COFFEE AND ITS CONSTITUENTS AS POTENTIAL TREATMENTS FOR METABOLIC SYNDROME

A Thesis submitted by

Nikhil Bhandarkar, MBiotech

For the award of

Doctor of Philosophy

2017

In loving memory of my late brother

Dr Rohit Bhandarkar

Abstract

Metabolic syndrome is the constellation of metabolic disorders such as central obesity, dyslipidaemia, insulin resistance, impaired glucose tolerance and hypertension. This combination increases the risk of development of cardiovascular disease, fatty liver and type 2 diabetes. The prevalence of metabolic syndrome is increasing worldwide.

Diet is important in the development of metabolic syndrome assisted by increased oxidative stress and inflammation. Plant-based diets provide potential therapeutic approaches to metabolic syndrome. Testing requires an appropriate animal model that mimics the human syndrome. In this project, I used a diet-induced obese rat model for examining the nutraceutical effects of some potential functional foods. To induce metabolic syndrome, young male Wistar rats were fed with a high-carbohydrate high-fat diet for 16 weeks while corn starch served as control diet. The high-carbohydrate high-fat diet induced an impaired glucose tolerance, insulin resistance, obesity, elevated blood pressure, dyslipidaemia, cardiovascular remodelling such as hypertrophy and fibrosis, increased cardiac stiffness, hepatic disorders such as inflammation and steatosis, along with elevated plasma markers of liver function.

Dietary interventions were given for the last 8 weeks only, as a reversal protocol. Interventions included green coffee extract (5%), decaffeinated green coffee extract (5%), chlorogenic acid (100 mg/kg/day), coffee pulp (5%), spent coffee (5%) and fish oils (3%). Green coffee with or without caffeine attenuated body weight and reduced cardiovascular disorders such blood pressure and cardiac stiffness, and improved heart and liver structure without improving glucose homeostasis or plasma lipid concentrations. Coffee pulp and spent coffee considered as waste products of coffee manufacturing industries attenuated cardiovascular remodelling and non-alcoholic fatty liver disease. Both waste products reduced body weight, improved glucose tolerance and decreased abdominal fat. Chlorogenic acid was present in all coffee products. Intervention with chlorogenic acid decreased body weight and visceral fat accumulation, improved heart and liver structure and function but did not improve glucose tolerance.

Prostate cancer patients treated with testosterone deprivation therapy, either through orchidectomy or leuprolide injection, show increased obesity. In high-

carbohydrate, high-fat fed rats, leuprolide treatment worsened metabolic syndrome symptoms and cardiovascular function, and orchidectomy produced greater responses. In H-fed leuprolide-treated rats, Omacor (a mixture of ethyl esters of EPA and DHA) decreased systolic blood pressure and left ventricular diastolic stiffness, reduced infiltration of inflammatory cells and collagen deposition in the heart, reduced lipid accumulation and inflammatory cell infiltration without improving liver damage. Thus, fish oils may provide an option to reduce metabolic syndrome while leuprolide treatment continues in patients with prostate cancer.

My studies show the promising potential of functional foods against life-style associated metabolic disorders. In particular, widely used beverage coffee showed relevant actions against most signs of metabolic syndrome. Further, waste products from coffee production are a potential source for new interventions in diet-induced cardiovascular and metabolic diseases.

Keywords

metabolic syndrome; obesity; cardiovascular remodelling; high-carbohydrate, high-fat diet; coffee; fish oil; coffee pulp; spent coffee; chlorogenic acid

Certification of Thesis

This Thesis is the work of **Nikhil Bhandarkar** except where otherwise acknowledged, with the majority of the authorship of the papers presented as a Thesis by Publication undertaken by the Student. The work is original and has not previously been submitted for any other award, except where acknowledged.

Principal Supervisor: Professor Lindsay Brown

Associate Supervisor: Dr Sunil K Panchal

Student and supervisors signatures of endorsement are held at the University.

Statement of Authorship

Chapter 1. Introduction - Metabolic syndrome, cardiovascular disease and coffee as a functional food. Nikhil Bhandarkar, Stephen Wanyonyi, Lindsay Brown, Sunil K Panchal

NB was responsible for 60% of conception and design of this chapter, 60% of analysis and interpretation of literature, 60% of drafting and writing and 20% of final important editorial input; SW was responsible for 10% of conception and design, 10% of analysis and interpretation of literature, 10% of drafting and writing and 20% of final important editorial input; LB was responsible for 10% of conception and design, 10% of analysis and interpretation of literature, 10% of drafting and writing and 20% of final important editorial input; SKP was responsible for 20% of conception and design, 20% of analysis and interpretation of literature, 20% of drafting and writing and 40% of final important editorial input.

Chapter 2. Green coffee improves diet-induced metabolic syndrome in rats. Nikhil Bhandarkar, Peter Mouatt, Sunil K Panchal, Lindsay Brown

NB was responsible for 40% of conception and design, 80% collection of data, 40% of analysis and interpretation of data, 30% of drafting and writing and 10% of final important editorial input; PM was responsible for 5% of conception and design, 10% collection of data, 10% of analysis and interpretation of data, 5% of drafting and writing and 5% of final important editorial input; SKP was responsible for 25% of conception and design, 10% collection of data, 30% of analysis and interpretation of literature, 25% of drafting and writing and 25% of final important editorial input; LB was responsible for 30% of conception and design, 20% of analysis and interpretation of literature, 30% of drafting and writing and 50% of final important editorial input.

Chapter 3. Chlorogenic acid attenuates metabolic syndrome in diet-induced obese rats. Nikhil Bhandarkar, Lindsay Brown, Sunil K Panchal

NB was responsible for 50% of conception and design, 80% collection of data, 50% of analysis and interpretation of data, 30% of drafting and writing and 20% of final important editorial input; LB was responsible for 20% of conception and design, 5% collection of data, 25% of analysis and interpretation of literature, 30% of drafting and

writing and 40% of final important editorial input; SKP was responsible for 30% of conception and design, 15% collection of data, 25% of analysis and interpretation of literature, 40% of drafting and writing and 40% of final important editorial input.

Chapter 4. Coffee pulp attenuates metabolic syndrome in diet-induced obese hypertensive rats. Nikhil Bhandarkar, Peter Mouatt, Lindsay Brown, Sunil K Panchal

NB was responsible for 50% of conception and design, 80% collection of data, 50% of analysis and interpretation of data, 40% of drafting and writing and 30% of final important editorial input; PM was responsible for 10% of conception and design, 10% collection of data, 10% of analysis and interpretation of data, 5% of drafting and writing and 5% of final important editorial input; LB was responsible for 20% of conception and design, 20% of analysis and interpretation of literature, 25% of drafting and writing and 30% of final important editorial input; SKP was responsible for 20% of conception and design, 10% collection of data, 20% of analysis and interpretation of literature, 30% of drafting and writing and 35% of final important editorial input.

Chapter 5. Spent coffee attenuates metabolic syndrome in diet-induced obese rats. Nikhil Bhandarkar, Peter Mouatt, Lindsay Brown, Sunil K Panchal

NB was responsible for 50% of conception and design, 80% collection of data, 50% of analysis and interpretation of data, 40% of drafting and writing and 30% of final important editorial input; PM was responsible for 10% of conception and design, 10% collection of data, 10% of analysis and interpretation of data, 5% of drafting and writing and 5% of final important editorial input; LB was responsible for 20% of conception and design, 20% of analysis and interpretation of literature, 25% of drafting and writing and 30% of final important editorial input; SKP was responsible for 20% of conception and design, 10% collection of data, 20% of analysis and interpretation of literature, 30% of drafting and writing and 35% of final important editorial input.

Chapter 6. Attenuation of metabolic syndrome by EPA/DHA ethyl esters in testosterone deficient high-carbohydrate, high-fat diet-fed rats. Nikhil Bhandarkar, Senthil Arun Kumar, Jared Martin, Lindsay Brown, Sunil K Panchal

NB was responsible for 20% of conception and design, 40% collection of data, 30% of analysis and interpretation of data, 40% of drafting and writing and 20% of final

important editorial input; SAK was responsible for 20% of conception and design, 40% collection of data, 30% of analysis and interpretation of data, 10% of drafting and writing and 10% of final important editorial input; JM was responsible for 10% of conception and design, 5% collection of data, 5% of analysis and interpretation of data, 5% of drafting and writing and 5% of final important editorial input; LB was responsible for 30% of conception and design, 5% collection of data, 15% of analysis and interpretation of data, 15% of drafting and writing and 30% of final important editorial input; SKP was responsible for 20% of conception and design, 10% collection of data, 20% of analysis and interpretation of data, 30% of drafting and writing and 35% of final important editorial input.

Acknowledgements

I would like to express my sincere gratitude for the support of the University of Southern Queensland for permission and access to mentorship and multidisciplinary expertise that was fundamental to the development of my technical and professional skills, and critical to the completion of my multidisciplinary research projects.

I would like to thank my colleagues from Functional Foods Research Group and outside this group who have worked alongside me on several projects. I would like to acknowledge that their assistance with repetitive tasks, encouragement and support were fundamental to this project's success. A big thanks to Peter Mouatt, Southern Cross University who performed many analytical analysis for my coffee samples.

I would like to thank past and present students from Functional Foods Research Group, namely Siti Raihanah Shafie, Anna Balzer, Sharyn Carnahan, Oliver John, Ryan du Preez, Jessica Pahl, Kiran Mahale, Ashwini Sharma, Kaitlyn Wieden and Edward Bliss. I also appreciate help and support from Dr Stephen Wanyonyi for learning techniques and providing expert advice whenever required.

To my Principal Supervisor Professor Lindsay Brown and Associate Supervisor Dr Sunil Panchal, I cannot thank you enough for your incredible support, encouragement, persistence, patience and extra strong coffee; particularly in times when I was lost, confused and feeling defeated over my health and personal losses. Your support and help are appreciated that can never be explained in words. A big thanks to my supervisors for providing me with tuition fee and living allowance.

I would like to dedicate this thesis to my late brother, Dr Rohit Bhandarkar, who played a major role in starting my studies in Australia and also supported me in achieving higher goals during my studies. Last but not least, I would like to thank my mother, Mrs Shevantibai Bhandarkar, and my father, Mr Suresh Bhandarkar, for their trust, faith and belief in me.

Table of Contents

Chapter 1. Introduction

Metabolic syndrome, cardiovascular disease and coffee as a functional food

Metabolic syndrome: prevalence and consequences

Obesity is characterised by the excessive accumulation of fat in adipose tissue [1]. The prevalence of obesity has escalated to epidemic levels globally. According to the World Health Organization (WHO), approximately 600 million people or 13% of the global population are categorised as obese and it is estimated that the number of newly diagnosed obese individuals has doubled since 1980 [2]. In the US, the prevalence of obesity was estimated at 35% among males and 40.4% among females in 2013-2014 [3]. Metabolic syndrome is the constellation of metabolic disorders such as dyslipidaemia, hypertension, visceral obesity, insulin resistance and impaired glucose tolerance [4]. Metabolic syndrome increases the risk of developing cardiovascular disease, type 2 diabetes and non-alcoholic fatty liver disease [5]. The association between obesity and metabolic syndrome was demonstrated in the National Health and Nutritional Examination Survey (NHANES) III study in which 5% of normal weight, 22% of overweight and 60% of obese subjects were classified as having metabolic syndrome in the United States [6].

Increasing poor nutrition, high-calorie diets, sedentary lifestyle and the comparative ageing of populations promotes an increase in the prevalence of metabolic syndrome [7]. Thus, targeting metabolism or energy homeostasis could be an approach to prevent or reverse metabolic syndrome and its consequences [8]. Due to the improvements in medical management, hypertriglyceridaemia and hypertension were reduced leading to reduction in metabolic syndrome from about 25.5% in 1999 to 22.9% in 2010 in the United States. However, abdominal circumference and hyperglycaemia steadily increased over the same period of time [9]. Cardiovascular diseases are one of the leading causes of mortality and morbidity worldwide [10, 11]. Patients with metabolic syndrome have higher risks to develop cardiovascular morbidity and mortality as well as type 2 diabetes. The prevalence of cardiovascular diseases was approximately three times higher in individuals with metabolic syndrome than individuals without it [12]. Despite some promising developments, metabolic syndrome remains one of the predominant causes of morbidity and mortality worldwide.

Chronic inflammation is closely linked to the development of obesity, insulin resistance, oxidative stress, cardiovascular disease and type 2 diabetes, characterised by abnormal secretion of cytokines and activation of the inflammatory signalling cascades [8]. Adipocytes modify metabolism by secreting non-esterified fatty acids, hormones such as leptin and adiponectin and pro-inflammatory cytokines [13, 14]. Excessive adipocyte accumulation triggers increased secretion of several pro-inflammatory adipokines such as interleukin (IL)-6, tumour necrosis factor (TNF), resistin, angiotensinogen, C-reactive protein (CRP), plasminogen activator inhibitor-1 (PAI-1) and fibrinogen causing prothrombotic alterations, increases in chronic low-grade inflammation and cardiovascular complications such as atherogenesis [15, 16]. These adipokines also regulate pathways that are associated with atherosclerosis, insulin resistance, hypertension and endothelial dysfunction [15, 16]. For instance, TNF is an adipokine that upregulates inflammatory variations in the vascular tissue, causing insulin resistance by inhibition of the insulin receptor signalling pathway and reduced the PPAR-γ expression level [15, 16].

Major components of metabolic syndrome

Metabolic syndrome is linked to hypertension and is responsible for myocardial dysfunctions, characterised by extended contraction and relaxation time, reduced rate of myocardial contraction and relaxation and reduced myocardial function [17, 18]. Several mechanisms have been proposed for cardiovascular abnormalities linked to metabolic syndromes, such as disrupted energy production because of reduced mitochondrial respiration and pyruvate dehydrogenase activity, oxidative stress, dyslipidaemia, mitochondrial leakage, reduced autophagy healing, endoplasmic reticulum stress and interrupted intracellular Ca^{2+} ion signalling [19, 20]. In a transgenic mouse model of lipotoxic diabetic cardiomyopathy, heart tissue showed elevated fatty acid intake, reduced diastolic sarcomere length, diminished myofilament Ca^{2+} response, higher β-MHC expression and extended diastole [21]. Several therapeutic options have been developed for the management of cardiovascular disease associated with metabolic syndrome, but they are not yet making a difference. This could be due to lack of clear understanding of metabolic syndrome associated pathogenesis in cardiovascular remodelling [22, 23].

Metabolic syndrome impairs cardiovascular function by the combination of atherogenic dyslipidaemia and hyperglycaemia due to the chronic pro-inflammatory conditions resulting in obesity, hypertension and prothrombotic state (Figure 1) [24, 25]. Subjects classified as having metabolic syndrome and diagnosed with at least 4 of 5 metabolic syndrome parameters have been shown to have 3-fold increased risk of cardiovascular diseases in a 5-year follow-up [26]. Atherogenic dyslipidaemia consists of elevations of lipoproteins such as apolipoprotein B (ApoB), elevated serum triglycerides, a high concentration of small LDL particles, and low concentration of high-density lipoproteins (HDL) [27]. The elevated levels of triglycerides and small LDL and low levels of HDL-cholesterol, collectively known as the lipid triad are the fundamental reasons for causing cardiovascular disorders such as angina pectoris, unstable angina, myocardial infarction or cardiac-related deaths [28].

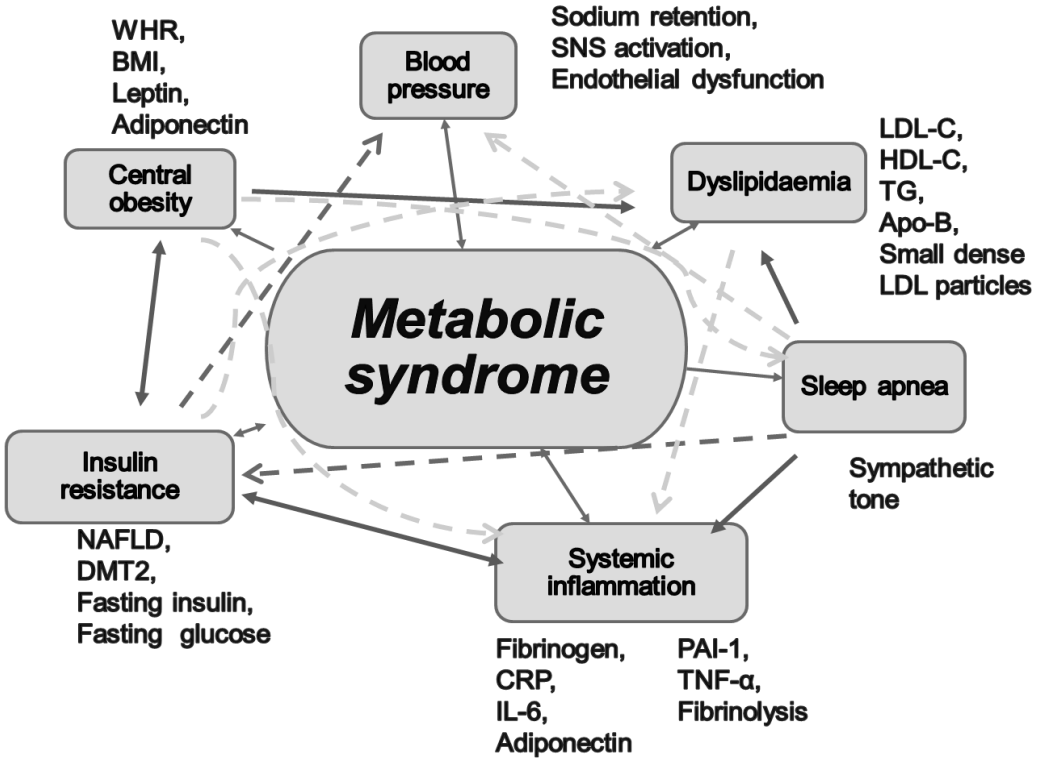


Figure 1. Components of metabolic syndrome [29]

Obesity

Obesity is responsible for triggering insulin resistance resulting in the pathogenesis of cardiovascular disease as insulin resistance promotes atherogenic dyslipidaemia [30, 31]. Insulin resistance is described as reduced sensitivity to circulating insulin. In diet-induced obesity, excess energy intake leads to an overload of adipocytes beyond their lipid storage capacity, causing excessive release of lipids into the bloodstream. High levels of circulating non-esterified fatty acids suppress glucose uptake by skeletal muscles [32]. Malfunction of fatty acid metabolism causes excess accumulation of lipids in myocytes, hepatocytes, and β-cells of pancreas, in turn, causing insulin resistance as well as impairment of β-cell function; these effects are noted as lipotoxicity [33, 34].

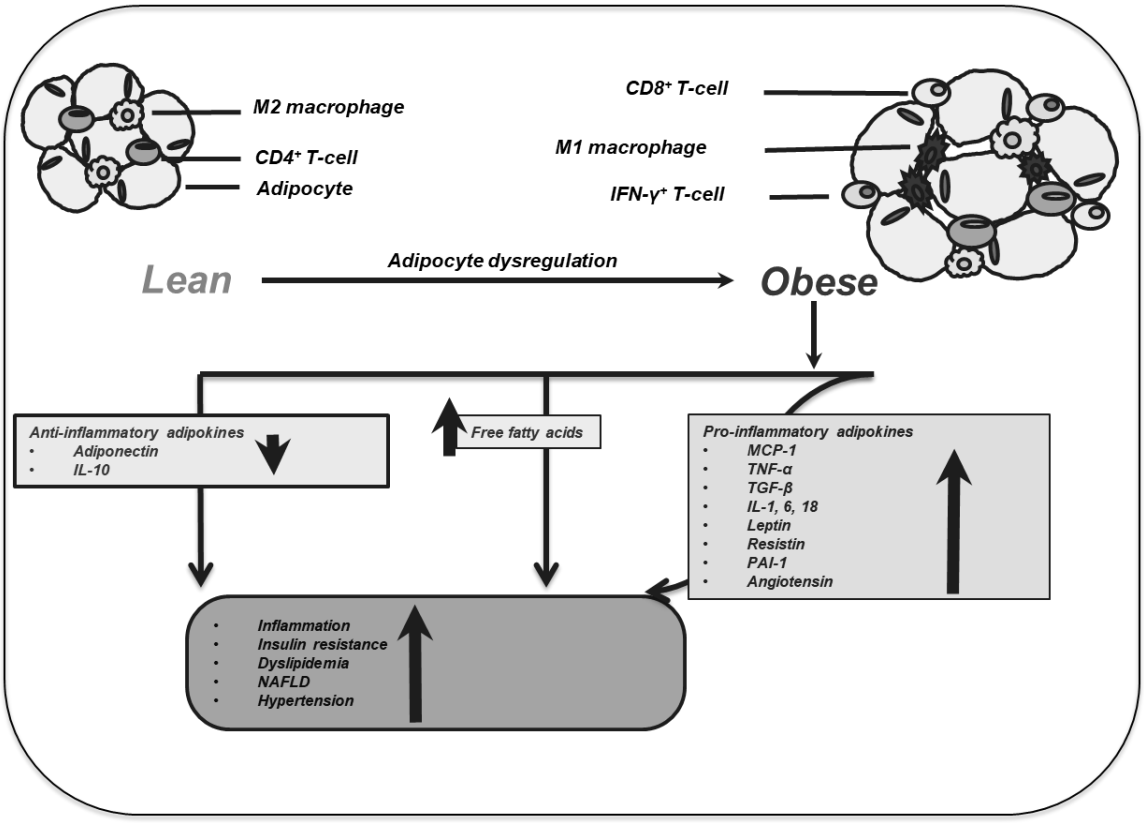


Figure 2. Chronic low-grade inflammation in obesity [35]

Lipotoxicity reduced insulin-mediated glucose metabolism in myocytes through inhibition of pyruvate dehydrogenase, consequently decreasing glucose oxidation and accumulation of glycolytic intermediates [36]. In such circumstances, pancreatic β-cells release more insulin to suppress the hyperglycaemia in the insulin-resistant state

essentially causing hyperinsulinemia. Sustained insulin resistance results in clinical symptoms of metabolic syndrome [37, 38] and may lead to cardiovascular events and type 2 diabetes [39]. Apart from glucose intolerance, dyslipidaemia and obesity play a crucial role in the excessive release of cytokines, leptin, TNF, IL-6 and decreased production of adiponectin (Figure 2) [40].

Insulin resistance

The insulin signalling pathway plays a fundamental role in glucose metabolism. Insulin binds to the receptor, a ligand-activated tyrosine kinase and insulin-associated tyrosine phosphorylation trigger activation of two corresponding pathways: the phosphoinositide 3-kinase (PI3K) pathway and the mitogen-activated protein (MAP) kinase pathway. In the insulin resistance state, the PI3K-Akt pathway is disturbed, while the MAP kinase pathway performs normally. Thus, insulin resistance creates disproportion between these two parallel pathways. Inhibition of the PI3K-Akt pathway causes a decrease in endothelial nitric oxide production, leading to endothelial dysfunction, and a decline in GLUT4 translocation, prominently resulting in reduced glucose uptake by myocytes [41]. Opposite to this, the MAP kinase pathway remains unaltered functionally. Consequently, there is a continuous release of endothelin-1 (ET-1), a stimulus of growth factors such as transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and vascular cell adhesion molecules (VCAMs), which are crucial for development of an atherogenic plaque [42]. Therefore, insulin resistance induces vascular abnormalities, predisposing to atherosclerosis [43]. However, an insulin-resistant subject is not always categorised as clinically obese and such a subject may have an irregular distribution of accumulated adipose tissue, described as upper body fat [44]. Irrespective of the relationship between body fat and insulin resistance, insulin resistance has a strong correlation with progression in central obesity and thus, with metabolic syndrome [43].

Oxidative stress

Oxidative stress is the common factor that occurs during obesity, insulin resistance, impaired glucose tolerance, type 2 diabetes and cardiovascular disease [45].

Oxidative stress is a disturbed equilibrium between tissue oxidants (free radicals or reactive oxygen species (ROSs)) and antioxidants. Oxidative stress plays an important role in the development of obesity-associated comorbidities such as cardiovascular disease and diabetes [46]. In conditions of excessive energy intake, the abundance of substrates stimulates the activity of the citric acid cycle leading to the production of excessive mitochondrial NADH (mNADH) and ROS [47]. Free fatty acids and glucose are the primary energy sources in the skeletal muscle cells and adipocytes. Breakdown of either substrates mitochondrial acetyl-CoA and NADH [48]. When this acetyl-CoA enters citric acid cycle, it produces isocitrate which then combines with NAD^+ generating NADH with the help of catalytic activity of isocitrate dehydrogenase. When excessive NADH is generated and not eliminated by oxidative phosphorylation, it causes an increased mitochondrial proton gradient and the shift of a single charged electron to oxygen, thereby destabilising it and consequently creating free radical species such as superoxide anion (Figure 3) [49].

The overproduction of ROS is the initial and significant step that stimulates other pathways engaged in the pathogenesis of endothelial dysfunction during hyperglycaemia [50, 51]. Excessive ROS such as superoxide combines with nitric oxide, triggering formation of the strong oxidant peroxynitrite [52] that can alter DNA sequences [51] and the damaged DNA initiates the activation of the nuclear enzyme poly(ADP-ribose) polymerase [51] which initiates DNA repair, a process that requires NAD^+ as a substrate. The process of DNA repair may cause scarcity of intracellular NAD^+. Restricted availability of NAD^+ slows the rate of glycolysis, electron transport and ATP generation, and promotes ADP-ribosylation of GAPDH [51], triggering acute endothelial dysfunction. Free fatty acids may function similarly [53] to increase oxidative stress and cause endothelial dysfunction. However, antioxidants can reverse consequences of oxidative stress in humans [54, 55].

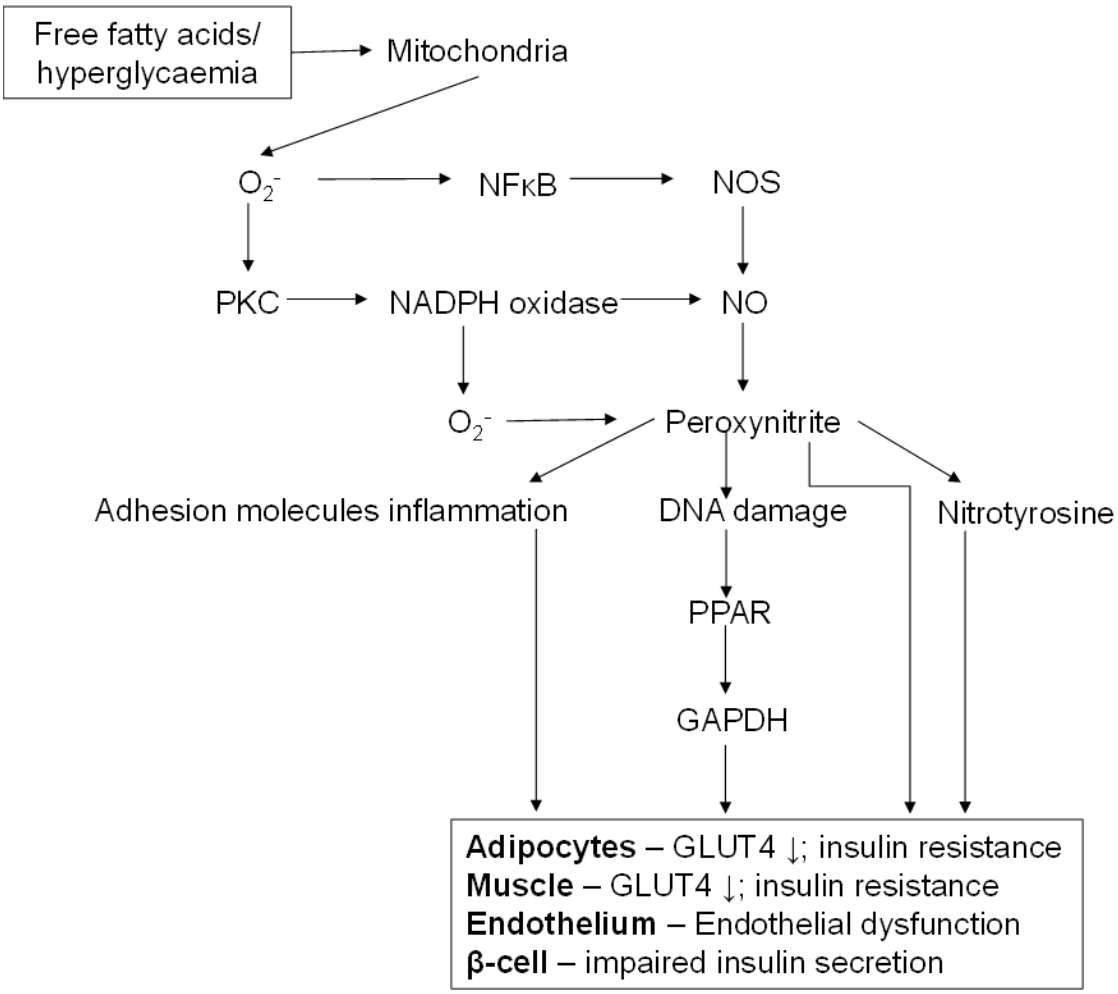


Figure 3. Association between oxidative stress and metabolic abnormalities [49].

Obesity and cardiovascular disease

Obesity has several severe impacts on cardiovascular structure and function and hence it is one of the prime reasons for cardiovascular disease-associated morbidity (Figure 4) [56]. Obesity escalates cardiac output, as well as total blood volume, thus increasing cardiac workload. In obesity, increased stroke volume causes an elevation in cardiac output associated with higher sympathetic activation which subsequently increases heart rate [57]. Obese subjects are more prone to develop hypertension than the lean persons as weight gain is associated with impaired control of arterial pressure [58]. Thus, obese and overweight subjects generally end up with left ventricular dilatation [56]. Regardless of age and sex, obesity increases the probability of left ventricular hypertrophy and other structural irregularities such as concentric remodelling and concentric left

ventricular hypertrophy [59]. Additionally, obesity causes left atrial enlargement because of elevated blood volume and eccentric left ventricular diastolic loading [60]. Such abnormalities contribute to impairments such as atrial fibrillation due to left atrial enlargement and heart failure [61]. Further, abnormal left ventricular structure increases the occurance of frequent and complex ventricular arrhythmias [62].

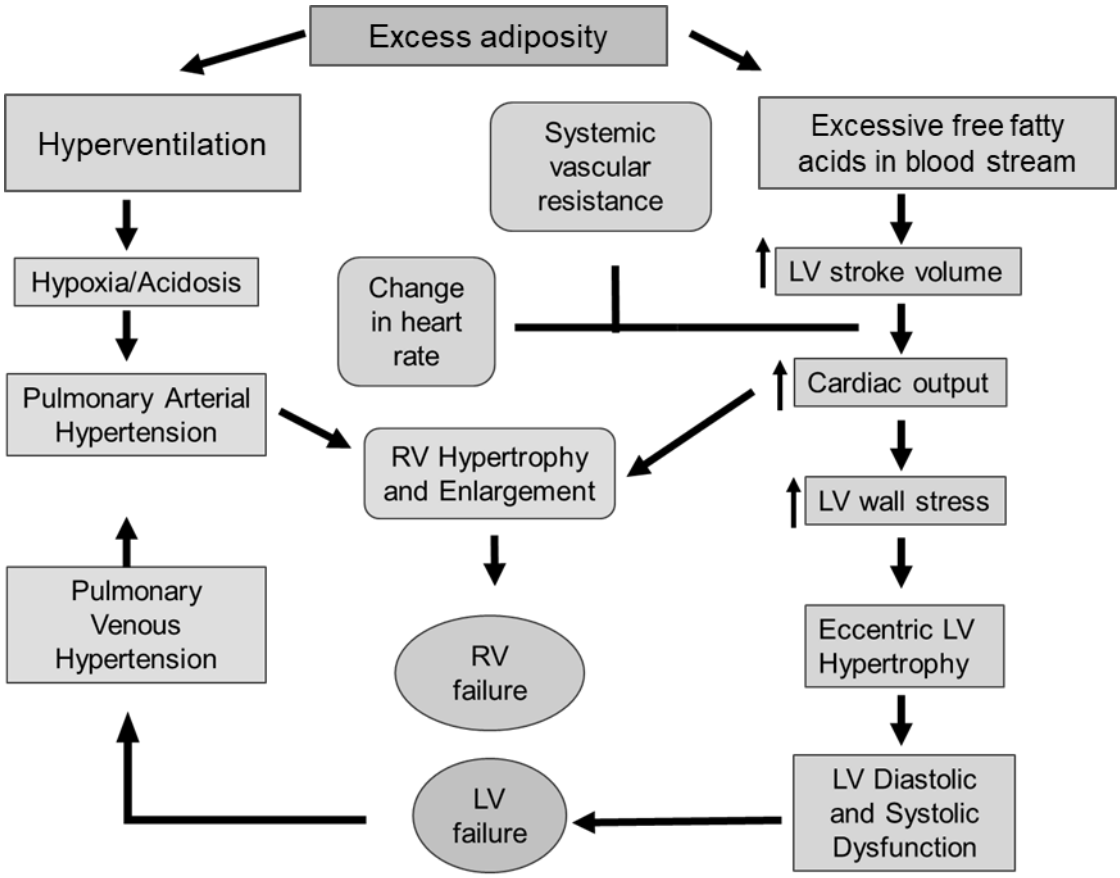


Figure. 4. Association of obesity and cardiomyopathy. LV = left ventricular; RV = right ventricular [56].

Thus, obesity as the major component of metabolic syndrome affects the cardiovascular system and increases the prevalence of hypertension, dyslipidaemia and type 2 diabetes. Similarly, obesity plays a crucial role in atherosclerosis and cardiovascular disease [63].

Cardiovascular disease

High circulating concentrations of lipids and inflammatory cytokines lead to the atherosclerotic condition. In cardiovascular conditions such as unstable angina and

myocardial infraction, elevated concentrations of CRP and IL-6 have been associated with worsening of prognosis [64]. Moreover, other inflammatory markers such as IL-7, IL-8, soluble CD40 ligand and CRP–related protein pentraxin 3 increased during cardiovascular events [65-67]. Following are the cardiovascular outcomes that can develop as a result of uncontrolled metabolic abnormalities.

Hypertension

Hypertension is one of the predominant disease conditions associated with metabolic disorders such as obesity, glucose intolerance and dyslipidaemia [68]. Renin-angiotensin-aldosterone (RAAS) system-associated pathway AT_1R (Angiotensin II type 1 receptor) and mineralocorticoid receptor initiate NADPH oxidase pathway and promote the production of ROS. NADPH oxidase, as well as the mitochondrial generation of ROS, causes oxidative stress in the myocardium. This ROS also triggers insulin resistance, endothelial dysfunction and atherosclerosis [69]. The RAAS pathway is activated by hyperglycaemia and hyperinsulinaemia, leading to increased expression of angiotensinogen, angiotensin II (AT II) and the AT_1 receptors. Mutually, these play a crucial role in the progress of hypertension in a subject with insulin resistance [70]. There is also evidence that insulin resistance and hyperinsulinaemia lead to activation of the sympathetic nervous system, causing higher sodium reabsorption in the nephron, higher cardiac output and vasoconstriction of arteries initiating severe hypertension [71]. Moreover, it has been recently been reported that adipose tissue also generates aldosterone in response to ATII [72]. Hence, the adipose tissue could be a location for renin-angiotensin-aldosterone system occurrence.

Chronic systemic inflammation or obesity has an important role in the development and progression of hypertension. A strong correlation exists between obesity and higher blood concentrations of inflammatory cytokines such as TNF-α, IL-1β, IL-6 and CRP. Subsequently higher concentrations of inflammatory cytokines (TNF-alpha and IL-1β) increase arterial stiffness, thus low-grade inflammation may play a role in the elevation of blood pressure [73, 74]. Increasing IL-6 concentrations in blood trigger elevation of soluble intercellular adhesion molecule-1 (sICAM-1) which is associated with elevated systolic and diastolic blood pressure, pulse pressure and mean arterial

pressure [75]. Plasma IL-6 was higher in women with elevated systolic and diastolic blood pressure, while in men, IL-6 was correlated with impaired glucose tolerance [74]. Several studies have shown that hypertension declined with the reduction in body weight regardless of the mechanisms involved [76]. Moreover, studies have reported the reduction in systolic blood pressure of 1-4 mmHg and diastolic blood pressure of 1-2 mmHg per kilogram of body weight lost in ~50% of the subjects tested [77].

Stroke

The published studies have shown that approximately 43% of people with metabolic syndrome are at the risk of stroke [78]. Obesity, insulin resistance and ROS are associated with increased inflammation that causes the development of the atherosclerotic cerebrovascular disease. Chronic low-grade inflammation present during atherogenesis of cerebral arteries is accelerated by several acute phase reactants such as CRP and fibrinogen. CRP is associated with an increasing risk of stroke events [79, 80]. Further, elevated CRP concentrations increased the incidence of systematic atrial fibrillation that escalates the risk of stroke [81, 82].

At cellular and molecular levels, insulin resistance has a role in the development of pathophysiology of vascular diseases such as stroke. Hyperglycaemia leads to increased oxidative stress by several mechanisms, for example, autoxidation of glucose produces ROS, a leading cause of oxidative stress [83]. In addition, advanced glycation end products, a heterogeneous group of compounds synthesised by non-enzymatic reactions between reducing sugars and free amino groups of proteins, are able to bind to cell surface receptors or cross-link with proteins, causing a modification of their structure and function [84]. Advanced glycation end products have the potential to induce insulin resistance and lead to increased oxidative stress and inflammation [85]. Furthermore, NADPH oxidase activation also initiates release of ROS [86]. NADPH oxidase catalyses the reduction of molecular oxygen causing superoxide radical formation [87]. In case of obesity, higher production of ROS occurs, especially in adipocytes, mediated by increased expression of NADPH oxidase. Further, treatment with NADPH oxidase inhibitors reduced ROS production in adipocytes, reduced the dysregulation of adipokines and improved metabolic syndrome parameters such as dyslipidaemia, obesity and insulin resistance

[88]. Nitric oxide is essential for prevention of cardiac ischaemia caused by oxidative stress and inhibits platelet aggregation thereby playing an important role in the response to cardiovascular disease [89]. Overproduction of nitric oxide can also cause adverse cardiac events such as atherosclerosis [90].

Hypertrophic cardiomyopathy

Obesity is strongly associated with heart failure and has been shown to cause deleterious effects on diastolic function [91, 92]. Cardiac hypertrophy is an adaptive response to physiological stress such as pressure or volume stress, mutations in myocyte proteins or metabolic disruptions. However, cardiac hypertrophy could be maladaptive and leads to cardiac dysfunction [93, 94]. Obesity could be a leading cause of severe hypertrophy by several mechanisms such as elevated sympathetic tone, elevated leptin concentrations, myocardial infiltration, insulin resistance and increased renin-angiotensin activity [95]. Obese individuals with hypertrophic cardiomyopathy had considerably higher left ventricular mass, left ventricular mass index and increased chances of progressing to severe cardiac events [96]. Moreover, the same study showed that obese subjects had a higher obstruction in the left ventricular outflow tract [96]. Prevalence of left ventricular obstruction was more than two-fold higher in obese individuals having hypertrophic cardiomyopathy compared to normal subjects and it was directly associated with elevated left ventricular mass [97, 98].

Coronary artery disease

Inflammation stimulates impaired arterial function both acutely as well as in chronic situation [99]. Chronic low-grade inflammation and oxidative stress contribute significantly to the development of atherosclerosis by contributing to the early stage of elevated endothelial permeability as well the creation of mature atherosclerotic plaque and plaque rupture [100]. Inflammatory biomarkers such as CRP have a crucial role in coronary artery disease risk [101]. Likewise, elevated concentrations of circulating soluble adhesion molecules such as sICAM-1, soluble VCAM-1 and soluble P-selectin contribute to coronary artery disease pathogenesis [102]. However, different inflammatory biomarkers cause an increased risk of coronary artery disease through various

mechanisms. Elevated CRP and IL-6 have been found in subjects with stable angina and myocardial infraction with worsening prognosis [67]. Moreover, other inflammatory biomarkers such as fibrinogen, IL-7, IL-8 and soluble CD40 ligand are also increased in cardiovascular disease patients [103].

Regardless of gender, weight-stable obese individuals have a higher risk of arrhythmias and sudden cardiac death [104]. In both genders, the rate of sudden cardiac arrest was approximately 40 times higher in obese individuals than lean individuals [95]. Further, for subjects aged between 35-44 years, mortality due to cardiac arrest is 6 times higher in men with severe obesity than in women of a similar age group. Hyperglycaemia associated with metabolic syndrome decreases nitric oxide availability resulting in elevated vasomotor tone and ventricular irregularity [105, 106]. Likewise, increased free fatty acids cause increased plasma catecholamine resulting in altered cardiac repolarisation [107]. Specifically, long-chain saturated fatty acids have an important role in the occurrence of ventricular arrhythmias leading to myocardial infarction [108]. The saturated fatty acid, palmitate, caused a reduction in mitochondrial respiration in cardiomyocytes and promoted production of excessive total cellular ROS as well as mitochondrial ROS [109]. Excessive ROS production caused partial depolarisation of the mitochondrial inner membrane and subsequently triggered mitochondrial calcium overload by raising sarcoplasmic reticulum calcium leakage, that leads to arrhythmias and heart failure [110].

Dietary interventions against metabolic syndrome

Previous studies have shown that moderately modified lifestyle and dietary interventions can reduce the risk for metabolic diseases such as diabetes [111-113]. Diet and physical activity promote weight loss, favourably modulating parameters of metabolic syndrome. Epidemiological studies have reported that polyphenol intake has a positive impact on cardiovascular and hepatic disorders [114-116]. In addition, human studies with polyphenol-rich diets have concluded a favorable effect of polyphenols on risk factors of cardiovascular health such as blood pressure [117], serum lipid profile [118] and insulin sensitivity [119]. Studies using animal models have demonstrated that polyphenols improve insulin sensitivity [120], have anti-obesity effects by reducing body fat [121] and

are suggested to reduce plasma concentrations of triglycerides [122, 123] and cholesterol [124].

Traditionally, coffee is recommended for health management as it is rich in bioactive substances with a wide range of physiological effects [125-127]. Key bioactive substances include caffeine, chlorogenic acid and the diterpenes, cafestol and kahweol. The chemistry and biochemistry of coffee have been extensively described [128]. Coffee undergoes a chemical transformation during the roasting process of green bean, and the type of bean (arabica vs robusta), the degree of roasting, and brewing techniques including coffee grind setting and brew type will all have an impact on the biochemical configuration of the final beverage [129, 130].

