Pan A, Cassidy A, Rimm EB, Sampson L, Rosner B, et al. Dietary flavonoid intakes and risk of type 2 diabetes in US men and women. The American Journal of Clinical Nutrition. 2012;95(4):925-33.

84.   Karlsen A, Retterstol L, Laake P, Paur I, Bohn SK, Sandvik L, et al. Anthocyanins inhibit nuclear factor-κB activation in monocytes and reduce plasma concentrations of pro-inflammatory mediators in healthy adults. Journal of Nutrition. 2007;137(8):1951-4.

85.   Poudyal H, Panchal SK, Waanders J, Ward L, Brown L. Lipid redistribution by alpha-linolenic acid-rich chia seed inhibits stearoyl-CoA desaturase-1 and induces cardiac and hepatic protection in diet-induced obese rats. The Journal of Nutritional Biochemistry. 2012;23(2):153-62.

86.   Poudyal H, Panchal SK, Ward LC, Brown L. Effects of ALA, EPA and DHA in high-carbohydrate, high-fat diet-induced metabolic syndrome in rats. The Journal of Nutritional Biochemistry. 2013;24(6):1041-52.

87.   Khitan Z, Kim DH. Fructose: a key factor in the development of metabolic syndrome and hypertension. Journal of Nutrition and Metabolism. 2013;2013:682673.

88.   Hu FB, Malik VS. Sugar-sweetened beverages and risk of obesity and type 2 diabetes: epidemiologic evidence. Physiology & Behaviour. 2010;100(1):47-54.

89.   Poudyal H, Brown L. Should the pharmacological actions of dietary fatty acids in cardiometabolic disorders be classified based on biological or chemical function? Progress in Lipid Research. 2015;59:172-200.

90.   Kopelman PG. Obesity as a medical problem. Nature. 2000;404(6778):635-43.

91.   Cheung BM, Cheung TT, Samaranayake NR. Safety of antiobesity drugs. Therapeutic Advances in Drug Safety. 2013;4(4):171-81.

92.   Pojer E, F M, Johnson J, Stockley CS. The case for anthocyanin consumption to promote human health: A review. Comprehensive Reviews in Food Science and Food Safety. 2013;12:483-508.

93.   Fernandesa I, Fariaa A, Calhaub C, de Freitasa V, Mateusa N. Bioavailability of anthocyanins and derivatives. Journal of Functional Foods. 2014;7:54-6.

94.   Hu C, Zawistowski J, Ling W, Kitts DD. Black rice (*Oryza sativa L. indica*) pigmented fraction suppresses both reactive oxygen species and nitric oxide in chemical and biological model systems. Journal of Agricultural and Food Chemistry. 2003;51(18):5271-7.

95.   Shields M, Tremblay MS, Connor Gorber S, Janssen I. Abdominal obesity and cardiovascular disease risk factors within body mass index categories. Health Reports. 2012;23(2):7-15.

96.   Carr MC, Brunzell JD. Abdominal obesity and dyslipidemia in the metabolic syndrome: importance of type 2 diabetes and familial combined hyperlipidemia in coronary artery disease risk. The Journal of Clinical Endocrinology & Metabolism. 2004;89(6):2601-7.

97.   Westphal SA. Obesity, abdominal obesity, and insulin resistance. Clinical Cornerstone. 2008;9(1):23-31.

98.   Panchal SK, Poudyal H, Waanders J, Brown L. Coffee extract attenuates changes in cardiovascular and hepatic structure and function without decreasing obesity in high-carbohydrate, high-fat diet-fed male rats. Journal of Nutrition. 2012;142(4):690-7.

99.   Valenti L, Riso P, Mazzocchi A, Porrini M, Fargion S, Agostoni C. Dietary anthocyanins as nutritional therapy for nonalcoholic fatty liver disease. Oxidative Medicine and Cellular Longevity. 2013;2013:145421.

100.   Prior RL. Fruits and vegetables in the prevention of cellular oxidative damage. The American Journal of Clinical Nutrition. 2003;78(3 Suppl):570S-8S.

101.   Scalbert A, Williamson G. Dietary intake and bioavailability of polyphenols. Journal of Nutrition. 2000;130(8S Suppl):2073S-85S.