Coffee as a functional food for metabolic syndrome

Coffee is one of the most commonly consumed beverages in the world. It is a healthier alternative to alcoholic beverages such as beer and spirits [131]. Coffee beans of two main species, *Coffea canephora* (also known as robusta) and *C. Arabica,* are commercially available for the preparation of coffee. Both beans are well-known for their pleasant aroma and enjoyable taste [132]. The range of compounds in coffee may provide beneficial effects against metabolic syndrome [133]. Coffee is the second-most traded commodity in the world after oil [134]. The consumption of coffee has doubled in the last four decades [135]. Coffee consumption was 6.947 million tonnes in 2010 [136] and it is estimated that consumption will increase to 9 million tonnes in 2019 [135]. In 2010, Brazil was the major producer of coffee contributing about 19% of the world's coffee production. The United States was the major coffee consumer with about 16% of world consumption [136].

The chemical composition of coffee is affected by factors including harvesting and post-harvest techniques, climate, physical conditions of soil and tree and genetic aspects of coffee seeds [137]. These components also vary between *C. canephora* and *C. arabica*. Coffee is a complex mixture of caffeine (0.5-2.8%), chlorogenic acid (4.80-6.41%), polyphenols, caffeic acid, lactones and diterpenoid alcohols including cafestol and kahweol, as well as niacin and the vitamin B_3 precursor, trigonelline. It also has ample

amounts of vitamin B_3, magnesium and potassium [137, 138]. During the roasting process of coffee beans, chlorogenic acid undergoes chemical transformations such as isomerisation, hydrolysis and degradation, causing reduction of chlorogenic acid concentrations and conversion into quinolactones and melanoidins [128, 139]. The roasting process initiates the browning reaction and gives various different compounds of varying molecular weights such as aldehydes, ketones, dicarbonyls, acrylamides, heterocyclic amines and melanoidins that impact flavour [140]. Melanoidins account for 25-29% (w/w of dry matter) of roasted coffee beans [141]. These compounds are high molecular weight, water-soluble, brown-colored nitrogenous compounds with antioxidant, antimicrobial and anti-hypertensive activities [142, 143]. Melanoidins link or bind with chlorogenic acid non-covalently and regulate gut microbiota growth to produce anti-inflammatory effects [139]. Although coffee is rich in caffeine, chlorogenic acid, trigonelline, diterpenes, and melanoidins, which collectively or individually can positively influence health, the availability of these compounds depends on how the coffee beans are processed and consumed.

There are several types of coffee beans generated depending upon processing of green coffee beans. A green coffee bean does not have the flavour qualities of a roasted bean and it's green in color with a grassy smell. Roasting process induced the aroma and flavour in beans depending on temperature and time taken for processing. It creates fresh roast flavour with different chemical components in roasted beans. The coffee extraction and brewing process only extracts part of the coffee components, so that the resulting spent coffee ground may be a source of useful products as healthy bioactive compounds, including trigonelline and melanoidins [139, 144].

Green coffee

Green coffee beverages accelerate glucose metabolism and lead to reduced body weight and prevent obesity [145]. Green coffee extract has the potential to inhibit fat absorption and control lipid metabolism in livers of mice [146]. Likewise, green coffee has anti-hypertensive properties against high blood pressure in rats [147].

Green coffee is the mixture of complex chemicals that impart flavour, aroma and texture to coffee beverages after roasting. Along with carbohydrates, proteins, lipids and minerals, it is also a rich source of phenolic acids such as chlorogenic acid and alkaloids such as caffeine [148]. Health beneficial effects from green coffee are mainly associated with the presence of phenolic compounds, especially chlorogenic acid, that have been recognised for their antioxidant properties [149] associated with its ability to scavenge ROS. In addition, chlorogenic acid and caffeine inhibited tumour promotion and inflammation cascade by restricting the pro-oxidant enzyme lipoxygenase for metabolising arachidonic acid [150]. Moreover, green coffee phytochemicals have demonstrated potential to decrease visceral adipose tissue accumulation and body weight gain with several studies supporting green coffee as a weight loss supplement in animal models [151, 152].

Chronic intake of coffee has been related inversely to body weight gain in humans [153]. Regular coffee intake has also been associated with modulation of glycaemic markers [154]. Likewise, green coffee intake reduced weight gain, where caffeine and other bioactive compounds present in coffee play a crucial role as thermogenic agents [155]. In human study, green coffee has shown potential for modulating hormone release and glucose tolerance [156]. The possible mechanism could be the delay in glucose absorption where green coffee causes the absorption of glucose in distal colon instead of the proximal colon. This results in the reduction in postprandial plasma concentrations of glucose and blood lipids [157]. Additionally, this modulation increased the diversion of the Na^+ electrochemical gradient, and consequently reduction in intestinal brush border membrane glucose uptake [156]. Green coffee was also shown to regulate glucose homeostasis by inhibiting hepatic glucose-6-phosphatase [158]. Through suppression of hepatic triglyceride accumulation, green coffee could also decrease obesity [151]. Green coffee also altered adipokines secretion, prevented fat accumulation via downregulation of lipogenesis while upregulating lipid oxidation and expression of PPAR-α in the liver [159].

Roasted coffee

While green coffee beverages have a mild, green and bean-like taste and aroma, the desirable properties of coffee are acquired during the roasting process. During the roasting process, the coffee beans are heated from 200-250°C and process duration can last between 0.75 to 25 minutes, the optimal period being 1.5 - 6 minutes depending upon the specifications of the end product i.e., light, medium or dark roasted beans. Roasting process causes several physical and chemical modifications leading to a shift in colour from green to brown. The chemical components such as protein, amino acids, polysaccharides, trigonelline, chlorogenic acid and water undergo complex processes to form melanoidins. Melanoidins are a product of Maillard reaction through caramelisation of polysaccharides and amino acids with other coffee components during heating of coffee beans [160]. Roasted coffee is a rich source of bioactive compounds especially methylxanthines and polyphenols. The roasted coffee extract has demonstrated anti-inflammatory and anti-oxidant properties with great potential for reducing ROS and inhibitory action against linoleic acid peroxidation and lipoxygenase [161]. In a human study, chlorogenic acid from roasted coffee caused a reduction in oxidative stress-induced DNA and macromolecular damage and increased antioxidant defense capacity [162]. In addition, the efficiency of antioxidant properties of roasted coffee is shown as the increases in glutathione S-transferase activity *in vitro* as well as in rats [163]. Further, roasted coffee components increased transcription and nuclear translocation of Nrf2 in HT-29 cells [164]; this modification induced by coffee components prevented colon cell damage from oxidative stress caused by ROS. Therefore, roasted coffee components reduced oxidative stress and restrict the progress of metabolic syndrome associated complications such as cardiovascular disease.

Decaffeinated coffee

Coffee beverage is generally popular for caffeine intake, and caffeine's stimulatory effect makes it the most recognised and most studied component in coffee. Studies have shown that caffeine has favorable effects on cardiovascular function [165, 166]. However, caffeine intake causes nervousness, irritability, headache and troubled sleeping patterns in many individuals [167]. Further, coffee consists of hundreds of other bioactive

compounds such as caffeic acid, chlorogenic acid and trigonelline, making it difficult to isolate the health effects of caffeine from those of the other ingredients.

Decaffeinated coffee is consumed by individuals who have problems in tolerating caffeine. According to the United States Department of Agriculture, decaffeinated coffee is not necessarily absolutely caffeine-free. In fact, coffee only requires being ~97% caffeine-free in order to be classified as decaffeinated coffee, although Brazilian regulations only permit a maximum of 0.1% of the residual caffeine in decaffeinated coffee [168]. The process of decaffeination causes an alteration in decaffeinated coffee bean and depending upon coffee variety, coffee components such as chlorogenic acid and other components increase or decrease [149]. Several studies have reported health benefits from the decaffeinated coffee intake. In a clinical trial, decaffeinated green coffee reduced weight in overweight volunteers treated with 400 mg/day for 60 days [169]. Another study stated that drinking 0.3% decaffeinated green coffee extract in water for 20 weeks reduced fat accumulation and insulin resistance in mice fed on a high-fat diet [170]. Decaffeinated coffee extract downregulates TLR4-mediated pro-inflammatory pathways and stimulates GLUT4 translocation to the plasma membrane in adipocytes [170]. Similarly, ingestion of decaffeinated coffee for 20 weeks after inducing obesity using a high-fat diet over a 10 week period resulted in a decrease in blood glucose concentrations and improvement in insulin tolerance. The coffee extract also promoted recovery from liver remodelling, showing a decrease in steatosis, and inhibited iNOS expression and restored the insulin-inducing Akt phosphorylation [171].

Coffee wastes

Almost 50% of the coffee bean is discarded as waste during the process of commercial production up to and including the consumption by the end-user. The waste is generated from production (e.g. coffee pulp, cherry husks and defective beans), from roasting industries (e.g. coffee silver skin), from soluble coffee industry (industrial spent coffee) and also directly by daily coffee consumers after coffee brewing (spent coffee) [172]. These coffee wastes have high concentrations of bioactive compounds including caffeine and polyphenols [173].

Coffee wastes have been used mainly for commercial processes such as the production of ethanol, butanol, hydrogen, organic acids, glycerol and hydroxymethylfurfural. Coffee wastes are also used for composting in several agro-industrial sectors [134]. Despite the high bioactive content of coffee wastes, only partial progress has been accomplished in its application as a functional food [174]. Coffee waste such as coffee husks, skin and pulp contain four major classes of polyphenols, flavan-3-ols, hydroxycinnamic acids, flavonols and anthocyanidins which include chlorogenic acid, epicatechin, catechin, rutin and ferulic acid. Coffee husks, skin, and pulp are potential sources of 5-feruloyl-quinic acid and anthocyanins such as cyanidin-3-rutinoside and cyanidin-3-glucoside [172].

Coffee mucilage and parchment include several bioactive components such as amino acids, polysaccharides, pectic constituents and a trace amount of ash in addition to α-cellulose, hemicellulose and lignin [134]. However, research on the functional characteristics of this coffee portion has not been performed yet. Roasted coffee silverskin, a by-product of roasted coffee beans, is a rich source of dietary fibre, accounting for up to 60% of the by-product, 14% of which are soluble dietary fibre [175]. The roasted coffee silverskin also contained a minor amount of phenolic compounds with demonstrated antioxidant properties. The antioxidant properties may be attributable to melanoidins. Additionally, roasted coffee silverskin stimulated the preferential growth of *Bifidobacterium* rather than *Clostridia* and *Bactericides* spp. Hence, the roasted coffee silverskin could be used as a functional ingredient due to its high content of soluble fibre, its potential antioxidant activity and the prospective prebiotic properties [175].

Coffee components

Caffeine

Caffeine is a well-known stimulant of the central nervous system, acting as an antagonist to adenosine receptors. Adenosine receptors are present and expressed in the central nervous system, the vascular endothelium, heart, liver, adipose tissues and muscles. Thus, caffeine triggers a broad range of responses in the body [138]. It produces many biological functions including an acute elevation of heart rate, stimulation of the

central nervous system, increased carbohydrate metabolism and decreased inflammation, as well as increasing metabolic rate and causing diuresis [176-178]. Caffeine (~30 mg/kg/day) in diet-induced obese rats reduced abdominal fat, total body fat, systolic blood pressure and improved glucose tolerance, cardiovascular and hepatic structure and function [179]. Caffeine promoted the gene expression of carnitine acyltransferase in the liver of obese mice [180]. This increase in the expression of carnitine acyltransferase may be responsible for the reduction of adipose tissue weight through increased fat oxidation and hence suppression of body weight gain. Caffeine also increased energy expenditure [181]. Regular intake of caffeine decreased the risk of insulin resistance by decreasing the sympathetic nervous system response [182].

Chlorogenic acid

Chlorogenic acids are a group of compounds that are esters of caffeic acid and quinic acid [183] and may have health benefits due to their antioxidant and anti-inflammatory activities. Only one-third of ingested chlorogenic acid is absorbed in the small intestine and some amount that escapes metabolism enters the colon [184]. In the colon, the microbiota hydrolyses chlorogenic acid into caffeic acid and quinic acid [184]. These microbial metabolites undergo absorption or are changed by human enzymes and finally get excreted in urine [183]. Negligible amounts (0.8%) of intact chlorogenic acid are found in human urine. Chlorogenic acid has bacteriostatic properties towards a wide range of Gram-positive and Gram-negative bacteria, such as *E. coli* and *Salmonella* spp. [185] while specifically promoting the growth of *Bifidobacterium* spp., *Lactobacillus*/*Enterococcus* spp., *Bactericides* spp. and *C. coccoides–E.rectale* [184]. As almost two-thirds of the dietary chlorogenic acid reaches the colon and undergoes metabolism, it may have some effects in the colon, especially on the gut microflora, which may help in attenuating obesity [186].

Chlorogenic acid inhibited liver glucose-6-phosphatase activity, suggesting that it can reduce liver glucose output [158]. Chlorogenic acid from decaffeinated coffee downgraded the expression of sodium-dependent glucose transporters in brush border membranes of the small intestine thus suggesting its potential to reduce glucose absorption [187]. Similarly, chlorogenic acid from green coffee extract (0.25 - 1% diet for 6 weeks)

lowered blood pressure in Spontaneously Hypertensive Rats [147]. Chlorogenic acid increased mRNA expression of PPAR-α and liver X receptor-α, the key mediators of lipid metabolism [188]. Chlorogenic acid (100 mg/kg/day in C57BL/6 mice for 15 weeks) prevented diet-induced obesity and metabolic syndrome by inhibiting expression of PPAR-α and by reducing inflammation [189]. Chlorogenic acid helps to delay glucose absorption from the gut into the bloodstream. Hence, chlorogenic acid could be a potential dietary candidate for the host to manage metabolic disorders such as obesity, increased blood glucose concentrations and cardiac complications.

Melanoidins

During the roasting of coffee beans, melanoidins are formed by the non-enzymatic browning reaction as a chemical reaction between reducing polysaccharides, amino acids and phenolic compounds [190]. Moreover, melanoidins are formed during the heating process of a broad range of foodstuffs including bread, malt, meat and tomato sauce [191]. The formation of melanoidins leads to increased antioxidant capacity for coffee beans, but this occurs only at a particular phase of coffee roasting [192]. Studies of coffee revealed that some positive effects on pathophysiology which are not due to caffeine can be credited to polyphenols and melanoidins [193]. The molecular structure of melonoidins are yet to be defined and thus, they are generally defined as macromolecular nitrogenous browning compounds [194]. However, some studies have shown that roasting process causes covalently linkage between arabinogalactans and proteins to produce arabinogalactan proteins [129] that have hydrophobic characteristics and ion-chelating abilities [195]. Roasting process leads to degradation of the proteins present in green coffee beans, including arginine, cysteine, lysine, and serine, and the degradation products contribute to the formation of complex melanoidins [196]. Moreover, chlorogenic acid and their derivatives are fused in coffee melanoidins [197]. The intact chlorogenic acids are incorporated into the melanoidin structures, combined through the caffeic acid moiety by mainly non-ester linkages [130]. In summary, polysaccharides, proteins and phenolic compounds such as chlorogenic acids are involved in the formation of coffee melanoidins.

An *in vitro* study by stimulating gastrointestinal enzymatic digestion showed that the coffee melanoidins are largely resistant to digestion in the human gut [198] suggesting that melanoidins could have prebiotic activity. Melanoidins have the potential to alter human gut microflora as fermentation of melanoidins supports the growth of residential bacteria [199]. The gut bacteria may also partly metabolise the melanoidins in the large intestine, hence, this might play a crucial role in the chelating and/or release of other, potentially harmful dietary components. For instance, a study of coffee melanoididns with human subjects has reported for growth in the number of *Bacteroides and Prevotella* bacteria identified in faecal samples [200]. In *in vivo* studies, melanoidins have increased the quantity of *Bifidobacterium spp*., known for their probiotic activity [201, 202]. *In vitro* fermentation of melanoidins for 24 hours with faecal bacteria from human subjects showed its anti-oxidant properties [203] indicating a potential role for coffee melanoidins in the protection against oxidative stress caused by free radical species in the colon, resulting in the reduction of gut dysbiosis.

The anti-oxidant properties of melanoidins have been implicated in their protective effects on human hepatoma HepG2 cells against oxidative stress where melanoidins reduced glutathione peroxidase and glutathione reductase [204], indicators of attenuation in oxidative stress, as melanoidins contain hydroxyl groups, which serves as a hydrogen donor, and are able to scavenge free radical species [205]. Melanoidins inhibits secondary lipid oxidation products that contribute to health benefits, products from lipid oxidation are involved in the development of cardiovascular diseases [206]. Thus, in addition to the antioxidant property of melanoidins, the available studies suggested that ingested melanoidins from regular coffee intake could possibly provided the protection against inflammation and promote the growth of selective gut microbiota [191].

Other coffee components

Along with chlorogenic acid, caffeine and melanoidins, coffee is also a source of other bioactive components such as trigonelline and diterpenes (cafestol and kahweol). These compounds from coffee have the ability to protect cells from ROS-induced oxidative stress by inducing the transcription of genes in the Nrf2 antioxidant response element signalling pathway [164].

Trigonelline is an alkaloid and derivative of nicotinic acid. After demethylation, it is converted to nicotinic acid which is present at approximately 1–3 mg/240 ml brewed coffee [187]. During roasting of coffee beans, trigonelline is also transformed into its intermediate, N-methylpyridinium, which improves the reducing and total antioxidant capacity of trigonelline [207]. N-methylpyridinium is converted to nicotinic acid which has the potential for anti-inflammatory properties through down-regulation of MCP-1 and has increased adiponectin in adipocytes treated with TNF [208]. Decomposition of trigonelline was greater at higher roasting temperature generating higher amounts of nicotinic acid [209].

Diterepens such as cafestol and kahweol have a negative impact on cholesterol concentrations. Diterpenes could be a possible reason for increased plasma lipid concentrations in coffee drinkers as a high concentration of diterpenes have been reported to induce increased total cholesterol and LDL-cholesterol [187]. Coffee also increased serum LDL through increased expression of cholesterol esterase transfer protein assisted by cafestol [210]. Diterpenes have shown antioxidant properties and cells treated with diterpenes showed decreased DNA damage because of the ROS scavenging activity of cafestol and kahweol [211]. Also, kahweol and cafestol treatment protected N1HT3T cells challenged with hydrogen peroxide [211]. Kahweol conferred an anti-inflammatory effect by down-reregulating the expression of cyclooxygenase-2 and MCP-1 in HUVECS and decreased the inducible nitric oxide synthase in rats treated with carrageenan [212]. A recent study has also shown beneficial effects of cafestol against diabetes in KKAy mice [213].

Thus, metabolic syndrome is strongly associated with the increasing prevalence of cardiovascular diseases and associated comorbidity. Cardiovascular diseases are reliant on reversible metabolic abnormalities such as oxidative stress, dyslipidaemia and impaired glucose tolerance, which are associated with metabolic syndrome. Coffee and its phytochemical components exhibit the potential against oxidative stress, inflammation and insulin resistance and thus, coffee beverages could be a potential dietary intervention for cardiovascular disease. It is difficult to determine what component of coffee could be responsible for beneficial health effects because of the complex chemical composition of

coffee beans as well as coffee waste. By investigating responses to coffee products and coffee components, beneficial effects of coffee waste and coffee components can be explored. Clearly, all investigations into the health benefits of coffee intake must consider several parameters such as coffee variety, coffee beans, roasting method, sample size, brewing course, and a dose of individual coffee components administered and the duration of treatment. In order to understand the specific roles of individual components of coffee in cardiovascular health, properly controlled experiments have been carried out to determine preclinical outcomes.

References

1. MacDonald Jr KG. Overview of the epidemiology of obesity and the early history of procedures to remedy morbid obesity. Archives of Surgery 2003; 138(4): 357-60.

2. Fuller J, Stevens L, Wang S, Group WMS. Risk factors for cardiovascular mortality and morbidity: The WHO multinational study of vascular disease in diabetes. Diabetologia 2001; 44(2): S54-S64.

3. Flegal KM, Kruszon-Moran D, Carroll MD, Fryar CD, Ogden CL. Trends in obesity among adults in the United States, 2005 to 2014. JAMA 2016; 315(21): 2284-91.

4. Hotamisligil GS. Inflammation and metabolic disorders. Nature 2006; 444(7121): 860-7.

5. 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.

6. Ford ES, Giles WH, Mokdad AH. Increasing prevalence of the metabolic syndrome among US adults. Diabetes Care 2004; 27(10): 2444-9.

7. Fall CH. Non-industrialised countries and affluence Relationship with type 2 diabetes. British Medical Bulletin 2001; 60(1): 33-50.

8. Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. Journal of Clinical Investigation 2005; 115(5): 1111-9.

9. Beltrán-Sánchez H, Harhay MO, Harhay MM, McElligott S. Prevalence and trends of metabolic syndrome in the adult US population, 1999–2010. Journal of the American College of Cardiology 2013; 62(8): 697-703.

10. Beaglehole R. International trends in coronary heart disease mortality, morbidity, and risk factors. Epidemiologic Reviews 1990; 12: 1-15.

11. AIHW. Trends in coronary heart disease mortality: age groups and populations. In: AIHW, editor. Cardiovascular disease. Canberra: AIHW; 2014.

12. Isomaa B, Almgren P, Tuomi T, Forsén B, Lahti K, Nissén M, et al. Cardiovascular Morbidity and Mortality Associated With the Metabolic Syndrome. Diabetes Care 2001; 24(4): 683-9.

13. Zimmermann R, Strauss JG, Haemmerle G, Schoiswohl G, Birner-Gruenberger R, Riederer M, et al. Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. Science 2004; 306(5700): 1383-6.

14. Scherer PE. Adipose tissue from lipid storage compartment to endocrine organ. Diabetes 2006; 55(6): 1537-45.

15. Lyon CJ, Law RE, Hsueh WA. Minireview: adiposity, inflammation, and atherogenesis. Endocrinology 2003; 144(6): 2195-200.

16. Trayhurn P, Wood IS. Adipokines: inflammation and the pleiotropic role of white adipose tissue. British Journal of Nutrition 2004; 92(3): 347-55.

17. Guyton JR, Slee AE, Anderson T, Fleg JL, Goldberg RB, Kashyap ML, et al. Relationship of lipoproteins to cardiovascular events: the AIM-HIGH trial (Atherothrombosis intervention in metabolic syndrome with low HDL/high triglycerides and impact on global health outcomes). Journal of the American College of Cardiology 2013; 62(17): 1580-4.

18. Thomsen M, Nordestgaard BG. Myocardial infarction and ischemic heart disease in overweight and obesity with and without metabolic syndrome. JAMA Internal Medicine 2014; 174(1): 15-22.

19. Wold LE, Ceylan-Isik AF, Ren J. Oxidative stress and stress signaling: menace of diabetic cardiomyopathy. Acta Pharmacologica Sinica 2005; 26(8): 908-17.

20. Ren J, Pulakat L, Whaley-Connell A, Sowers JR. Mitochondrial biogenesis in the metabolic syndrome and cardiovascular disease. Journal of Molecular Medicine (Berlin, Germany) 2010; 88(10): 993-1001.

21. Flagg TP, Cazorla O, Remedi MS, Haim TE, Tones MA, Bahinski A, et al. Ca2+-independent alterations in diastolic sarcomere length and relaxation kinetics in a mouse model of lipotoxic diabetic cardiomyopathy. Circulation Research 2009; 104(1): 95-103.

22. Sowers JR, Bakris GL, Black HR, Giles TD. The cardiometabolic syndrome and calcium channel blocker combination drugs. Journal of the Cardiometabolic Syndrome 2007; 2(3): 207-12.

23. Whaley-Connell A, Sowers JR. Hypertension and insulin resistance. Hypertension 2009; 54(3): 462-4.

24. Rosenson RS. Assessing risk across the spectrum of patients with the metabolic syndrome. The American Journal of Cardiology 2005; 96(4): 8-10.

25. Juhan-Vague I, Alessi M, Vague P. Increased plasma plasminogen activator inhibitor 1 levels. A possible link between insulin resistance and atherothrombosis. Diabetologia 1991; 34(7): 457-62.

26. Sattar N, Gaw A, Scherbakova O, Ford I, O'Reilly DSJ, Haffner SM, et al. Metabolic syndrome with and without C-reactive protein as a predictor of coronary heart disease and diabetes in the West of Scotland Coronary Prevention Study. Circulation 2003; 108(4): 414-9.

27. National Cholesterol Education Program (NCEP) Expert Panel on Detection E, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). Third report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III) final report. Circulation 2002; 106(25): 3143.

28. Vinik AI. The metabolic basis of atherogenic dyslipidemia. Clinical Cornerstone 2005; 7(2): 27-35.

29. Eckel RH, Grundy SM, Zimmet PZ. The metabolic syndrome. Lancet 2005; 365(9468): 1415-28.

30. Després J-P, Lamarche B, Mauriège P, Cantin B, Dagenais GR, Moorjani S, et al. Hyperinsulinemia as an independent risk factor for ischemic heart disease. New England Journal of Medicine 1996; 334(15): 952-8.

31. Pyörälä M, Miettinen H, Laakso M, Pyörälä K. Hyperinsulinemia predicts coronary heart disease risk in healthy middle-aged men the 22-year follow-up results of the helsinki policemen study. Circulation 1998; 98(5): 398-404.

32. Liu C, Yang Q, Fang G, Li BS, Wu DB, Guo WJ, et al. Collagen metabolic disorder induced by oxidative stress in human uterosacral ligamentderived fibroblasts: A possible pathophysiological mechanism in pelvic organ prolapse. Molecular Medicine Reports 2016; 13(4): 2999-3008.

33. Savage DB, Petersen KF, Shulman GI. Mechanisms of insulin resistance in humans and possible links with inflammation. Hypertension 2005; 45(5): 828-33.

34. McGarry JD. Banting lecture 2001 Dysregulation of fatty acid metabolism in the etiology of type 2 diabetes. Diabetes 2002; 51(1): 7-18.

35. Jung UJ, Choi MS. Obesity and its metabolic complications: the role of adipokines and the relationship between obesity, inflammation, insulin resistance, dyslipidemia and nonalcoholic fatty liver disease. Int J Mol Sci 2014; 15(4): 6184-223.

36. Okere IC, Chandler MP, McElfresh TA, Rennison JH, Sharov V, Sabbah HN, et al. Differential effects of saturated and unsaturated fatty acid diets on cardiomyocyte apoptosis, adipose distribution, and serum leptin. American Journal of Physiology - Heart and Circulatory Physiology 2006; 291(1): H38-44.

37. Gill H, Mugo M, Whaley-Connell A, Stump C, Sowers JR. The key role of insulin resistance in the cardiometabolic syndrome. American Journal of the Medical Sciences 2005; 330(6): 290-4.

38. Hoy AJ, Brandon AE, Turner N, Watt MJ, Bruce CR, Cooney GJ, et al. Lipid and insulin infusion-induced skeletal muscle insulin resistance is likely due to metabolic feedback and not changes in IRS-1, Akt, or AS160 phosphorylation. American Journal of Physiology - Endocrinology and Metabolism 2009; 297(1): E67-E75.

39. Petersen KF, Shulman GI. Etiology of insulin resistance. American Journal of Medicine 2006; 119(5 Suppl 1): S10-6.

40. Havel PJ. Control of energy homeostasis and insulin action by adipocyte hormones: leptin, acylation stimulating protein, and adiponectin. Current Opinion in Lipidology 2002; 13(1): 51-9.

41. Nandipati KC, Subramanian S, Agrawal DK. Protein kinases: mechanisms and downstream targets in inflammation-mediated obesity and insulin resistance. Molecular and Cellular Biochemistry 2016: 1-19.

42. Goettsch W, Lattmann T, Amann K, Szibor M, Morawietz H, Münter K, et al. Increased expression of endothelin-1 and inducible nitric oxide synthase isoform II in aging arteries in vivo: implications for atherosclerosis. Biochemical and Biophysical Research Communications 2001; 280(3): 908-13.

43. Jensen MD, Haymond MW, Rizza RA, Cryer PE, Miles JM. Influence of body fat distribution on free fatty acid metabolism in obesity. Journal of Clinical Investigation 1989; 83(4): 1168-73.

44. Björntorp P. Body fat distribution, insulin resistance, and metabolic diseases. Nutrition 1997; 13(9): 795-803.

45. Odegaard AO, Jacobs DR, Sanchez OA, Goff DC, Reiner AP, Gross MD. Oxidative stress, inflammation, endothelial dysfunction and incidence of type 2 diabetes. Cardiovascular Diabetology 2016; 15: 51.

46. Higdon JV, Frei B. Obesity and oxidative stress: a direct link to CVD? Arteriosclerosis, Thrombosis, and Vascular Biology 2003; 23(3): 365-7.

47. Maddux BA, See W, Lawrence JC, Goldfine AL, Goldfine ID, Evans JL. Protection against oxidative stress—induced insulin resistance in rat l6 muscle cells by micromolar concentrations of α-lipoic acid. Diabetes 2001; 50(2): 404-10.

48. He W, Miao FJ-P, Lin DC-H, Schwandner RT, Wang Z, Gao J, et al. Citric acid cycle intermediates as ligands for orphan G-protein-coupled receptors. Nature 2004; 429(6988): 188-93.

49. Ceriello A, Motz E. Is oxidative stress the pathogenic mechanism underlying insulin resistance, diabetes, and cardiovascular disease? The common soil hypothesis revisited. Arteriosclerosis, Thrombosis, and Vascular Biology 2004; 24(5): 816-23.

50. Nishikawa T, Edelstein D, Du XL, Yamagishi S-i, Matsumura T, Kaneda Y, et al. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. Nature 2000; 404(6779): 787-90.

51. Garcia Soriano F, Virag L, Jagtap P, Szabo E, Mabley JG, Liaudet L, et al. Diabetic endothelial dysfunction: the role of poly(ADP-ribose) polymerase activation. Nature Medicine 2001; 7(1): 108-13.

52. Hink U, Li H, Mollnau H, Oelze M, Matheis E, Hartmann M, et al. Mechanisms underlying endothelial dysfunction in diabetes mellitus. Circulation Research 2001; 88(2): e14-e22.

53. Evans JL, Goldfine ID, Maddux BA, Grodsky GM. Are oxidative stress-activated signaling pathways mediators of insulin resistance and beta-cell dysfunction? Diabetes 2003; 52(1): 1-8.

54. Krauss S, Zhang C-Y, Scorrano L, Dalgaard LT, St-Pierre J, Grey ST, et al. Superoxide-mediated activation of uncoupling protein 2 causes pancreatic β cell dysfunction. The Journal of Clinical Investigation 2003; 112(12): 1831-42.

55. Li H, Li H, Bao Y, Zhang X, Yu Y. Free fatty acids induce endothelial dysfunction and activate protein kinase C and nuclear factor-κB pathway in rat aorta. International Journal of Cardiology 2011; 152(2): 218-24.

56. Alpert MA. Obesity cardiomyopathy: pathophysiology and evolution of the clinical syndrome. American Journal of the Medical Sciences 2001; 321(4): 225-36.

57. Foppa M, Arora G, Gona P, Ashrafi A, Salton CJ, Yeon SB, et al. Right ventricular volumes and systolic function by cardiac magnetic resonance and the impact of sex, age, and obesity in a longitudinally followed cohort free of pulmonary and cardiovascular disease: The framingham heart study. Circulation: Cardiovascular Imaging 2016; 9(3).

58. Di Bello V, Fabiani I, Conte L, Barletta V, Delle Donne MG, Cuono C, et al. New echocardiographic techniques in the evaluation of left ventricular function in obesity. Obesity 2013; 21(5): 881-92.

59. Lavie CJ, Milani RV, Ventura HO, Cardenas GA, Mehra MR, Messerli FH. Disparate effects of left ventricular geometry and obesity on mortality in patients with preserved left ventricular ejection fraction. American Journal of Cardiology 2007; 100(9): 1460-4.

60. Zhang Y, Ren J. Role of cardiac steatosis and lipotoxicity in obesity cardiomyopathy. Hypertension 2011; 57(2): 148-50.

61. Goudis CA, Korantzopoulos P, Ntalas IV, Kallergis EM, Ketikoglou DG. Obesity and atrial fibrillation: A comprehensive review of the pathophysiological mechanisms and links. International Journal of Cardiology 2015; 66(5): 361-9.

62. Draper TS, Jr., Silver JS, Gaasch WH. Adverse structural remodeling of the left ventricle and ventricular arrhythmias in patients with depressed ejection fraction. Journal of Cardiac Failure 2015; 21(2): 97-102.

63. Lavie CJ, Milani RV. Metabolic syndrome, hostility, and cardiac rehabilitation. American Journal of Cardiology 2005; 96(11): 1615.

64. Gruzdeva O, Uchasova E, Dyleva Y, Akbasheva O, Matveeva V, Karetnikova V, et al. Relationship key factor of inflammation and the development of complications in the late period of myocardial infarction in patients with visceral obesity. BMC Cardiovascular Disorders 2017; 17: 36.

65. Liuzzo G, Biasucci LM, Gallimore JR, Grillo RL, Rebuzzi AG, Pepys MB, et al. The prognostic value of C-reactive protein and serum amyloid a protein in severe unstable angina. New England Journal of Medicine 1994; 331(7): 417-24.

66. Biasucci LM, Vitelli A, Liuzzo G, Altamura S, Caligiuri G, Monaco C, et al. Elevated levels of interleukin-6 in unstable angina. Circulation 1996; 94(5): 874-7.

67. Lindahl B, Toss H, Siegbahn A, Venge P, Wallentin L. Markers of myocardial damage and inflammation in relation to long-term mortality in unstable coronary artery disease. New England Journal of Medicine 2000; 343(16): 1139-47.

68. Fujibayashi K, Gunji T, Yokokawa H, Naito T, Sasabe N, Okumura M, et al. The relationships between metabolic disorders (hypertension, dyslipidemia, and impaired glucose tolerance) and computed tomography-based indices of hepatic steatosis or visceral fat accumulation in middle-aged japanese men. PLoS One 2016; 11(3): e0149689.

69. Manrique C, Lastra G, Gardner M, Sowers JR. The renin angiotensin aldosterone system in hypertension: Roles of insulin resistance and oxidative stress. Medical Clinics of North America 2009; 93(3): 569-82.

70. Malhotra A, Kang BP, Cheung S, Opawumi D, Meggs LG. Angiotensin II promotes glucose-induced activation of cardiac protein kinase C isozymes and phosphorylation of troponin I. Diabetes 2001; 50(8): 1918-26.

71. Morse SA, Zhang R, Thakur V, Reisin E. Hypertension and the metabolic syndrome. American Journal of the Medical Sciences 2005; 330(6): 303-10.

72. Briones AM, Cat AND, Callera GE, Yogi A, Burger D, He Y, et al. Adipocytes produce aldosterone through calcineurin-dependent signaling pathways implications in diabetes mellitus–associated obesity and vascular dysfunction. Hypertension 2012; 59(5): 1069-78.

73. Bastard J-P, Jardel C, Bruckert E, Blondy P, Capeau J, Laville M, et al. Elevated levels of interleukin 6 are reduced in serum and subcutaneous adipose tissue of obese women after weight loss 1. Journal of Clinical Endocrinology & Metabolism 2000; 85(9): 3338-42.

74. Ridker PM, Buring JE, Cook NR, Rifai N. C-reactive protein, the metabolic syndrome, and risk of incident cardiovascular events: an 8-year follow-up of 14 719 initially healthy American women. Circulation 2003; 107(3): 391-7.

75. Kwaśniewska M, Kozińska J, Dziankowska-Zaborszczyk E, Kostka T, Jegier A, Rębowska E, et al. The impact of long-term changes in metabolic status on cardiovascular biomarkers and microvascular endothelial function in middle-aged men: A 25-year prospective study. Diabetology and Metabolic Syndrome 2015; 7(1).

76. Montani J, Antic V, Yang Z, Dulloo A. Pathways from obesity to hypertension: from the perspective of a vicious triangle. International Journal of Obesity 2002; 26(Suppl 2): S28.

77. Xie X, Atkins E, Lv J, Bennett A, Neal B, Ninomiya T, et al. Effects of intensive blood pressure lowering on cardiovascular and renal outcomes: updated systematic review and meta-analysis. Lancet 2016; 387(10017): 435-43.

78. Olijhoek JK, van der Graaf Y, Banga J-D, Algra A, Rabelink TJ, Visseren FL. The metabolic syndrome is associated with advanced vascular damage in patients with coronary heart disease, stroke, peripheral arterial disease or abdominal aortic aneurysm. European Heart Journal 2004; 25(4): 342-8.

79. Ridker PM, Rifai N, Rose L, Buring JE, Cook NR. Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. New England Journal of Medicine 2002; 347(20): 1557-65.

80. Sesso HD, Buring JE, Rifai N, Blake GJ, Gaziano JM, Ridker PM. C-reactive protein and the risk of developing hypertension. JAMA 2003; 290(22): 2945-51.

81. Sesso HD, Wang L, Buring JE, Ridker PM, Gaziano JM. Comparison of interleukin-6 and C-reactive protein for the risk of developing hypertension in women. Hypertension 2007; 49(2): 304-10.

82. Chung MK, Martin DO, Sprecher D, Wazni O, Kanderian A, Carnes CA, et al. C-reactive protein elevation in patients with atrial arrhythmias inflammatory mechanisms and persistence of atrial fibrillation. Circulation 2001; 104(24): 2886-91.

83. Newairy A-SA, Mansour HA, Yousef M, Sheweita S. Alterations of lipid profile in plasma and liver of diabetic rats: effect of hypoglycemic herbs. Journal of Environmental Science and Health, Part B 2002; 37(5): 475-84.

84. Schmidt AM, Hasu M, Popov D, Zhang JH, Chen J, Yan SD, et al. Receptor for advanced glycation end products (AGEs) has a central role in vessel wall interactions and gene activation in response to circulating AGE proteins. Proceedings of the National Academy of Sciences of the United States of America 1994; 91(19): 8807-11.

85. Macias-Cervantes MH, Rodriguez-Soto JM, Uribarri J, Diaz-Cisneros FJ, Cai W, Garay-Sevilla ME. Effect of an advanced glycation end product-restricted diet and exercise on metabolic parameters in adult overweight men. Nutrition 2015; 31(3): 446-51.

86. Kuwabara WM, Zhang L, Schuiki I, Curi R, Volchuk A, Alba-Loureiro TC. NADPH oxidase-dependent production of reactive oxygen species induces endoplasmatic reticulum stress in neutrophil-like HL60 cells. PLoS One 2015; 10(2): e0116410.

87. Winiarska K, Jarzyna R, Dzik JM, Jagielski AK, Grabowski M, Nowosielska A, et al. ERK1/2 pathway is involved in renal gluconeogenesis inhibition under conditions

of lowered NADPH oxidase activity. Free Radical Biology and Medicine 2015; 81: 13-21.

88. Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, et al. Increased oxidative stress in obesity and its impact on metabolic syndrome. Journal of Clinical Investigation 2004; 114(12): 1752-61.

89. Cosentino F, Rubattu S, Savoia C, Venturelli V, Pagannonne E, Volpe M. Endothelial dysfunction and stroke. Journal of Cardiovascular Pharmacology 2001; 38: S75-S8.

90. Higashi Y, Sasaki S, Nakagawa K, Matsuura H, Chayama K, Oshima T. Effect of obesity on endothelium-dependent, nitric oxide–mediated vasodilation in normotensive individuals and patients with essential hypertension. American Journal of Hypertension 2001; 14(10): 1038-45.

91. Kenchaiah S, Sesso HD, Gaziano JM. Body mass index and vigorous physical activity and the risk of heart failure among men. Circulation 2009; 119(1): 44-52.

92. Çil H, Bulur S, Türker Y, Kaya A, Alemdar R, Karabacak A, et al. Impact of body mass index on left ventricular diastolic dysfunction. Echocardiography 2012; 29(6): 647-51.

93. Skurk C, Izumiya Y, Maatz H, Razeghi P, Shiojima I, Sandri M, et al. The FOXO3a transcription factor regulates cardiac myocyte size downstream of AKT signaling. Journal of Biological Chemistry 2005; 280(21): 20814-23.

94. Fiedler B, Wollert KC. Targeting calcineurin and associated pathways in cardiac hypertrophy and failure. Expert Opinion on Therapeutic Targets 2005; 9(5): 963-73.

95. Poirier P, Giles TD, Bray GA, Hong Y, Stern JS, Pi-Sunyer FX, et al. Obesity and cardiovascular disease: pathophysiology, evaluation, and effect of weight loss an update of the 1997 American Heart Association Scientific statement on obesity and heart disease from the obesity committee of the council on nutrition, physical activity, and metabolism. Circulation 2006; 113(6): 898-918.

96. Olivotto I, Maron BJ, Tomberli B, Appelbaum E, Salton C, Haas TS, et al. Obesity and its association to phenotype and clinical course in hypertrophic cardiomyopathy. Journal of the American College of Cardiology 2013; 62(5): 449-57.

97. Maron MS, Olivotto I, Zenovich AG, Link MS, Pandian NG, Kuvin JT, et al. Hypertrophic cardiomyopathy is predominantly a disease of left ventricular outflow tract obstruction. Circulation 2006; 114(21): 2232-9.

98. Olivotto I, Maron MS, Autore C, Lesser JR, Rega L, Casolo G, et al. Assessment and significance of left ventricular mass by cardiovascular magnetic resonance in hypertrophic cardiomyopathy. Journal of the American College of Cardiology 2008; 52(7): 559-66.

99. Vlachopoulos C, Dima I, Aznaouridis K, Vasiliadou C, Ioakeimidis N, Aggeli C, et al. Acute systemic inflammation increases arterial stiffness and decreases wave reflections in healthy individuals. Circulation 2005; 112(14): 2193-200.

100. Harrison D, Griendling KK, Landmesser U, Hornig B, Drexler H. Role of oxidative stress in atherosclerosis. American Journal of Cardiology 2003; 91(3): 7-11.

101. Tzoulaki I, Murray GD, Lee AJ, Rumley A, Lowe GD, Fowkes FG. C-reactive protein, interleukin-6, and soluble adhesion molecules as predictors of progressive peripheral atherosclerosis in the general population: Edinburgh Artery Study. Circulation 2005; 112(7): 976-83.

102. Blake GJ, Ridker PM. Inflammatory bio-markers and cardiovascular risk prediction. Journal of Internal Medicine 2002; 252(4): 283-94.

103. Damas JK, Waehre T, Yndestad A, Otterdal K, Hognestad A, Solum NO, et al. Interleukin-7-mediated inflammation in unstable angina: possible role of chemokines and platelets. Circulation 2003; 107(21): 2670-6.

104. Kimura Y, Noda T, Otsuka Y, Wada M, Nakajima I, Ishibashi K, et al. Potentially lethal ventricular arrhythmias and heart failure in arrhythmogenic right ventricular

cardiomyopathy: What are the differences between men and women? JACC: Clinical Electrophysiology 2016; 2(5): 546-55.

105. Giardina EGV, Mull L, Sciacca RR, Akabas S, Flink LE, Moise N, et al. Relationship between cardiovascular disease knowledge and race/ethnicity, education, and weight status. Clinical Cardiology 2012; 35(1): 43-8.

106. D'Amico M, Marfella R, Nappo F, Di Filippo C, De Angelis L, Berrino L, et al. High glucose induces ventricular instability and increases vasomotor tone in rats. Diabetologia 2001; 44(4): 464-70.

107. Rider OJ, Francis JM, Ali MK, Holloway C, Pegg T, Robson MD, et al. Effects of catecholamine stress on diastolic function and myocardial energetics in obesity. Circulation 2012; 125(12): 1511-9.

108. Lemaitre RN, McKnight B, Fretts A, Qureshi WT, Siscovick DS, Sotoodehnia N, et al. Circulating very long-chain saturated fatty acids and heart failure. Circulation 2016; 134(Suppl 1): A11369.

109. Gambert S, Vergely C, Filomenko R, Moreau D, Bettaieb A, Opie LH, et al. Adverse effects of free fatty acid associated with increased oxidative stress in postischemic isolated rat hearts. Molecular and Cellular Biochemistry 2006; 283(1-2): 147-52.

110. Joseph LC, Barca E, Subramanyam P, Komrowski M, Pajvani U, Colecraft HM, et al. Inhibition of NAPDH Oxidase 2 (NOX2) Prevents Oxidative Stress and Mitochondrial Abnormalities Caused by Saturated Fat in Cardiomyocytes. PLoS One 2016; 11(1).

111. Tuomilehto J, Lindström J, Eriksson JG, Valle TT, Hämäläinen H, Ilanne-Parikka P, et al. Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance. New England Journal of Medicine 2001; 344(18): 1343-50.

112. Drori A, Rotnemer-Golinkin D, Avni S, Drori A, Danay O, Levanon D, et al. Attenuating the rate of total body fat accumulation and alleviating liver damage by oral

administration of vitamin D-enriched edible mushrooms in a diet-induced obesity murine model is mediated by an anti-inflammatory paradigm shift. BMC Gastroenterology 2017; 17(1): 130.

113. Roager HM, Vogt JK, Kristensen M, Hansen LBS, Ibrugger S, Maerkedahl RB, et al. Whole grain-rich diet reduces body weight and systemic low-grade inflammation without inducing major changes of the gut microbiome: a randomised cross-over trial. Gut 2017; doi:10.1136/gutjnl-2017-314786.

114. Walle T, Hsieh F, DeLegge MH, Oatis JE, Walle UK. High absorption but very low bioavailability of oral resveratrol in humans. Drug Metabolism and Disposition 2004; 32(12): 1377-82.

115. Covas MI, Nyyssönen K, Poulsen HE, Kaikkonen J, Zunft HJF, Kiesewetter H, et al. The effect of polyphenols in olive oil on heart disease risk factors: A randomized trial. Annals of Internal Medicine 2006; 145(5): 333-41.

116. Hou X, Xu S, Maitland-Toolan KA, Sato K, Jiang B, Ido Y, et al. SIRT1 regulates hepatocyte lipid metabolism through activating AMP-activated protein kinase. Journal of Biological Chemistry 2008; 283(29): 20015-26.

117. Hooper L, Kroon PA, Rimm EB, Cohn JS, Harvey I, Le Cornu KA, et al. Flavonoids, flavonoid-rich foods, and cardiovascular risk: a meta-analysis of randomized controlled trials. American Journal of Clinical Nutrition 2008; 88(1): 38-50.

118. Chen C-C, Hsu J-D, Wang S-F, Chiang H-C, Yang M-Y, Kao E-S, et al. Hibiscus sabdariffa extract inhibits the development of atherosclerosis in cholesterol-fed rabbits. Journal of Agricultural and Food Chemistry 2003; 51(18): 5472-7.

119. Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A, et al. Resveratrol improves health and survival of mice on a high-calorie diet. Nature 2006; 444(7117): 337-42.

120. Takikawa M, Inoue S, Horio F, Tsuda T. Dietary anthocyanin-rich bilberry extract ameliorates hyperglycemia and insulin sensitivity via activation of AMP-activated protein kinase in diabetic mice. Journal of Nutrition 2010; 140(3): 527-33.

121. Choo JJ. Green tea reduces body fat accretion caused by high-fat diet in rats through β-adrenoceptor activation of thermogenesis in brown adipose tissue. Journal of Nutritional Biochemistry 2003; 14(11): 671-6.

122. Zern TL, West KL, Fernandez ML. Grape polyphenols decrease plasma triglycerides and cholesterol accumulation in the aorta of ovariectomized guinea pigs. Journal of Nutrition 2003; 133(7): 2268-72.

123. Aprikian O, Duclos V, Guyot S, Besson C, Manach C, Bernalier A, et al. Apple pectin and a polyphenol-rich apple concentrate are more effective together than separately on cecal fermentations and plasma lipids in rats. Journal of Nutrition 2003; 133(6): 1860-5.

124. Qin B, Polansky MM, Harry D, Anderson RA. Green tea polyphenols improve cardiac muscle mRNA and protein levels of signal pathways related to insulin and lipid metabolism and inflammation in insulin-resistant rats. Molecular Nutrition & Food Research 2010; 54(S1): S14-S23.

125. Gokcen BB, Sanlier N. Coffee consumption and disease correlations. Critical Reviews in Food Science and Nutrition 2017; doi:10.1080/10408398.2017.1369391: 1-13.

126. Grosso G, Godos J, Galvano F, Giovannucci EL. Coffee, caffeine, and health outcomes: An umbrella review. Annual Review of Nutrition 2017; 37: 131-56.

127. Nieber K. The impact of coffee on health. Planta Medica 2017; 83(16): 1256-63.

128. Ludwig IA, Clifford MN, Lean ME, Ashihara H, Crozier A. Coffee: biochemistry and potential impact on health. Food & function 2014; 5(8): 1695-717.

129. Bekedam EK, Loots MJ, Schols HA, Van Boekel MA, Smit G. Roasting effects on formation mechanisms of coffee brew melanoidins. Journal of Agricultural and Food Chemistry 2008; 56(16): 7138-45.

130. Bekedam EK, Schols HA, Van Boekel MA, Smit G. Incorporation of chlorogenic acids in coffee brew melanoidins. Journal of Agricultural and Food Chemistry 2008; 56(6): 2055-63.

131. Bonita JS, Mandarano M, Shuta D, Vinson J. Coffee and cardiovascular disease: In vitro, cellular, animal, and human studies. Pharmacological Research 2007; 55(3): 187-98.

132. Grigg D. The worlds of tea and coffee: Patterns of consumption. GeoJournal 2002; 57(4): 283-94.

133. Richelle M, Tavazzi I, Offord E. Comparison of the antioxidant activity of commonly consumed polyphenolic beverages (coffee, cocoa, and tea) prepared per cup serving. Journal of Agricultural and Food Chemistry 2001; 49(7): 3438-42.

134. Mussatto SI, Machado EMS, Martins S, Teixeira JA. Production, composition, and application of coffee and its industrial residues. Food and Bioprocess Technology 2011; 4(5): 661-72.

135. Paterson RRM, Lima N, Taniwaki MH. Coffee, mycotoxins and climate change. Food Research International 2014; 61(0): 1-15.

136. FAO. Medium-term prospects for agricultural commodities. Rome: Food And Agriculture Organization Of The United Nations, Department EaSD; 2010.

137. Leroy T, Ribeyre F, Bertrand B, Charmetant P, Dufour M, Montagnon C, et al. Genetics of coffee quality. Brazilian Journal of Plant Physiology 2006; 18: 229-42.

138. Cano-Marquina A, Tarín JJ, Cano A. The impact of coffee on health. Maturitas 2013; 75(1): 7-21.

139. Panusa A, Zuorro A, Lavecchia R, Marrosu G, Petrucci R. Recovery of natural antioxidants from spent coffee grounds. Journal of Agricultural and Food Chemistry 2013; 61(17): 4162-8.

140. Alves G, Perrone D. Breads enriched with guava flour as a tool for studying the incorporation of phenolic compounds in bread melanoidins. Food Chemistry 2015; 185(0): 65-74.

141. Anklam E. H.-D. Belitz, W. Grosch, P. Schieberle: Food Chemistry, Third Edition. Analytical and Bioanalytical Chemistry 2005; 382(1): 10-1.

142. Jiménez-Zamora A, Pastoriza S, Rufián-Henares JA. Revalorization of coffee by-products. Prebiotic, antimicrobial and antioxidant properties. LWT - Food Science and Technology 2015; 61(1): 12-8.

143. Pastoriza S, Rufián-Henares JA. Contribution of melanoidins to the antioxidant capacity of the Spanish diet. Food Chemistry 2014; 164(0): 438-45.

144. Bravo J, Juaniz I, Monente C, Caemmerer B, Kroh LW, De Pena MP, et al. Evaluation of spent coffee obtained from the most common coffeemakers as a source of hydrophilic bioactive compounds. Journal of Agricultural and Food Chemistry 2012; 60(51): 12565-73.

145. Onakpoya I, Terry R, Ernst E. The use of green coffee extract as a weight loss supplement: A systematic review and meta-analysis of randomised clinical trials. Gastroenterology Research and Practice 2011; 2011: 382852.

146. Beam JR, Gibson AL, Kerksick CM, Conn CA, White AC, Mermier CM. Effect of post-exercise caffeine and green coffee bean extract consumption on blood glucose and insulin concentrations. Nutrition 2015; 31(2): 292-7.

147. Suzuki A, Kagawa D, Ochiai R, Tokimitsu I, Saito I. Green coffee bean extract and its metabolites have a hypotensive effect in spontaneously hypertensive rats. Hypertension Research 2002; 25(1): 99-107.

148. Wei F, Furihata K, Koda M, Hu F, Kato R, Miyakawa T, et al. 13C NMR-based metabolomics for the classification of green coffee beans according to variety and origin. Journal of Agricultural and Food Chemistry 2012; 60(40): 10118-25.

149. Jeszka-Skowron M, Sentkowska A, Pyrzyńska K, De Peña MP. Chlorogenic acids, caffeine content and antioxidant properties of green coffee extracts: influence of green coffee bean preparation. European Food Research and Technology 2016; 242(8): 1403-9.

150. Gawlik-Dziki U, Świeca M, Dziki D, Kowalska I, Pecio T, Durak A, et al. Lipoxygenase inhibitors and antioxidants from green coffee-mechanism of action in the light of potential bioaccessibility. Food Research International 2014; 61: 48-55.

151. Shimoda H, Seki E, Aitani M. Inhibitory effect of green coffee bean extract on fat accumulation and body weight gain in mice. BMC Complementary and Alternative Medicine 2006; 6: 9.

152. Cho AS, Jeon SM, Kim MJ, Yeo J, Seo KI, Choi MS, et al. Chlorogenic acid exhibits anti-obesity property and improves lipid metabolism in high-fat diet-induced-obese mice. Food and Chemical Toxicology 2010; 48(3): 937-43.

153. Lopez-Garcia E, van Dam RM, Rajpathak S, Willett WC, Manson JE, Hu FB. Changes in caffeine intake and long-term weight change in men and women. American Journal of Clinical Nutrition 2006; 83(3): 674-80.

154. Hiltunen L. Are there associations between coffee consumption and glucose tolerance in elderly subjects? European Journal of Clinical Nutrition 2006; 60(10): 1222-5.

155. Tunnicliffe JM, Shearer J. Coffee, glucose homeostasis, and insulin resistance: physiological mechanisms and mediators. Applied Physiology, Nutrition, and Metabolism 2008; 33(6): 1290-300.

156. Johnston KL, Clifford MN, Morgan LM. Coffee acutely modifies gastrointestinal hormone secretion and glucose tolerance in humans: glycemic effects of chlorogenic acid and caffeine. American Journal of Clinical Nutrition 2003; 78(4): 728-33.

157. Thom E. The effect of chlorogenic acid enriched coffee on glucose absorption in healthy volunteers and its effect on body mass when used long-term in overweight and obese people. Journal of International Medical Research 2007; 35(6): 900-8.

158. Ong KW, Hsu A, Tan BK. Chlorogenic acid stimulates glucose transport in skeletal muscle via AMPK activation: a contributor to the beneficial effects of coffee on diabetes. PLoS One 2012; 7(3): e32718.

159. Zheng G, Qiu Y, Zhang QF, Li D. Chlorogenic acid and caffeine in combination inhibit fat accumulation by regulating hepatic lipid metabolism-related enzymes in mice. British Journal of Nutrition 2014; 112(6): 1034-40.

160. del Castillo MD, Ames JM, Gordon MH. Effect of roasting on the antioxidant activity of coffee brews. Journal of Agricultural and Food Chemistry 2002; 50(13): 3698-703.

161. Zlotek U, Karas M, Gawlik-Dziki U, Szymanowska U, Baraniak B, Jakubczyk A. Antioxidant activity of the aqueous and methanolic extracts of coffee beans (Coffea arabica L.). Acta Scientiarum Polonorum Technologia Alimentaria 2016; 15(3): 281-8.

162. Hoelzl C, Knasmüller S, Wagner KH, Elbling L, Huber W, Kager N, et al. Instant coffee with high chlorogenic acid levels protects humans against oxidative damage of macromolecules. Molecular Nutrition & Food Research 2010; 54(12): 1722-33.

163. Somoza V, Lindenmeier M, Wenzel E, Frank O, Erbersdobler HF, Hofmann T. Activity-guided identification of a chemopreventive compound in coffee beverage using in vitro and in vivo techniques. Journal of Agricultural and Food Chemistry 2003; 51(23): 6861-9.

164. Boettler U, Sommerfeld K, Volz N, Pahlke G, Teller N, Somoza V, et al. Coffee constituents as modulators of Nrf2 nuclear translocation and ARE (EpRE)-dependent gene expression. Journal of Nutritional Biochemistry 2011; 22(5): 426-40.

165. Higgins JP, Babu KM. Caffeine reduces myocardial blood flow during exercise. American Journal of Medicine 2013; 126(8): 730.e1-e8.

166. El Agaty S, Seif A. Cardiovascular effects of long-term caffeine administration in aged rats. Irish Journal of Medical Science (1971-) 2015; 184(2): 265-72.

167. Rogers PJ, Heatherley SV, Mullings EL, Smith JE. Faster but not smarter: effects of caffeine and caffeine withdrawal on alertness and performance. Psychopharmacology 2013; 226(2): 229-40.

168. Meinhart AD, Bizzotto CS, Ballus CA, Prado MA, Bruns RE, Teixeira Filho J, et al. Optimisation of a CE method for caffeine analysis in decaffeinated coffee. Food Chemistry 2010; 120(4): 1155-61.

169. Dellalibera O, Lemaire B, Lafay S. Svetol, green coffee extract, induces weight loss and increases the lean to fat mass ratio in volunteers with overweight problem. Phytotherapie 2006; 4(4): 194-7.

170. Song SJ, Choi S, Park T. Decaffeinated green coffee bean extract attenuates diet-induced obesity and insulin resistance in mice. Evidence-Based Complementary and Alternative Medicine 2014; 2014: 718379.

171. Caria CReP, de Oliveira CC, Gotardo ÉFM, de Souza VT, Rocha T, Macedo JA, et al. Caffeinated and decaffeinated instant coffee consumption partially reverses high-fat diet-induced metabolic alterations in mice. Food Research International 2014; 61: 120-6.

172. Esquivel P, Jimenez VM. Functional properties of coffee and coffee by-products. Food Research International 2012; 46(2): 488-95.

173. Cruz R, Cardoso MM, Fernandes L, Oliveira M, Mendes E, Baptista P, et al. Espresso coffee residues: a valuable source of unextracted compounds. Journal of Agricultural and Food Chemistry 2012; 60(32): 7777-84.

174. Heimbach JT, Marone PA, Hunter JM, Nemzer BV, Stanley SM, Kennepohl E. Safety studies on products from whole coffee fruit. Food and Chemical Toxicology 2010; 48(8-9): 2517-25.

175. Borrelli RC, Esposito F, Napolitano A, Ritieni A, Fogliano V. Characterization of a new potential functional ingredient: coffee silverskin. Journal of Agricultural and Food Chemistry 2004; 52(5): 1338-43.

176. Ohta A, Sitkovsky M. Methylxanthines, inflammation, and cancer: Fundamental mechanisms. Methylxanthines. Handbook of Experimental Pharmacology. 200: Springer Berlin Heidelberg; 2011. p. 469-81.

177. Beaudoin M-S, Graham T. Methylxanthines and human health: Epidemiological and experimental evidence. Methylxanthines. Handbook of Experimental Pharmacology. 200: Springer Berlin Heidelberg; 2011. p. 509-48.

178. Bae J-H, Park J-H, Im S-S, Song D-K. Coffee and health. Integrative Medicine Research 2014; 3(4): 189-91.

179. Panchal SK, Wong WY, Kauter K, Ward LC, Brown L. Caffeine attenuates metabolic syndrome in diet-induced obese rats. Nutrition 2012; 28(10): 1055-62.

180. Zheng G, Qiu Y, Zhang Q-F, Li D. Chlorogenic acid and caffeine in combination inhibit fat accumulation by regulating hepatic lipid metabolism-related enzymes in mice. British Journal of Nutrition 2014; 112(06): 1034-40.

181. Riedel A, Pignitter M, Hochkogler CM, Rohm B, Walker J, Bytof G, et al. Caffeine dose-dependently induces thermogenesis but restores ATP in HepG2 cells in culture. Food & function 2012; 3(9): 955-64.

182. Sacramento JF, Ribeiro MJ, Yubero S, Melo BF, Obeso A, Guarino MP, et al. Disclosing caffeine action on insulin sensitivity: Effects on rat skeletal muscle. European Journal of Pharmaceutical Sciences 2015; 70(0): 107-16.

183. Tomas-Barberan F, Garcia-Villalba R, Quartieri A, Raimondi S, Amaretti A, Leonardi A, et al. In vitro transformation of chlorogenic acid by human gut microbiota. Molecular Nutrition & Food Research 2014; 58(5): 1122-31.

184. Mills CE, Tzounis X, Oruna-Concha M-J, Mottram DS, Gibson GR, Spencer JPE. In vitro colonic metabolism of coffee and chlorogenic acid results in selective changes in human faecal microbiota growth. British Journal of Nutrition 2015; FirstView: 1-8.

185. Couteau D, McCartney AL, Gibson GR, Williamson G, Faulds CB. Isolation and characterization of human colonic bacteria able to hydrolyse chlorogenic acid. Journal of Applied Microbiology 2001; 90(6): 873-81.

186. Lafay S, Gil-Izquierdo A, Manach C, Morand C, Besson C, Scalbert A. Chlorogenic acid is absorbed in its intact form in the stomach of rats. Journal of Nutrition 2006; 136(5): 1192-7.

187. Higdon JV, Frei B. Coffee and health: A review of recent human research. Critical Reviews in Food Science and Nutrition 2006; 46(2): 101-23.

188. Huang K, Liang XC, Zhong YL, He WY, Wang Z. 5-Caffeoylquinic acid decreases diet-induced obesity in rats by modulating PPARα and LXRα transcription. Journal of the Science of Food and Agriculture 2015; 95(9): 1903-10.

189. Ma Y, Gao M, Liu D. Chlorogenic acid improves high fat diet-induced hepatic steatosis and insulin resistance in mice. Pharmaceutical Research 2015; 32(4): 1200-9.

190. Borrelli RC, Visconti A, Mennella C, Anese M, Fogliano V. Chemical characterization and antioxidant properties of coffee melanoidins. Journal of Agricultural and Food Chemistry 2002; 50(22): 6527-33.

191. Moreira AS, Nunes FM, Domingues MR, Coimbra MA. Coffee melanoidins: structures, mechanisms of formation and potential health impacts. Food & function 2012; 3(9): 903-15.

192. Paur I, Balstad TR, Blomhoff R. Degree of roasting is the main determinant of the effects of coffee on NF-κB and EpRE. Free Radical Biology and Medicine 2010; 48(9): 1218-27.

193. Corrao G, Zambon A, Bagnardi V, D'Amicis A, Klatsky A, Collaborative SG. Coffee, caffeine, and the risk of liver cirrhosis. Annals of Epidemiology 2001; 11(7): 458-65.

194. Moreira ASP, Nunes FM, Simoes C, Maciel E, Domingues P, Domingues MRM, et al. Data on coffee composition and mass spectrometry analysis of mixtures of coffee related carbohydrates, phenolic compounds and peptides. Data Brief 2017; 13: 145-61.

195. Gniechwitz D, Reichardt N, Ralph J, Blaut M, Steinhart H, Bunzel M. Isolation and characterisation of a coffee melanoidin fraction. Journal of the Science of Food and Agriculture 2008; 88(12): 2153-60.

196. Bekedam EK, Schols HA, Van Boekel MA, Smit G. High molecular weight melanoidins from coffee brew. Journal of Agricultural and Food Chemistry 2006; 54(20): 7658-66.

197. Delgado-Andrade C, Morales FJ. Unraveling the contribution of melanoidins to the antioxidant activity of coffee brews. Journal of Agricultural and Food Chemistry 2005; 53(5): 1403-7.

198. Rufián-Henares JA, Morales FJ. Effect of in vitro enzymatic digestion on antioxidant activity of coffee melanoidins and fractions. Journal of Agricultural and Food Chemistry 2007; 55(24): 10016-21.

199. Ames JM, Wynne A, Hofmann A, Plos S, Gibson GR. The effect of a model melanoidin mixture on faecal bacterial populations in vitro. British Journal of Nutrition 1999; 82(06): 489-95.

200. Jaquet M, Rochat I, Moulin J, Cavin C, Bibiloni R. Impact of coffee consumption on the gut microbiota: a human volunteer study. International journal of food microbiology 2009; 130(2): 117-21.

201. Obretenov TD, Ivanova SD, Kuntcheva MJ, Somov GT. Melanoidin formation in cooked meat products. Journal of Agricultural and Food Chemistry 1993; 41(4): 653-6.

202. Adams A, Borrelli RC, Fogliano V, De Kimpe N. Thermal degradation studies of food melanoidins. Journal of Agricultural and Food Chemistry 2005; 53(10): 4136-42.

203. Reichardt N, Gniechwitz D, Steinhart H, Bunzel M, Blaut M. Characterization of high molecular weight coffee fractions and their fermentation by human intestinal microbiota. Molecular Nutrition & Food Research 2009; 53(2): 287-99.

204. Goya L, Delgado-Andrade C, Rufián-Henares JA, Bravo L, Morales FJ. Effect of coffee Melanoidin on human hepatoma HepG2 cells. Protection against oxidative stress induced by tert-butylhydroperoxide. Molecular Nutrition & Food Research 2007; 51(5): 536-45.

205. Valls-Bellés V, Torres MC, Muñiz P, Boix L, González-Sanjose ML, Codoñer-Franch P. The protective effects of melanoidins in adriamycin-induced oxidative stress in isolated rat hepatocytes. Journal of the Science of Food and Agriculture 2004; 84(13): 1701-7.

206. Uchida K. Role of reactive aldehyde in cardiovascular diseases. Free Radical Biology and Medicine 2000; 28(12): 1685-96.

207. Votavová L, Voldrich M, Sevcik R, Cizkova H, Mlejnecká J, Stolar M, et al. Changes of antioxidant capacity of robusta coffee during roasting. Czech Journal of Food Science 2009; 27: S49-S52.

208. Digby JE, McNeill E, Dyar OJ, Lam V, Greaves DR, Choudhury RP. Anti-inflammatory effects of nicotinic acid in adipocytes demonstrated by suppression of fractalkine, RANTES, and MCP-1 and upregulation of adiponectin. Atherosclerosis 2010; 209(1): 89-95.

209. Stadler RH, Varga N, Milo C, Schilter B, Vera FA, Welti DH. Alkylpyridiniums. 2. Isolation and quantification in roasted and ground coffees. Journal of Agricultural and Food Chemistry 2002; 50(5): 1200-6.

210. Ranheim T, Halvorsen B. Coffee consumption and human health - beneficial or detrimental? - Mechanisms for effects of coffee consumption on different risk factors for

cardiovascular disease and type 2 diabetes mellitus. Molecular Nutrition & Food Research 2005; 49(3): 274-84.

211. Lee KJ, Jeong HG. Protective effects of kahweol and cafestol against hydrogen peroxide-induced oxidative stress and DNA damage. Toxicology Letters 2007; 173(2): 80-7.

212. Kim JY, Kim DH, Jeong HG. Inhibitory effect of the coffee diterpene kahweol on carrageenan-induced inflammation in rats. Biofactors 2006; 26(1): 17-28.

213. Mellbye FB, Jeppesen PB, Shokouh P, Laustsen C, Hermansen K, Gregersen S. Cafestol, a Bioactive Substance in Coffee, Has Antidiabetic Properties in KKAy Mice. Journal of Natural Products 2017; 80(8): 2353-9.

Chapter 2.

Green coffee improves diet-induced metabolic syndrome in rats

Green coffee improves diet-induced metabolic syndrome in rats

Nikhil **Bhandarkar**[1, 2], Peter **Mouatt**[3], Lindsay **Brown**[1, 2], Sunil K **Panchal**[2]

[1]School of Health and Wellbeing, Faculty of Health, Engineering and Sciences, and [2]Functional Foods Research Group, Institute for Agriculture and the Environment, University of Southern Queensland, Toowoomba, QLD 4350, Australia; [3]Southern Cross Plant Science, Southern Cross University, Lismore, NSW 2480, Australia.

Running title: Green coffee and obesity

Corresponding author: Dr Sunil K Panchal

Functional Foods Research Group, Institute for Agriculture and the Environment

University of Southern Queensland, Toowoomba, QLD 4350, Australia

Email: Sunil.Panchal@usq.edu.au; Tel.: +61 7 4631 1012

Abstract

Abdominal obesity is associated with the development of metabolic disorders, type 2 diabetes and cardiovascular diseases. This study assessed the effects of green coffee on metabolic variables in obesity induced by high-carbohydrate, high-fat diet in rats. Male Wistar rats (8-9 weeks old, 340 ± 5 g, n = 72) were divided into 6 groups and fed with either corn starch diet (16 weeks) (C), corn starch diet with 5% green coffee (CGC) or decaffeinated green coffee extract (CDC) in food for the last 8 weeks, high-carbohydrate high-fat diet (16 weeks) (H), or high-carbohydrate high-fat diet with 5% green coffee (HGC) or decaffeinated green coffee extract (HDC) in food for the last 8 weeks. The high-carbohydrate high-fat diet-fed rats showed signs of metabolic syndrome as changes in cardiovascular and liver structure and function. Green coffee attenuated body weight (C 385 ± 5g, H 563 ± 14g, HGC 515 ± 16g, HDC 522 ± 15g), feed efficiency (C 0.11 ± 0.01kJ/g, H 0.37 ± 0.02kJ/g, HGC 0.31 ± 0.02kJ/g, HDC 0.32 ± 0.02kJ/g), systolic blood pressure (C 128 ± 1mmHg, H 147 ± 1mmHg, HGC 130 ± 2mmHg, HDC 128 ± 2mmHg) and improved structure of heart and liver without improving obesity, glucose sensitivity or dyslipidaemia. The chlorogenic acid in green coffee could be the major bioactive compound to attenuate diet-induced abnormalities in heart and liver structure and function.

Keywords: Green coffee, Obesity, Metabolic syndrome, High-carbohydrate, high-fat diet

Introduction

Functional foods have been proposed as interventions to prevent or reverse metabolic syndrome [1], a constellation of metabolic disorders including obesity, hypertension, impaired glucose tolerance, insulin resistance, dyslipidaemia and fatty liver as a major risk factor for cardiovascular disease and type 2 diabetes [1]. Obesity and insulin resistance are strongly linked with metabolic syndrome in the general population [2, 3]. As part of a healthy diet, green coffee could be a potential functional food for metabolic syndrome [4]. Green coffee extract decreased obesity and blood lipid changes by mechanisms including inhibition of adipogenesis, scavenging of reactive oxygen species, and reduced triglycerides and glucose concentrations in rats and adipocytes in culture [5]. Coffee components such as chlorogenic acids, caffeine, trigonelline, and diterpenes reach active concentrations in the human body [6]. A cross-sectional study in humans concluded that green coffee intake was inversely associated with some parameters of metabolic syndrome and cardiovascular mortality [7]. Likewise, green coffee improved weight loss [8] and protected against the development of non-alcoholic fatty liver disease (NAFLD) [9]. A meta-analysis of studies on weight loss with green coffee showed an average loss of 2.47 kg, but all studies had a high risk of bias and were of poor methodological quality [10]. However, the extent of the responses on the range of disorders in metabolic syndrome and the compounds responsible for those effects have not been determined.

The processing of coffee berries into coffee involves six main steps of pulping, fermentation, drying, hulling, roasting then grinding [11]. Green coffee products, usually sold as capsules, come from coffee beans that have not been roasted, so these products

contain different proportions of components compared to roasted or black coffee [12]. The moderate intake of green coffee, 4-6 cups/day, delivers bioactivity of some coffee components such as caffeine [13], chlorogenic acids [13], trigonelline [14], and diterepenes [15] in human body [16,6]. Caffeine intake has been demonstrated to decrease metabolic syndrome [17], however, caffeine consumption is also reported for adverse outcomes consistent with the physiologic effects among adults and young adults [18,19]. Esters of caffeic acid and chlorogenic acid (caffeoylquinic acids) are well demonstrated for health benefits such as improvement in cardiovascular disease and type 2 diabetes [20]. Chlorogenic acids show potential anti-oxidant and anti-inflammatory effects through reducing liver mRNA expression of TNF-alpha, IL-6 and IL-1beta that provide protection against lipid accumulation of Sprague-Dawley rats fed a high-fat diet (HFD) [21]. Chlorogenic acids (50 mg/kg for 42 days) increased the plasma lipid metabolism in rats by reducing the concentrations of free fatty acids and triglycerides and regulating the multiple components in hepatocytes through AMPK pathway [22], which indicates that chlorogenic acids could be a promising ingredient in diet for obesity management. Along with chlorogenic acids, caffeic acids, trigonelline and cafestol provided the synergistic effects and improved the insulin sensitivity in the treated rats, and increased plasma concentrations of adiponectin [23]. Further, cafestol (a diterpene present in coffee brew) has insulinotropic effects on pancreatic β-cells, induces glucose uptake in human myoblasts [24], and increases glycaemic regulation *in vivo* [25].

This study has determined whether green coffee and its decaffeinated product can improve signs of metabolic syndrome when given as 8-week dietary interventions in rats fed a high-carbohydrate, high-fat diet for 16 weeks to mimic human metabolic syndrome

[13]. Cardiovascular function was studied *in vivo* by measurement of systolic blood pressure and *ex vivo* in isolated Langendorff heart preparations, isolated thoracic aortic rings together with histology. Liver structure and function were measured by histology and plasma biochemistry. Metabolic parameters related to obesity and glucose tolerance were also evaluated.

Methods

Analysis of green coffee

Extractions were prepared in 3:2 ethanol:water mixture. Briefly, 1 g of coffee (*Arabica spp*.) was dissolved in 50 mL of ethanol:water mixture, sonicated for 15 minutes and an aliquot of the supernatant was taken for analysis by the HPLC using an Agilent 1100 series system coupled with a mass spectrometer for further peak confirmation or identification as required. The HPLC system consisted of a diode array detector (G4212B), binary pump (G4220A), an autosampler (G4226A), a vacuum degasser and a column oven with an MSD (G1946D) detector also present. The chromatography was performed on a Phenomenex luna C18 (2) HPLC column (100 x 4.6 mm) using a gradient method of water and acetonitrile with 0.005% trifluoroacetic acid over 28 minutes. The optimal solvent gradient for separation of target constituents were starting at 10% acetonitrile which was increased as a gradient to 30% acetonitrile over 10 minutes, then to 95% acetonitrile over 8 minutes, at a flow rate of 0.75 mL/minute and an injection volume of 5 µL. Calibration standards of trigonelline, caffeine and chlorogenic acid were prepared in 60:40 ethanol:water, at concentrations from 0.01 to 1 mg/mL, 0.005 to 0.5 mg/mL and 0.004 to 1 mg/mL for each of these standards, respectively. Specific detection and calibration

curves for each compound were performed at 254 nm, 280 nm and 330 nm, respectively. Quantification was performed using the Chemstation Software based on reference standards, peak area and sample dilution at specific wavelengths for each compound [14].

Rats, diets and treatment

Male Wistar rats (8–9 weeks old, 330-340 g, n = 72) were obtained from the Animal Resource Centre, Perth. Rats were divided into 6 groups (n = 12 rats/group): C, corn starch diet-fed rats; CGC, corn starch diet + regular green coffee extract (5% in food); CDC, corn starch diet + decaffeinated green coffee extract (5% in food); H, high-carbohydrate, high-fat diet-fed rats; HGC, high-carbohydrate, high-fat diet-fed rats + regular green coffee extract (5% in food); and HDC, high-carbohydrate, high-fat diet-fed rats + decaffeinated green coffee extract (5% in food).

C and H rats were fed with corn starch and high-carbohydrate, high-fat diets for 16 weeks, respectively [13]. Treatment groups were fed with either corn starch (C) or high-carbohydrate, high-fat diets (H) for the first 8 weeks and then the respective diets were supplemented with treatments for the last 8 weeks. Extracts of regular green coffee or decaffeinated green coffee were prepared by mixing ground coffee (50 g) with hot water but not boiling water (100 mL) and then filtering after 5 minutes of mixing to obtain 50 mL of extract. This extract was then mixed with the food by replacing 50 mL water per kg food.

Cornstarch diet contained 570 g of corn starch, 155 g of powdered rat food, 25 g of HMW salt mixture and 250 mL of water per kilogram of diet. High-carbohydrate, high-fat diet contained 175 g of fructose, 395 g of sweetened condensed milk, 200 g of beef

tallow, 155 g of powdered rat food, 25 g of HMW salt mixture and 50 mL of water per kilogram of diet [13]. Drinking water containing 25 % (w/v) fructose was provided to all H groups. C rat groups received normal drinking water. Rats were individually housed under temperature controlled, 12-hour-light/dark conditions and given free access to food and water [13].

Physiological parameters

Body weight and food and water intakes were measured daily [13]. Abdominal circumference and body length were measured using a standard measuring tape under light anaesthesia with Zoletil (tiletamine 10 mg/kg and zolazepam 10 mg/kg, intraperitoneal; Virbac, Peakhurst, Australia) [13]. Body mass index was calculated as body weight (in grams)/[body length (in cm)]^2 [13]. Feed efficiency was calculated as [mean body weight gain (in grams)/daily energy intake (in kJ)] [13].

Systolic blood pressure was determined under light sedation with Zoletil (tiletamine 10 mg/kg and zolazepam 10 mg/kg, intraperitoneal; Virbac, Peakhurst, Australia), using an MLT1010 Piezo-Electric Pulse Transducer and inflatable tail-cuff connected to an MLT844 Physiological Pressure Transducer and PowerLab data acquisition unit (ADInstruments, Sydney, Australia) [13].

Dual-energy X-ray absorptiometric measurements were carried out at the end of the protocol with a Norland XR46 DXA instrument (Norland Corp, Fort Atkinson, WI). These scans were evaluated using the manufacturer's suggested software for use in laboratory animals (Small Subject Analysis Software, version 2.5.3/1.3.1; Norland Corp)

[13]. The precision error of lean mass for replicate measurements, with repositioning, is 3.2%.

Oral glucose tolerance tests were performed after determining overnight fasting blood glucose concentrations in tail vein blood using Medisense Precision Q.I.D. glucose meters (Abbot Laboratories, Bedford, MA). For overnight fasting, rats were deprived of all types of diets for 12 hours. Fructose-supplemented drinking water in H diet-fed groups was replaced with normal drinking water for the overnight food deprivation period. Rats were given a glucose load of 2 g/(kg body weight) as 40% glucose solution via oral gavage and blood glucose concentrations were measured again 30, 60, 90 and 120 minutes after oral glucose administration [13].

Euthanasia

Rats were euthanized with Lethabarb (pentobarbitone sodium, 100 mg/kg, intraperitoneal; Virbac, Peakhurst, Australia). After euthanasia, heparin (200 IU; Sigma-Aldrich Australia, Sydney, NSW, Australia) was injected through the right femoral vein. The abdomen was then opened and blood (~5 mL) was withdrawn from the abdominal aorta and collected into heparinised tubes. Blood was centrifuged at 5000 × g for 10 minutes to obtain plasma. Plasma samples were stored at -20°C.

Hearts were then removed from rats for isolated Langendorff heart preparation [13]. These hearts were perfused with modified Krebs–Henseleit bicarbonate buffer 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. Left ventricular end-diastolic pressure values were measured during pacing of the heart at 250 beats per minute using an electrical stimulator. End-diastolic pressures were obtained from 0 to 30 mmHg for the calculation of diastolic stiffness constant (κ, dimensionless) [13].

Thoracic aortic rings (~ 4 mm in length) were suspended in an organ bath filled with Tyrode physiological salt solution bubbled with 95% O_2–5% CO_2, maintained at 35°C and allowed to stabilise at a resting tension of ~10 mN. Cumulative concentration-response curves (contraction) were obtained for noradrenaline and cumulative concentration-response curves (relaxation) were obtained for acetylcholine and sodium nitroprusside after submaximal (~70%) contraction to noradrenaline [13].

After isolated heart perfusion studies, hearts were separated into left ventricles (with septum) and right ventricles and weighed. Livers were isolated and weighed. Retroperitoneal, epididymal and omental abdominal fats were removed separately and weighed. These organ weights were normalised against the tibial length at the time of organ removal and expressed as mg/mm of tibial length [13].

Histology

Hearts and livers were removed from the rats (n = 4) soon after euthanasia and these organs were fixed in 10% neutral buffered formalin. The samples were then dehydrated and embedded in paraffin wax. Thin sections (5 µm) were cut and stained with haematoxylin and eosin stain to study infiltration of inflammatory cells (×20) and for determining fat vacuoles (×40) in the liver. Picrosirius red stain was used to study left ventricular collagen deposition (×20) [13].

Plasma biochemistry

Plasma activities of aspartate transaminase, alanine transaminase and alkaline phosphatase, and plasma concentrations of total cholesterol, triglycerides and non-esterified fatty acids were measured [13].

Statistical analysis

Data are presented as mean ± SEM. Results were tested for variance using Bartlett's test and variables that were not normally distributed were transformed (using log 10 functions) prior to statistical analyses. Groups were tested for effects of diet, treatment and their interactions by two-way analysis of variance. When the interaction and/or the main effects were significant, means were compared using Newman-Keuls multiple comparison post hoc tests. A P value <0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism version 5.0 for Windows (San Diego, CA, USA).

Results

Green coffee analysis

The contents and doses of chlorogenic acid, caffeine and trigonelline in green coffee are given in Table 1.

Table 1: Green and decaffeinated green coffee

Components	***Green coffee (mg/ml)***	***Decaffeinated green coffee (mg/ml)***	***CGC (mg/kg/ day)***	***CDC (mg/kg/ day)***	***HGC (mg/kg/ day)***	***HDC (mg/kg/ day)***
Chlorogenic acid	2.58	1.3	118 ± 5	55 ± 5	69 ± 2	34 ± 1
Caffeine	0.7	0	32 ± 2	0	19 ± 2	0
Trigonelline	0.85	0.9	39 ± 2	38 ± 4	23 ± 1	24 ± 1

Dietary intake, body composition and metabolic parameters

Green coffee treatment in corn starch rats (CGC, CDC rats) did not alter physiological parameters or plasma biochemistry compared to C rats (Tables 2 and 3). Green coffee treatment in HGC and HDC rats decreased the rate of body weight gain without affecting food, water or energy intakes, by reducing feed efficiency compared to H rats (Table 2). Body mass index reduced in HGC rats but did not change in HDC rats compared to H rats. However, abdominal circumference, basal or final blood glucose

concentrations, the area under the curve, plasma lipid concentrations, and total lean and fat mass did not change in HGC and HDC rats compared to H rats. Retroperitoneal fat was decreased while epididymal fat and omental fat were unchanged in HGC and HDC rats compared to H rats (Table 2).

Cardiovascular and liver function

Green coffee did not change cardiovascular or liver parameters in CGC and CGC compared to C rats (Table 2). Systolic blood pressure, left ventricular stiffness, fibrosis and inflammation were increased in H rats compared to C rats; in HGC and HDC rats, both parameters were reduced compared to H rats (Table 2). Green coffee treatments did not change fibrosis or inflammation in hearts of CGC (Figure 1B and 1H) or CDC (Figure 1C and 1I) rats compared to C rats (Figure 1A and 1G) while decreasing fibrosis and inflammation in HGC rats (Figure 1E and 1K) and HDC rats (Figure 1F and 1L) compared to H rats (Figure 1D and 1J). Green coffee treatment did not alter aortic responses in C or H rats (Figure 2A, 2B and 2C). Green coffee treatments did not change inflammation and fat deposition in livers of CGC (Figure 3B and 3H) or CDC (Figure 3C and 3I) rats compared to C rats (Figure 3A and 3G) while decreasing inflammation and fat deposition in livers of HGC rats (Figure 3E and 3K) and HDC rats (Figure 3F and 3L) compared to H rats (Figure 3D and 3J).