102.   Drossard C, Bolzenius K, Kunz C, Kersting M. Anthocyanins in the diet of children and adolescents: intake, sources and trends. European Journal of Nutrition. 2013;52(2):667-76.

103.   Galvano F, La Fauci L, Lazzarino G, Fogliano V, Ritieni A, Ciappellano S, et al. Cyanidins: metabolism and biological properties. The Journal of Nutritional Biochemistry. 2004;15(1):2-11.

104.   Little RC, Little WC. Cardiac preload, afterload, and heart failure. Archives of Internal Medicine. 1982;142(4):819-22.

105.   Bartlett K, Eaton S. Mitochondrial beta-oxidation. European Journal of Biochemistry. 2004;271(3):462-9.

106.   Barter PJ, Brewer HB, Jr., Chapman MJ, Hennekens CH, Rader DJ, Tall AR. Cholesteryl ester transfer protein: a novel target for raising HDL and inhibiting atherosclerosis. Arteriosclerosis, Thrombosis, and Vascular Biology. 2003;23(2):160-7.

107.   Xu JW, Ikeda K, Yamori Y. Upregulation of endothelial nitric oxide synthase by cyanidin-3-glucoside, a typical anthocyanin pigment. Hypertension. 2004;44(2):217-22.

108.   Hollenberg SM. Vasodilators in acute heart failure. Heart Failure Reviews. 2007;12(2):143-7.

109.   Cheng J, Zhang W, Zhang X, Han F, Li X, He X, et al. Effect of angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers on all-cause mortality, cardiovascular deaths, and cardiovascular events in patients with diabetes mellitus: a meta-analysis. JAMA Internal Medicine. 2014;174(5):773-85.

110.   Miyazawa T, Nakagawa K, Kudo M, Muraishi K, Someya K. Direct intestinal absorption of red fruit anthocyanins, cyanidin-3-glucoside and cyanidin-3,5-diglucoside, into rats and humans. Journal of Agricultural and Food Chemistry. 1999;47(3):1083-91.

111.   Ling WH, Cheng QX, Ma J, Wang T. Red and black rice decrease atherosclerotic plaque formation and increase antioxidant status in rabbits. Journal of Nutrition. 2001;131(5):1421-6.

112.   Ichiyanagi T, Shida Y, Rahman MM, Hatano Y, Matsumoto H, Hirayama M, et al. Metabolic pathway of cyanidin 3-O-beta-D-glucopyranoside in rats. Journal of Agricultural and Food Chemistry. 2005;53(1):145-50.

113.   de Pascual-Teresa S, Sanchez-Ballesta MT. Anthocyanins: from plant to health. Phytochemistry Reviews. 2008;7:281-99.

114.   Domitrovic R. The molecular basis for the pharmacological activity of anthocyans. Current Medicinal Chemistry. 2011;18(29):4454-69.

115.   Mohamed S. Functional foods against metabolic syndrome (obesity, diabetes, hypertension and dyslipidemia) and cardiovasular disease. Trends in Food Science & Technology. 2014;35(2):114-28.

116.   Ojeda D, Jimenez-Ferrer E, Zamilpa A, Herrera-Arellano A, Tortoriello J, Alvarez L. Inhibition of angiotensin convertin enzyme (ACE) activity by the anthocyanins delphinidin- and cyanidin-3-O-sambubiosides from *Hibiscus sabdariffa*. Journal of Ethnopharmacology. 2010;127(1):7-10.

117.   Upadhyay S, Dixit M. Role of polyphenols and other phytochemicals on molecular signaling. Oxidative Medicine and Cellular Longevity. 2015;2015:504253.

118.   Tsuda T, Horio F, Kato Y, Osawa T. Cyanidin 3-O-beta-D-glucoside attenuates the hepatic ischemia-reperfusion injury through a decrease in the neutrophil chemoattractant production in rats. Journal of Nutritional Science and Vitaminology (Tokyo). 2002;48(2):134-41.

119.   Chiang AN, Wu HL, Yeh HI, Chu CS, Lin HC, Lee WC. Antioxidant effects of black rice extract through the induction of superoxide dismutase and catalase activities. Lipids. 2006;41(8):797-803.