Table 2: Green and decaffeinated green coffee on physiological and metabolic variables

Variables	***C***	***CGC***	***CDC***	***H***	***HGC***	***HDC***	***P* value**		
							Diet	***Treatment***	***Diet* × *Treatment***
Initial body weight, g	340 ± 1	339 ± 1	340 ± 1	340 ± 1	337 ± 1	339 ± 1	0.23	0.12	0.61
Final body weight, g	385 ± 5^{c}	384 ± 6^{c}	402 ± 9^{c}	563 ± 14^{a}	515 ± 16^{b}	522 ± 15^{b}	<0.0001	0.12	0.035
Body weight gain (8-16 weeks), %	7.7 ± 1.4^{c}	10.5 ± 0.8^{c}	7.6 ± 1.3^{c}	20.7 ± 1.8^{a}	16.0 ± 1.0^{b}	16.7 ± 1.6^{b}	<0.0001	0.33	0.028
Water intake, mL/d	25.1 ± 3.5	27.5 ± 2.6	30.2 ± 1.9	32.2 ± 2.5	34.6 ± 2.7	31.5 ± 1.1	0.054	0.56	0.42
Food intake, g/d	35.2 ± 1.3^{a}	34.2 ± 1.8^{a}	33.0 ± 0.9^{a}	27.2 ± 0.7^{b}	26.5 ± 0.9^{b}	25.5 ± 0.6^{b}	<0.0001	0.22	0.98
Energy intake, kJ/d	395 ± 15^{b}	365 ± 6^{b}	371 ± 10^{b}	612 ± 17^{a}	621 ± 22^{a}	578 ± 10^{a}	<0.0001	0.13	0.20

Feed efficiency, kJ/g	0.11 ± 0.01^{c}	0.11 ± 0.01^{c}	0.15 ± 0.02^{c}	0.37 ± 0.02^{a}	0.31 ± 0.02^{b}	0.32 ± 0.02^{b}	<0.0001	0.19	0.038
Body mass index, g/cm^2	0.63 ± 0.02^{d}	0.65 ± 0.01^{d}	0.71 ± 0.02^{c}	0.85 ± 0.02^{a}	0.77 ± 0.03^{b}	0.81 ± 0.02^{a}	<0.0001	0.19	0.038
Abdominal circumference, cm	18.8 ± 0.3^{b}	18.3 ± 0.1^{b}	18.8 ± 0.3^{b}	23.3 ± 0.7^{a}	21.7 ± 0.7^{a}	22.0 ± 0.6^{a}	<0.0001	0.19	0.038
Whole-body lean mass, g	297 ± 4	273 ± 7	312 ± 11	278 ± 8	278 ± 9	295 ± 18	0.20	0.051	0.39
Whole-body fat mass, g	68.7 ± 4.8^{b}	89.6 ± 6.9^{b}	74.1 ± 4.8^{b}	252 ± 17^{a}	209 ± 17^{a}	224 ± 29^{a}	<0.0001	0.70	0.12
Bone mineral density, g/cm^2	0.174 ± 0.003^{b}	0.168 ± 0.003^{b}	0.176 ± 0.002^{ab}	0.185 ± 0.003^{a}	0.182 ± 0.002^{a}	0.183 ± 0.004^{a}	<0.0001	0.24	0.52
Bone mineral content, g	11.2 ± 0.2^{c}	11.3 ± 0.3^{c}	11.7 ± 0.3^{c}	17.4 ± 0.5^{a}	15.5 ± 0.7^{b}	15.4 ± 0.8^{b}	<0.0001	0.16	0.034

Basal blood glucose concentrations, mmol/L	4.1 ± 0.2^{b}	4.3 ± 0.2^{b}	4.0 ± 0.2^{b}	5.1 ± 0.2^{a}	5.4 ± 0.3^{a}	4.8 ± 0.2^{a}	<0.0001	0.13	0.79
Blood glucose concentrations at 120 minutes, mmol/L	4.9 ± 0.1^{b}	4.7 ± 0.1^{b}	5.0 ± 0.1^{b}	5.7 ± 0.3^{a}	5.8 ± 0.1^{a}	6.0 ± 0.1^{a}	<0.0001	0.22	0.62
Area under the curve, mmol/L·minute	715 ± 20^{b}	661 ± 10^{b}	707 ± 17^{b}	805 ± 18^{a}	832 ± 12^{a}	778 ± 20^{a}	<0.0001	0.52	0.009
Retroperitoneal fat, mg/mm tibial length	136 ± 12^{c}	192 ± 16^{c}	179 ± 15^{c}	484 ± 54^{a}	380 ± 28^{b}	393 ± 29^{b}	<0.0001	0.59	0.010

Epididymal fat, mg/mm tibial length	87.2 ± 6[b]	112 ± 9[b]	97.2 ± 10.7[b]	226 ± 23[a]	220 ± 21[a]	246 ± 15[a]	<0.0001	0.59	0.37
Omental fat, mg/mm tibial length	130 ± 8[b]	136 ± 8[b]	126 ± 7[b]	251 ± 16[a]	224 ± 16[a]	252 ± 20[a]	<0.0001	0.67	0.27
Total abdominal fat, mg/mm tibial length	353 ± 18[b]	439 ± 30[b]	402 ± 27[b]	961 ± 89[a]	824 ± 56[a]	892 ± 52[a]	<0.0001	0.86	0.08
Plasma total cholesterol, mmol/L	1.64 ± 0.08	1.64 ± 0.08	1.53 ± 0.04	1.58 ± 0.08	1.60 ± 0.06	1.55 ± 0.05	0.64	0.45	0.83

Plasma triglycerides, mmol/L	0.53 ± 0.06^{d}	0.54 ± 0.07^{d}	0.80 ± 0.06^{cd}	1.30 ± 0.16^{b}	1.23 ± 0.16^{bc}	2.80 ± 0.22^{a}	<0.0001	<0.0001	<0.0001
Plasma NEFA, mmol/L	1.4 ± 0.2^{b}	1.4 ± 0.2^{b}	1.9 ± 0.3^{b}	3.8 ± 0.3^{a}	3.7 ± 0.3^{a}	4.4 ± 0.2^{a}	<0.0001	0.052	0.93

Values are mean ± SEM, n=6-12. Means in a row with superscripts without a common letter differ significantly, *P<0.05.* C, corn starch diet-fed rats; CGC, corn starch diet-fed rats treated with green coffee extract; CDC, corn starch diet-fed rats treated with decaffeinated green coffee extract; H, high-carbohydrate, high-fat diet-fed rats; HGC, high-carbohydrate, high-fat diet-fed rats treated with green coffee extract; HDC, high-carbohydrate, high-fat diet-fed rats treated with decaffeinated green coffee extract.

Table 3: Green and decaffeinated green coffee on cardiovascular and liver function

Variables	***C***	***CGC***	***CDC***	***H***	***HGC***	***HDC***	***P* value**		
							Diet	***Treatment***	***Diet* × *Treatment***
Systolic blood pressure, mmHg	128 ± 1^{b}	129 ± 1^{b}	127 ± 2^{b}	147 ± 1^{a}	130 ± 2^{b}	128 ± 2^{b}	<0.0001	<0.0001	<0.0001
Left ventricular diastolic stiffness constant (κ)	24.1 ± 1.2^{b}	22.1 ± 0.4^{b}	22.1 ± 0.5^{b}	27.5 ± 2.2^{a}	22.4 ± 0.6^{b}	21.5 ± 0.6^{b}	0.28	0.002	0.20
Left ventricular + Septum wet weight, mg/mm tibial length	20.6 ± 1	20.1 ± 1.1	22.1 ± 0.8	24.1 ± 2.0	20.3 ± 0.8	22.7 ± 0.6	0.12	0.09	0.29

Right ventricular wet weight, mg/mm tibial length	4.8 ± 0.4	4.9 ± 0.4	5.1 ± 0.2	5.5 ± 0.8	4.6 ± 0.4	4.6 ± 0.4	0.89	0.62	0.33
Liver wet weight, mg/mm	220 ± 7[b]	225 ± 7[b]	254 ± 5[b]	336 ± 19[a]	308 ± 17[a]	329 ± 8[a]	<0.0001	0.11	0.17
Plasma aspartate transaminase activity, U/L	69.1 ± 1.8	76.7 ± 3.2	75.2 ± 3	81.6 ± 6.1	70.7 ± 5.8	74.9 ± 5.1	0.58	0.93	0.12
Plasma alanine transaminase activity, U/L	29.8 ± 2.6	31.8 ± 2.5	30.5 ± 2.3	37.6 ± 3.4	31.9 ± 2.7	31.1 ±2	0.19	0.54	0.26

[1]Values are mean ± SEM, n=6-10. Means in a row with superscripts without a common letter differ significantly, *P<0.05.* C, corn starch diet-fed rats; CGC, corn starch diet-fed rats treated with green coffee extract; CDC, corn starch diet-fed rats treated with decaffeinated green coffee extract; H, high-carbohydrate, high-fat diet-fed rats; HGC, high-carbohydrate, high-fat diet-fed rats treated with green coffee extract; HDC, high-carbohydrate, high-fat diet-fed rats treated with decaffeinated green coffee extract.

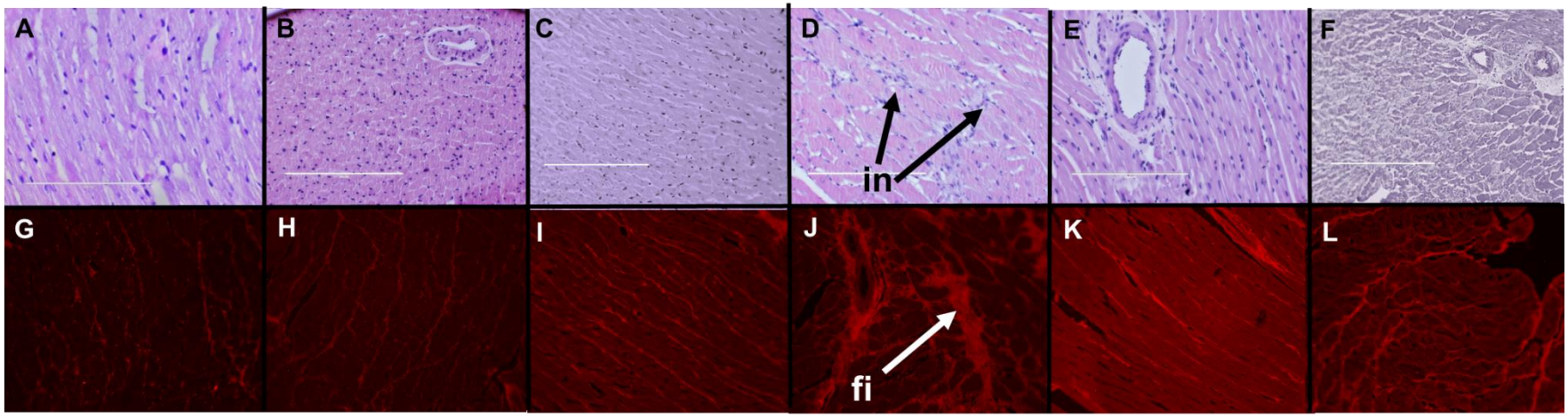


Figure 1. Effects of green coffee extracts on inflammation and fibrosis in the heart. Haematoxylin and eosin staining of left ventricle showing infiltration of inflammatory cells (A–F, inflammatory cells marked as "in") from C (A), CGC (B), CDC (C), H (D), HGC (E) and HDC (F) rats. Picrosirius red staining of left ventricle showing collagen deposition (G-L, fibrosis marked as "fi") from C (G), CGC (H), CDC (I), H (J), HGC (K) and HDC (L) rats. C, corn starch diet-fed rats; CGC, corn starch diet-fed rats treated with regular green coffee extract; CDC, corn starch diet-fed rats treated with decaffeinated green coffee extract; H, high-carbohydrate, high-fat diet-fed rats; HGC, high-carbohydrate, high-fat diet-fed rats treated with regular green coffee extract; HDC, high-carbohydrate, high-fat diet-fed rats treated with decaffeinated green coffee extract.

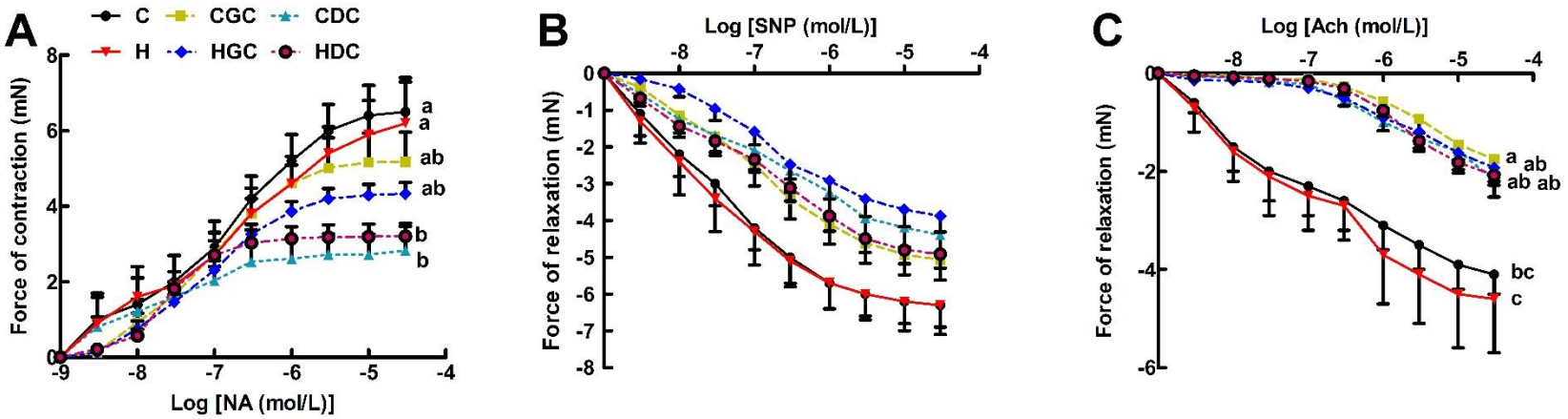


Figure 2. Effects of regular green coffee and decaffeinated green coffee extract on (A) noradrenaline-induced contraction, (B) sodium nitroprusside-induced relaxation and (C) acetylcholine-induced relaxation in thoracic aortic preparation from C, CGC, CDC, H, HGC and HDC rats. Values are mean ± SEM, n = 8-12. Endpoint means without a common letter differ, $P < 0.05$. C, corn starch diet-fed rats; CGC, corn starch diet-fed rats treated with regular green coffee extract; CDC, corn starch diet-fed rats treated with decaffeinated green coffee extract; H, high-carbohydrate, high-fat diet-fed rats; HGC, high-carbohydrate, high-fat diet-fed rats treated with regular green coffee extract; HDC, high-carbohydrate, high-fat diet-fed rats treated with decaffeinated green coffee extract.

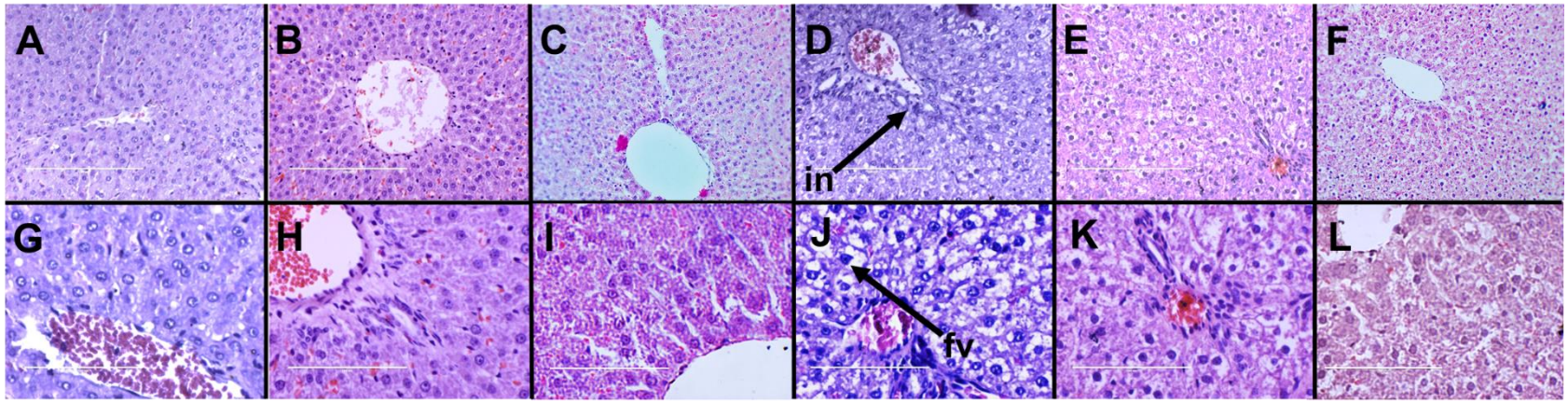


Figure 3. Effects of green coffee extracts on inflammation and fat deposition in the liver. Haematoxylin and eosin staining of liver showing infiltration of inflammatory cells (A–F, inflammatory cells marked as "in") from C (A), CGC (B), CDC (C), H (D), HGC (E) and HDC (F) rats and fat deposition (G-L, enlarged fat vacuoles as "fv") from C (G), CGC (H), CDC (I), H (J), HGC (K) and HDC (L) rats. C, corn starch diet-fed rats; CGC, corn starch diet-fed rats treated with regular green coffee extract; CDC, corn starch diet-fed rats treated with decaffeinated green coffee extract; H, high-carbohydrate, high-fat diet-fed rats; HGC, high-carbohydrate, high-fat diet-fed rats treated with regular green coffee extract; HDC, high-carbohydrate, high-fat diet-fed rats treated with decaffeinated green coffee extract.

Discussion

In this study, green coffee extract and decaffeinated green coffee extract decreased body weight and visceral fat accumulation, and improved heart and liver structure and function in obese, hypertensive rats fed a high-carbohydrate, high-fat diet, without improving glucose tolerance or dyslipidaemia. High-carbohydrate, high-fat diet-fed rats are an appropriate preclinical model for mimicking human metabolic syndrome as they develop relevant symptoms including visceral obesity, impaired glucose tolerance, dyslipidaemia, hypertension, cardiovascular remodelling and liver abnormalities [13].

Obesity is the critical factor of metabolic syndrome associated with high calorie intake and sedentary life lifestyle [15, 16]. In contrast to the few studies in green coffee, roasted (black) coffee has been more widely studied so that it has been proposed as a functional food to reverse the health complications caused by obesity [17]. Coffee contains around 1000 possibly biologically active components, including caffeine, chlorogenic acid, caffeic acid, hydroxyhydroquinone, trigonelline, diterpenes such as kahweol and cafestol, lignin and melanoidins [18]. Chronic black coffee consumption has been associated with a reduction in the development of chronic diseases such as cardiac disorders [19], hepatic diseases [20] and type 2 diabetes [21]. The anti-obesity effect of black coffee has been associated with reduced lipid accumulation in cells by reduction of adipogenesis and modifications of transcription factors and lipogenesis-related proteins in the adipose tissue of animal models [22] but could also be associated with altered glucose homeostasis, antioxidant activity and inflammatory biomarkers [21]. Individual components including caffeine [23], chlorogenic acid [24] and trigonelline [25] may improve glucose tolerance and reduce the risk of type 2 diabetes progression [26] but the

diterpenes may increase serum lipid concentrations in humans [27]. However, the type of coffee and the method of preparation determines the effects of coffee on lipoproteins [28]. In our previous study, roasted coffee extract in obese rats improved cardiovascular and hepatic structure and function with no improvements in obesity or blood concentrations of triglycerides and non-esterified fatty acids [23]. In contrast, caffeine at the same dose as in coffee extract decreased body weight and abdominal obesity [29]. This clearly indicated that caffeine was only partly contributing to the responses from coffee extract.

This study determined whether the unroasted green coffee has potential for health improvement, rather than black coffee. Decaffeinated green coffee supplementation reduced postprandial hyperglycaemia in rats and humans [30]. An intake of 200 mg/kg/day green coffee extract for 6 weeks reduced obesity and dyslipidaemia in mice [31] and anti-inflammatory effects of green coffee extract in rats were demonstrated by decreasing lipopolysaccharide-induced leukocyte migration in the peritonitis test [32]. Likewise, decaffeinated green coffee reduced obesity and fat accumulation with improving insulin sensitivity by 0.3% diet supplementation for 11 weeks in high-fat diet-fed mice [33]. In humans, a randomised crossover study showed that green coffee consumption reduced body weight, body mass index, fat accumulation and hypertension [34]. A 22-week crossover study of green coffee extract in overweight humans reduced body weight and heart rate, and body mass index shifted back to normal [34]. Meta-analysis of human clinical trials showed reduced body weight with green coffee extract compared with placebo [10].

The observation in our study was that green coffee extract reduced feed efficiency. The reduced feed efficiency indicates higher satiety level from diet supplemented with

green coffee. This outcome pointed out that green coffee extract delivered a similar energy intake as their control, and revealed a decrease in feed efficiency and hence, body weight. The decreased feed efficiency could result from elevated energy expenditure and thermogenesis. Chlorogenic acid, the hydoxycinnamic acid derivative present in green coffee extract, enhanced energy expenditure through the upregulation of adenosine monophosphate activated protein kinase (AMPK) by inhibiting cAMP phosphodiesterase, with the subsequent increase in fatty acid oxidation [35]. The proposed mechanism of action is the inhibition of the enzyme pancreatic lipase, which leads in a decrease in lipid absorption [36] resulting in weight reduction.

Green coffee contains a wide range of potentially bioactive compounds. In this study, green coffee contained higher concentrations of chlorogenic acid compared to the decaffeinated green coffee with no caffeine present in decaffeinated green coffee. Trigonelline was equivalent in the two types of green coffee. As the responses of green and decaffeinated green coffee were quite similar, caffeine could be ruled out for contribution towards the responses observed in obese rats. Further, the dose of chlorogenic acid was almost half in decaffeinated green coffee (~34 mg/kg/day) compared to green coffee (~69 mg/kg/day). In addition, previous studies used a dose of 100 mg/kg/day of chlorogenic acid to suggest its beneficial effects [37, 38]. This suggests that chlorogenic acid is one of several active components in green coffee to attenuate metabolic syndrome.

Trigonelline is also a putative bioactive compound in green coffee with the dose around 25 mg/kg/day from both types of green coffee interventions. A higher dose of trigonelline (40 mg/kg/day) for 48 weeks in a high-carbohydrate, high-fat diet reduced body weight, lowered blood glucose and HbA1c via increasing serum GLP-1

concentrations and GLP-1R expression in rats [39]. At the same dose, trigonelline for 4 weeks in rats improved insulin sensitivity by reducing the availability of free fatty acids, TNFα and IL-6 in serum [40]. A limitation of our study was the limited availability and high cost of pure trigonelline to test its responses.

The responses of green and decaffeinated coffee suggest that chlorogenic acid and trigonelline may have additive or synergistic effects towards attenuating symptoms of metabolic syndrome. Further, coffee also contains diterpenoids, cafestol and kahweol, that were not measured and may have their role in either improving metabolic complications [41] or have detrimental effects as in previous study where non-filtered, boiled coffee containing increased diterpenoids increased serum cholesterol concentrations and hence the risk of cardiovascular disease [42].

Conclusions

Green coffee with or without caffeine attenuated obesity and reduced cardiovascular disease components without changes in glucose homeostasis and plasma lipid concentrations in high-carbohydrate, high-fat diet-induced metabolic syndrome in rats. Both green coffee products improved structure and function of the heart and liver in these rats. Results from this study suggest that chlorogenic acid and trigonelline, rather than caffeine, may serve as the bioactive components of green coffee. Following well-designed human studies with green coffee [8, 43], green coffee may serve as a simple and safe dietary supplement to manage weight and blood pressure that may prove very effective in combination with healthy lifestyles in obese hypertensive patients.

Acknowledgment: This study was supported by the University of Southern Queensland Research and Innovation Division. We would like to thank Mr. Brain Bynon, School of Veterinary Sciences, The University of Queensland Gatton, for plasma biochemical analyses.

Authors' contribution: S.K.P. and L.B. designed the research protocol; S.K.P., L.B., and N.B. interpreted the results; S.K.P. and N.B. conducted the animal experiments and analysed the data; S.K.P., L.B., and N.B. wrote the manuscript; P.M. analysed the components of coffee samples; and S.K.P. had primary responsibility for the final content. All authors read and approved the final manuscript.

Compliance with ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Statement on the Welfare of Animals All experimental protocols were approved by the University of Southern Queensland Animal Ethics Committee under the guidelines of the National Health and Medical Research Council of Australia. The approval number for this project was 13REA005. This article does not contain any studies with human participants performed by any of the authors.

References

1. Armani A, Berry A, Cirulli F, Caprio M. Molecular mechanisms underlying metabolic syndrome: the expanding role of the adipocyte. FASEB Journal 2017; 31(10): 4240-55.
2. Yubero-Serrano EM, Delgado-Lista J, Tierney AC, Perez-Martinez P, Garcia-Rios A, Alcala-Diaz JF, et al. Insulin resistance determines a differential response to

changes in dietary fat modification on metabolic syndrome risk factors: the LIPGENE study. American Journal of Clinical Nutrition 2015; 102(6): 1509-17.

3. Cornier MA, Dabelea D, Hernandez TL, Lindstrom RC, Steig AJ, Stob NR, et al. The metabolic syndrome. Endocrine Reviews 2008; 29(7): 777-822.

4. Roshan H, Nikpayam O, Sedaghat M, Sohrab G. Effects of green coffee extract supplementation on anthropometric indices, glycaemic control, blood pressure, lipid profile, insulin resistance and appetite in patients with the metabolic syndrome: a randomised clinical trial. Br J Nutr 2018; 119(3): 250-8.

5. Budryn G, Zaklos-Szyda M, Zaczynska D, Zyzelewicz D, Grzelczyk J, Zdunczyk Z, et al. Green and roasted coffee extracts as antioxidants in beta TC3 cells with induced oxidative stress and lipid accumulation inhibitors in 3T3L1 cells, and their bioactivity in rats fed high fat diet. European Food Research and Technology 2017; 243(8): 1323-34.

6. Takahashi S, Saito K, Jia H, Kato H. An integrated multi-omics study revealed metabolic alterations underlying the effects of coffee consumption. PLoS One 2014; 9(3): e91134.

7. Takami H, Nakamoto M, Uemura H, Katsuura S, Yamaguchi M, Hiyoshi M, et al. Inverse correlation between coffee consumption and prevalence of metabolic syndrome: baseline survey of the Japan Multi-Institutional Collaborative Cohort (J-MICC) Study in Tokushima, Japan. J Epidemiol 2013; 23(1): 12-20.

8. Vinson JA, Burnham BR, Nagendran MV. Randomized, double-blind, placebo-controlled, linear dose, crossover study to evaluate the efficacy and safety of a green coffee bean extract in overweight subjects. Diabetes Metab Syndr Obes 2012; 5: 21-7.

9. Marventano S, Salomone F, Godos J, Pluchinotta F, Del Rio D, Mistretta A, et al. Coffee and tea consumption in relation with non-alcoholic fatty liver and metabolic syndrome: A systematic review and meta-analysis of observational studies. Clinical Nutrition 2016; 35(6): 1269-81.

10. Onakpoya I, Terry R, Ernst E. The use of green coffee extract as a weight loss supplement: a systematic review and meta-analysis of randomised clinical trials. Gastroenterology Research and Practice 2011; 2011.

11. Brando CHJ. Harvesting and green coffee processing. Coffee: Growing, Processing, Sustainable Production: Wiley-VCH Verlag GmbH; 2008. p. 604-715.

12. Jeszka-Skowron M, Sentkowska A, Pyrzyńska K, De Peña MP. Chlorogenic acids, caffeine content and antioxidant properties of green coffee extracts: influence of green coffee bean preparation. European Food Research and Technology 2016; 242(8): 1403-9.
13. Panchal SK, Poudyal H, Iyer A, Nazer R, Alam MA, Diwan V, et al. High-carbohydrate, high-fat diet-induced metabolic syndrome and cardiovascular remodeling in rats. Journal of Cardiovascular Pharmacology 2011; 57(5): 611-24.
14. Nongmaithem BD, Mouatt P, Smith J, Rudd D, Russell M, Sullivan C, et al. Volatile and bioactive compounds in opercula from Muricidae molluscs supports their use in ceremonial incense and traditional medicines. Scientific Reports 2017; 7.
15. Yary T, Voutilainen S, Tuomainen TP, Ruusunen A, Nurmi T, Virtanen JK. Omega-6 polyunsaturated fatty acids, serum zinc, delta-5- and delta-6-desaturase activities and incident metabolic syndrome. Journal of Human Nutrition and Dietetics 2017; 30(4): 506-14.
16. Wong W-Y, Ward LC, Fong CW, Yap WN, Brown L. Anti-inflammatory γ-and δ-tocotrienols improve cardiovascular, liver and metabolic function in diet-induced obese rats. European Journal of Nutrition 2017; 56(1): 133-50.
17. Santos RMM, Lima DRA. Coffee consumption, obesity and type 2 diabetes: a mini-review. European Journal of Nutrition 2016; 55(4): 1345-58.
18. Butt MS, Sultan MT. Coffee and its consumption: Benefits and risks. Critical Reviews in Food Science and Nutrition 2011; 51(4): 363-73.
19. Grosso G, Micek A, Godos J, Sciacca S, Pajak A, Martinez-Gonzalez MA, et al. Coffee consumption and risk of all-cause, cardiovascular, and cancer mortality in smokers and non-smokers: a dose-response meta-analysis. European Journal of Epidemiology 2016; 31(12): 1191-205.
20. Liu F, Wang XW, Wu G, Chen L, Hu P, Ren H, et al. Coffee consumption decreases risks for hepatic fibrosis and cirrhosis: A meta-analysis. PLoS One 2015; 10(11): e0142457.
21. Casal S, Rebelo I. Coffee: A dietary intervention on type 2 diabetes? Current Medicinal Chemistry 2017; 24(4): 376-83.

22. Pan MH, Tung YC, Yang GL, Li SM, Ho CT. Molecular mechanisms of the anti-obesity effect of bioactive compounds in tea and coffee. Food & Function 2016; 7(11): 4481-91.
23. 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.
24. Naveed M, Hejazi V, Abbas M, Kamboh AA, Khan GJ, Shumzaid M, et al. Chlorogenic acid (CGA): A pharmacological review and call for further research. Biomedicine & Pharmacotherapy 2018; 97: 67-74.
25. Yoshinari O, Igarashi K. Anti-diabetic effect of trigonelline and nicotinic acid, on KK-A(Y) mice. Current Medicinal Chemistry 2010; 17(20): 2196-202.
26. Baspinar B, Eskici G, Ozcelik AO. How coffee affects metabolic syndrome and its components. Food & Function 2017; 8(6): 2089-101.
27. Penson P, Serban MC, Ursoniu S, Banach M, Lipid, Blood Pressure Meta-analysis Collaboration G. Does coffee consumption alter plasma lipoprotein(a) concentrations? A systematic review. Critical Reviews in Food Science and Nutrition 2017: 1-9.
28. Pirich C, Ogrady J, Sinzinger H. Coffee, lipoproteins and cardiovascular-disease. Wiener Klinische Wochenschrift 1993; 105(1): 3-6.
29. Panchal SK, Wong WY, Kauter K, Ward LC, Brown L. Caffeine attenuates metabolic syndrome in diet-induced obese rats. Nutrition 2012; 28(10): 1055-62.
30. Iwai K, Narita Y, Fukunaga T, Nakagiri O, Kamiya T, Ikeguchi M, et al. Study on the postprandial glucose responses to a chlorogenic acid-rich extract of decaffeinated green coffee beans in rats and healthy human subjects. Food Science and Technology Research 2012; 18(6): 849-60.
31. Choi BK, Park SB, Lee DR, Lee HJ, Jin YY, Yang SH, et al. Green coffee bean extract improves obesity by decreasing body fat in high-fat diet-induced obese mice. Asian Pacific Journal of Tropical Medicine 2016; 9(7): 616-24.
32. de Castro Moreira ME, Pereira RGFA, Dias DF, Gontijo VS, Vilela FC, de Moraes GdOI, et al. Anti-inflammatory effect of aqueous extracts of roasted and green *Coffea arabica* L. Journal of Functional Foods 2013; 5(1): 466-74.

33. Song SJ, Choi S, Park T. Decaffeinated green coffee bean extract attenuates diet-induced obesity and insulin resistance in mice. Evidence-Based Complementary and Alternative Medicine 2014; 2014: 718379.
34. Vinson JA, Burnham BR, Nagendran MV. Randomized, double-blind, placebo-controlled, linear dose, crossover study to evaluate the efficacy and safety of a green coffee bean extract in overweight subjects. Diabetes Metabolic Syndrome and Obesity-Targets and Therapy 2012; 5: 21-7.
35. Bruckbauer A, Zemel MB. Synergistic effects of polyphenols and methylxanthines with leucine on AMPK/Sirtuin-mediated metabolism in muscle cells and adipocytes. Plos One 2014; 9(2): e89166.
36. Narita Y, Iwai K, Fukunaga T, Nakagiri O. Inhibitory activity of chlorogenic acids in decaffeinated green coffee beans against porcine pancreas lipase and effect of a decaffeinated green coffee bean extract on an emulsion of olive oil. Bioscience, biotechnology, and biochemistry 2012; 76(12): 2329-31.
37. Sudeep HV, Venkatakrishna K, Patel D, Shyamprasad K. Biomechanism of chlorogenic acid complex mediated plasma free fatty acid metabolism in rat liver. BMC Complementary & Alternative Medicine 2016; 16: 274.
38. Ali N, Rashid S, Nafees S, Hasan SK, Shahid A, Majed F, et al. Protective effect of chlorogenic acid against methotrexate induced oxidative stress, inflammation and apoptosis in rat liver: An experimental approach. Chemico-Biological Interactions 2017; 272: 80-91.
39. Zhou J-Y, Zhou S-W. Protection of trigonelline on experimental diabetic peripheral neuropathy. Evidence-Based Complementary and Alternative Medicine 2012; 2012: 164219.
40. Tharaheswari M, Reddy NJ, Kumar R, Varshney K, Kannan M, Rani SS. Trigonelline and diosgenin attenuate ER stress, oxidative stress-mediated damage in pancreas and enhance adipose tissue PPARγ activity in type 2 diabetic rats. Molecular and Cellular Biochemistry 2014; 396(1-2): 161-74.
41. Mellbye FB, Jeppesen PB, Shokouh P, Laustsen C, Hermansen K, Gregersen S. Cafestol, a bioactive substance in coffee, has antidiabetic properties in KKAy mice. J Nat Prod 2017; 80(8): 2353-9.

42. Aro A, Tuomilehto J, Kostiainen E, Uusitalo U, Pietinen P. Bioled coffee increases serum low density lipoprotein concentration. Metabolism 1987; 36(11): 1027-30.

43. Tajik N, Tajik M, Mack I, Enck P. The potential effects of chlorogenic acid, the main phenolic components in coffee, on health: a comprehensive review of the literature. Eur J Nutr 2017; 56(7): 2215-44.

Chapter 3.

Chlorogenic acid attenuates metabolic syndrome in diet-induced obese rats

Chlorogenic acid attenuates metabolic syndrome in diet-induced obese rats

Nikhil **Bhandarkar**[1], Lindsay **Brown**[1,2], Sunil K **Panchal**[2]

[1]School of Health and Wellbeing, Faculty of Health, Engineering and Sciences, and [2]Functional Foods Research Group, Institute for Agriculture and the Environment, University of Southern Queensland, Toowoomba, QLD 4350, Australia

Keywords: Chlorogenic acid, Obesity, Metabolic syndrome, Cardiovascular disease, Coffee, High-carbohydrate, high-fat diet

Running title: Chlorogenic acid and obesity

Corresponding author:

Dr Sunil K Panchal

Functional Foods Research Group, Institute for Agriculture and the Environment

University of Southern Queensland, Toowoomba, QLD 4350, Australia

Email: Sunil.Panchal@usq.edu.au; Tel.: +61 7 4631 1012

Word count:

Abstract = 220

Main text = Introduction (394) + Methods (1009) + Results (622) + Discussion (1113) + References (1411) + Figure legends (208) = 4672

Number of tables – 1; Number of figures – 2; Number of references – 50

Abstract

Chlorogenic acid as a constituent of coffee is consumed regularly in the human diet. Chlorogenic acid intake has been associated with decreased risk of cardiovascular disease and type 2 diabetes. This study investigated whether chlorogenic acid improves cardiovascular, liver and metabolic responses in a diet-induced rat model of metabolic syndrome induced by a high-carbohydrate, high-fat diet. Male Wistar rats (8-9 week old, 335 ± 5 g, n = 48) were divided into 4 groups and fed with either corn starch diet (16 weeks), corn starch diet with 2 g/kg chlorogenic acid in food for the last 8 weeks, high-carbohydrate, high-fat diet (16 weeks), or high-carbohydrate, high-fat diet with 2 g/kg chlorogenic acid in food for the last 8 weeks. In high-carbohydrate, high-fat diet-fed rats, chlorogenic acid reduced energy intake and food efficiency to reduce visceral fat (especially retroperitoneal fat) and abdominal circumference; reversed the elevated systolic blood pressure and attenuated left ventricular diastolic stiffness while reducing collagen deposition and infiltration of inflammatory cells in the left ventricle. Chlorogenic acid decreased inflammation and fat deposition in the liver along with reduction in plasma liver enzyme activities of obese rats but did not change the plasma lipid profile. These results suggest that chronic dietary chlorogenic acid may attenuate inflammation as well as cardiovascular, liver, and metabolic abnormalities induced by high-carbohydrate, high-fat diet.

Introduction

Coffee, a complex mixture of almost 1000 compounds, may decrease the risk of developing metabolic syndrome, for example, reducing the risk of type 2 diabetes and decreasing triglyceride concentrations [1]. An important component of coffee is a class of compounds known as chlorogenic acids, which are isomeric hydroxycinnamic esters of quinic acid [2]; one of the major compounds from this class is 5-caffeoyl-quinic acid [3]. Closely related compounds are also present in coffee beans, including the 3- and 4-isomers (isochlorogenic acids A and B), as well as 3,4-, 3,5-, and 4,5-diquinyl esters of cinnamic acid [4]. Chlorogenic acid intake is common and the content in one cup of coffee varies between 70 and 350 mg [3]. In the stomach, chlorogenic acid is absorbed directly whereas in the small and large intestines, it is broken down to caffeic acid and quinic acid before absorption [3].

Patients with metabolic syndrome have an increased risk of developing cardiovascular disease and diabetes [5]. Consumption of chlorogenic acids has been associated with decreased risk of these two chronic and common diseases [3]. These therapeutic responses to chlorogenic acids have been attributed to their antioxidant and anti-inflammatory properties [3, 6], modulation of glucose and lipid metabolism [7] by increased mRNA expression of PPAR-α and liver X receptor α as key mediators of lipid metabolism [8, 9], inhibition of glucose-6-phosphatase activity to reduce hepatic glucose output [10], and decreased expression of sodium-dependent glucose transporters in brush border membranes of the small intestine to reduce glucose absorption [11].

Our previous study with coffee extract in high-carbohydrate, high-fat diet-fed rats did not show any changes in obesity but improved heart and liver structure and function [12]. Following this, we matched the dose of caffeine from the coffee extract study to show that this dose of caffeine reversed obesity along with other improvements from coffee extract [13].

Thus, the current study investigated whether chlorogenic acid has beneficial or opposing effects to caffeine during an 8-week diet intervention in rats fed a high-carbohydrate, high-fat diet. After treatment with chlorogenic acid, metabolic parameters related to obesity and glucose tolerance, and the structure and function of the cardiovascular system and liver were examined. Cardiovascular function was studied in isolated Langendorff heart preparations, isolated thoracic aortic rings and by measurement of systolic blood pressure together with histopathology. Liver structure and function were measured by plasma biochemistry and histopathology.

Methods and materials

Rats, diets, and treatments

All experimental protocols were approved by the University of Southern Queensland Animal Ethics Committee under the guidelines of the National Health and Medical Research Council of Australia. Male Wistar rats (8–9 weeks old, 340 ± 2 g, n = 48) were obtained from Animal Resource Centre, Murdoch, WA, Australia. Rats were randomly divided into 4 groups for the 16 week feeding protocol: corn starch diet-fed rats (C, *n* = 12), corn starch diet + chlorogenic acid (CC, *n* = 12; 2 g/kg of food for the final 8 weeks); high-carbohydrate, high-fat diet-fed rats (H; *n* = 12); and high-carbohydrate, high-fat diet + chlorogenic acid (HC; *n* = 12, 2 g/kg of food for the final 8 weeks). C diet contained 570 g corn starch, 155 g powdered rat food, 25 g HMW salt mixture, and 250 g water per kilogram of diet. H diet contained 175 g fructose, 395 g sweetened condensed milk, 200 g beef tallow, 155 g powdered rat food, 25 g HMW salt mixture, and 50 g water per kilogram of diet [14]. Drinking water with 25% w/v fructose was provided to H and HC groups. C and CC rats were given normal drinking water. All the rats were individually housed under temperature-controlled, 12-hour-light/dark conditions and given *ad libitum* access to food and water [14].

Physiological parameters

Body weight, food, and water intakes were measured daily [14]. Abdominal circumference and body length (nose to anus) were measured using a standard measuring tape under light anesthesia with Zoletil (tiletamine 10 mg/kg, zolazepam 10 mg/kg, intraperitoneally) [14]. Body mass index was calculated as body weight (in grams)/[body length (in cm)]^2 [14]. Feed efficiency was calculated as [mean body weight gain (in grams)/daily energy intake (in kJ)] [14].

Systolic blood pressure measurements

Systolic blood pressure was determined under light sedation with Zoletil (tiletamine 10 mg/kg, zolazepam 10 mg/kg, intraperitoneally), using an MLT1010 Piezo-Electric Pulse Transducer and inflatable tail-cuff connected to an MLT844 Physiological Pressure Transducer and PowerLab data acquisition unit [14].

Body composition measurement

Dual-energy X-ray absorptiometric measurements were carried out with a Norland XR36 DXA instrument (Norland Corp, Fort Atkinson, WI). These scans were evaluated using the manufacturer's suggested 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% [15]. Visceral adiposity index (%) was analyzed as ([retroperitoneal fat (g) + omental fat (g) + epididymal fat (g)]/ [body weight (g)]) × 100.

Oral glucose tolerance test

Oral glucose tolerance tests were performed on rats following a 12-hour food deprivation during which fructose-supplemented drinking water in H and HC groups was

replaced with normal drinking water [14]. After determining basal blood glucose concentrations in tail vein blood using Medisense Precision Q.I.D. glucose meters, rats were given a glucose load of 2 g/kg body weight as 40% glucose solution via oral gavage and blood glucose concentrations were measured again 30, 60, 90, and 120 minutes after oral glucose administration [14].

Terminal experiments

Rats were euthanased with Lethabarb (pentobarbitone sodium, 100 mg/kg, intraperitoneally). After euthanasia, heparin (200 IU) was injected through the right femoral vein. The abdomen was then opened and blood (~5 mL) was withdrawn from the abdominal aorta and collected into heparinized tubes. Blood was centrifuged at 5000 × g for 15 minutes to obtain plasma. Plasma was stored at –20°C for further characterization. Hearts were then removed from rats for isolated Langendorff heart preparation [14].

Isolated Langendorff heart preparation

Hearts isolated from euthanased rats were perfused with modified Krebs–Henseleit bicarbonate buffer 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 per minute using an electrical stimulator. End-diastolic pressures were obtained from 0 to 30 mmHg for the calculation of diastolic stiffness constant (κ, dimensionless) [14].

Isolated tissue reactivity

Thoracic aortic rings (~4 mm in length) were suspended in an organ bath filled with Tyrode physiological salt solution bubbled with 95% O_2–5% CO_2 maintained at 35°C and allowed to stabilize at a resting tension of ~10 mN. Cumulative concentration–response curves (contraction) were obtained for noradrenaline and cumulative concentration–response curves (relaxation) were obtained for acetylcholine and sodium nitroprusside after submaximal (~70%) contraction to noradrenaline [14].

Organ weights

After isolated heart perfusion studies, the heart was separated into left ventricle (with septum) and right ventricle and weighed separately. The liver was isolated and weighed. Retroperitoneal, epididymal, and omental abdominal fat pads were removed separately and weighed. These organ weights were normalized against the tibial length at the time of organ removal and expressed as mg/mm of tibial length [14].

Histology

Heart and liver were removed from rats soon after euthanasia and fixed in 10% neutral buffered formalin. The samples were then dehydrated and embedded in paraffin wax. Thin sections (~5 μm) of heart and liver were cut and stained with hematoxylin and eosin to study infiltration of inflammatory cells and for determining fat vacuoles in liver. Heart sections were also stained with picrosirius red stain to study collagen distribution [14].

Plasma biochemistry

Plasma activities of aspartate transaminase (AST) and alanine transaminase (ALT) and concentrations of total cholesterol, triglycerides, and non-esterified fatty acids (NEFA) in plasma were measured [14].

Statistical analysis

All data are presented as mean ± SEM. Results were tested for variance using Bartlett's test and variables that were not normally distributed were transformed (using log 10 functions) prior to statistical analyses. C, CC, H, and HC groups were tested for effects of diet, treatment, and their interactions by 2-way Analysis of Variance. When the interaction and/or the main effects were significant, means were compared using Newman-Keuls multiple comparison *post hoc* tests. A P value <0.05 was considered significant. All statistical analyses were performed using GraphPad Prism version 5.0 for Windows (San Diego, CA, USA).

Results

Dietary intake, body composition, and plasma biochemistry

At the end of 16 weeks, H rats had higher body weight compared to C rats while chlorogenic acid reduced the body weight in HC rats (Table 1). Water intakes were similar between C and CC rats and between H and HC rats. Food intakes was lower in H rats compared to C rats. Chlorogenic acid increased food intake in CC rats compared to C rats while it did not change food intake in HC rats compared to H rats. The food intake in CC rats was higher than HC rats; hence, the chlorogenic acid intake was higher in CC rats compared to HC rats. Energy intake was higher in H rats than in C rats. Chlorogenic acid increased energy intake in CC rats compared to C rats while it did not change energy intake in HC rats compared to H rats (Table 1). Feed efficiency, body mass index, and abdominal circumference were higher in H rats than

in C rats whereas chlorogenic acid reduced these parameters in HC rats compared to H rats with no effects in CC rats compared to C rats on these parameters at the end of 16 weeks (Table 1). Whole-body lean mass was similar in all diet groups. Whole-body fat mass was higher in H rats than in C rats and was decreased with chlorogenic acid in HC rats compared to H rats (Table 1). Basal blood glucose concentrations were higher in H rats than in C rats and reduced by chlorogenic acid in both HC and CC rats compared to H and C rats, respectively. However, chlorogenic acid failed to reduce the 120-minute blood glucose concentrations and hence area under the curve during oral glucose tolerance test (Table 1). Plasma total cholesterol concentrations were unchanged between the groups while chlorogenic acid was unable to reduce the plasma concentrations of triglycerides and non-esterified fatty acids in CC or HC rats (Table 1). Retroperitoneal, omental, epididymal, and total abdominal fat pads were higher in H rats than in C rats. These fat depots were unchanged in CC rats compared to C rats while chlorogenic acid reduced total abdominal fat through reduction in retroperitoneal fat in HC rats compared to H rats. Visceral adiposity followed the same trend between the groups as total abdominal fat (Table 1).

Cardiovascular structure and function

Histopathological analysis of left ventricle indicated increased infiltration of inflammatory cells in H rats (Figure 1C) compared to C rats (Figure 1A). Chlorogenic acid reduced infiltration of inflammatory cells into the left ventricle of HC rats (Figure 1D) than in H rats. Picrosirius red staining of left ventricle suggested higher collagen deposition in H rats (Figure 1G) compared to C rats (Figure 1E). Chlorogenic acid reduced collagen deposition in the left ventricle of HC rats (Figure 1H) than in H rats. Noradrenaline-induced contraction and acetylcholine-induced relaxation were lower in H rats than in C rats and chlorogenic acid treatment did not change these responses (Figure 2A and 2C). There were no differences in sodium nitroprusside-induced vascular relaxation in isolated thoracic aortic rings between the

groups (Figure 2B). Systolic blood pressure and diastolic stiffness were higher in H rats than in C rats and chlorogenic acid reduced both systolic blood pressure and diastolic stiffness in HC rats compared to H rats (Table 1). LV and RV wet weights were unchanged between the groups (Table 1).

Liver structure and function

Increased inflammatory cell infiltration and fat deposition were observed in H rats (Figure 1K) than in C rats (Figure 1I). Chlorogenic acid reduced infiltration of inflammatory cells and fat deposition in livers of HC rats (Figure 1L) compared to H rats. Liver wet weight was higher in H rats than in C rats and it was unchanged by chlorogenic acid treatment in both CC and HC rats (Table 1). Plasma ALT activities were higher in H rats compared to C rats and reduced in HC rats. Plasma AST activity was unchanged between H and C rats and reduced in HC rats compered to H rats (Table 1).

Discussion

In this study, chlorogenic acid decreased body weight and visceral fat accumulation, and improved heart and liver structure and function in obese, hypertensive rats fed a high-carbohydrate, high-fat diet, without improving glucose tolerance. These responses of chlorogenic acid, one of the major components of coffee, are an important aspect in functional foods research as many people consume chlorogenic acid and related compounds on a daily basis through diet, mainly coffee. Further, the responses to chlorogenic acid in this study were obtained in a high-carbohydrate, high-fat diet-fed rats as an appropriate model for human metabolic syndrome developing similar symptoms including visceral obesity along with impaired glucose tolerance, dyslipidemia, hypertension, endothelial dysfunction, and liver abnormalities [14].

Coffee consumption in human studies, mostly cross-sectional analyses, has been associated with decreases in components of metabolic syndrome including waist circumference, systolic and diastolic blood pressures, and blood concentrations of triglyceride and fasting glucose [1]. Coffee consumption has also been associated with decreased risk of type 2 diabetes and cardiovascular disease [1]. Coffee is a widely-used source of bioactive compounds such as caffeine, chlorogenic acid, trigonelline, cafetstol, and kahweol [1]. Caffeine and chlorogenic acid are two major components of coffee that have been associated with beneficial health effects, especially against metabolic syndrome [1]. Our previous study with coffee extract treatment in obese rats (5% coffee extract in food for 8 weeks) showed improved cardiovascular and hepatic structure and function with no improvements in obesity or blood concentrations of triglycerides and NEFA [12]. Caffeine treatment (~28 mg/kg/day) in obese rats for 8 weeks showed improved structure and function of heart and liver, with reduction in obesity but no improvements in blood concentrations of cholesterol, triglycerides, and NEFA [13]. Caffeine inhibited A_1-adenosine receptors in the hypothalamus to suppress appetite and increase energy expenditure in order to reduce diet-induced obesity in mice [16].

Chlorogenic acid has been associated with decreases in blood pressure and improvements in endothelial function, lipid metabolism, and carbohydrate metabolism [3, 17, 18]. The dose of chlorogenic acid in the high-carbohydrate, high-fat diet-fed rats was ~100 mg/kg in this study. In mice, no improvements in body weight or insulin resistance were observed with lower doses of ~50 mg/kg/day for 12 weeks or ~30 mg/kg/day for 8 weeks [19, 20]. The doses of CGA used in our study are comparable with those obtained in a typical cup of coffee. However, the relationship is not straightforward as these values fluctuate markedly depending on the type of coffee and the preparation method [21]. Typical values in green coffee bean are of the order of 8 % for CGA so the doses used in our study are achievable from the daily consumption levels.

Intestinal absorption of chlorogenic acid has been controversial. In some studies, chlorogenic acid has been found in urine after coffee or pure chlorogenic acid consumption [22-26] while chlorogenic acid was not detected in other studies in plasma, urine, or bile [27-33]. One study suggested direct absorption of chlorogenic acid in the gut of 8% [27]. Further, metabolism of chlorogenic acid is quite variable in different individuals and it may cause the variation in the responses to chlorogenic acid [34]. After ingestion of coffee, various derivatives of chlorogenic acid were found in the urine. Further, chlorogenic acid was broken down to caffeic acid and hence derivatives of caffeic acid were also found in the urine [35]. These metabolites derived from chlorogenic acid are further metabolized into compounds including *m*-coumaric acid and hydroxy-derivatives of phenylpropionic, benzoic and hippuric acids [26, 36, 37]. Chlorogenic acids and their metabolites scavenge free radical oxygen species, with dihydrocaffeic acid showing the strongest effect against oxidative stress-induced inflammation [38]. Further, metabolites of chlorogenic acid, but not chlorogenic acid itself, improved the growth of *Bifidobacterium* spp. and moderated the *Firmicutes*/*Bacteroidetes* ratio [39]. These changes in gut microbiota may modulate the metabolic changes observed in obesity. This may be one of mechanisms of action of chlorogenic acid in improving metabolic changes in obesity in this study.

In our study, chlorogenic acid did not reverse the glucose intolerance in obese rats. In a previous study, 90 mg/kg/day chlorogenic acid in Sprague−Dawley rats improved fasting glucose, glucose tolerance, and fasting serum insulin concentrations through differential expression of SGLT1, GLUT2, and proglucagon in the intestinal segments [40]. However, this study confirmed the outcomes from our study in terms of body weight and abdominal fat reduction [40].

We suggest that the reduced inflammation following chlorogenic acid treatment decreased the signs of the metabolic syndrome in diet-induced obese rats. In animal models, high-fat diets are associated with increased chylomicrons in the intestine, which promote systemic uptake of gut microbiota-derived lipopolysaccharides causing metabolic endotoxemia to trigger the low-grade inflammation that characterizes obesity [41, 42]. In obese humans, circulating endotoxin was 20% higher than in lean patients and 125% higher in a type 2 diabetic patient compared to lean individual [43]. Diets rich in fat or carbohydrate triggered systemic lipopolysaccharides secretion and increased expression of TLR-4, NF-κB, TNF-α, and IL-6, which are components engaged in pathways that also participate in the regulation of insulin secretion [44]. Thus, further studies on finding specific targets for the suppression of inflammation by chlorogenic acid are warranted.

Increased plasma ALT and AST activities, inflammatory cell infiltration and lipid deposition in the liver are important markers of fatty liver [45]; these changes were reduced by chlorogenic acid in this study. A possible explanation could be the activation of AMP kinase [46]. AMP kinase is an energy-sensing enzyme with a crucial role in the regulation of carbohydrate and lipid metabolism in response to cellular stress [47]. Chlorogenic acid as an anti-inflammatory agent reduced liver fibrosis by decreasing inflammatory cytokines, TLR-4, iNOS, COX-2, and NF-κB [48]. Further, chlorogenic acid intervention attenuated diet-induced liver abnormalities by downregulating the expression of the PPAR-γ to reduce fibrosis and accumulation of fat [49] with decreased expression of macrophage mRNA such as TNF-α, MCP-1, and chemokines associated with inflammatory cascade pathways. Consistent with our results, chlorogenic acid reduced fat deposition in adipose tissue by decreasing mRNA expression of the lipogenic enzymes (fatty acid synthase, acetyl-CoA carboxylase, and stearoyl-CoA desaturase) in the liver [50]. Overall, the reduction of expression in

inflammation-associated proteins could attenuate lipid-related disorders such as obesity, hypertension, and metabolic abnormalities.

In conclusion, high-carbohydrate, high-fat-diet-induced metabolic syndrome in rats was attenuated by chlorogenic acid. As coffee is the major dietary source of chlorogenic acid, this compound could be one of the most effective bioactive constituents in coffee leading to improved cardiovascular, liver, and metabolic functions. Production of chlorogenic acid-enriched coffee could be an effective intervention strategy with improved compliance. Thus, a clinical trial with chlorogenic acid–enriched coffee in mildly hypertensive, overweight, or obese patients is warranted.

References

1. Baspinar B, Eskici G, Ozcelik AO. How coffee affects metabolic syndrome and its components. Food Funct 2017; 8(6): 2089-101.

2. Tomas-Barberan F, Garcia-Villalba R, Quartieri A, Raimondi S, Amaretti A, Leonardi A, et al. *In vitro* transformation of chlorogenic acid by human gut microbiota. Mol Nutr Food Res 2014; 58(5): 1122-31.

3. Tajik N, Tajik M, Mack I, Enck P. The potential effects of chlorogenic acid, the main phenolic components in coffee, on health: a comprehensive review of the literature. Eur J Nutr 2017; 56(7): 2215-44.

4. Ludwig IA, Mena P, Calani L, Cid C, Del Rio D, Lean ME, et al. Variations in caffeine and chlorogenic acid contents of coffees: what are we drinking? Food Funct 2014; 5(8): 1718-26.

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

6. Narita Y, Inouye K. Degradation kinetics of chlorogenic acid at various pH values and effects of ascorbic acid and epigallocatechin gallate on its stability under alkaline conditions. J Agric Food Chem 2013; 61(4): 966-72.

7. Meng S, Cao J, Feng Q, Peng J, Hu Y. Roles of chlorogenic acid on regulating glucose and lipids metabolism: A review. Evid Based Complement Alternat Med 2013; 2013: 801457.

8. Huang K, Liang XC, Zhong YL, He WY, Wang Z. 5-Caffeoylquinic acid decreases diet-induced obesity in rats by modulating PPARα and LXRα transcription. J Sci Food Agric 2014; 95(9): 1903-10.

9. Wan CW, Wong CN, Pin WK, Wong MH, Kwok CY, Chan RY, et al. Chlorogenic acid exhibits cholesterol lowering and fatty liver attenuating properties by up-regulating the gene expression of PPAR-α in hypercholesterolemic rats induced with a high-cholesterol diet. Phytother Res 2013; 27(4): 545-51.

10. Ong KW, Hsu A, Tan BK. Chlorogenic acid stimulates glucose transport in skeletal muscle via AMPK activation: a contributor to the beneficial effects of coffee on diabetes. PLoS One 2012; 7(3): e32718.

11. Higdon JV, Frei B. Coffee and health: A review of recent human research. Crit Rev Food Sci Nutr 2006; 46(2): 101-23.

12. 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. J Nutr 2012; 142(4): 690-7.

13. Panchal SK, Wong WY, Kauter K, Ward LC, Brown L. Caffeine attenuates metabolic syndrome in diet-induced obese rats. Nutrition 2012; 28(10): 1055-62.

14. 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. J Cardiovasc Pharmacol 2011; 57(5): 611-24.

15. Ward LC, Battersby KJ. Assessment of body composition of rats by bioimpedance spectroscopy: Validation against dual-energy x-ray absorptiometry. Scand J Lab Anim Sci 2009; 36(3): 9.

16. Wu L, Meng J, Shen Q, Zhang Y, Pan S, Chen Z, et al. Caffeine inhibits hypothalamic A_1R to excite oxytocin neuron and ameliorate dietary obesity in mice. Nat Commun 2017; 8: 15904.

17. Loader TB, Taylor CG, Zahradka P, Jones PJ. Chlorogenic acid from coffee beans: evaluating the evidence for a blood pressure-regulating health claim. Nutr Rev 2017; 75(2): 114-33.

18. Santana-Galvez J, Cisneros-Zevallos L, Jacobo-Velazquez DA. Chlorogenic acid: Recent advances on its dual role as a food additive and a nutraceutical against metabolic syndrome. Molecules 2017; 22(3): 358.

19. Mubarak A, Hodgson JM, Considine MJ, Croft KD, Matthews VB. Supplementation of a high-fat diet with chlorogenic acid is associated with insulin resistance and hepatic lipid accumulation in mice. J Agric Food Chem 2013; 61(18): 4371-8.

20. Cho A-S, Jeon S-M, Kim M-J, Yeo J, Seo K-I, Choi M-S, et al. Chlorogenic acid exhibits anti-obesity property and improves lipid metabolism in high-fat diet-induced-obese mice. Food Chem Toxicol 2010; 48(3): 937-43.

21. Niseteo T, Komes D, Belščak-Cvitanović A, Horžić D, Budeč M. Bioactive composition and antioxidant potential of different commonly consumed coffee brews affected by their preparation technique and milk addition. Food chemistry 2012; 134(4): 1870-7.

22. Cremin P, Kasim-Karakas S, Waterhouse AL. LC/ES-MS detection of hydroxycinnamates in human plasma and urine. J Agric Food Chem 2001; 49(4): 1747-50.

23. Olthof MR, Hollman PCH, Buijsman MNCP, van Amelsvoort JMM, Katan MB. Chlorogenic acid, quercetin-3-rutinoside and black tea phenols are extensively metabolized in humans. J Nutr 2003; 133(6): 1806-14.

24. Gonthier MP, Verny MA, Besson C, Rémésy C, Scalbert A. Chlorogenic acid bioavailability largely depends on its metabolism by the gut microflora in rats. J Nutr 2003; 133(6): 1853-9.

25. Ito H, Gonthier MP, Manach C, Morand C, Mennen L, Remesy C, et al. Polyphenol levels in human urine after intake of six different polyphenol-rich beverages. Br J Nutr 2005; 94(4): 500-9.

26. Olthof MR, Hollman PC, Katan MB. Chlorogenic acid and caffeic acid are absorbed in humans. J Nutr 2001; 131(1): 66-71.

27. Lafay S, Morand C, Manach C, Besson C, Scalbert A. Absorption and metabolism of caffeic acid and chlorogenic acid in the small intestine of rats. Br J Nutr 2006; 96(1): 39-46.

28. Booth AN, Emerson OH, Jones FT, Deeds F. Urinary metabolites of caffeic and chlorogenic acids. J Biol Chem 1957; 229(1): 51-9.

29. Bourne LC, Rice-Evans CA. Urinary detection of hydroxycinnamates and flavonoids in humans after high dietary intake of fruit. Free Radic Res 1998; 28(4): 429-38.

30. Choudhury R, Srai SK, Debnam E, Rice-Evans CA. Urinary excretion of hydroxycinnamates and flavonoids after oral and intravenous administration. Free Radic Biol Med 1999; 27(3-4): 278-86.

31. Azuma K, Ippoushi K, Nakayama M, Ito H, Higashio H, Terao J. Absorption of chlorogenic acid and caffeic acid in rats after oral administration. J Agric Food Chem 2000; 48(11): 5496-500.

32. Nardini M, Cirillo E, Natella F, Scaccini C. Absorption of phenolic acids in humans after coffee consumption. J Agric Food Chem 2002; 50(20): 5735-41.

33. Rechner AR, Kuhnle G, Bremner P, Hubbard GP, Moore KP, Rice-Evans CA. The metabolic fate of dietary polyphenols in humans. Free Radic Biol Med 2002; 33(2): 220-35.

34. Monteiro M, Farah A, Perrone D, Trugo LC, Donangelo C. Chlorogenic acid compounds from coffee are differentially absorbed and metabolized in humans. J Nutr 2007; 137(10): 2196-201.

35. Del Rio D, Stalmach A, Calani L, Crozier A. Bioavailability of coffee chlorogenic acids and green tea flavan-3-ols. Nutrients 2010; 2(8): 820-33.

36. Gonthier MP, Remesy C, Scalbert A, Cheynier V, Souquet JM, Poutanen K, et al. Microbial metabolism of caffeic acid and its esters chlorogenic and caftaric acids by human faecal microbiota *in vitro*. Biomed Pharmacother 2006; 60(9): 536-40.

37. Ogawa M, Suzuki Y, Endo Y, Kawamoto T, Kayama F. Influence of coffee intake on urinary hippuric acid concentration. Ind Health 2011; 49(2): 195-202.

38. Jang H, Choi Y, Ahn HR, Jung SH, Lee CY. Effects of phenolic acid metabolites formed after chlorogenic acid consumption on retinal degeneration *in vivo*. Mol Nutr Food Res 2015; 59(10): 1918-29.

39. Parkar SG, Trower TM, Stevenson DE. Fecal microbial metabolism of polyphenols and its effects on human gut microbiota. Anaerobe 2013; 23: 12-9.

40. Peng BJ, Zhu Q, Zhong YL, Xu SH, Wang Z. Chlorogenic acid maintains glucose homeostasis through modulating the expression of SGLT-1, GLUT-2, and PLG in different intestinal segments of Sprague-Dawley rats fed a high-fat diet. Biomed Environ Sci 2015; 28(12): 894-903.

41. Vijay-Kumar M, Aitken JD, Carvalho FA, Cullender TC, Mwangi S, Srinivasan S, et al. Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5. Science 2010; 328(5975): 228-31.

42. Rainone V, Schneider L, Saulle I, Ricci C, Biasin M, Al-Daghri NM, et al. Upregulation of inflammasome activity and increased gut permeability are associated with obesity in children and adolescents. Int J Obes 2016; 40(6): 1026-33.

43. Harte AL, Varma MC, Tripathi G, McGee KC, Al-Daghri NM, Al-Attas OS, et al. High fat intake leads to acute postprandial exposure to circulating endotoxin in type 2 diabetic subjects. Diabetes Care 2012; 35(2): 375-82.

44. Ghanim H, Abuaysheh S, Sia CL, Korzeniewski K, Chaudhuri A, Fernandez-Real JM, et al. Increase in plasma endotoxin concentrations and the expression of toll-like receptors and suppressor of cytokine signaling-3 in mononuclear cells after a high-fat, high-carbohydrate meal implications for insulin resistance. Diabetes Care 2009; 32(12): 2281-7.

45. Sanal MG. Biomarkers in nonalcoholic fatty liver disease-the emperor has no clothes? World J Gastroenterol 2015; 21(11): 3223-31.

46. Ong KW, Hsu A, Tan BKH. Anti-diabetic and anti-lipidemic effects of chlorogenic acid are mediated by ampk activation. Biochem Pharmacol 2013; 85(9): 1341-51.

47. Steinberg GR, Jorgensen SB. The AMP-activated protein kinase: role in regulation of skeletal muscle metabolism and insulin sensitivity. Mini Rev Med Chem 2007; 7(5): 519-26.

48. Shi H, Dong L, Jiang J, Zhao J, Zhao G, Dang X, et al. Chlorogenic acid reduces liver inflammation and fibrosis through inhibition of toll-like receptor 4 signaling pathway. Toxicology 2013; 303: 107-14.

49. Ma Y, Gao M, Liu D. Chlorogenic acid improves high fat diet-induced hepatic steatosis and insulin resistance in mice. Pharm Res 2015; 32(4): 1200-9.

50. Murase T, Misawa K, Minegishi Y, Aoki M, Ominami H, Suzuki Y, et al. Coffee polyphenols suppress diet-induced body fat accumulation by downregulating SREBP-1c and related molecules in C57BL/6J mice. Am J Physiol Endocrinol Metab 2011; 300(1): E122-33.

Acknowledgments

We thank Mr Brian Bynon for the assistance with plasma analyses. We would also like to thank the University of Southern Queensland Research and Innovation Division for funding support to complete this study.

Figure legends

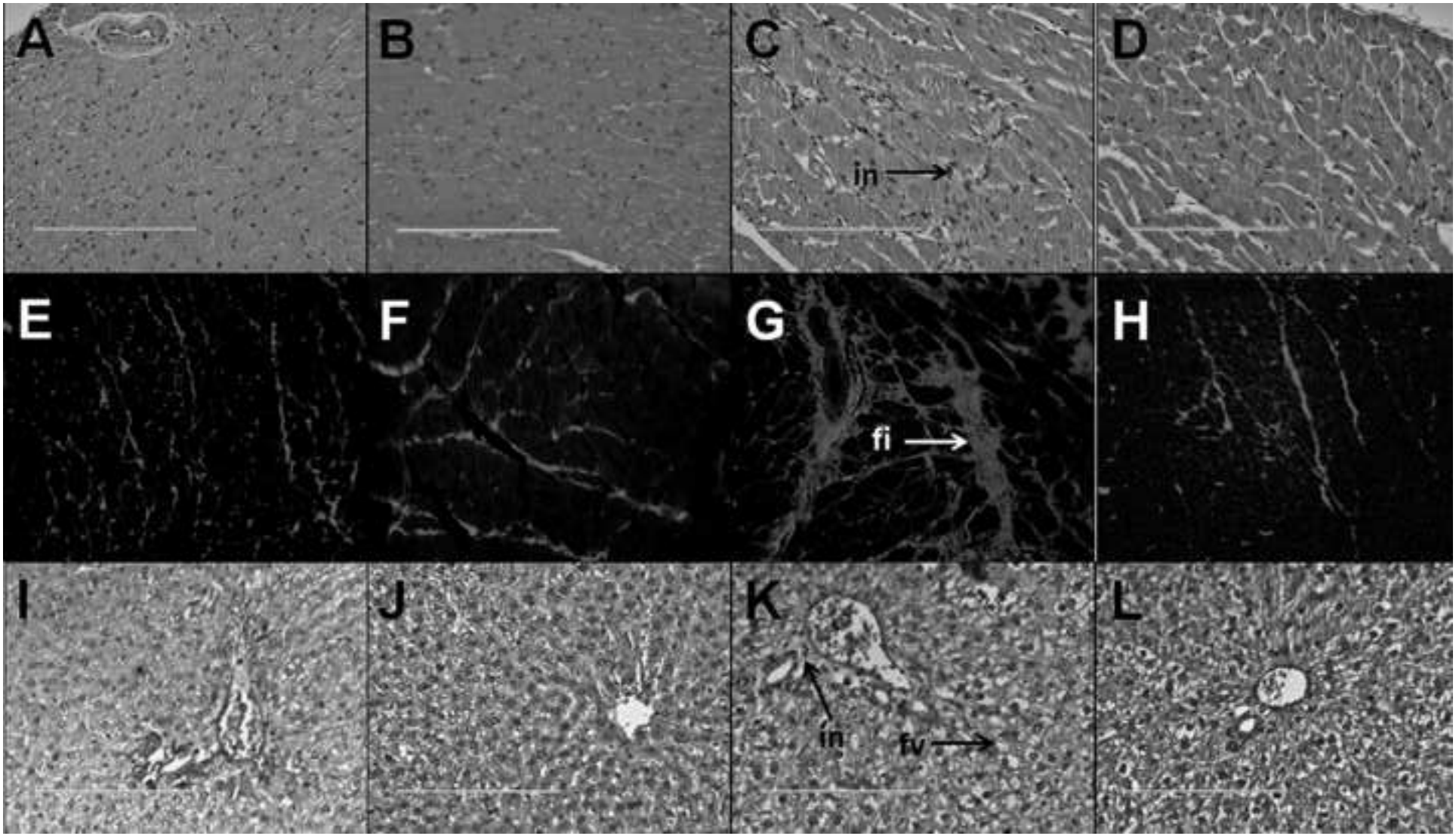
Figure 1. Effects of chlorogenic acid on structure of heart and liver at the end of 16 weeks. Hematoxylin and eosin staining of left ventricle showing inflammatory cells ("in") in corn starch diet-fed rats (*A*), corn starch diet-fed rats supplemented with chlorogenic acid (*B*), high-carbohydrate, high-fat diet-fed rats (*C*), and high-carbohydrate, high-fat diet-fed rats supplemented with chlorogenic acid (*D*). Picrosirius red staining of left ventricle showing fibrosis ("fi") in corn starch diet-fed rats (*E*), corn starch diet-fed rats supplemented with chlorogenic acid (*F*), high-carbohydrate, high-fat diet-fed rats (*G*), and high-carbohydrate, high-fat diet-fed rats supplemented with chlorogenic acid (*H*). Hematoxylin and eosin staining of liver showing enlarged fat vacuoles ("fv") and inflammatory cells ("in") from rats fed with corn starch diet (*I*), corn starch diet-fed rats treated with chlorogenic acid (*J*), high-carbohydrate, high-fat diet-fed rats (*K*), and high-carbohydrate, high-fat diet-fed rats treated with chlorogenic acid (*L*).

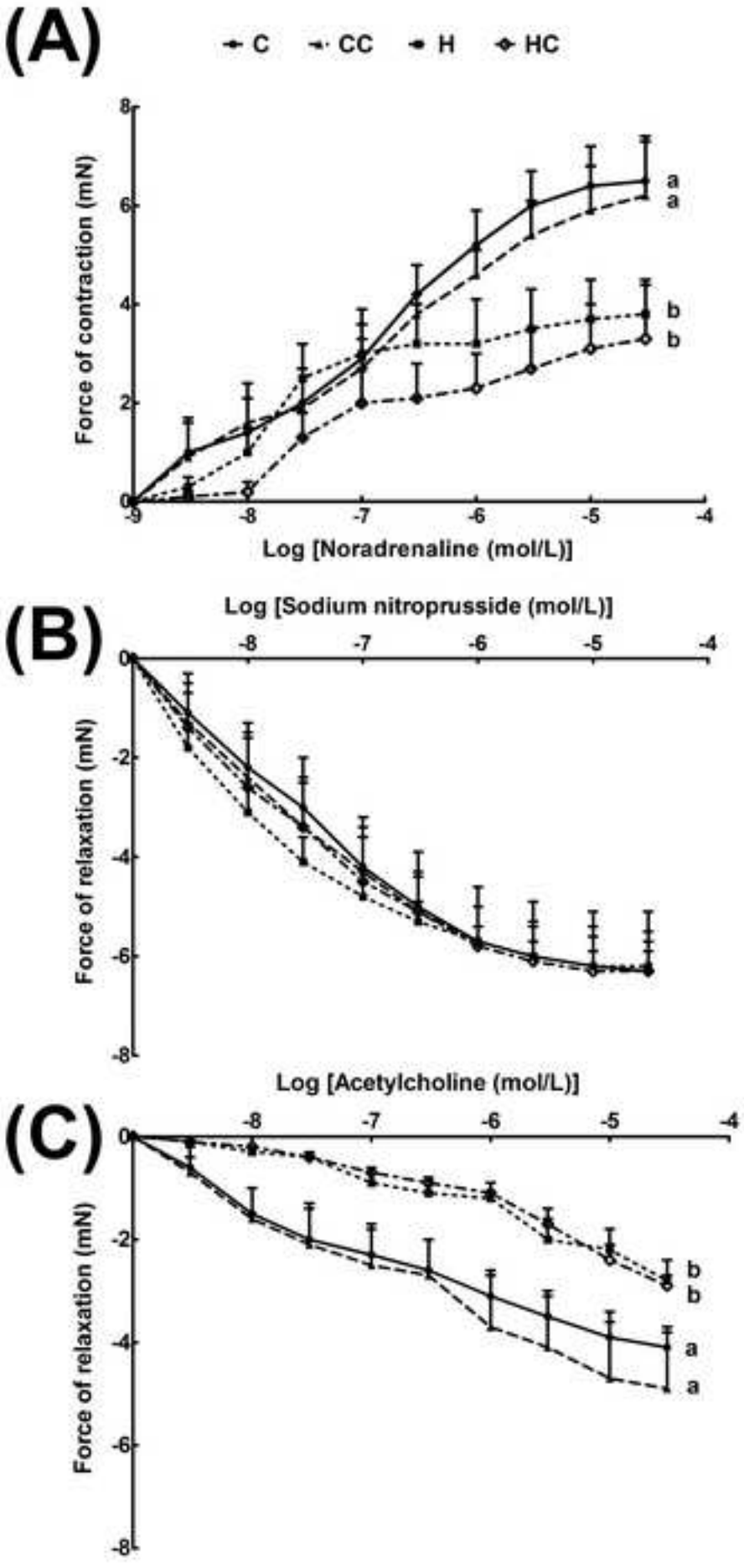
Figure 2. Effects of chlorogenic acid on thoracic aortic responses to noradrenaline (A), sodium nitroprusside (B), and acetylcholine (C) at the end of 16 weeks. Values are presented as mean ± SEM (n = 8–10). End-point means without a common alphabet significantly differ, $P < 0.05$. *C*, corn starch diet-fed rats; *CC*, corn starch diet-fed rats treated with chlorogenic acid; *H*, high-carbohydrate, high-fat diet-fed rats; *HC*, high-carbohydrate, high-fat diet-fed rats treated with chlorogenic acid.

Table 1. Effects of Chlorogenic acid treatment on physiological, compositional, and metabolic parameters at the end of 16 weeks

Variables	*C*	*CC*	*H*	*HC*	***P* value**		
					Diet	***Chlorogenic acid***	***Diet* × *Chlorogenic acid***
Initial body weight, g	342 ± 1	341 ± 2	340 ± 1	340 ± 2	0.34	0.75	0.75
Final body weight, g	385 ± 5^{c}	380 ± 5^{c}	563 ± 14^{a}	498 ± 13^{b}	<0.0001	0.002	0.006
Water intake, mL/d	27.3 ± 0.8^{a}	27.2 ± 1.1^{a}	24.7 ± 0.8^{ab}	23.6 ± 0.8^{b}	0.001	0.50	0.58
Food intake, g/d	32.3 ± 0.8^{b}	36.1 ± 0.9^{a}	25.1 ± 0.9^{c}	23.4 ± 0.6^{c}	<0.0001	0.20	0.002
Chlorogenic acid intake, mg/kg/day	-	173 ± 3	-	103 ± 2	-	-	-
Energy intake, kJ/d	363 ± 9^{c}	406 ± 11^{b}	541 ± 13^{a}	508 ± 14^{a}	<0.0001	0.68	0.003
Feed efficiency, kJ/g	0.12 ± 0.01^{c}	0.10 ± 0.01^{c}	0.41 ± 0.03^{a}	0.31 ± 0.02^{b}	<0.0001	0.003	0.045
Body mass index, g/cm^{2}	0.63 ± 0.02^{c}	0.63 ± 0.01^{c}	0.85 ± 0.02^{a}	0.75 ± 0.02^{b}	<0.0001	0.008	0.008
Abdominal circumference, cm	18.8 ± 0.3^{b}	18.6 ± 0.2^{a}	23.3 ± 0.5^{a}	21.8 ± 0.2^{a}	0.002	0.007	<0.0001
Whole-body lean mass, g	297 ± 4	293 ± 9	291 ± 6	300 ± 15	0.96	0.79	0.50
Whole-body fat mass, g	69 ± 5^{c}	55 ± 8^{c}	223 ± 11^{a}	187 ± 10^{b}	<0.0001	0.008	0.22
Basal blood glucose, mmol/L	4.14 ± 0.24^{c}	3.65 ± 0.14^{d}	5.25 ± 0.16^{a}	4.66 ± 0.06^{b}	<0.0001	0.002	0.76
Blood glucose at 120 minutes, mmol/L	4.86 ± 0.14^{b}	4.90 ± 0.16^{b}	5.82 ± 0.31^{a}	5.62 ± 0.09^{a}	<0.0001	0.68	0.54
Blood glucose AUC, mmol/L × minutes	715 ± 20^{b}	761 ± 23^{ab}	805 ± 18^{a}	819 ± 23^{a}	0.001	0.16	0.45
Plasma total cholesterol, mmol/L	1.70 ± 0.10	1.70 ± 0.06	1.58 ± 0.08	1.56 ± 0.09	0.1242	0.9045	0.9045
Plasma triglyceride, mmol/L	0.56 ± 0.06^{b}	0.64 ± 0.06^{b}	1.30 ± 0.16^{a}	1.23 ± 0.16^{a}	<0.0001	0.9661	0.5249
Plasma NEFA, mmol/L	1.54 ± 0.22^{b}	1.86 ± 0.23^{b}	3.83 ± 0.27^{a}	3.37 ± 0.27^{a}	<0.0001	0.7784	0.1224
Retroperitoneal fat, mg/mm tibial length	136 ± 12^{c}	146 ± 12^{c}	484 ± 54^{a}	279 ± 18^{b}	<0.0001	0.0012	0.0004
Epididymal fat, mg/mm tibial length	87.2 ± 6^{b}	97.0 ±8.0^{b}	226 ± 23^{a}	189 ± 16^{a}	<0.0001	0.3550	0.1168
Omental fat, mg/mm tibial length	130 ± 8^{b}	127 ± 9^{b}	251 ± 16^{a}	226 ± 13^{a}	<0.0001	0.2401	0.3485
Total abdominal fat, mg/mm tibial length	353 ± 18^{c}	371 ± 27^{c}	961 ± 89^{a}	694 ± 30^{b}	<0.0001	0.0101	0.0038
Visceral adiposity index, %	4.33 ± 0.19^{c}	4.65 ± 0.28^{c}	8.91 ± 0.65^{a}	6.90 ± 0.30^{b}	<0.0001	0.038	0.0052
Systolic blood pressure, mmHg	134 ± 1^{b}	131 ± 1^{b}	142 ± 2^{a}	132 ± 1^{b}	0.002	<0.0001	0.012
LV + septum wet weight, mg/mm tibial length	20.6 ± 1.0	20.5 ± 1.0	24.1 ± 2.0	22.7 ± 1.1	0.051	0.58	0.63
RV wet weight, mg/mm tibial length	4.85 ± 0.45	4.48 ± 0.37	5.49 ± 0.75	5.25 ± 0.38	0.18	0.56	0.89
LV diastolic stiffness constant (κ)	22.5 ± 0.8^{b}	23.0 ± 0.8^{b}	28.4 ± 1.1^{a}	24.2 ± 0.9^{b}	0.0004	0.049	0.014
Liver, mg/mm tibial length	220 ± 7^{b}	209 ± 9^{b}	336 ± 19^{a}	304 ± 10^{a}	<0.0001	0.0676	0.3565
Plasma ALT activity, mmol/L	28.6 ± 2.6^{b}	32.5 ± 3.7^{b}	41.7 ± 3.8^{a}	29.5 ± 1.8^{b}	0.031	0.039	0.013
Plasma AST, mmol/L	71.6 ± 3.0^{a}	71.0 ± 3.7^{a}	81.1 ± 6.2^{a}	60.5 ± 2.3^{b}	0.5812	0.0074	0.0090

Values are mean ± SEM, n = 8–10. Mean values within a row with unlike superscript letters are significantly different, P<0.05. *ALT*, alanine transaminase; *AST*, aspartate transaminase; *AUC*, area under the curve; *C*, corn starch diet-fed rats; *CC*, corn starch diet-fed rats treated with chlorogenic acid; *H*, high-carbohydrate, high-fat diet-fed rats; *HC*, high-carbohydrate, high-fat diet-fed rats treated with chlorogenic acid; *LV*, left ventricle; *NEFA*, non-esterified fatty acids; *RV*, right ventricle.


A
B
C
in
D
E
F
G
fi
H
I
J
K
in
fv
L


(A)
C
CC
H
HC
Force of contraction (mN)
Log [Noradrenaline (mol/L)]
a
a
b
b
(B)
Log [Sodium nitroprusside (mol/L)]
Force of relaxation (mN)
(C)
Log [Acetylcholine (mol/L)]
Force of relaxation (mN)
b
b
a
a

Chapter 4.

Coffee pulp attenuates metabolic syndrome in diet-induced obese hypertensive rats

Abstract

Coffee pulp is a major by-product of the coffee industry and is a rich source of chlorogenic acid, caffeine and dietary fibre. However, there is very limited data available on applications of coffee pulp in human health. In this study, we demonstrated the pathophysiological and metabolic effects of coffee pulp on a rat model of human metabolic syndrome. 8-9 weeks old male Wistar rats were divided into four groups. Two groups of rats were fed on corn starch diet while the other two groups were fed on high-carbohydrate, high-fat diet for 16 weeks. One group from each diet was given 5% freeze-dried coffee pulp mixed in the food for the last 8 weeks of the protocol. High-carbohydrate, high-fat diet-fed rats developed the symptoms of metabolic syndrome including abdominal obesity, dyslipidaemia, impaired glucose tolerance, and cardiovascular and hepatic complications. Coffee pulp attenuated obesity and hypertension, and improved glucose tolerance, cardiovascular and hepatic dysfunction while reducing blood lipid concentrations without affecting plasma total cholesterol concentrations or vascular reactivity. This study suggested that coffee pulp could be used as a functional food for managing obesity-associated metabolic, cardiovascular and hepatic abnormalities.

Introduction

Obesity has become a major health concern globally [1]. Sedentary lifestyle, lack of physical activity and chronic consumption of excess energy are the prominent causes of the escalating prevalence of obesity [2] as approximately 38% of the world's population is overweight and 20% is obese [3]. Obesity as a component of metabolic syndrome, a constellation of metabolic disorders such as hypertension, central obesity, dyslipidaemia, impaired glucose tolerance and insulin resistance, further increases the risk of developing type 2 diabetes, cardiovascular diseases and non-alcoholic fatty liver disease [4]. Under chronic excess energy intake, excess energy is stored in adipocytes leading to an imbalance in metabolic functions, causing oxidative stress through the disproportionate production of reactive oxygen species, subsequently activating low-grade chronic inflammatory signaling pathways and insulin resistance [5]. Previous studies with functional foods and nutraceuticals have shown antioxidant and anti-inflammatory responses against obesity and metabolic syndrome leading to decreased risks of developing type 2 diabetes, cardiovascular diseases and non-alcoholic fatty liver disease [6].

Coffee is a highly consumed beverage around the world [7], and reports show that coffee is associated with improved glucose tolerance [8], obesity [9], oxidative stress [10], impaired glucose tolerance [11], dyslipidaemia [12], type 2 diabetes [13] and cardiovascular disease [14]. Coffee is an excellent source of phytochemicals including caffeine, chlorogenic acid and fibre that are responsible for delivering the health beneficial effects [15, 16]. High consumption of coffee requires the processing of a large number of coffee berries which generates coffee pulp as the waste from processing. Coffee pulp accounts for ~29% (w/w) of the dry weight of the whole berry. This waste leads to the challenge of disposing of it without environmental contamination [17]. The dry matter of coffee pulp contains carbohydrates (15.7%), proteins (17.4%), crude fibre (14.1%), cellulose (20.7%), hemicellulose (3.6%), lignin (14.3%) [18] and minerals (especially potassium), along with tannins, polyphenols such as chlorogenic acid, and caffeine [17]. HPLC analysis of coffee pulp showed phenolic compounds such as chlorogenic acid (5-caffeoylquinic acid, 42.2%), epicatechin (21.6%), isochlorogenic acid I (5.7%),

isochlorogenic acid II (19.3%), isochlorogenic acid III (4.4%), catechin (2.2%), rutin (2.1%), protocatechuic acid (1.6%) and ferulic acid (1.0%) [19]. Hence, the use of coffee pulp may be considered as a potential approach for management of metabolic complications associated with obesity as there are many reports of health benefits from these individual components of coffee pulp. However, there is insufficient evidence for the health effects of coffee pulp as a dietary supplement.

This study investigated the effects of freeze-dried coffee pulp as an intervention for the last 8 weeks to rats fed a high-carbohydrate, high-fat diet for 16 weeks to mimic human metabolic syndrome [20]. After treatment with coffee pulp, the structure and the function of the cardiovascular system and liver were investigated. Cardiovascular function was studied in isolated Langendorff heart preparations, isolated thoracic aortic rings and by measurement of systolic blood pressure together with histology. Liver structure and function were measured by plasma biochemistry and histology. Metabolic parameters related to obesity and glucose tolerance were also evaluated [20].

Methods

Preparation of coffee pulp powder

Coffee pulp sample was collected from Mountain Top Coffee Farm and Mill, Nimbin, Asutralia in December 2016. The sample was freeze-dried at School of Agriculture and Food Sciences, University of Queensland, Gatton, Australia and stored at 4°C until analysis.