120.   Zhu W, Jia Q, Wang Y, Zhang Y, Xia M. The anthocyanin cyanidin-3-O-beta-glucoside, a flavonoid, increases hepatic glutathione synthesis and protects hepatocytes against reactive oxygen species during hyperglycemia: Involvement of a cAMP-PKA-dependent signaling pathway. Free Radical Biology and Medicine. 2012;52(2):314-27.

121.   Guo H, Li D, Ling W, Feng X, Xia M. Anthocyanin inhibits high glucose-induced hepatic mtGPAT1 activation and prevents fatty acid synthesis through PKCzeta. The Journal of Lipid Research. 2011;52(5):908-22.

122.   Dembinska-Kiec A, Mykkanen O, Kiec-Wilk B, Mykkanen H. Antioxidant phytochemicals against type 2 diabetes. British Journal of Nutrition. 2008;99 E Suppl 1:ES109-17.

123.   Pergola C, Rossi A, Dugo P, Cuzzocrea S, Sautebin L. Inhibition of nitric oxide biosynthesis by anthocyanin fraction of blackberry extract. Nitric Oxide. 2006;15(1):30-9.

124.   Baynes JW, Thorpe SR. Role of oxidative stress in diabetic complications: a new perspective on an old paradigm. Diabetes. 1999;48(1):1-9.

125.   Poudyal H. Mechanisms for the cardiovascular effects of glucagon-like peptide-1. Acta Physiologica (Oxford). 2015.

126.   Min SW, Ryu SN, Kim DH. Anti-inflammatory effects of black rice, cyanidin-3-O-beta-D-glycoside, and its metabolites, cyanidin and protocatechuic acid. International Immunopharmacology. 2010;10(8):959-66.

127.   Demrow HS, Slane PR, Folts JD. Administration of wine and grape juice inhibits in vivo platelet activity and thrombosis in stenosed canine coronary arteries. Circulation. 1995;91(4):1182-8.

128.   Wixon J. Website review: The Jackson Laboratory Mouse Genome Informatics Site: version 2.3.2. Yeast. 2000;17(2):134-45.

129.   Sasaki R, Nishimura N, Hoshino H, Isa Y, Kadowaki M, Ichi T, et al. Cyanidin 3-glucoside ameliorates hyperglycemia and insulin sensitivity due to downregulation of retinol binding protein 4 expression in diabetic mice. Biochemical Pharmacology. 2007;74(11):1619-27.

130.   Han SJ, Ryu SN, Trinh HT, Joh EH, Jang SY, Han MJ, et al. Metabolism of cyanidin-3-O-beta-D-glucoside isolated from black colored rice and its antiscratching behavioral effect in mice. Journal of Food Science. 2009;74(8):H253-8.

131.   Hwang YP, Choi JH, Han EH, Kim HG, Wee JH, Jung KO, et al. Purple sweet potato anthocyanins attenuate hepatic lipid accumulation through activating adenosine monophosphate-activated protein kinase in human HepG2 cells and obese mice. Nutrition Research. 2011;31(12):896-906.

132.   Jang HH, Park MY, Kim HW, Lee YM, Hwang KA, Park JH, et al. Black rice (*Oryza sativa L.*) extract attenuates hepatic steatosis in *C57BL/6 J* mice fed a high-fat diet via fatty acid oxidation. Nutrition and Metabolism. 2012;9(1):27.

133.   Shah N, Tang H, Doak TG, Ye Y. Comparing bacterial communities inferred from 16S rRNA gene sequencing and shotgun metagenomics. Pacific Symposium on Biocomputing. 2011:165-76.

134.   Guinane CM, Cotter PD. Role of the gut microbiota in health and chronic gastrointestinal disease: understanding a hidden metabolic organ. Therapeutic Advances in Gastroenterology. 2013;6(4):295-308.

135.   Mahmood T, Yang PC. Western blot: technique, theory, and trouble shooting. North American Journal of Medical Sciences. 2012;4(9):429-34.

136.   Masuzaki H, Ogawa Y, Isse N, Satoh N, Okazaki T, Shigemoto M, et al. Human obese gene expression. Adipocyte-specific expression and regional differences in the adipose tissue. Diabetes. 1995;44(7):855-8.