Characterisation of coffee pulp freeze-dried powder

Extracts of coffee pulp powder were prepared in 3:2 ethanol:water mixture. Briefly, 1 g of powder was dissolved in 50 mL of ethanol:water mixture, sonicated for 15 minutes and an aliquot of the supernatant was taken for analysis by HPLC using an Agilent 1100 series system coupled with a mass spectrometer for further peak confirmation or identification as required. The HPLC system consisted of a diode array detector (G4212B), binary pump (G4220A), an autosampler (G4226A), a vacuum degasser and a column oven with an MSD (G1946D) detector also present. The chromatography was

performed on a Phenomenex luna C18 (2) HPLC column (100 x 4.6 mm) using a gradient method of water and acetonitrile with 0.005% trifluoroacetic acid over 28 minutes. The optimal solvent gradient for separation of target constituents started with 10% acetonitrile which was increased as a gradient to 30% acetonitrile over 10 minutes, then to 95% acetonitrile over 8 minutes, at a flow rate of 0.75 mL/minute and an injection volume of 5 µL. Calibration standards of trigonelline, caffeine and chlorogenic acid were prepared in 3:2 ethanol:water, at concentrations from 0.01 to 1 mg/mL, 0.005 to 0.5 mg/mL and 0.004 to 1 mg/mL for each of these standards, respectively. Specific detection and calibration curves for each compound were performed at 254 nm, 280 nm and 330 nm, respectively. Quantification was performed using the Chemstation Software based on reference standards, peak area and sample dilution at specific wavelengths for each compound. Mr Peter Mouatt, Senior Analytical Officer from Southern Cross University, played a major role in the analysis of components.

Rats, diets and treatments

All experimental protocols were approved by the University of Southern Queensland Animal Ethics Committee under the guidelines of the National Health and Medical Research Council of Australia. Male Wistar rats (8–9 weeks old, 340 ± 1 g, n = 48) were obtained from Animal Resource Centre, Perth, Australia. Rats were divided into 4 groups for the 16 week feeding protocol: corn starch diet-fed rats (C; n = 12), corn starch diet + coffee pulp powder (CCP, n=12; 5% in food for the final 8 weeks); high-carbohydrate, high-fat-diet-fed rats (H; n = 12) and high-carbohydrate, high-fat diet + coffee pulp powder (HCP, n=12; 5% in food for the final 8 weeks). Corn starch diet contained 570 g corn starch, 155 g powdered rat food, 25 g Hubbel, Mendel & Wakeman salt mixture and 250 g water per kilogram of diet. High-carbohydrate, high-fat diet contained 175 g fructose, 395 g sweetened condensed milk, 200 g beef tallow, 155 g powdered rat food, 25 g Hubbel, Mendel & Wakeman salt mixture and 50 g water per kilogram of diet [20]. Drinking water with 25% (w/v) fructose was provided to H and HCP groups. C and CCP groups were given normal drinking water. The rats were individually housed under temperature-controlled, 12-hour light/dark conditions and given *ad libitum* access to food and water [20].

Physiological parameters

Body weight, food and water intakes were measured daily. Abdominal circumference and body length (nose to anus) were measured using a standard measuring tape under light anaesthesia with Zoletil (tiletamine 10 mg/kg, zolazepam 10 mg/kg, intraperitoneal). Body mass index was calculated as body weight (in grams)/[body length (in cm)]^2. Feed efficiency was calculated as [mean body weight gain (in grams)/daily energy intake (in kJ)] [20].

Systolic blood pressure

Systolic blood pressure was measured under light anaesthesia with Zoletil (tiletamine 10 mg/kg, zolazepam 10 mg/kg, intraperitoneally), using an MLT1010 Piezo-Electric Pulse Transducer and inflatable tail-cuff connected to an MLT844 Physiological Pressure Transducer and PowerLab data acquisition unit [20].

Body composition measurement

Dual-energy X-ray absorptiometric measurements were carried out at the end of the protocol with a Norland XR46 DXA instrument (Norland Corp, Fort Atkinson, WI). These scans were evaluated using the manufacturer's suggested software for use in laboratory animals (Small Subject Analysis Software, version 2.5.3/1.3.1; Norland Corp) [21]. The precision error of lean mass for replicate measurements, with repositioning, is 3.2%.

Oral glucose tolerance test

Oral glucose tolerance tests were performed after determining overnight fasting blood glucose concentrations in tail vein blood using Medisense Precision Q.I.D. glucose meters. For overnight fasting, rats were deprived of food for 12 hours. Fructose-supplemented drinking water in all H diet-fed groups was replaced with normal drinking water for the overnight food deprivation period. Basal blood glucose concentrations were measured followed by administration of glucose load 2 g/kg body weight as 40% glucose

solution via oral gavage. Blood glucose concentrations were then measured 30, 60, 90 and 120 minutes after oral glucose administration [20].

Metabolic rate assessment

Indirect calorimetry was applied to determine oxygen consumption and carbon dioxide production using a 4-chamber Oxy-Max system (Columbus Instruments, Columbus, OH) with one rat per chamber. Rats were given *ad libitum* access to food and water during the measurements. Oxygen consumption (V_{O2}) and carbon dioxide production (V_{CO2}) were measured individually from each chamber. The respiratory exchange ratio was calculated by Oxy-Max software (v. 4.86). Energy expenditure was determined by assessment of the exchange of oxygen for carbon dioxide that occurs during the metabolic processing of food [22].

Terminal experiments

Rats were euthanased with Lethabarb (pentobarbitone sodium, 100 mg/kg, intraperitoneally). After euthanasia, heparin (200 IU) was injected through the right femoral vein. The abdomen was then opened and blood (~5 mL) was withdrawn from the abdominal aorta and collected into heparinised tubes. Blood was centrifuged at 5000 × *g* for 10 minutes to obtain plasma. Plasma was stored at -20°C for further characterisation. Hearts were then removed from rats for isolated Langendorff heart preparation [20].

Isolated Langendorff heart preparation

Hearts isolated from euthanased rats were perfused with modified Krebs–Henseleit bicarbonate buffer 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. Left ventricular end-diastolic pressure values were measured during pacing of the heart at 250 beats per minute using an electrical stimulator. End-diastolic pressures were obtained from 0 to 30 mmHg for the calculation of diastolic stiffness constant (κ, dimensionless) [20].

Vascular reactivity

Thoracic aortic rings (~4 mm in length) were suspended in an organ bath filled with Tyrode physiological salt solution bubbled with 95% O_2–5% CO_2 maintained at 35°C and allowed to stabilise at a resting tension of ~10 mN. Cumulative concentration-response curves (contraction) were obtained for noradrenaline (1×10^{-9} – 3×10^{-6} M) and cumulative concentration-response curves (relaxation) were obtained for acetylcholine (1×10^{-9} – 3×10^{-6} M) and sodium nitroprusside (1×10^{-9} – 3×10^{-6} M) after submaximal (~70%) contraction to noradrenaline [20].

Analysis of intestinal transit time

Rats were orally gavaged with 3 mL of 0.05% phenol red solution 20 minutes before euthanasia. After euthanasia, the entire region from the stomach to small intestine was removed from its mesenteric attachment immediately. The length of small intestine was measured from the pyloric sphincter to the ileocecal junction. The endpoint of phenol red transit in the small intestine was visualised using a few drops of 0.1 M sodium hydroxide [23]. The intestinal transit for each rat was determined by the following formula:

Intestinal transit (%) = (the total distance traveled by phenol red solution/total length of small intestine) × 100

Organ weights

After isolated heart perfusion studies, the heart was separated into left ventricle (with septum) and right ventricle and weighed. The liver was isolated and weighed. Retroperitoneal, epididymal and omental fat pads were removed separately and weighed. These organ weights were normalised against tibial length at the time of organ removal and expressed as mg/mm of tibial length [20].

Histology

Hearts and livers were removed from the rats soon after euthanasia and fixed in 10% neutral buffered formalin. These samples were then dehydrated and embedded in

paraffin wax. Thin sections (5 µm) were cut and stained with hematoxylin and eosin to study infiltration of inflammatory cells (heart and liver) and fat deposition (liver) and with picrosirius red (heart) to study collagen deposition [20].

Plasma biochemistry

Plasma activities of aspartate transaminase, alanine transaminase and alkaline phosphatase, and plasma concentrations of total cholesterol, triglycerides and non-esterified fatty acids were measured [20].

Statistical analysis

All data are presented as mean ± SEM. Results were tested for variance using Bartlett's test and variables that were not normally distributed were transformed (using log 10 functions) prior to statistical analyses. C, CCP, H and HCP groups were tested for effects of diet, treatment and their interactions by 2-way analysis of variance. When the interaction and/or the main effects were significant, means were compared using Newman-Keuls multiple comparison *post hoc* tests. A P value of <0.05 was considered significant. All statistical analyses were performed using GraphPad Prism version 5.0 for Windows (San Diego, CA, USA).

Results

Coffee pulp intake

The caffeine and trigonelline doses (mg/kg) were higher in CCP rats than in HCP rats due to higher intake of food by rats fed with corn-starch supplemented with treatments (Table 1).

Table 1. Intakes in coffee pulp powder diets

Component	*CCP*	*HCP*
Caffeine intake, mg/kg/day	24.5 ± 2.2	17.5 ± 2.9
Chlorogenic acid intake, mg/kg/day	4.2 ± 0.4	3.0 ± 0.5
Trigonelline intake, mg/kg/day	14.4 ± 1.3	10.2 ± 1.7

Dietary intake, body composition and plasma biochemistry

Body weight was similar between C and CCP rats. H rats had increased body weight compared to C rats while coffee pulp treatment decreased the body weight in HCP rats compared to H rats (Table 2). Food intake was lower in H rats compared to C rats and coffee pulp did not change food intake in both CCP and HCP rats (Table 2). Water intake was similar between C, H and HCP rats while CCP rats had higher water intake. Energy intake was higher in H rats compared to C rats. Coffee pulp did not change energy intake in CCP rats compared to C rats whereas it was higher in HCP rats compared to H rats (Table 2). Feed efficiency and abdominal circumference were similar between C and CCP rats but coffee pulp increased body mass index in CCP rats compared to C rats. Feed efficiency, body mass index and abdominal circumference were higher in H rats than in C rats. Coffee pulp reduced these parameters in HCP rats compared to H rats (Table 2). Heat produced and respiratory exchange ratio were unchanged by coffee pulp in both CCP and HCP rats. Basal blood glucose concentrations were higher in H rats compared to C rats and coffee pulp reduced basal blood glucose concentrations in both CCP and HCP rats compared to C and H rats, respectively. Area under the curve was higher in H rats compared to C rats while it was decreased in both CCP and HCP rats compared to C and

H rats, respectively (Table 2). Retroperitoneal, epididymal, omental and total abdominal fat pads were unchanged in CCP rats compared to C rats but were higher in H rats than in C rats. Coffee pulp reduced total abdominal fat in HCP rats through reduction in omental and epididymal fat pads (Table 2). Whole-body lean mass was similar between C and H rats and coffee pulp only increased it in HCP rats. Whole-body fat mass was higher in H rats than in C rats and it was decreased with coffee pulp in HCP rats compared to H rats (Table 2). Plasma total cholesterol concentrations were unchanged between the groups while coffee pulp reduced plasma concentrations of triglycerides and non-esterified fatty acids in CCP and HCP rats compared to C and H rats, respectively (Table 2).

Cardiovascular structure and function

Histopathological analysis of left ventricle indicated increased infiltration of inflammatory cells in H rats (Figure 1C) compared to C rats (Figure 1A). Coffee pulp reduced infiltration of inflammatory cells into the left ventricle of HCP rats (Figure 1D) than in H rats. Picrosirius red staining of left ventricle showed higher collagen deposition in H rats (Figure 1G) compared to C rats (Figure 1E). Coffee pulp reduced collagen deposition in the left ventricle of HCP rats (Figure 1H) compared to H rats. Noradrenaline-induced contraction and acetylcholine-induced relaxation were lower in H rats than in C rats and coffee pulp treatment did not change these responses (Figure 2A and 2C). There were no differences in sodium nitroprusside-induced vascular relaxation in isolated thoracic aortic rings between the groups (Figure 2B). Systolic blood pressure and left ventricular diastolic stiffness were higher in H rats than in C rats and coffee pulp reduced these parameters in HCP rats compared to H rats (Table 3). LV and RV wet weights were unchanged by coffee pulp treatment (Table 3).

Table 2. Coffee pulp on physiological and metabolic variables

Variables	***C***	***CCP***	***H***	***HCP***	***P values***		
					Diet	***Intervention***	***Diet × Intervention***
Initial body weight, g	340 ± 1	338 ± 1	339 ± 1	339 ± 1	1.00	0.27	0.27
Final body weight, g	383 ± 2^{c}	386 ± 6^{c}	555 ± 9^{a}	494 ± 9^{b}	<0.0001	0.0002	<0.0001
Body weight gain (8-16 week), %	6.0 ± 2.0^{c}	-1.0 ± 1.0^{d}	23.0 ± 2.0^{a}	10.0 ± 1.0^{b}	<0.0001	<0.0001	0.06
Food intake, g/day	35.2 ± 1.3^{a}	34.2 ± 0.6^{a}	27.2 ± 0.7^{b}	28.0 ± 1.5^{b}	<0.0001	0.93	0.42
Water intake, g/day	31.0 ± 2.0^{ab}	36.8 ± 2.1^{a}	26.6 ± 1.6^{b}	32.2 ± 1.7^{ab}	0.02	0.004	0.96
Energy intake, kJ/d	405 ± 15^{c}	384 ± 10^{c}	556 ± 19^{b}	616 ± 17^{a}	<0.0001	0.22	0.013
Feed efficiency, kJ/g	0.10 ± 0.01^{c}	0.12 ± 0.02^{c}	0.39 ± 0.02^{a}	0.25 ± 0.02^{b}	<0.0001	0.001	0.0001
Heat, kcal	3.36 ± 0.17^{b}	3.14 ± 0.31^{b}	4.14 ± 0.10^{a}	4.51 ± 0.16^{a}	<0.0001	0.71	0.13
Respiratory exchange ratio	1.01 ± 0.01^{a}	0.95 ± 0.04^{ab}	0.89 ± 0.01^{b}	0.89 ± 0.01^{b}	0.0003	0.19	0.29
Body mass index, g/cm^{2}	0.61 ± 0.01^{d}	0.66 ± 0.01^{c}	0.83 ± 0.02^{a}	0.75 ± 0.01^{b}	<0.0001	0.26	<0.0001
Abdominal circumference, cm	18.4 ± 0.2^{c}	17.8 ± 0.2^{c}	23.2 ± 0.4^{a}	20.2 ± 0.2^{b}	<0.0001	<0.0001	<0.0001
Basal blood glucose concentrations, mmol/L	3.9 ± 0.2^{b}	2.9 ± 0.1^{c}	4.9 ± 0.3^{a}	3.2 ± 0.1^{c}	0.002	<0.0001	0.08
Area under the curve, mmol/L.min	692 ± 28^{b}	421 ± 13^{d}	790 ± 23^{a}	571 ± 13^{c}	<0.0001	<0.0001	0.21
Retroperitoneal fat (mg/mm)	182 ± 21^{b}	196 ± 14^{b}	534 ± 53^{a}	458 ± 22^{a}	<0.0001	0.30	0.14
Epididymal fat (mg/mm)	90 ± 8^{c}	86 ± 10^{c}	222 ± 17^{a}	183 ± 10^{b}	<0.0001	0.07	0.13
Omental fat (mg/mm)	130 ± 8^{c}	117 ± 11^{c}	263 ± 17^{a}	213 ± 12^{b}	<0.0001	0.015	0.14
Total Fat pads (mg/mm)	402 ± 30^{c}	399 ± 28^{c}	1019 ± 81^{a}	855 ± 33^{b}	<0.0001	0.08	0.09
Whole-body lean mass, g	283 ± 5^{b}	295 ± 4^{ab}	286 ± 11^{b}	314 ± 7^{a}	0.15	0.01	0.30
Whole-body fat mass, g	83 ± 7^{c}	77 ± 7^{c}	235 ± 17^{a}	168 ± 11^{b}	<0.0001	0.003	0.01
Bone mineral content, g	11.8 ± 0.4^{b}	12.2 ± 0.5^{b}	17.4 ± 0.6^{a}	15.8 ± 0.5^{a}	<0.0001	0.24	0.06
Bone mineral density, g/cm^{2}	0.172 ± 0.002^{c}	0.188 ± 0.002^{b}	0.185 ± 0.002^{b}	0.197 ± 0.005^{a}	0.0002	<0.0001	0.47
Intestinal transit, %	70.4 ± 7.2^{b}	52.2 ± 7.5^{b}	89.1 ± 4.9^{a}	58.2 ± 8.0^{b}	0.010	0.002	0.40

Plasma total cholesterol concentrations, mmol/L	1.84 ± 0.08	1.71 ± 0.07	1.71 ± 0.10	1.84 ± 0.13	1.0000	1.0000	0.18
Plasma triglyceride concentrations , mmol/L	0.57 ± 0.05^{b}	0.33 ± 0.04^{c}	1.17 ± 0.10^{a}	0.61 ± 0.07^{b}	<0.0001	<0.0001	0.03
Plasma non-esterified fatty acids concentrations, mmol/L	1.37 ± 0.16^{b}	0.76 ± 0.14^{c}	3.57 ± 0.18^{a}	1.53 ± 0.20^{b}	<0.0001	<0.0001	<0.0001

Values are mean ± SEM, n = 8–12. Means in a row with unlike superscripts differ significantly, P<0.05. C, corn starch diet-fed rats; CCP, corn starch diet-fed rats supplemented with coffee pulp; H, high-carbohydrate, high-fat diet-fed rats; HCP, high-carbohydrate, high-fat diet-fed rats treated with coffee pulp.

Table 3. Coffee pulp on cardiovascular and hepatic parameters

Variable	***C***	***CCP***	***H***	***HCP***	***P values***		
					Diet	***Intervention***	***Diet × Intervention***
Systolic blood pressure, mmHg	125 ± 1^{b}	125 ± 1^{b}	145 ± 2^{a}	129 ± 1^{b}	<0.0001	<0.0001	<0.0001
Diastolic stiffness constant, κ	21.9 ± 0.5^{b}	20.48 ± 0.4^{b}	29.3 ± 1.9^{a}	21.0 ± 0.3^{b}	<0.0001	0.0004	0.002
LV + septum wet weight, mg/mm tibial length	21.1 ± 0.7^{b}	20.4 ± 0.7^{b}	24.7 ± 1.5^{a}	23.5 ± 0.8^{ab}	0.002	0.34	0.80
RV wet weight, mg/mm tibial length	4.97 ± 0.38	4.44 ± 0.20	5.64 ± 0.52	5.15 ± 0.20	0.06	0.16	0.96
Liver wet weight, mg/mm tibial length	230 ± 6^{c}	245 ± 10^{c}	336 ± 13^{a}	293 ± 10^{b}	<0.0001	0.17	0.006
Plasma aspartate transaminase activity, mmol/L	79.1 ± 4.8^{b}	94.7 ± 3.9^{ab}	82.2 ± 4.4^{b}	103 ± 6.7^{a}	0.25	0.0007	0.60
Plasma alanine transaminase activity, mmol/L	33.0 ± 4.2	37.4 ± 2.8	35.2 ± 3.2	42.1 ± 3.5	0.33	0.12	0.72

Values are mean ± SEM, n = 6–10. Means in a row with unlike superscripts differ significantly, P<0.05. C, corn starch diet-fed rats; CCP, corn starch diet-fed rats treated with coffee pulp; H, high-carbohydrate, high-fat diet-fed rats; HCP, high-carbohydrate, high-fat diet-fed rats treated with coffee pulp.

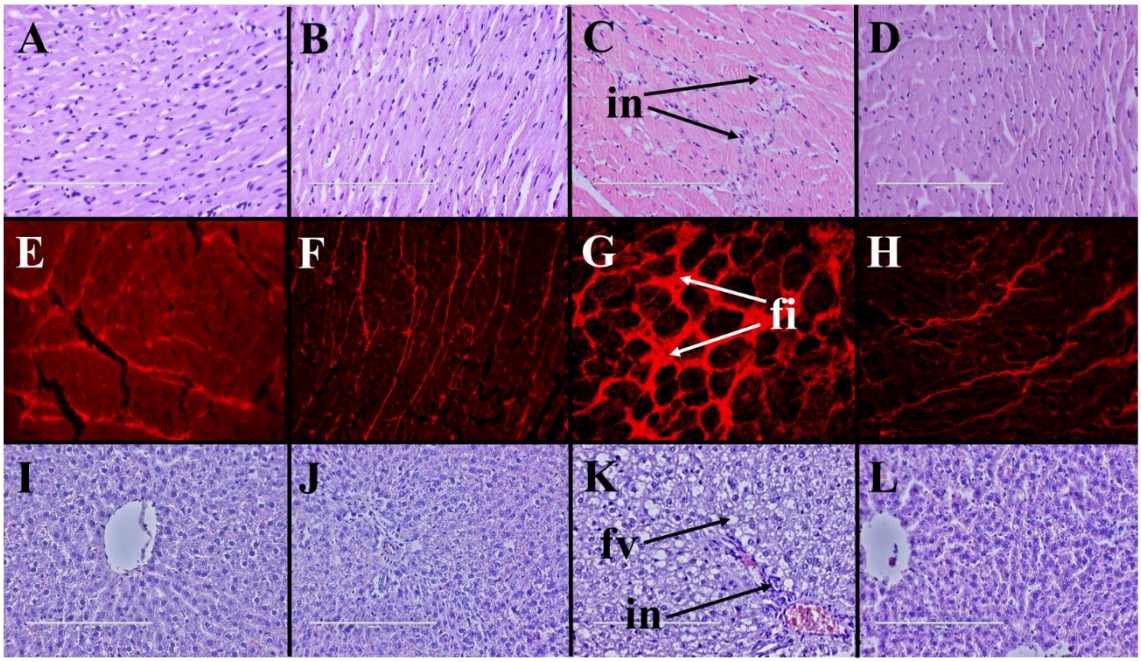


Figure 1. Effects of coffee pulp on the heart and liver structure. Haematoxylin & eosin staining showing infiltration of inflammatory cells ("in") in hearts from C (A), CCP (B), H (C) and HCP (D) and enlarged fat vacuoles ("fv") in livers from C (I), CCP (J), H (K) and HCP (L) (×20). Picrosirius red staining showing fibrosis ("fi") in heart from C (E), CCP (F), H (G) and HCP (H) (×20). C, corn starch diet-fed rats; CCP, corn starch diet-fed rats treated with coffee pulp; H, high-carbohydrate, high-fat diet-fed rats; HCP, high-carbohydrate, high-fat diet-fed rats treated with coffee pulp.

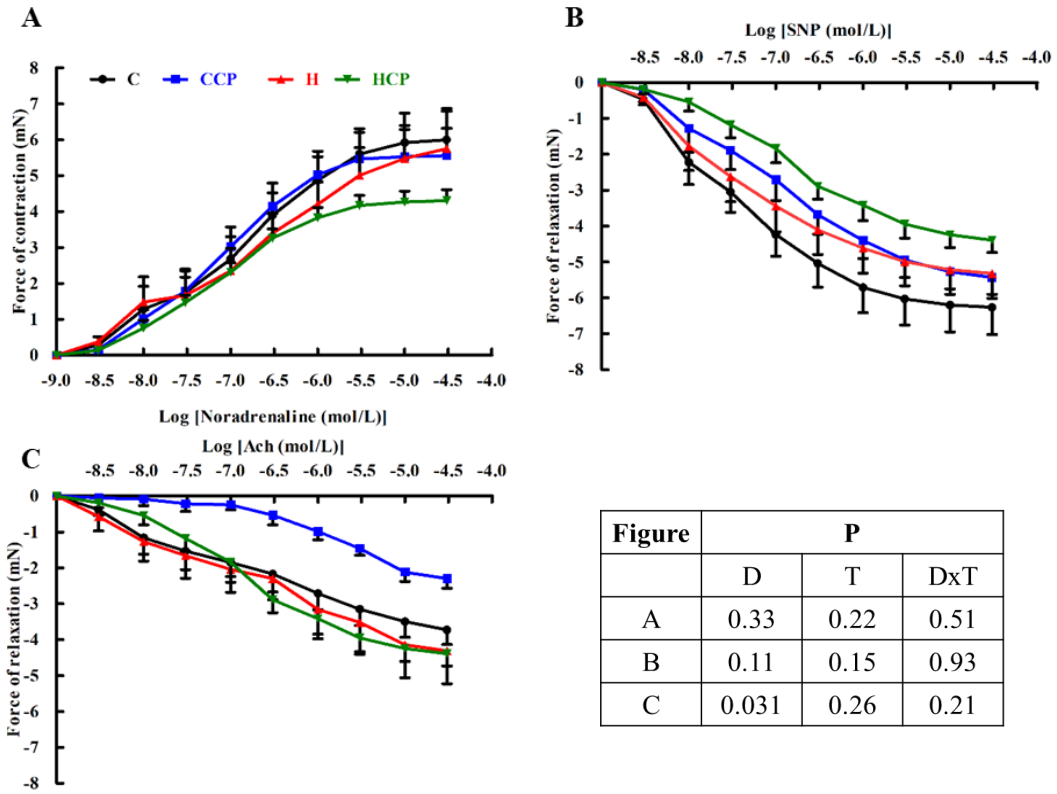


Figure	P		
	D	T	DxT
A	0.33	0.22	0.51
B	0.11	0.15	0.93
C	0.031	0.26	0.21

Figure 2. Effects of coffee pulp on noradrenaline-induced contraction (A), sodium nitroprusside-induced relaxation (B) and acetylcholine-induced relaxation (C) in thoracic aorta ring, prepared from C, CCP, H and HCP rats. Values are mean ± SEM, n = 8-10. End-point means without a common letter differ, $P<0.05$. C, corn starch diet-fed rats; CCP, corn starch diet-fed rats treated with coffee pulp; H, high-carbohydrate, high-fat diet-fed rats; HCP, high-carbohydrate, high-fat diet-fed rats treated with coffee pulp.

Liver structure and function

Liver wet weight was higher in H rats than in C rats and coffee pulp decreased the liver wet weight of HCP rats compared to H rats (Table 3). Liver wet weight was unchanged by coffee pulp treatment between C and CCP rats. Increased inflammatory cell infiltration and fat deposition were observed in livers from H rats (Figure 1K) compared to C rats (Figure 1I). Coffee pulp reduced infiltration of inflammatory cells and fat deposition in livers of HCP rats (Figure 1L) compared to H rats. Plasma alanine transaminase activity was unchanged between the groups. Plasma aspartate transaminase activity was unchanged between H and C rats and increased in HCP rats compared to H rats (Table 3).

Discussion

In this study, coffee pulp reduced body weight and central obesity, improved glucose tolerance along with improved heart and liver structure and function on diet-induced obese rats, fed with the high-carbohydrate high-fat diet. Moreover, coffee pulp increased lean mass and bone mineral density in obese rats. These responses of coffee pulp, a major waste product of the coffee industry, demonstrated the potential application of coffee pulp as a functional food containing bioactive components such as caffeine, chlorogenic acid and trigonelline. These effects of coffee pulp in our diet-induced rat model of metabolic syndrome suggest that this intervention may provide benefits in the complex condition of human metabolic syndrome. The rat model of diet-induced metabolic syndrome used in this study has been validated for the signs of metabolic syndrome including abdominal obesity, impaired glucose tolerance, dyslipidaemia, elevated blood pressure, cardiovascular remodelling and fatty liver [20].

During coffee processing, by-products such as pulp are generated and every 2 tons of coffee creates 1 ton of coffee pulp, a leading cause of serious environmental issue [24, 25]. Therefore, alternative application of coffee pulp is attracting interest. Coffee pulp has been used for production of cascara, a refreshing beverage containing 226 mg/L caffeine [26]. Dry mass of coffee pulp has been used for bioethanol production as it contained polysaccharides including arabinose, galactose, glucose, xylose and mannose at concentrations of 5.8, 5.2, 20.2, 4.2 and 4.7% in dry mass, respectively [27]. Moreover,

coffee pulp consisting of proteins 12%, fibre 21%, caffeine 1.3% and 1% polyphenol [28] and coffee pulp components such as caffeine and chlorogenic acid have antioxidant properties that may counterbalance oxidative stress [29].

Meta-analysis of coffee consumption has reported the inverse relationship with components of metabolic syndrome including abdominal obesity, hypertension, insulin resistance, impaired glucose tolerance, dyslipidaemia and liver complications [30, 31]. Treatment with coffee extract attenuated high-fat diet-induced metabolic disorders and decreased body weight, adipose tissue, and plasma concentrations of glucose, free fatty acid, cholesterol and insulin in mice [32]. In the high-carbohydrate, high-fat fed rat model used in this study, coffee extract [33] and caffeine [34] have previously attenuated metabolic syndrome in these obese hypertensive rats. This study was conducted based on the concept that coffee pulp is a rich source of the same bioactive compounds that are present in coffee prepared as a beverage. This study supported the inverse relationship between consumption of coffee bioactive compounds and cardiovascular disease and type 2 diabetes [35].

Caffeine treatment (~28 mg/kg/day) in obese rats for 8 weeks improved structure and function of heart and liver, with a reduction in obesity except for dyslipidaemia [34]. Caffeine antagonised A_1-adenosine receptors in the hypothalamus to suppress appetite and promote energy use that reduced diet-induced obesity in mice [36]. Chlorogenic acid treatment (5 mg/kg/day) for 3 weeks in obese rats improved glucose tolerance, decreased plasma and liver lipid profile, and recovered mineral pool distribution [37]. Chlorogenic acid (5 mg/kg/day) for 45 days in rats reduced blood lipids including cholesterol, free fatty acids, triglycerides, phospholipids, LDL-cholesterol, VLDL- cholesterol and increased concentrations of HDL- cholesterol and decreased HMG-CoA reductase activity and lipid synthesising enzymes [38]. The study has suggested that diet-induced inflammation in obesity could be managed by the anti-inflammatory property of chlorogenic acid [39] through the increased production of adiponectin and PPARγ. Likewise, chlorogenic acid reduced the intracellular ROS production and genes associated with ROS-producing enzymes such NOX2, NOX4 and iNOS [40]. Similarly, chlorogenic acid improved lipid metabolism disorders by modifying the expression of PPARα and LXRα, which contribute

to several intracellular signalling pathways [40]. Consistent with our study, coffee pulp has been reported as a rich source of chlorogenic acid and caffeine, where coffee pulp showed anti-oxidant properties due to their presence [24]. However, in *Chapter* 3, I showed that a much higher dose of chlorogenic acid (approximately 100 mg/kg/day) was required to attenuate inflammation as well as cardiovascular, liver and metabolic abnormalities induced by the high-carbohydrate, high-fat diet. This suggests that chlorogenic acid is not the major bioactive component of coffee pulp.

Coffee pulp used in this study also contained trigonelline, the second most abundant alkaloid in green coffee beans. Trigonelline, as an anti-oxidant, attenuated endoplasmic reticulum-associated stress and oxidative stress-triggered damage in pancreas and adipocytes [41]. Trigonelline (40 mg/kg/day) treatment in the diet for 8 weeks reduced serum activity of aspartate transaminase and aspartate transaminase, and serum concentrations of total cholesterol and LDL-cholesterol and decreased non-alcoholic fatty liver diseases in rats fed with a high fat diet [42]. Trigonelline reduced lipid accumulation by restricting adipocyte differentiation by the PPARgamma cascade. This study suggested that trigonelline inhibits adipocyte differentiation by downregulating fatty acid synthase and GLUT-4 transporter in muscles and adipocytes of mice [43]. These studies suggest that components from coffee pulp as a waste product of the coffee industry, especially trigonelline, have the potential to attenuate metabolic syndrome.

Besides bioactive compounds, the coffee pulp also contained fibre [18] and fibre reduced obesity-associated health disorders through modulating the gut microbiota [44] and reduced body fat, an improved hepatic function that is advantageous to host health [45]. Altered gut microbiota composition disrupts gut barrier junction and promotes the production of lipopolysaccharides by gram-negative bacteria in the gut. This modulation induces gut permeability that enables the translocation of whole bacteria or endotoxic bacterial components into metabolic active tissues and leads to endotoxaemia condition [46]. Endotoxaemia triggers the chronic low-grade inflammation that characterises obesity [47]. Endotoxaemia also induces increased production of nitric oxide metabolites, nitrite and nitrate, increases reactive oxygen species, and increases plasma free fatty acids, tumor necrosis factor and interleukin-6 [48]. Our study with coffee pulp intervention

reduced plasma concentrations of triglycerides and non-esterified fatty acids along with the reduction in body fat mass. These changes may be assisted by healthy gut microbiota that modulate the metabolic changes observed in obesity. Crude fibre accounted for approximately 33.6% of coffee pulp [49] and fibre has prebiotic effects on gut microbiota leading to decreased food intake, weight gain and adiposity, and increased circulating satiety hormones GLP-1 and PYY and colonic fermentation [50]. Colonic fermentation of fibre produces butyrate, acetate and propionate and other dietary secondary metabolites that serve as a substrate for *de novo* lipogenesis in the liver, whereas propionate can be used for gluconeogenesis [51]. Moreover, short-chain fatty acids are also ligands of free fatty acid receptors [52]. Activation of free fatty acid receptors promotes expression and secretion of enteroendocrine hormones such as glucagon-like-peptide 1 or peptide YY which is responsible for satiety [53]. Using the same rat model as in this chapter, treatment with the prebiotic inulin attenuated abdominal obesity, hypertension, increases in inflammatory cell infiltration in the heart and liver, left ventricular diastolic stiffness, lipid droplets in the liver and plasma lipids as well as impaired glucose and insulin tolerance [54]. Dietary fibre has demonstrated prebiotic and anti-bacterial properties, leading to growth of health beneficial gut microbiota and their metabolites [55]. Therefore, I suggest that reduced inflammatory markers and increased colonic production of short-chain fatty acids with the increased intake of dietary fibre from coffee pulp could have contributed to the reduction of the signs of metabolic syndrome in this study.

In conclusion, this chapter provides evidence that coffee pulp can deliver similar results on both pathophysiology and metabolic variables as products from coffee beans. These positive outcomes correlated with reduced plasma lipid concentrations and improvement of glucose tolerance and contributed to reduced obesity, dyslipidaemia and hyperglycaemia. This chapter suggests that dietary intervention with coffee pulp has therapeutic potential to reverse the signs of metabolic syndrome such as obesity, cardiovascular diseases and hepatic abnormalities. Further, the most plausible bioactive components of coffee pulp are trigonelline and fibre. Human clinical trials are necessary to determine whether coffee pulp supplementation in the diet will attenuate or reverse metabolic disorders associated with metabolic syndrome, particularly obesity and hypertension.

References

1. Hurt RT, Kulisek C, Buchanan LA, McClave SA. The obesity epidemic: challenges, health initiatives, and implications for gastroenterologists. Clinical Gastroenterology and Hepatology 2010; 6(12): 780-92.

2. Dabelea D, Harrod CS. Role of developmental overnutrition in pediatric obesity and type 2 diabetes. Nutrition Reviews 2013; 71(Suppl 1): S62-7.

3. Kelly T, Yang W, Chen C-S, Reynolds K, He J. Global burden of obesity in 2005 and projections to 2030. International Journal of Obesity 2008; 32(9): 1431-7.

4. Miller GE, Chen E, Yu TY, Brody GH. Metabolic syndrome risks following the great recession in rural black young adults. Journal of the American Heart Association 2017; 6(9): e006052.

5. Lin Y, Berg AH, Iyengar P, Lam TK, Giacca A, Combs TP, et al. The hyperglycemia-induced inflammatory response in adipocytes: the role of reactive oxygen species. Journal of Biological Chemistry 2005; 280(6): 4617-26.

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

7. Mussatto SI, Machado EM, Martins S, Teixeira JA. Production, composition, and application of coffee and its industrial residues. Food and Bioprocess Technology 2011; 4(5): 661.

8. Lee AH, Tan LB, Hiramatsu N, Ishisaka A, Alfonso H, Tanaka A, et al. Plasma concentrations of coffee polyphenols and plasma biomarkers of diabetes risk in healthy Japanese women. Nutrition & Diabetes 2016; 6: e212.

9. Choi BK, Park SB, Lee DR, Lee HJ, Jin YY, Yang SH, et al. Green coffee bean extract improves obesity by decreasing body fat in high-fat diet-induced obese mice. Asian Pacific Journal of Tropical Medicine 2016; 9(7): 616-24.

10. Han SH, Ko BS, Ahn SH, Noh DO, Suh HJ. Comparison of the antioxidant activities of roasted and explosive puffed coffees. International Journal of Food Science and Technology 2017; 52(6): 1417-24.

11. Rustenbeck I, Lier-Glaubitz V, Willenborg M, Eggert F, Engelhardt U, Jorns A. Effect of chronic coffee consumption on weight gain and glycaemia in a mouse model of obesity and type 2 diabetes. Nutrition & Diabetes 2014; 4: e123.

12. Chang CW, Hsu YJ, Chen YM, Huang WC, Huang CC, Hsu MC. Effects of combined extract of cocoa, coffee, green tea and garcinia on lipid profiles, glycaemic markers and inflammatory responses in hamsters. BMC Complementary and Alternative Medicine 2015; 15: 269.

13. Akash MSH, Rehman K, Chen S. Effects of coffee on type 2 diabetes mellitus. Nutrition 2014; 30(7): 755-63.

14. Miranda AM, Steluti J, Fisberg RM, Marchioni DM. Association between coffee consumption and its polyphenols with cardiovascular risk factors: A population-based study. Nutrients 2017; 9(3): 276.

15. van Dam RM. Coffee and type 2 diabetes: from beans to beta-cells. Nutrition, Metabolism & Cardiovascular Diseases 2006; 16(1): 69-77.

16. Loader TB, Taylor CG, Zahradka P, Jones PJ. Chlorogenic acid from coffee beans: evaluating the evidence for a blood pressure-regulating health claim. Nutrition Reviews 2017; 75(2): 114-33.

17. Murthy PS, Naidu MM. Sustainable management of coffee industry by-products and value addition-A review. Resources Conservation and Recycling 2012; 66: 45-58.

18. Dias M, Melo M, Schwan R, Silva C. A new alternative use for coffee pulp from semi-dry process to β-glucosidase production by *Bacillus subtilis*. Letters in Applied Microbiology 2015; 61(6): 588-95.

19. Ramirez-Martinez JR. Phenolic compounds in coffee pulp: quantitative determination by HPLC. Journal of the Science of Food and Agriculture 1988; 43(2): 135-44.

20. Panchal SK, Poudyal H, Iyer A, Nazer R, Alam MA, Diwan V, et al. High-carbohydrate, high-fat diet-induced metabolic syndrome and cardiovascular remodeling in rats. Journal of Cardiovascular Pharmacology 2011; 57(5): 611-24.

21. Ward LC, Battersby KJ. Assessment of body composition of rats by bioimpedance spectroscopy : Validation against dual-energy x-ray absorptiometry. Scandinavian Journal of Laboratory Animal Science 2009; 36(3): 9.

22. Sekar S, Shafie SR, Prasadam I, Crawford R, Panchal SK, Brown L, et al. Saturated fatty acids induce development of both metabolic syndrome and osteoarthritis in rats. Scientific Reports 2017; 7: 46457.

23. Fu XY, Li Z, Zhang N, Yu HT, Wang SR, Liu JR. Effects of gastrointestinal motility on obesity. Nutrition & Metabolism 2014; 11(1): 3.

24. Duangjai A, Suphrom N, Wungrath J, Ontawong A, Nuengchamnong N, Yosboonruang A. Comparison of antioxidant, antimicrobial activities and chemical profiles of three coffee (Coffea arabica L.) pulp aqueous extracts. Integrative Medicine Research 2016; 5(4): 324-31.

25. Esquivel P, Jiménez VM. Functional properties of coffee and coffee by-products. Food Research International 2012; 46(2): 488-95.

26. Heeger A, Kosinska-Cagnazzo A, Cantergiani E, Andlauer W. Bioactives of coffee cherry pulp and its utilisation for production of Cascara beverage. Food Chemistry 2017; 221: 969-75.

27. Gurram R, Al-Shannag M, Knapp S, Das T, Singsaas E, Alkasrawi M. Technical possibilities of bioethanol production from coffee pulp: a renewable feedstock. Clean Technologies and Environmental Policy 2016; 18(1): 269-78.

28. Pandey A, Soccol CR, Nigam P, Brand D, Mohan R, Roussos S. Biotechnological potential of coffee pulp and coffee husk for bioprocesses. Biochemical Engineering Journal 2000; 6(2): 153-62.

29. Arellano-González MA, Ramírez-Coronel M, Torres-Mancera M, Pérez-Morales GG, Saucedo-Castañeda G. Antioxidant activity of fermented and nonfermented coffee (Coffea arabica) pulp extracts. Food Technology and Biotechnology 2011; 49(3): 374-8.

30. Marventano S, Salomone F, Godos J, Pluchinotta F, Del Rio D, Mistretta A, et al. Coffee and tea consumption in relation with non-alcoholic fatty liver and metabolic syndrome: A systematic review and meta-analysis of observational studies. Clinical Nutrition 2016; 35(6): 1269-81.

31. Shang F, Li X, Jiang X. Coffee consumption and risk of the metabolic syndrome: A meta-analysis. Diabetes & Metabolism 2016; 42(2): 80-7.

32. Maki C, Funakoshi-Tago M, Aoyagi R, Ueda F, Kimura M, Kobata K, et al. Coffee extract inhibits adipogenesis in 3T3-L1 preadipocyes by interrupting insulin signaling through the downregulation of IRS1. PloS One 2017; 12(3): e0173264.

33. 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.

34. Panchal SK, Wong WY, Kauter K, Ward LC, Brown L. Caffeine attenuates metabolic syndrome in diet-induced obese rats. Nutrition 2012; 28(10): 1055-62.

35. Baspinar B, Eskici G, Ozcelik A. How coffee affects metabolic syndrome and its components. Food & Function 2017; 8(6): 2089-101.

36. Wu L, Meng J, Shen Q, Zhang Y, Pan S, Chen Z, et al. Caffeine inhibits hypothalamic A1R to excite oxytocin neuron and ameliorate dietary obesity in mice. Nature Communications 2017; 8: 15904.

37. de Sotillo DVR, Hadley M. Chlorogenic acid modifies plasma and liver concentrations of: cholesterol, triacylglycerol, and minerals in (fa/fa) Zucker rats. Journal of Nutritional Biochemistry 2002; 13(12): 717-26.

38. Karthikesan K, Pari L, Menon V. Antihyperlipidemic effect of chlorogenic acid and tetrahydrocurcumin in rats subjected to diabetogenic agents. Chemico-Biological Interactions 2010; 188(3): 643-50.

39. Hwang SJ, Kim YW, Park Y, Lee HJ, Kim KW. Anti-inflammatory effects of chlorogenic acid in lipopolysaccharide-stimulated RAW 264.7 cells. Inflammation Research 2014; 63(1): 81-90.

40. Huang K, Liang XC, Zhong YL, He WY, Wang Z. 5-Caffeoylquinic acid decreases diet-induced obesity in rats by modulating PPARα and LXRα transcription. Journal of the Science of Food and Agriculture 2015; 95(9): 1903-10.

41. Tharaheswari M, Reddy NJ, Kumar R, Varshney KC, Kannan M, Rani SS. Trigonelline and diosgenin attenuate ER stress, oxidative stress-mediated damage in pancreas and enhance adipose tissue PPAR gamma activity in type 2 diabetic rats. Molecular and Cellular Biochemistry 2014; 396(1-2): 161-74.

42. Zhang DF, Zhang F, Zhang J, Zhang RM, Li R. Protection effect of trigonelline on liver of rats with non-alcoholic fatty liver diseases. Asian Pacific Journal of Tropical Medicine 2015; 8(8): 639-42.

43. Ilavenil S, Arasu MV, Lee J-C, Kim DH, Roh SG, Park HS, et al. Trigonelline attenuates the adipocyte differentiation and lipid accumulation in 3T3-L1 cells. Phytomedicine 2014; 21(5): 758-65.

44. Chen T, Kim CY, Kaur A, Lamothe L, Shaikh M, Keshavarzian A, et al. Dietary fibre-based SCFA mixtures promote both protection and repair of intestinal epithelial barrier function in a Caco-2 cell model. Food & Function 2017; 8(3): 1166-73.

45. Parnell JA, Raman M, Rioux KP, Reimer RA. The potential role of prebiotic fibre for treatment and management of non-alcoholic fatty liver disease and associated obesity and insulin resistance. Liver International 2012; 32(5): 701-11.

46. Scheithauer TPM, Dallinga-Thie GM, de Vos WM, Nieuwdorp M, van Raalte DH. Causality of small and large intestinal microbiota in weight regulation and insulin resistance. Molecular Metabolism 2016; 5(9): 759-70.

47. Guerville M, Leroy A, Sinquin A, Laugerette F, Michalski MC, Boudry G. Western-diet consumption induces alteration of barrier function mechanisms in the ileum that correlates with metabolic endotoxemia in rats. American Journal of Physiology - Endocrinology and Metabolism 2017; 313(2): E107-20.

48. Dandona P, Ghanim H, Bandyopadhyay A, Korzeniewski K, Sia CL, Dhindsa S, et al. Insulin suppresses endotoxin-induced oxidative, nitrosative, and inflammatory stress in humans. Diabetes Care 2010; 33(11): 2416-23.

49. Núñez AC, Rentería IDCD, Villagómez MÁL, Elorza P, Martínez CEMS, Pulido SAA, et al. The use of coffee pulp as a potential alternative supplement in ruminant diets. Journal of Agricultural Science and Technology 2015; 5: 214-7.

50. Adam CL, Williams PA, Dalby MJ, Garden K, Thomson LM, Richardson AJ, et al. Different types of soluble fermentable dietary fibre decrease food intake, body weight gain and adiposity in young adult male rats. Nutrition & Metabolism 2014; 11: 36.

51. Andoh A. Physiological role of gut microbiota for maintaining human health. Digestion 2016; 93(3): 176-81.

52. Miyamoto J, Hasegawa S, Kasubuchi M, Ichimura A, Nakajima A, Kimura I. Nutritional signaling via free fatty acid receptors. International Journal of Molecular Sciences 2016; 17(4): 450.

53. Perrin P, Pierre F, Patry Y, Champ M, Berreur M, Pradal G, et al. Only fibres promoting a stable butyrate producing colonic ecosystem decrease the rate of aberrant crypt foci in rats. Gut 2001; 48(1): 53-61.

54. Kumar SA, Ward LC, Brown L. Inulin oligofructose attenuates metabolic syndrome in high-carbohydrate, high-fat diet-fed rats. British Journal of Nutrition 2016; 116(9): 1502-11.

55. Hussain MB. Role of Honey in Topical and Systemic Bacterial Infections. J Altern Complement Med 2018; 24(1): 15-24.

Chapter 5.

Spent coffee attenuates metabolic syndrome in diet-induced obese rats

Abstract

Spent coffee is the by-product of coffee brewing process containing chlorogenic acid, caffeine, trigonelline and melanoidins. However, limited data has been reported for its health benefits. In this study, we investigated responses to spent coffee in a diet-induced rat model of metabolic syndrome. 8-9 weeks old male Wistar rats were divided into four groups. Two groups of rats were fed on corn starch diet with normal drinking water while the other two groups were fed on high-carbohydrate, high-fat diet with 25% fructose in drinking water for 16 weeks. One group from each diet was supplemented with 5% dried spent coffee in the food for the last 8 weeks of protocol. High-carbohydrate, high-fat diet-fed rats developed symptoms of metabolic syndrome including abdominal obesity, impaired glucose tolerance, dyslipidaemia and cardiovascular and hepatic damages. Spent coffee intervention reduced body weight, abdominal fat pads, total body fat mass, systolic blood pressure and concentrations of plasma triglycerides and non-esterified fatty acids along with improvements in glucose tolerance, heart and liver structure and function. Spent coffee increased the diversity of gut microbiota and ratio between Bacteroidetes/Firmicutes. Thus, spent coffee has potential for further testing as a functional food in managing human obesity and metabolic syndrome.

Introduction

Coffee is one of the most consumed beverages and it is the second most important commodity of world trade after crude oil (1). Green coffee beans are an excellent source of compounds such as carbohydrates (59–61%), lipids (10–16%), proteins (10%), chlorogenic acid (7–10%), minerals (4%), caffeine (1–2%), trigonelline (1%) and free amino acids (<1%). The roasting process of green coffee beans causes significant loss of some bioactive compounds including carbohydrates, proteins, chlorogenic acid, free amino acids, lipids, minerals, caffeine and trigonelline (2). During the roasting process, melanoidins are formed as the products of Maillard reaction (3). Melanoidins are nitrogenous non-enzymatic browning compounds that consist of several components contributing to polymerisation reactions (4). The exact chemical structure of coffee melanoidins is still unknown due to variations based on temperature and chemical components present in the complex structure of melanoidins. A potential structure of coffee melanoidins has been provided using sugars, glycoside linkages, amino acids and phenolic compounds (5). Melanoidins may serve as a prebiotic as the melanoidins reach the colon without metabolism in the small intestine. In colon, melanoidins are fermented by gut microbiota, potentially supporting the growth of important gut microbiota (6, 7).

Following the coffee brewing process, spent grains are discarded as a by-product. This discarded spent coffee contributes to approximately 50% of the total coffee weight (8). Thus, a huge quantity of spent coffee is generated as waste during the making of coffee brew. Spent coffee consists of chlorogenic acid, caffeine, trigonelline and melanoidins and hence it may have potential against metabolic syndrome (9). Studies have shown the presence of other bioactive compounds such as diterpenes, lignins, cellulose,

hemicellulose and other polysaccharides in spent coffee (10, 11). The current applications of spent coffee include biodiesel production, as a precursor for the production of activated carbon, as compost and as a sorbent for metal ions removal (12, 13). Very few studies have demonstrated health benefits of spent coffee. Spent coffee exhibited free radical scavenging properties against oxidative stress developed as an outcome of mitochondrial dysfunction (14). Spent coffee restricted the inflammatory activity in J774A.1 mouse macrophage cell line and tumourigenesis activity in P388 leukaemia cell line (15). Melanoidins have previously shown antioxidant and anti-inflammatory activities along with some prebiotic actions on gut microbiota (16). Caffeine, trigonellne and chlorogenic acid found in spent coffee, have already shown potential against various metabolic diseases (17-22). Thus, this project identified the combined effects of these components with melanoidins as spent coffee is generated in large quantities throughout the world and currently is thrown away as a waste material.

The aim of this study was to investigate whether the spent coffee can reverse obesity-associated metabolic changes and changes in structure and function of the heart and liver in high-carbohydrate, high-fat diet-fed rats. High-carbohydrate, high-fat diet-fed rats demonstrated the symptoms of metabolic syndrome with metabolic abnormalities, cardiovascular remodelling and non-alcoholic steatohepatitis (23). Following spent coffee intervention for the last 8 weeks of the 16 week protocol, the structure and function of the heart were characterised through isolated Langendorff heart preparation and histopathology whereas the structure and function of the liver were characterised through histopathology and plasma biochemical studies. Metabolic changes were also characterised through measurement of glucose tolerance, plasma lipids and body

composition. Further, gut microbiota composition was analysed through diversity profiling of faeces isolated from rat colon.

Methods and materials

Spent coffee collection and storage

Spent coffee was collected from the Campus Catering People café at the University of Southern Queensland, Toowoomba, Australia in September 2016. Spent coffee was dried at 60°C until constant weight was achieved. Dried spent coffee was stored at 4°C in dry conditions until analysis of samples and use as dietary intervention.

Characterisation of spent coffee

Spent coffee was prepared in 3:2 ethanol:water mixture. Briefly, 1 g of spent coffee was dissolved in 50 mL of ethanol:water mixture, sonicated for 15 minutes and an aliquot of the supernatant was taken for analysis by HPLC using an Agilent 1100 series system coupled with a mass spectrometer for further peak confirmation or identification as required. The HPLC system consisted of a diode array detector (G4212B), binary pump (G4220A), an autosampler (G4226A), a vacuum degasser and a column oven with an MSD (G1946D) detector also present. The chromatography was performed on a Phenomenex luna C18 (2) HPLC column (100 x 4.6 mm) using a gradient method of water and acetonitrile with 0.005% trifluoroacetic acid over 28 minutes. The optimal solvent gradient for separation of target constituents started with 10% acetonitrile which was increased as a gradient to 30% acetonitrile over 10 minutes, then to 95% acetonitrile over 8 minutes, at a flow rate of 0.75 mL/minute and an injection volume of 5 µL. Calibration

standards of trigonelline, caffeine and chlorogenic acid were prepared in 60:40 ethanol:water, at concentrations from 0.01 to 1 mg/mL, 0.005 to 0.5 mg/mL and 0.004 to 1 mg/mL, respectively. Specific detection and calibration curves for each compound were performed at 254 nm, 280 nm and 330 nm, respectively. Quantification was performed using the Chemstation Software based on reference standards, peak area and sample dilution at specific wavelengths for each compound. Mr Peter Mouatt, Senior Analytical Officer from Southern Cross University, played a major role in the analysis of components.

Rats, diets and treatments

All experimental protocols were approved by the University of Southern Queensland Animal Ethics Committee under the guidelines of the National Health and Medical Research Council of Australia. Male Wistar rats (8–9 weeks old, 340 ± 1 g, n = 48) were obtained from Animal Resource Centre, Perth, Australia. Rats were divided into 4 groups for the 16 week feeding protocol: corn starch diet-fed rats (C; n = 12), corn starch diet + spent coffee (CSC, n = 12; 5% in food for the final 8 weeks); high-carbohydrate, high-fat diet-fed rats (H; n = 12), high-carbohydrate high-fat diet + spent coffee (HSC, n = 12; 5% in food for the final 8 weeks).

Corn starch diet contained 570 g cornstarch, 155 g powdered rat food, 25 g HMW salt mixture and 250 g water per kilogram of diet. High-carbohydrate, high-fat diet contained 175 g fructose, 395 g sweetened condensed milk, 200 g beef tallow, 155 g powdered rat food, 25 g HMW salt mixture and 50 g water per kilogram of diet. Drinking water with 25% (w/v) fructose was provided to H and HSC groups. C and CSC groups

were given normal drinking water. Rats were individually housed under temperature-controlled, 12-hour-light/dark conditions and given *ad libitum* access to food and water (23).

Physiological parameters

Body weight, food and water intakes were measured daily. Abdominal circumference and body length were measured using a standard measuring tape under light anaesthesia with Zoletil (tiletamine 10 mg/kg, zolazepam 10 mg/kg, intraperitoneal). Body mass index was calculated as body weight (in grams)/[body length (in cm)]^2. Feed efficiency was calculated as [mean body weight gain (in grams)/daily energy intake (in kJ)] (23).

Systolic blood pressure

Systolic blood pressure was measured under light anaesthesia with Zoletil (tiletamine 10 mg/kg, zolazepam 10 mg/kg, intraperitoneally), using an MLT1010 Piezo-Electric Pulse Transducer and inflatable tail-cuff connected to an MLT844 Physiological Pressure Transducer and PowerLab data acquisition unit (23).

Body composition measurement

Dual-energy X-ray absorptiometric measurements were carried out at the end of the protocol with a Norland XR46 DXA instrument (Norland Corp, Fort Atkinson, WI). These scans were evaluated using the manufacturer's suggested software for use in laboratory animals (Small Subject Analysis Software, version 2.5.3/1.3.1; Norland Corp)

(24). The precision error of lean mass for replicate measurements, with repositioning, is 3.2%.

Oral glucose tolerance test

Oral glucose tolerance tests were performed after determining overnight fasting blood glucose concentrations in tail vein blood using Medisense Precision Q.I.D. glucose meters. For overnight fasting, rats were deprived of food for 12 hours. Fructose-supplemented drinking water for H and HSC groups was replaced with normal drinking water for the overnight food deprivation period. Following basal blood glucose concentration measurement, rats were administered 2 g/kg body weight of glucose as 40% glucose solution via oral gavage. Blood glucose concentrations were measured 30, 60, 90 and 120 minutes after oral glucose administration (23).

Metabolic rate assessment

Indirect calorimetry was applied to determine oxygen consumption and carbon dioxide production using a 4-chamber OxyMax system (Columbus Instruments, Columbus, OH) with one rat per chamber. Rats were given *ad libitum* access to food and water during the measurement. Oxygen consumption and carbon dioxide production were measured individually from each chamber. The respiratory exchange ratio was evaluated by OxyMax software (v. 4.86). Energy expenditure was determined by assessment of the exchange of oxygen for carbon dioxide that occurs during the metabolic processing of food (25).

Terminal experiments

Rats were euthanased with Lethabarb (pentobarbitone sodium, 100 mg/kg, intraperitoneally). After euthanasia, heparin (~200 IU) was injected into the right femoral vein. The abdomen was then opened and blood (~5 mL) was withdrawn from the abdominal aorta and collected into heparinised tubes. Blood was centrifuged at 5000 × *g* for 10 minutes to obtain plasma. Plasma was stored at -20°C for further characterisation. Hearts were then removed from rats for isolated Langendorff heart preparation (23).

Isolated Langendorff heart preparation

Hearts isolated from euthanased rats were perfused with modified Krebs–Henseleit bicarbonate buffer 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 per minute using an electrical stimulator. End-diastolic pressures were obtained from 0 to 30 mmHg for the calculation of diastolic stiffness constant (κ, dimensionless) (23).

Vascular reactivity

Thoracic aortic rings (~4 mm in length) were suspended in an organ bath filled with Tyrode physiological salt solution bubbled with 95% O_2–5% CO_2 maintained at 35°C and allowed to stabilise at a resting tension of ~10 mN. Cumulative concentration–response curves (contraction) were obtained for noradrenaline (1×10^{-9} – 3×10^{-5} M) and cumulative concentration–response curves (relaxation) were obtained for acetylcholine

(1×10^{-9} – 3×10^{-5} M) and sodium nitroprusside (1×10^{-9} – 3×10^{-5} M) after submaximal (~70%) contraction to noradrenaline (23).

Analysis of intestinal transit

Rats from each diet group were orally gavaged with 3 mL of 0.05% phenol red solution 20 minutes before euthanasia. After euthanasia, the entire region from the stomach to small intestine was removed from its mesenteric attachment immediately. The length of small intestine was measured from the pyloric sphincter to the ileo-caecal junction. The endpoint of phenol red transit in small intestine was visualised by adding a few drops of 0.5 M NaOH. The intestinal transit for each rat was determined by following formula (26):

Intestinal transit (%) = (the total distance traveled by phenol red solution/ total length of small intestine) × 100

Organ weights

After isolated heart perfusion studies, the heart was separated into left ventricle (with septum) and right ventricle and weighed. The liver was isolated and weighed. Retroperitoneal, epididymal and omental fat contents were removed separately and weighed. These organ weights were normalised against tibial length at the time of organ removal and expressed as mg/mm of tibial length (23).

Histology

Hearts and livers were removed from the rats soon after euthanasia and fixed in 10% neutral buffered formalin. These samples were then dehydrated and embedded in paraffin wax. Thin sections (5 μm) were cut and stained with haematoxylin and eosin to study infiltration of inflammatory cells (heart and liver) and fat deposition (liver) and with picrosirius red (heart) to study collagen deposition (23).

Plasma biochemistry

Plasma activities of aspartate transaminase, alanine transaminase and alkaline phosphatase, and plasma concentrations of total cholesterol, triglycerides and non-esterified fatty acids were measured (23).

Gut microbiota diversity profiling

Immediately following euthanasia and organ removal, two to three fecal pellets were collected from the colon of rats and stored at –80°C in nuclease-free tubes. Diversity profiling was performed as described previously (25). In brief, the V3-V4 region of the 16S rRNA gene was chosen for amplification. The primers applied were F341 (5′-CCTAYGGGRBGCASCAG-3′) and R806 (5′-GGACTACNNGGGTATCTAAT-3′). PCR amplicons were created using AmpliTaq Gold 360 mastermix (Life Technologies, Scoresby, VIC, Australia) for the primary PCR. A secondary PCR to index the amplicons was operated with TaKaRa Taq DNA Polymerase (Clontech, Mountain View, CA, USA). The subsequent amplicons were measured by fluorometry (Invitrogen Picogreen, Mount Waverley, VIC, Australia) and normalized. The equimolar pool was then calculated by

qPCR (KAPA) followed by sequencing on the Illumina MiSeq (San Diego, CA, USA) with 2 x 300 base pairs paired-end chemistry.

Paired-ends reads were assembled by aligning the forward and reverse reads using PEAR (version 0.9.5) (27). Primers were detected and trimmed. Trimmed sequences were processed using Quantitative Insights into Microbial Ecology (QIIME 1.8) (28) USEARCH (version 7.1.1090) (29, 30) and UPARSE software (31). Using USEARCH, sequences were quality filtered, full length duplicate sequences were eliminated and organized by abundance. Singletons or unique reads in the data set were discarded. Sequences were clustered followed by chimera filtered using "rdp_gold" database as the reference. To obtain the number of reads in each OTU, reads were mapped back to OTUs with a minimum identity of 97%. Using QIIME, taxonomy was assigned using Greengenes database (version 13_8, Aug 2013) (32).

A heat map was constructed using R statistical software according to the developer's instructions to visualise the relative abundance of each bacterial species and their respective phyla.

Statistical analysis

All data are presented as mean ± SEM. Results were tested for variance using Bartlett's test and variables that were not normally distributed were transformed (using log 10 functions) prior to statistical analyses. C, CSC, H and HSC groups were tested for effects of diet, treatment and their interactions by two-way analysis of variance. When the interaction and/or the main effects were significant, means were compared using Newman-Keuls multiple comparison *post hoc* test. A *P* value of less than 0.05 was

considered significant. All statistical analyses were performed using GraphPad Prism version 5.0 for Windows (San Diego, CA, USA).

Results

Spent coffee composition

Spent coffee contained caffeine (7 mg/g *w/w*), chlorogenic acid (4 mg/g *w/w*) and trigonelline (1.3 mg/g *w/w*). The average intake of these compounds over the 8-16 week period was in CSC rats was caffeine (41.9 ± 0.5 mg/kg/day), chlorogenic acid (8.9 ± 0.1 mg/kg/day) and trigonelline (12.1 ± 0.2 mg/kg/day) and in HSC rats was caffeine (17.7 ± 0.4 mg/kg/day), chlorogenic acid (3.8 ± 0.1 mg/kg/day) and trigonelline (5.1 ± 0.1 mg/kg/day).

Dietary intake, body composition and plasma biochemistry

Spent coffee treatment in CSC rats did not change body weight even after increasing food, water and energy intakes compared to C rats. Thus, feed efficiency and body mass index were unchanged between C and CSC rats (Table 1). Abdominal circumference and abdominal fat pads did not change between C and CSC rats while basal blood glucose concentrations and area under the curve were reduced with spent coffee treatment in CSC rats compared to C rats (Table 1). Whole-body lean mass, whole-body fat mass, bone mineral content and bone mineral density did not change between C and CSC rats (Table 1). Spent coffee did not change plasma concentrations of total cholesterol, triglycerides and non-esterified fatty acids in CSC rats compared to C rats (Table 1). Respiratory exchange ratio and heat produced did not change between C and CSC rats (Table 1).

Spent coffee treatment in HSC rats decreased body weight even after increasing food, water and energy intakes compared to H rats. Thus, feed efficiency and body mass index were decreased in HSC rats compared to H rats (Table 1). Abdominal circumference, abdominal fat pads, basal blood glucose concentrations and area under the curve were reduced in HSC rats compared to H rats (Table 1). Whole-body lean mass, bone mineral content, bone mineral density, respiratory exchange ratio and heat produced did not change between H and HSC rats while whole body fat mass was reduced in HSC rats compared to H rats (Table 1). Spent coffee did not change plasma concentrations of total cholesterol but reduced plasma concentrations of triglycerides and non-esterified fatty acids in HSC rats compared to H rats (Table 1).

Cardiovascular and hepatic function

In CSC rats, systolic blood pressure and left ventricular diastolic stiffness constant did not change with spent coffee treatment compared to C rats (Table 2). Spent coffee treatment did not induce inflammation or fibrosis in hearts of CSC rats (Figure 1B and 1F) compared to C rats (Figure 1A and 1E). Spent coffee did not change aortic responses to noradrenaline, sodium nitroprusside or acetylcholine in CSC rats compared to C rats (Figure 2A, 2B and 2C). Left ventricular wet weight decreased while right ventricular wet weights were unchanged in CSC rats compared to C rats (Table 2). Liver wet weight, and plasma activities of aspartate transaminase and alanine transaminase were unchanged between C and CSC rats (Table 2). Spent coffee did not induce inflammation and fat deposition in livers of CSC rats (Figure 3B and 3F) compared to C rats (Figure 3A and 3E).

In HSC rats, systolic blood pressure and left ventricular diastolic stiffness constant decreased with spent coffee treatment compared to H rats (Table 2). Spent coffee treatment reduced inflammation and fibrosis in hearts of HSC rats (Figure 1D and 1H) compared to H rats (Figure 1C and 1G). Spent coffee did not change aortic responses to noradrenaline, sodium nitroprusside or acetylcholine in HSC rats compared to H rats (Figure 2A, 2B and 2C). Left ventricular wet weight decreased while right ventricular wet weight was unchanged in HSC rats compared to H rats (Table 2). Liver wet weight decreased while plasma activities of aspartate transaminase and alanine transaminase were unchanged between H and HSC rats (Table 2). Spent coffee reduced inflammation and fat deposition in livers of HSC rats (Figure 3D and 3H) compared to H rats (Figure 3C and 3G).

Table 1. Response to spent coffee on physiological and metabolic variables

Variables	**C**	**CSC**	**H**	**HSC**	***P***		
					Diet	**Spent coffee**	**Diet × spent coffee**
Initial body weight, g	340 ± 1	341 ± 1	339 ± 1	339 ± 1	0.13	0.61	0.61
Final body weight, g	383 ± 2^{c}	374 ± 5^{c}	555 ± 9^{a}	481 ± 12^{b}	<0.0001	<0.0001	<0.0001
Food intake, g/day	35.7 ± 0.6^{c}	44.7 ± 0.4^{a}	26.0 ± 0.4^{d}	36.7 ± 0.6^{b}	<0.0001	<0.0001	0.01
Water intake, g/day	26.8 ± 0.4^{c}	34.6 ± 0.4^{b}	27.7 ± 0.5^{c}	36.5 ± 0.5^{a}	<0.0001	<0.0001	0.01
Energy intake, kJ/day	378 ± 8^{d}	443 ± 7^{c}	558 ± 7^{b}	640 ± 10^{a}	<0.0001	<0.0001	0.30
Feed efficiency, kJ/g	0.11 ± 0.01^{c}	0.07 ± 0.01^{c}	0.39 ± 0.02^{a}	0.22 ± 0.03^{b}	<0.0001	<0.0001	0.002
BMI, g/cm^{2}	0.61 ± 0.01^{c}	0.63 ± 0.01^{c}	0.83 ± 0.02^{a}	0.76 ± 0.02^{b}	<0.0001	0.13	0.01
Abdominal circumference, cm	18.4 ± 0.2^{c}	18.8 ± 0.2^{c}	23.2 ± 0.4^{a}	21.5 ± 0.2^{b}	<0.0001	0.015	0.0002

Basal blood glucose concentrations, mmol/L	3.9 ± 0.2^{b}	2.6 ± 0.1^{d}	4.9 ± 0.3^{a}	3.3 ± 0.1^{c}	0.0002	<0.0001	0.47
Area under the curve, mmol/L·minutes	692 ± 28^{b}	400 ± 10^{c}	790 ± 23^{a}	682 ± 15^{b}	<0.0001	<0.0001	<0.0001
Retroperitoneal fat, mg/mm	182 ± 21^{c}	165 ± 13^{c}	534 ± 53^{a}	404 ± 33^{b}	<0.0001	0.04	0.10
Epididymal fat, mg/mm	90 ± 8^{c}	66 ± 8^{c}	222 ± 17^{a}	142 ± 16^{b}	<0.0001	0.0003	0.04
Omental fat, mg/mm	130 ± 8^{c}	108 ± 3^{c}	263 ± 17^{a}	221 ± 20^{b}	<0.0001	0.031	0.49
Total abdominal fat, mg/mm	402 ± 30^{c}	339 ± 21^{c}	1019 ± 81^{a}	767 ± 63^{b}	<0.0001	0.007	0.091
Whole-body lean mass, g	283 ± 5	308 ± 6	286 ± 11	309 ± 8	0.80	0.052	0.90
Whole-body fat mass, g	83 ± 7^{c}	49 ± 4^{c}	235 ± 17^{a}	180 ± 21^{b}	<0.0001	0.002	0.45
Bone mineral content, g	11.8 ± 0.4^{b}	11.4 ± 0.2^{b}	17.4 ± 0.6^{a}	15.9 ± 0.8^{a}	<0.0001	0.08	0.31
Bone mineral density, g/cm^2	0.172 ± 0.002^{b}	0.175 ± 0.002^{b}	0.185 ± 0.002^{a}	0.188 ± 0.003^{a}	<0.0001	0.19	1.00
Respiratory exchange ratio	1.01 ± 0.01^{a}	1.01 ± 0.01^{a}	0.89 ± 0.01^{b}	0.88 ± 0.02^{b}	<0.0001	0.70	0.70
Heat, kJ	3.36 ± 0.17^{bc}	2.94 ± 0.09^{c}	4.14 ± 0.10^{a}	3.61 ± 0.19^{b}	<0.0001	0.003	0.70

Plasma total cholesterol, mmol/L	1.84 ± 0.08	1.85 ± 0.06	1.71 ± 0.10	1.92 ± 0.08	0.72	0.20	0.24
Plasma triglycerides, mmol/L	0.57 ± 0.05^{c}	0.45 ± 0.05^{c}	1.17 ± 0.10^{a}	0.81 ± 0.1^{b}	<0.0001	0.004	0.12
Plasma non-esterified fatty acids, mmol/L	1.37 ± 0.16^{bc}	0.94 ± 0.15^{c}	3.57 ± 0.18^{a}	1.76 ± 0.18^{b}	<0.0001	<0.0001	0.0002

Values are mean ± SEM, n = 8–10. Means in a row with superscripts without a common letter differ significantly, $P < 0.05$. C, corn starch diet-fed rats; CSC, corn starch diet-fed rats treated with spent coffee; H, high-carbohydrate, high-fat diet-fed rats; HSC, high-carbohydrate, high-fat diet-fed rats treated with spent coffee.

Table 2. Effects of spent coffee on cardiovascular and liver function

Variable	C	CCR	H	HCR	*p*		
					Diet	Spent coffee	Diet × Spent coffee
Systolic blood pressure, mmHg	125 ± 1^{b}	127 ± 2^{b}	145 ± 2^{a}	127 ± 2^{b}	<0.0001	<0.0001	<0.0001
Left ventricular diastolic stiffness constant (κ)	20.7 ± 0.4^{b}	20.8 ± 0.4^{b}	26.7 ± 1.3^{a}	20.8 ± 0.2^{b}	0.0002	0.0002	0.0002
Left ventricle + septum wet weight, mg/mm	21.6 ± 0.8^{a}	19.0 ± 1.3^{b}	24.7 ± 1.5^{a}	19.5 ± 1.2^{b}	0.16	0.004	0.30
Right ventricle wet weight, mg/mm	4.97 ± 0.38	4.91 ± 0.78	5.64 ± 0.52	6.02 ± 0.76	0.15	0.80	0.72
Liver wet weight, mg/mm	230 ± 6^{c}	245 ± 10^{c}	336 ± 13^{a}	293 ± 10^{b}	<0.0001	0.17	0.01
Plasma aspartate transaminase, U/L	79.1 ± 4.8	80.7 ± 3.0	82.2 ± 4.4	81.1 ± 5.7	0.73	0.96	0.79
Plasma alanine transaminase, U/L	27.7 ± 1.8^{ab}	24.7 ± 1.9^{b}	35.2 ± 3.2^{a}	29.1 ± 1.8^{ab}	0.02	0.07	0.52

Values are mean ± SEM, n = 8–10. Means in a row with superscripts without a common letter differ significantly, $P < 0.05$. C, corn starch diet-fed rats; CSC, corn starch diet-fed rats treated with spent coffee; H, high-carbohydrate, high-fat diet-fed rats; HSC, high-carbohydrate, high-fat diet-fed rats treated with spent coffee.

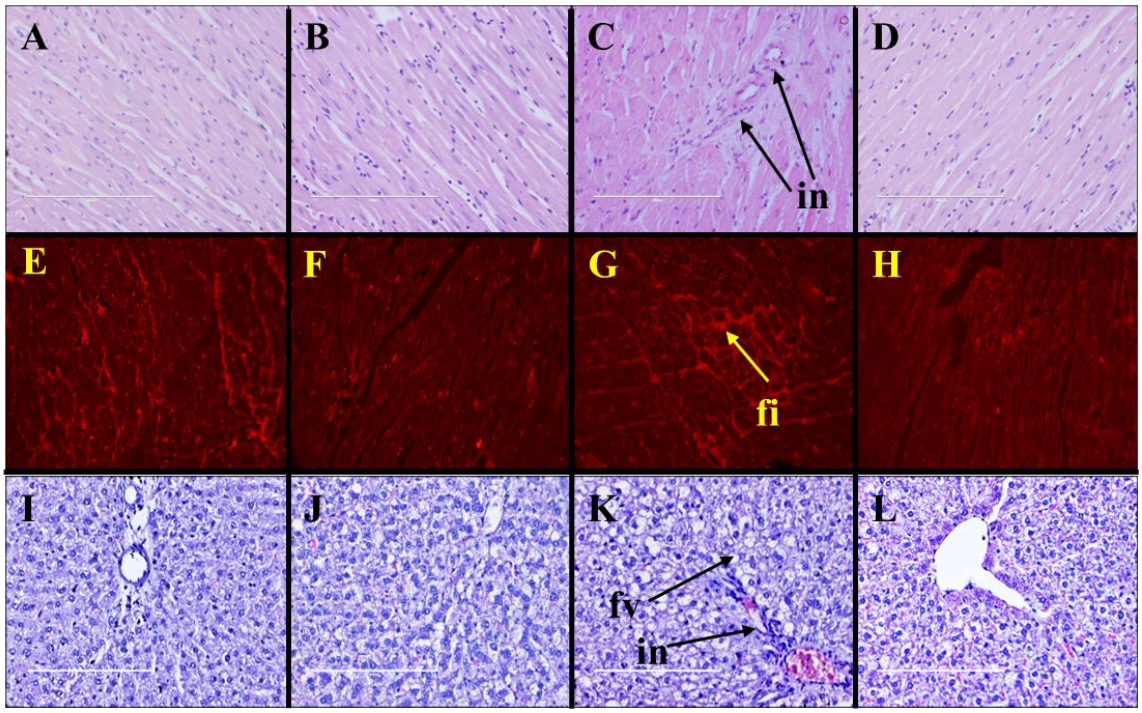


Figure 1. Effects of spent coffee in the heart and liver. H&E staining showing infiltration of inflammatory cells (in) and enlarged fat vacuoles (fv) in heart (A-D) from C (A), CSC (B), H (C), and HSC (D) and liver (I-L) from C (I), CSC (J), H (K) and HSC (L) (×20). Picrosirius red staining showing fibrosis (fi) in heart (E-H) from C (E), CSC (F), H (G), and HSC (H) (×20). C, corn starch diet-fed rats; CSC, corn starch diet-fed rats treated with spent coffee; H, high-carbohydrate, high-fat diet-fed rats; HSC, high-carbohydrate, high-fat diet-fed rats treated with spent coffee.

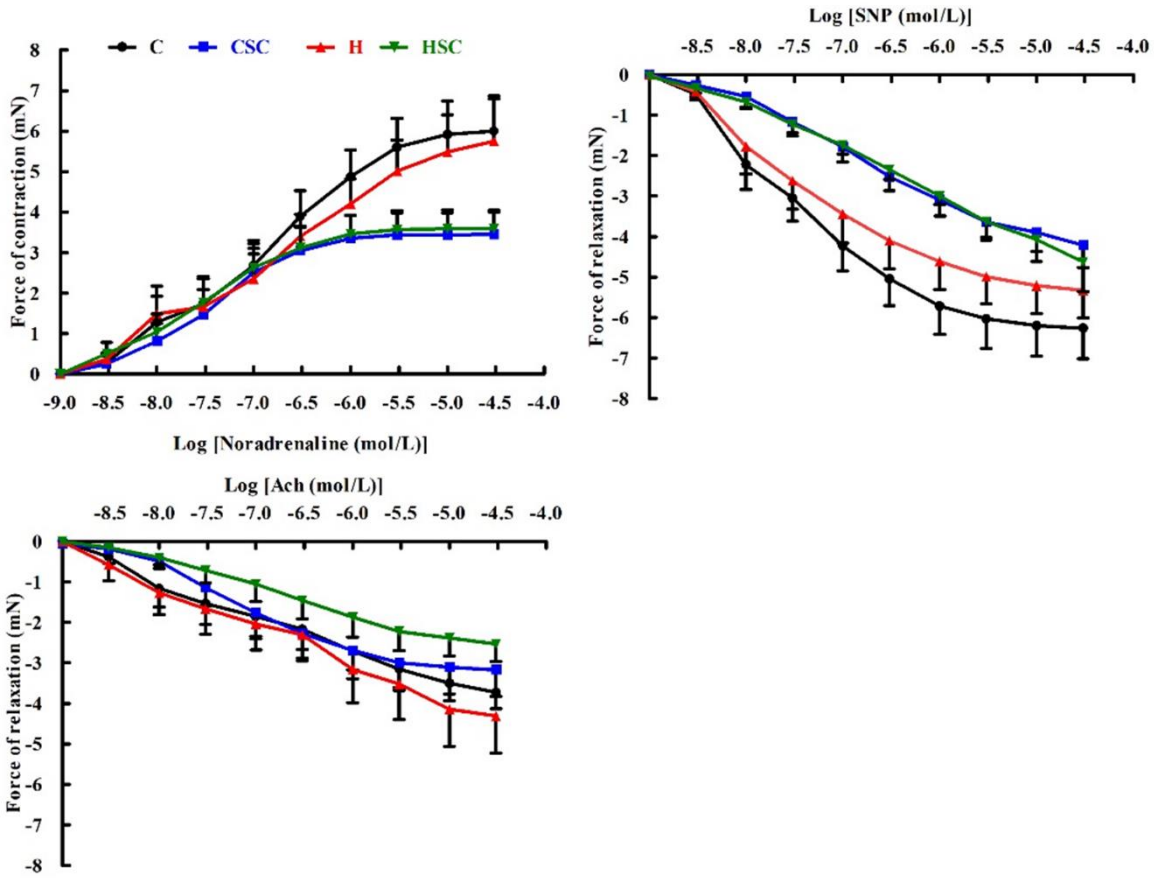


Figure 2. Effects of spent coffee on noradrenaline-induced contraction (A), sodium nitroprusside-induced relaxation (B) and acetylcholine-induced relaxation (C) in thoracic aorta ring, prepared from C, CSC, H and HSC rats. Values are mean ± SEM, n = 6-10. End-point means without a common letter differ, $P<0.05$. C, corn starch diet-fed rats; CSC, corn starch diet-fed rats treated with spent coffee; H, high-carbohydrate, high-fat diet-fed rats; HSC, high-carbohydrate, high-fat diet-fed rats treated with spent coffee.

Gut microbiome

Figure 3 shows the heat map of bacterial species abundance. In all groups, the major gastrointestinal bacterial phyla, Firmicutes and Bacteroidetes, were predominant (Figure 4). Gut bacterial composition of groups are shown in Figure 4. HSC rats had higher abundance of Bacteroidetes compared to H rats while it did not change between C and CSC rats. Conversely, abundance of Firmicutes remained unchanged among all diet groups (Figure 5A). Spent coffee increased the Bacteroidetes/Firmicutes ratio in CSC and HSC rats compared to C and H rats respectively (Figure 5B). Based on the Shannon diversity index, there was an increase in diversity between the treatment groups CSC and HSC rats compared to control groups C and H rats. (Figure 5C).

The relative abundance of all species was compared between the treatment and control groups to evaluate the effect of spent coffee at species level. A cut-off point of 1% abundance in C rats was applied to enhance confidence. The abundance of two species from phylum Bacteroidetes (Bacteroides sp., and an unspecified species from the S24-7 family) was increased and two species from phylum Firmicutes (Turicibacter sp. and an unspecified species from Clostridiaceae family) was decreased by spent coffee (Figure 5D). Compared to C rats, there was significant increase in Bacteroides sp without changing in the S24-7 family species, Turicibacter sp. and an unspecified species from Clostridiaceae family in CSC rats. HSC rats had higher abundance of Bacteroides sp., S24-7 family species, Turicibacter sp. and an unspecified species from Clostridiaceae family in compared to H rats (Figure 5D).

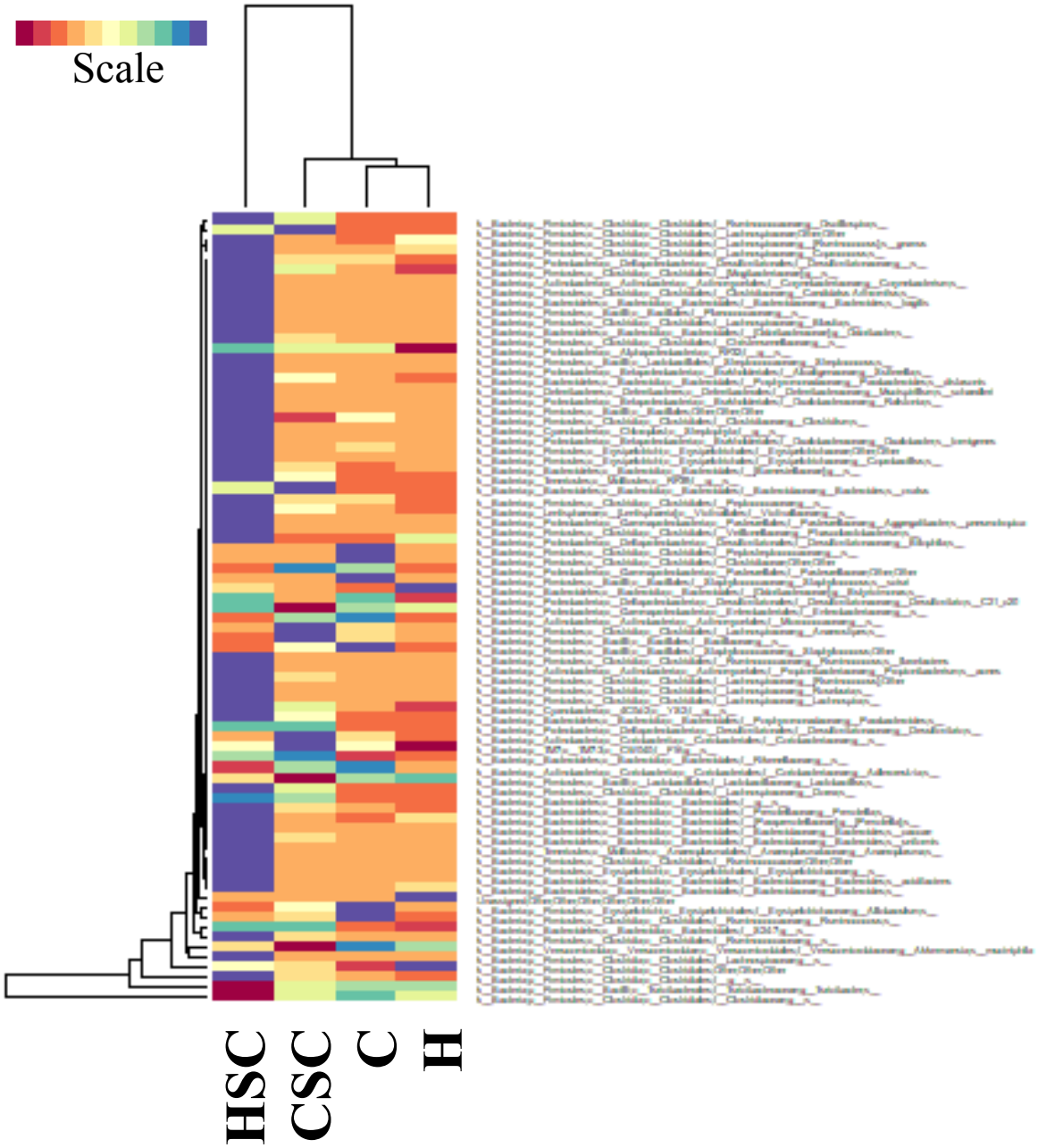


Figure 3. Heat map of bacterial species abundance. Abundance values for each species were plotted as a percentile with the most abundant species represented by violet, the 50 percentile species represented with yellow and the lowest value with dark brown; C, corn starch diet-fed rats; CSC, corn starch diet-fed rats treated with spent coffee; H, high-

carbohydrate, high-fat diet-fed rats; HSC, high-carbohydrate, high-fat diet-fed rats treated with spent coffee.

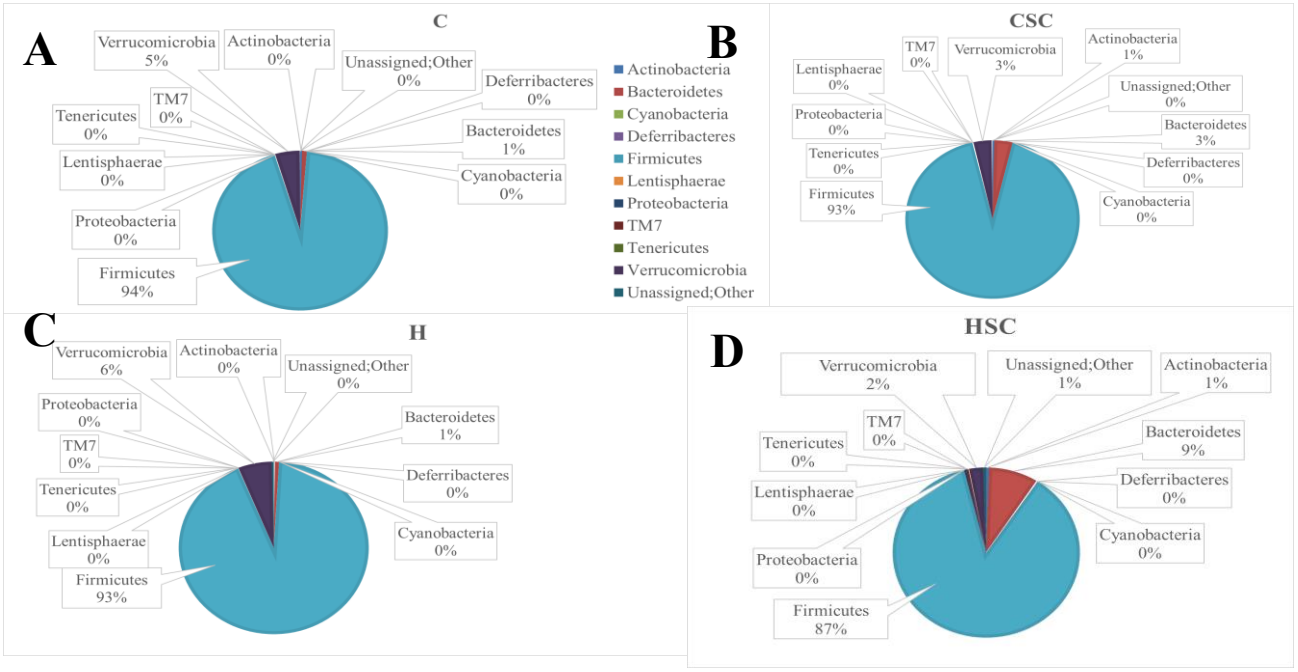


Figure 4. Effect of spent coffee on gut microbiota diversity profiles. Alpha diversity with the diet-group. The relative abundance of each phylum presented as a percentage of the total population for each treatment group. C, corn starch diet-fed rats; CSC, corn starch diet-fed rats treated with spent coffee; H, high-carbohydrate, high-fat diet-fed rats; HSC, high-carbohydrate, high-fat diet-fed rats treated with spent coffee.

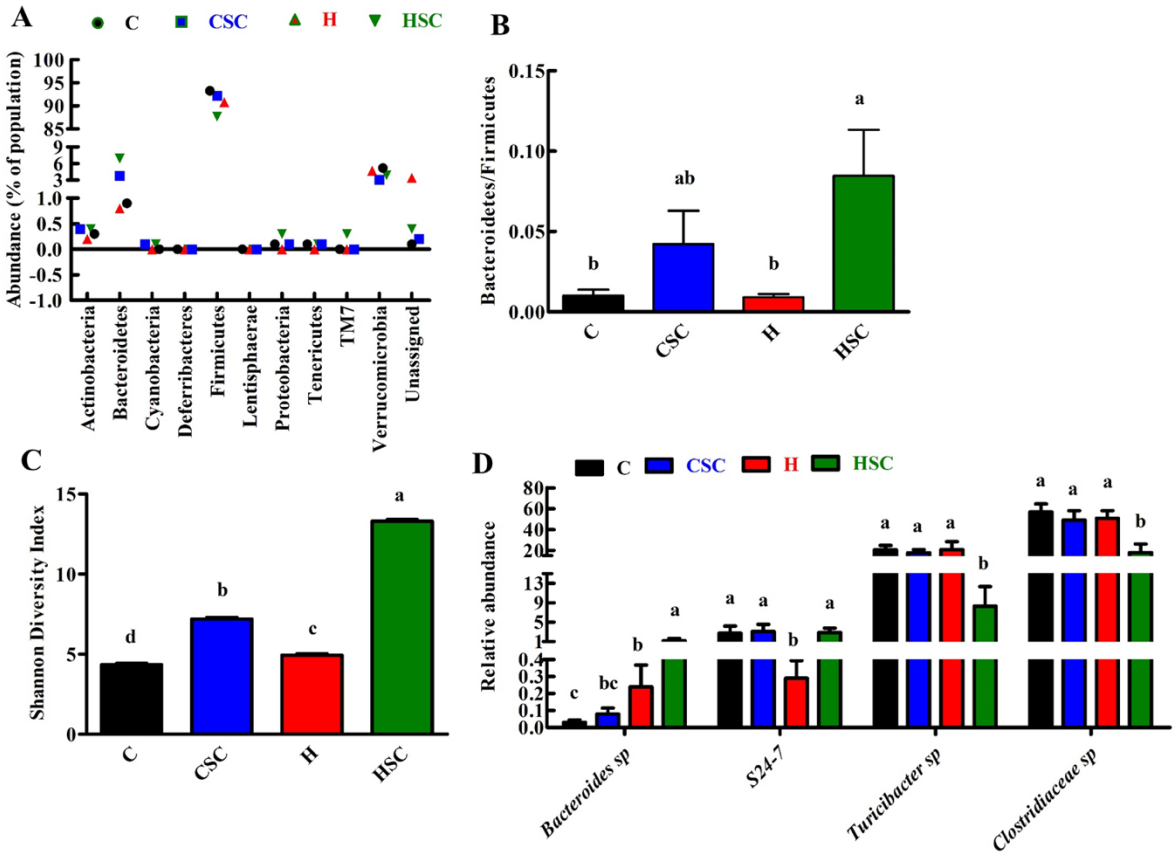


Figure 5. Effect of spent coffee on gut microbiota diversity profiles. A: The relative abundance of each phylum presented as a percentage of the total population for each treatment group. B: Bacteroidetes/Firmicutes ratio. C. Shannon diversity index. The index was determined from the means of abundance for each treatment group. D: Relative abundance of species that were differentially regulated by diet. Only species whose mean abundance for the C diet group was equal to or higher than 1% were plotted in order to enhance confidence. C, corn starch-rich diet-fed rats; CSC, corn starch-rich diet-fed rats treated with spent coffee; H, high-carbohydrate, high-fat diet-fed rats; HSC, high-carbohydrate, high-fat diet-fed rats treated with spent coffee..

Discussion

In this study, spent coffee that is usually discarded after brewing was used as the possible intervention in diet-induced metabolic syndrome in rats. Spent coffee decreased abdominal obesity, systolic blood pressure, plasma triglycerides and plasma non-esterified fatty acids along with improvements in glucose tolerance, cardiovascular and hepatic structure and function in diet-induced obese rat. In our previous studies, we reported that coffee extract (33) and caffeine (34), improved glucose tolerance and cardiovascular structure and function. In these studies, caffeine decreased obesity while coffee extract failed to decrease obesity. Caffeine, through its inhibitory effects on A_1R adenosine receptors (34, 35), decreased obesity while coffee extract was unable to decrease obesity.

Spent coffee contained significant amounts of caffeine, chlorogenic acid and trigonelline, thus suggesting potential of these components in attenuating obesity and metabolic syndrome. However, the dose of caffeine in obese rats (~18 mg/kg/day) was lower than the dose of the caffeine in our previous study (~28 mg/kg/day) (34). This suggests that these 3 components may play a role in attenuating metabolic syndrome but are not the component causing significant response. Spent coffee has also been shown to contain melanoidins, large molecular complexes that still requires further work for elucidating their structure. Roasting process of coffee beans reduced chlorogenic acid (36) while producing melanoidins (37). Melanoidins are formed as the product of Maillard reactions during roasting process using amino acids, polysaccharides and phenolic acids such as chlorogenic acid (5, 38). Similarly, spent coffee includes melanoidins with polysaccharides such as arabinogalactans and galactomannans, which have been shown to have prebiotic activity (39). Melanoidins from roasted coffee have shown several

biological activities such as antioxidant (40), anti-inflammatory (41) and antihypertensive (42). Coffee melanoidins are metabolised by the gut microbiota and produces fermentation products in the colon (43). Previous studies have concluded that coffee melanoidins, through their prebiotic activity, produced acetate and propionate from polysaccharides including galactomannans and arabinogalactans (44, 45). These fermentation products may have a role in improving the quality of gut microbiota. Previous *in vitro* studies have supported the improvements in gut microbiota with the use of melanoidins (46). In this study, we have identified the role of melanoidins from spent coffee in improving the gut microbiota in obese rats thus attenuating metabolic complications. Gut microbiota diversity has increased in spent coffee-treated group of rats. The changes observed in gut microbiota at the species level included increased Bacteroidetes species - *Bacteroides sp, S24-7*, and decreased Firmicutes species - *Turicibacter sp, and Clostridiaceae sp* in spent coffee-treated group of H rats. Obesity is associated with the modulation of gut microbiota where free fatty acids promotes growth of Firmicutes and consequently reduces Bacteroidetes count (47). The positive modulation in gut microbiota by coffee pulp treatment could be associated with the dietary fibres present in coffee pulp as dietary fibres have been reported for delivering health beneficial (48). Dietary fibre improved the water content of faeces, decreased intestinal transit time and shifted the composition of the gut microbiota positively and enhanced the concentration of short-chain fatty acids in the faeces of mice (48). Thus, fibre from spent coffee could be considered as a prebiotic which could be possible reason for the positive modulation in gut microbiota. Further, this study with spend coffee showed reduction in blood lipids and these changes were not seen in any of our previous studies with coffee (33) or caffeine (34).

Spent coffee, generated in large amounts throughout the world on a daily basis, has served as a functional food against diet-induced obesity in rats in this study. Spent coffee reduced decreased systolic blood pressure as found in our previous study with coffee extract and caffeine (33, 34). This may suggest a role of caffeine and other phenolic acids such as chlorogenic acid in causing these effects. Further, spent coffee also improved glucose tolerance in treatment groups. This study supported our previous studies where coffee (49) and caffeine (34) diet-supplements improved glucose tolerance and insulin sensitivity. Spent coffee still includes an appreciable amount of caffeine. Caffeine induced thermogenesis effects that facilitate energy expenditure and weight management (50). Moreover, the combination of caffeine and chlorogenic acid produced synergistic effects against lipogenesis in mice where caffeine + chlorogenic acid was found to reduce serum and hepatic total cholesterol and triglycerides concentrations (51). This synergetic effect occurs by inhibition of FAS and down-regulation of FAS protein in liver as well as upregulation of AMPK protein expression in adipose tissue (51). Thus, the higher energy expenditure triggered by synergistic effects of caffeine and chlorogenic acid could be the possible reason in our study where elevated food intake delivered the reduction in body weight, feed efficiency and whole-body fat mass.

A high-fat diet is associated with inflammation and modulating energy homeostasis, particularly diets rich in saturated fatty acids (C14:0, C16:0 and C18:0), and caused higher availability of free fatty acids that serve as a ligand to TLR4 (52). Activated TLRs trigger phosphorylation of JNK and binding of activator protein-1 that serve as a transcriptional activator of pro-inflammatory cytokines such as TNF, MCP-1, IFN-γ and IL-6 (53). Thus, we speculated that spent coffee may reduce activation of TLR4-mediated

signalling pathway and accordingly prevented the expression of pro-inflammatory cytokines, confirmed by reduced inflammation in heart and liver. The phosphorylated JNK inhibits IRS-1 by phosphorylating its serine unit. Phosphorylated IRS-1 prevents GLUT4 translocation resulting in insulin resistance state (54). In this study, spent coffee may have improved glucose tolerance by reducing JNK activation and stimulating GLUT4 translocation leading to decreased basal blood glucose concentration and improved glucose response at the end of glucose tolerance test.

Spent coffee improved lipid profile by reducing triglycerides and non-esterified fatty acids in plasma. Increased concentrations of these lipid components are associated with development of obesity, cardiovascular complications and fatty liver (55). Reduction in the lipid components in the blood may suggest the role of spent coffee in improving overall metabolic status thus contributing towards the improvement in metabolic syndrome-associated complications. These improvements in the metabolic status can be linked to the improved gut microbiota observed with the spent coffee treatment. This is the first study to demonstrate the potential of spent coffee in decreasing obesity, and improving obesity-related complication. As we are throwing away useful bioactive components that are present in spent coffee, this is an important finding to warrant future human studies for using food wastes in improving human metabolic health, and so possibly decrease the incidence of obesity in the world.

References

1. Yeretzian C, Jordan A, Badoud R, Lindinger W. From the green bean to the cup of coffee: Investigating coffee roasting by on-line monitoring of volatiles. European Food Research and Technology. 2002;214(2):92-104.

2. Illy A, Viani R. Espresso coffee: The science of quality: Academic Press; 2005.

3. Ludwig IA, Clifford MN, Lean ME, Ashihara H, Crozier A. Coffee: Biochemistry and potential impact on health. Food Function. 2014;5(8):1695-717.

4. Opitz SEW, Goodman BA, Keller M, Smrke S, Wellinger M, Schenker S, et al. Understanding the effects of roasting on antioxidant components of coffee brews by coupling on-line abts assay to high performance size exclusion chromatography. Phytochemical Analysis. 2017;28(2):106-14.

5. Moreira ASP, Nunes FM, Domingues MR, Coimbra MA. Coffee melanoidins: Structures, mechanisms of formation and potential health impacts. Food Function. 2012;3(9):903-15.

6. Tagliazucchi D, Bellesia A. The gastro-intestinal tract as the major site of biological action of dietary melanoidins. Amino Acids. 2015;47(6):1077-89.

7. Borrelli RC, Fogliano V. Bread crust melanoldins as potential prebiotic ingredients. Molecular Nutrition & Food Research. 2005;49(7):673-8.

8. Tsai W-T, Liu S-C, Hsieh C-H. Preparation and fuel properties of biochars from the pyrolysis of exhausted coffee residue. Journal of Analytical and Applied Pyrolysis. 2012;93:63-7.

9. Jiménez-Zamora A, Pastoriza S, Rufián-Henares JA. Revalorization of coffee by-products. Prebiotic, antimicrobial and antioxidant properties. LWT-Food Science and Technology. 2015;61(1):12-8.

10. Tian T, Freeman S, Corey M, German JB, Barile D. Chemical characterization of potentially prebiotic oligosaccharides in brewed coffee and spent coffee grounds. Journal of Agricultural and Food Chemistry. 2017;65(13):2784-92.

11. Clifford M. Chemical and physical aspects of green coffee and coffee products. Coffee: Springer; 1985. p. 305-74.

12. Mussatto SI, Machado EM, Martins S, Teixeira JA. Production, composition, and application of coffee and its industrial residues. Food and Bioprocess Technology. 2011;4(5):661.

13. Pujol D, Liu C, Gominho J, Olivella M, Fiol N, Villaescusa I, et al. The chemical composition of exhausted coffee waste. Industrial Crops and Products. 2013;50:423-9.

14. Bravo J, Arbillaga L, de Pena MP, Cid C. Antioxidant and genoprotective effects of spent coffee extracts in human cells. Food Chem Toxicol. 2013;60:397-403.

15. Ramalakshmi K, Rao LJM, Takano-Ishikawa Y, Goto M. Bioactivities of low-grade green coffee and spent coffee in different in vitro model systems. Food Chemistry. 2009;115(1):79-85.

16. Mesías M, Delgado-Andrade C. Melanoidins as a potential functional food ingredient. Current Opinion in Food Science. 2017;14(Supplement C):37-42.

17. Harland BF. Caffeine and nutrition. Nutrition. 2000;16(7-8):522-6.

18. Dorea JG, da Costa THM. Is coffee a functional food? British Journal of Nutrition. 2005;93(6):773-82.

19. Dai J, Mumper RJ. Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. Molecules. 2010;15(10):7313-52.

20. Wu CM, Luan H, Zhang X, Wang S, Zhang XP, Sun XB, et al. Chlorogenic acid protects against atherosclerosis in apoe(-/-) mice and promotes cholesterol efflux from raw264.7 macrophages. Plos One. 2014;9(9).

21. Ong KW, Hsu A, Tan BKH. Chlorogenic acid stimulates glucose transport in skeletal muscle via ampk activation: A contributor to the beneficial effects of coffee on diabetes. Plos One. 2012;7(3).

22. Ghadieh HE, Smiley ZN, Kopfman MW, Najjar MG, Hake MJ, Najjar SM. Chlorogenic acid/chromium supplement rescues diet-induced insulin resistance and obesity in mice. Nutrition & Metabolism. 2015;12.

23. Panchal SK, Poudyal H, Iyer A, Nazer R, Alam MA, Diwan V, et al. High-carbohydrate, high-fat diet-induced metabolic syndrome and cardiovascular remodeling in rats. J Cardiovasc Pharmacol. 2011;57(5):611-24.

24. Ward LC, Battersby KJ. Assessment of body composition of rats by bioimpedance spectroscopy : Validation against dual-energy x-ray absorptiometry. Scandinavian Journal of Laboratory Animal Science. 2009;36(3):9.

25. Wanyonyi S, Preez Rd, Brown L, Paul NA, Panchal SK. Kappaphycus alvarezii as a food supplement prevents diet-induced metabolic syndrome in rats. Nutrients. 2017;9(11):1261.

26. Fu XY, Li Z, Zhang N, Yu HT, Wang SR, Liu JR. Effects of gastrointestinal motility on obesity. Nutr Metab (Lond). 2014;11(1):3.

27. Zhang J, Kobert K, Flouri T, Stamatakis A. Pear: A fast and accurate illumina paired-end read merger. Bioinformatics. 2014;30(5):614-20.

28. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. Qiime allows analysis of high-throughput community sequencing data. Nat Methods. 2010;7(5):335-6.

29. Edgar RC. Search and clustering orders of magnitude faster than blast. Bioinformatics. 2010;26(19):2460-1.

30. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. Uchime improves sensitivity and speed of chimera detection. Bioinformatics. 2011;27(16):2194-200.

31. Edgar RC. Uparse: Highly accurate otu sequences from microbial amplicon reads. Nature Methods. 2013;10(10):996-+.

32. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, et al. Greengenes, a chimera-checked 16s rrna gene database and workbench compatible with arb. Applied and Environmental Microbiology. 2006;72(7):5069-72.

33. 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. The Journal of Nutrition. 2012;142(4):690-7.

34. Panchal SK, Wong WY, Kauter K, Ward LC, Brown L. Caffeine attenuates metabolic syndrome in diet-induced obese rats. Nutrition. 2012;28(10):1055-62.

35. Wu L, Meng J, Shen Q, Zhang Y, Pan S, Chen Z, et al. Caffeine inhibits hypothalamic a1r to excite oxytocin neuron and ameliorate dietary obesity in mice. Nature Communications. 2017;8:15904.

36. Moon J-K, Yoo HS, Shibamoto T. Role of roasting conditions in the level of chlorogenic acid content in coffee beans: Correlation with coffee acidity. Journal of agricultural and food chemistry. 2009;57(12):5365-9.

37. Bekedam EK, Loots MJ, Schols HA, Van Boekel MAJS, Smit G. Roasting effects on formation mechanisms of coffee brew melanoidins. Journal of Agricultural and Food Chemistry. 2008;56(16):7138-45.

38. Pilipczuk T, Kusznierewicz B, Zielińska D, Bartoszek A. The influence of roasting and additional processing on the content of bioactive components in special purpose coffees. Journal of food science and technology. 2015;52(9):5736-44.

39. Nunes FM, Coimbra MA. Melanoidins from coffee infusions. Fractionation, chemical characterization, and effect of the degree of roast. Journal of Agricultural and Food Chemistry. 2007;55(10):3967-77.

40. Gorjanovic S, Komes D, Lalicic-Petronijevic J, Pastor FT, Belscak-Cvitanovic A, Veljovic M, et al. Antioxidant efficiency of polyphenols from coffee and coffee substitutes-electrochemical versus spectrophotometric approach. Journal of Food Science and Technology-Mysore. 2017;54(8):2324-31.

41. Jung S, Kim MH, Park JH, Jeong Y, Ko KS. Cellular antioxidant and anti-inflammatory effects of coffee extracts with different roasting levels. Journal of Medicinal Food. 2017;20(6):626-35.

42. Patrignani M, Rinaldi GJ, Lupano CE. In vivo effects of maillard reaction products derived from biscuits. Food Chemistry. 2016;196:204-10.

43. Gniechwitz D, Reichardt N, Blaut M, Steinhart H, Bunzel M. Dietary fiber from coffee beverage: Degradation by human fecal microbiota. Journal of Agricultural and Food Chemistry. 2007;55(17):6989-96.

44. Gniechwitz D, Reichardt N, Meiss E, Ralph J, Steinhart H, Blaut M, et al. Characterization and fermentability of an ethanol soluble high molecular weight coffee fraction. Journal of Agricultural and Food Chemistry. 2008;56(14):5960-9.

45. Gniechwitz D, Reichardt N, Ralph J, Blaut M, Steinhart H, Bunzel M. Isolation and characterisation of a coffee melanoidin fraction. Journal of the Science of Food and Agriculture. 2008;88(12):2153-60.

46. Helou C, Denis S, Spatz M, Marier D, Rame V, Alric M, et al. Insights into bread melanoidins: Fate in the upper digestive tract and impact on the gut microbiota using in vitro systems. Food & Function. 2015;6(12):3737-45.

47. De Bandt JP, Waligora-Dupriet AJ, Butel MJ. Intestinal microbiota in inflammation and insulin resistance: Relevance to humans. Curr Opin Clin Nutr Metab Care. 2011;14(4):334-40.

48. Wang L, Hu L, Yan S, Jiang T, Fang S, Wang G, et al. Effects of different oligosaccharides at various dosages on the composition of gut microbiota and short-chain fatty acids in mice with constipation. Food Funct. 2017;8(5):1966-78.

49. 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. J Nutr. 2012;142(4):690-7.

50. Stohs SJ, Badmaev V. A review of natural stimulant and non-stimulant thermogenic agents. Phytotherapy Research. 2016;30(5):732-40.

51. Zheng G, Qiu Y, Zhang Q-F, Li D. Chlorogenic acid and caffeine in combination inhibit fat accumulation by regulating hepatic lipid metabolism-related enzymes in mice. British Journal of Nutrition. 2014;112(6):1034-40.

52. Kim JK. Fat uses a toll-road to connect inflammation and diabetes. Cell Metabolism. 2006;4(6):417-9.

53. Mishra J, Verma RK, Alpini G, Meng F, Kumar N. Role of janus kinase 3 in predisposition to obesity-associated metabolic syndrome. Journal of Biological Chemistry. 2015;290(49):29301-12.

54. Oh JS, Riek AE, Darwech I, Funai K, Shao JS, Chin K, et al. Deletion of macrophage vitamin d receptor promotes insulin resistance and monocyte cholesterol transport to accelerate atherosclerosis in mice. Cell Reports. 2015;10(11):1872-86.

55. Ahmed MH, Barakat S, Almobarak AO. Nonalcoholic fatty liver disease and cardiovascular disease: Has the time come for cardiologists to be hepatologists? J Obes. 2012;2012:483135.

Chapter 6.

Attenuation of metabolic syndrome by EPA/DHA ethyl esters in testosterone-deficient high-carbohydrate, high-fat diet-fed rats

Attenuation of metabolic syndrome by EPA/DHA ethyl esters in testosterone-deficient high-carbohydrate, high-fat diet-fed rats

Nikhil **Bhandarkar**[a,b], Senthil Arun **Kumar**[a,b#], Jarad **Martin**[c,d], Lindsay **Brown**[a,b], Sunil K **Panchal**[b,*]

[a]School of Health and Wellbeing, Faculty of Health, Engineering and Sciences, and [b]Functional Foods Research Group, Institute for Agriculture and the Environment, University of Southern Queensland, Toowoomba, QLD 4350, Australia; [c]Department of Radiation Oncology, Calvary Mater Newcastle, Waratah, NSW 2298, Australia; [d]Genesis Cancer Care, Lake Macquarie Private Hospital, Gateshead NSW 2290, Australia

***Keywords*:** Testosterone, Obesity, Metabolic syndrome, Leuprolide acetate, EPA/DHA ethyl esters

***Running title*:** EPA/DHA ethyl esters, testosterone and obesity

Corresponding author

Dr Sunil K Panchal
Functional Foods Research Group, Institute for Agriculture and the Environment
University of Southern Queensland, Toowoomba, QLD 4350, Australia
Email: Sunil.Panchal@usq.edu.au; Tel.: +61 7 4631 1012

[#]Current address for Dr Senthil Arun Kumar: School of Biotechnology and Bioinformatics, DY Patil University, Nerul, Navi Mumbai, Maharasthra 400706, India

Word Count

Abstract (224) + Introduction (497) + Methods (1590) + Results (1542) + Discussion (1491) + Conclusions (102) + References (1898) + Figure legends (377) = 7721
Number of tables – 4; Number of figures – 7; Number of references – 58

Abstract

Inducing testosterone deficiency by orchidectomy or gonadotrophin-releasing hormone agonists is the standard treatment of prostate cancer but this treatment may cause metabolic disorders including insulin resistance, hyperinsulinemia, dyslipidemia, central obesity, cardiovascular diseases, and type 2 diabetes. This study measured responses to testosterone deficiency in a diet-induced model of metabolic syndrome induced by a high-carbohydrate, high-fat (H) diet in rats. We then tested whether eicosapentaenoic acid (EPA)/docosahexaenoic acid (DHA) ethyl esters (Omacor) reversed these metabolic changes. Male Wistar rats (8-9 weeks old) were divided into 8 groups with 4 groups fed corn starch (C) and 4 groups fed H diet. For each diet, 1 group received diet only; 1 group was orchidectomized, 1 group was given leuprolide acetate (2 mg/kg every 4^{th} week) and the last group was treated with leuprolide and their diet was supplemented with 3% Omacor. The protocol was for 16 weeks. Leuprolide treatment worsened the metabolic syndrome symptoms and cardiovascular function, and orchidectomy produced greater responses. In H-fed leuprolide-treated rats, Omacor decreased systolic blood pressure and left ventricular diastolic stiffness, reduced infiltration of inflammatory cells and collagen deposition in the heart, reduced lipid accumulation and inflammatory cell infiltration without improving liver damage. These results suggest that Omacor supplementation is associated with decreased inflammation associated with H diets and so could attenuate metabolic complications in prostate cancer patients with induced testosterone deprivation.

Introduction

Metabolic syndrome is the constellation of insulin resistance, impaired glucose tolerance, dyslipidemia, hypertension, and obesity which increases risk for cardiovascular disease and type 2 diabetes, with an increasing prevalence in the last few decades (O'Neill & O'Driscoll, 2015). Metabolic syndrome, potentially caused by imbalances in energy intake and expenditure, increases morbidity and mortality and is one of the leading preventable causes of death (Zhang*, et al.*, 2017). Further, low testosterone concentrations have been associated with many of these complications including insulin resistance, hyperinsulinemia, dyslipidemia, and cardiovascular disorders (Dockery*, et al.*, 2003; Haffner*, et al.*, 1993; Simon*, et al.*, 1997; Stellato*, et al.*, 2000). Prostate cancer is the second most commonly diagnosed cancer world-wide; in men with prostate cancer, the presence of metabolic syndrome was associated with worse oncologic outcomes, in particular with more aggressive tumor features and biochemical recurrence (Di Francesco & Tenaglia, 2017; Gacci*, et al.*, 2017). The major therapies for prostate cancer rely on induction of testosterone deficiency by orchidectomy or gonadotrophin-releasing hormone (GnRH) agonists such as leuprolide acetate (Lepor & Shore, 2012). However, GnRH agonists increased the risk of development of diabetes and cardiovascular disease, increased fat mass, and decreased lean mass (Goldenberg*, et al.*, 2011; Smith*, et al.*, 2008; Van Londen*, et al.*, 2008).

Diet interventions targeting these metabolic syndrome parameters would be a potential approach to decrease the risk of cardiovascular disease in prostate cancer survivors treated with induced testosterone deficiency, and possibly slow down tumor progression. The health benefits of omega-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in metabolic syndrome and cardiovascular diseases have been reviewed (Bennacer*, et al.*, 2017; Guo*, et al.*, 2017; Tortosa-Caparros*, et al.*, 2017). Omega 3-

fatty acids (EPA and DHA, 1.8 g/day for 26 weeks) decreased expression of genes associated with inflammation and atherogenesis-associated pathways (Bouwens*, et al.*, 2009). Omega-3 fatty acids reduced inflammatory markers and cardiovascular disease risk factors (Calder, 2013; Mori & Beilin, 2004). Mixed EPA:DHA interventions decreased inflammation and cardiovascular disease risk components with elevated antioxidant enzymes activities (Dasilva*, et al.*, 2016). In a randomized controlled trial, daily doses of 300 mg EPA and 200 mg of DHA for 8 weeks reduced high-sensitivity C reactive protein, fasting blood glucose and triglyceride concentrations in hypertensive and/or diabetic obese patients (Ellulu*, et al.*, 2016).

In this study, we firstly investigated whether orchidectomy worsened metabolic syndrome in rats fed a high-carbohydrate, high-fat diet. This diet mimics the changes observed in humans with metabolic syndrome (Panchal*, et al.*, 2011). Secondly, we investigated whether rats with testosterone deficiency following 4-weekly leuprolide acetate injections developed similar pathophysiological changes to the high-carbohydrate, high-fat diet as the rats with orchidectomy. Thirdly, we investigated whether a commercially-available mixture of ethyl esters of EPA and DHA (Omacor) reversed the cardiovascular, hepatic, and metabolic parameters in these leuprolide-treated high-carbohydrate, high-fat diet-fed rats. Our hypothesis was that this mixture of EPA and DHA esters has potential as a treatment for metabolic syndrome in prostate cancer patients treated long-term with leuprolide acetate.

Materials and methods

Rats and diets

All experimental procedures were approved by the Animal Ethics Committee of the University of Southern Queensland under the guidelines of the National Health and Medical

Research Council of Australia. 8–9 weeks old male Wistar rats (338 ± 1 g, n = 96) were obtained from Animal Resource Centre (Murdoch, WA, Australia). Rats were randomly divided into 8 groups each of 12 rats, with 4 groups fed a corn starch diet and 4 groups fed a high-carbohydrate, high-fat diet, as follows:

- **C**: Corn starch diet-fed rats for 16 weeks
- **COr**: Orchidectomized rats fed corn starch diet for 16 weeks
- **CL**: Rats treated with leuprolide acetate and fed corn starch diet for 16 weeks
- **CLOm**: Rats treated with leuprolide acetate for 16 weeks and fed corn starch diet for first 8 weeks followed by corn starch diet supplemented with 3% Omacor for the final 8 weeks
- **H**: High-carbohydrate, high-fat diet-fed rats for 16 weeks
- **HOr**: Orchidectomized rats fed high-carbohydrate, high-fat diet for 16 weeks
- **HL**: Rats treated with leuprolide acetate and fed high-carbohydrate, high-fat diet for 16 weeks
- **HLOm**: Rats treated with leuprolide acetate for 16 weeks and fed high-carbohydrate, high-fat diet for first 8 weeks followed by high-carbohydrate, high-fat diet supplemented with 3% Omacor for the final 8 weeks

Induction of testosterone deficiency

24 male rats were orchidectomized at the age of 8 weeks. Bilateral orchidectomy was performed under anesthesia induced by intraperitoneal injection of Zoletil (tiletamine 15 mg/kg, zolazepam 15 mg/kg; Virbac, Peakhurst, NSW, Australia) combined with Rompun (xylazine 10 mg/kg; Troy Laboratories, Smithfield, NSW, Australia). An incision was made at the midpoint of the scrotum and the underlying tissue, followed by excision of the testicles and part of the spermatic cord. The incision site was sutured and rats were allowed to recover with administration of carprofen (1 mg/kg for 3 days). Before initiation of the experimental

diet, the orchidectomized rats were given standard laboratory chow diet and monitored daily. In a further 24 age-matched male rats, testosterone deficiency was induced by subcutaneous injection of 2 mg/kg leuprolide acetate (Lupron Depot, AbbVie, Sydney, NSW, Australia) at 0, 4, 8, and 12 weeks of the protocol.

Rats, diets, and treatments

All rats were individually housed at the University of Southern Queensland animal house under temperature-controlled, 12-hour-light/dark conditions and were fed *ad libitum* with their respective diets. C, COr, and CL rats were fed with corn starch diet for 16 weeks while CLOm rats were fed with corn starch diet for first 8 weeks and then 3% Omacor-supplemented corn starch diet for the last 8 weeks. C, COr, CL, and CLOm rats received normal drinking water for the duration of the protocol. H, HOr, and HL rats were fed with high-carbohydrate, high-fat diet for 16 weeks while HLOm rats were fed with high-carbohydrate, high-fat diet for first 8 weeks and then 3% Omacor-supplemented high-carbohydrate, high-fat diet for the last 8 weeks. H, HOr, HL, and HLOm rats received drinking water with 25% fructose (w/v) for the duration of the protocol. Corn starch diet contained 570 g corn starch, 155 g powdered rat food, 25 g Hubble, Mendel and Wakeman salt mixture, and 250 mL water per kilogram of diet. High-carbohydrate, high-fat diet contains 175 g fructose, 395 g sweetened condensed milk, 200 g beef tallow, 155 g powdered rat food, 25 g Hubble, Mendel and Wakeman salt mixture, and 50 mL water per kilogram of diet (Panchal*, et al.*, 2011). Each gram of Omacor contained 840 mg of the omega-3 fatty acid ethyl esters comprising 460 mg of EPA ethyl ester and 380 mg of DHA ethyl esters.

Physiological parameters

Body weight, and food and water intakes of all rats were measured daily (Panchal*, et al.*, 2011). Abdominal circumference and body length (nose to anus) were measured using a

standard measuring tape under light sedation with Zoletil (tiletamine 10 mg/kg, zolazepam 10 mg/kg, intraperitoneal). Feed efficiency was calculated as [mean body weight gain (in grams)/daily energy intake (in kJ)] (Panchal*, et al.*, 2011).

Systolic blood pressure measurements

Systolic blood pressure was determined every fourth week under light sedation with Zoletil (tiletamine 10 mg/kg, zolazepam 10 mg/kg, intraperitoneal), using an MLT1010 Piezo-Electric Pulse Transducer and inflatable tail-cuff connected to an MLT844 Physiological Pressure Transducer and PowerLab data acquisition unit (Panchal*, et al.*, 2011). After blood pressure measurements, a small volume of blood was collected from the tail vein for measuring plasma total testosterone concentrations.

Echocardiography

Echocardiographic examination (Phillips iE33, 12-MHz transducer) was performed in rats at the end of protocol (Panchal*, et al.*, 2011). Briefly, rats were anesthetized using Zoletil (tiletamine 10 mg/kg and zolazepam 10 mg/kg, intraperitoneal) and Ilium Xylazil (xylazine 6 mg/kg, intraperitoneal) and positioned in dorsal recumbency before scanning (Panchal*, et al.*, 2011).

Body composition measurement

Dual-energy X-ray absorptiometric (DXA) measurements were carried out at the end of the protocol with a Norland XR36 DXA instrument (Norland Corp, Fort Atkinson, WI, USA). These scans were evaluated using the manufacturer's suggested software for use in laboratory animals (Small Subject Analysis Software, version 2.5.3/1.3.1; Norland Corp) (Ward & Battersby, 2009). The precision error of lean mass for replicate measurements, with repositioning, was 3.2%.

Oral glucose tolerance test

Oral glucose tolerance tests were performed on rats every fourth week following a 12-hour food deprivation when fructose-supplemented drinking water in all H diet-fed groups was replaced with normal drinking water (Panchal*, et al.*, 2011). After determining basal blood glucose concentrations in tail vein blood using Medisense Precision Q.I.D. glucose meters, rats were given a glucose load of 2 g/kg body weight as 40% glucose solution via oral gavage and blood glucose concentrations were measured again 30, 60, 90, and 120 minutes after oral glucose administration (Panchal*, et al.*, 2011).

Terminal experiments

Rats were euthanized with Lethabarb (pentobarbitone sodium, 100 mg/kg, intraperitoneal) before injection of heparin (200 IU) through the right femoral vein. The abdomen was then opened and blood (~5 mL) was withdrawn from the abdominal aorta and collected into heparinized tubes. Blood was centrifuged at 5000 × g for 15 minutes to obtain plasma. Plasma was stored at –20°C for further biochemical characterization. Hearts were then removed for isolated Langendorff heart studies.

Left ventricular function

Isolated Langendorff heart preparations were used to assess left ventricular function of rats (Panchal*, et al.*, 2011). Hearts isolated from euthanized rats were perfused with modified Krebs–Henseleit bicarbonate buffer 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. Left ventricular end-diastolic pressure values were measured during pacing of the heart at 250 beats per minute using an

electrical stimulator. End-diastolic pressures were obtained from 0 to 30 mmHg for the calculation of diastolic stiffness constant (κ, dimensionless) (Panchal*, et al.*, 2011).

Vascular reactivity

Thoracic aortic rings (~4 mm in length) were suspended in an organ bath filled with Tyrode physiological salt solution bubbled with 95% O_2 – 5% CO_2 maintained at 35°C and the rings were allowed to stabilise at a resting tension of ~10 mN. Cumulative concentration–response curves (contraction) were obtained for noradrenaline and cumulative concentration–response curves (relaxation) were obtained for acetylcholine and sodium nitroprusside after submaximal (~70%) contraction to noradrenaline (Panchal*, et al.*, 2011).

Organ weights

After isolated heart perfusion studies, hearts were separated into left ventricles (with septum) and right ventricles and weighed. Livers were isolated and weighed. Retroperitoneal and omental fat pads were removed separately and weighed; epididymal fat pads were removed from rats except orchidectomized rats. Organ weights were normalized against the tibial length at the time of organ removal and expressed as mg/mm of tibial length (Panchal*, et al.*, 2011).

Histology

Heart and liver portions were collected and fixed in 10% neutral buffered formalin. The samples were then dehydrated and embedded in paraffin wax. Thin sections (~5 μm) of heart and liver were cut and stained with hematoxylin and eosin to study infiltration of inflammatory cells and for determining fat vacuoles in liver. Heart sections were also stained with picrosirius red stain to study collagen distribution in the heart (Panchal*, et al.*, 2011).

Plasma biochemistry

Activities of aspartate transaminase (AST) and alanine transaminase (ALT), and concentrations of total cholesterol, triglycerides, and non-esterified fatty acids (NEFA) in plasma were measured (Panchal*, et al.*, 2011). Plasma total testosterone concentrations were measured using commercial kits (Enzo Life Sciences, Farmingdale, NY) according to protocols provided by the manufacturer.

Statistical analysis

All data are presented as mean ± SEM. Rats were divided in groups of 12 as this group size produced statistically robust data to answer the hypotheses of this project, as in our previous studies (Panchal*, et al.*, 2011; Poudyal*, et al.*, 2013). Results were tested for variance using Bartlett's test and variables that were not normally distributed were transformed (using log 10 function) prior to statistical analyses. C, COr, HL, H, HL, and HOr rats were tested for effects of diet, testosterone deficiency and their interactions by two-way analysis of variance. When the interaction and/or the main effects were significant, means were compared using Newman-Keuls multiple comparison post hoc test. CL, CLOm, HL, and HLOm groups were tested for effects of diet + leuprolide, Omacor treatment and their interactions by two-way analysis of variance. When the interaction and/or the main effects were significant, means were compared using Newman-Keuls multiple comparison *post hoc* test. A P value of <0.05 was considered significant. All statistical analyses were performed using GraphPad Prism version 6.1 for Windows (San Diego, CA, USA).

Results

Bilateral orchidectomy

Dietary intakes, body composition, and metabolic parameters

Orchidectomy rapidly reduced plasma total testosterone concentrations in both COr and HOr rats compared to C and H rats, respectively, confirming the effectiveness of orchidectomy (Figure 1A). Orchidectomy did not change body weight, feed efficiency, or intakes of food, water, and energy after 16 weeks of feeding C or H diet compared to their respective non-orchidectomized rats (Table 1). Abdominal circumference, basal blood glucose concentrations, and area under the curve for glucose were higher in orchidectomized rats fed either C or H diet compared to their respective controls (Table 1). Plasma concentrations of total cholesterol, triglycerides, and NEFA were unchanged between the controls and their respective orchidectomized rats (Table 1). Orchidectomy reduced total lean mass in COr and HOr rats compared to C and H rats, respectively. In contrast, the total fat mass was unchanged in C and COr rats but increased in HOr rats compared to H rats (Table 1). Bone mineral content increased in HOr rats compared to H rats but was unchanged in COr and C rats while bone mineral density was unchanged. Retroperitoneal fat increased in COr and HOr rats compared to C and H rats, respectively, while omental fat did not change between C and COr or H and HOr rats (Table 1).

Cardiovascular and liver functions

Orchidectomy in COr rats increased systolic blood pressure and left ventricular diastolic stiffness while all other cardiovascular parameters were similar to C rats (Table 2). Orchidectomy induced inflammation and fibrosis in hearts of COr rats (Figure 2B and 2H) compared to C rats (Figure 2A and 2G). Orchidectomy reduced aortic responses to noradrenaline, sodium nitroprusside, and acetylcholine in COr rats compared to C rats (Figure 3A, 3B, and 3C). HOr rats had lower heart rate and cardiac output along with higher systolic blood pressure than H rats. H diet induced cardiac changes including inflammation (Figure 2D) and fibrosis (Figure 2J) while orchidectomy worsened these changes in HOr rats (Figure 2E and 2K). H diet reduced the aortic responses to noradrenaline, sodium

nitroprusside, and acetylcholine compared to C rats while HOr rats had similar aortic response as in H rats (Figure 3A, 3B, and 3C).

C diet did not induce inflammation or fat deposition in liver (Figure 4A and 4G), while orchidectomy induced inflammation and fat deposition in livers of COr rats (Figure 4B and 4H). H diet induced inflammation and fat deposition in livers (Figure 4D and 4J) whereas HOr rats showed similar changes to H rats (Figure 4E and 4K). No changes in liver weight and ALT and AST activities were observed between C and COr or H and HOr rats (Table 2).

Leuprolide treatment

Dietary intake, body composition, and metabolic parameters

Leuprolide reduced plasma total testosterone concentrations after 8 weeks from initial injection in CL rats compared to C rats (Figure 1A). Leuprolide treatment did not change body weight, feed efficiency, or water and food intakes in CL rats compared to C rats; however, energy intakes were increased in CL rats compared to C rats after 16 weeks (Table 1). Abdominal circumference, basal blood glucose concentrations, and area under the curve for glucose were higher in CL rats compared to C rats (Table 1). Plasma concentrations of total cholesterol, triglycerides, and NEFA were unchanged between CL and C rats. Leuprolide treatment did not change fat mass of CL rats compared to C rats while the lean mass was reduced in CL rats compared to C rats. Bone mineral content and bone mineral density did not change between CL and C rats. Retroperitoneal fat increased in CL rats compared to C rats, while omental and epididymal fat did not change between C and CL rats (Table 1).

Leuprolide reduced plasma testosterone concentrations after 8 weeks from initial injection in HL rats compared to H rats (Figure 1A). Leuprolide treatment did not change body weight, feed efficiency, or water and food intakes in HL rats compared to H rats.

Energy intakes were increased in HL groups compared to H rats after 16 weeks (Table 1). Abdominal circumference and area under the curve for glucose were higher in HL rats compared to H rats while basal blood glucose concentrations did not change in HL rats compared to H rats (Table 1). Plasma concentrations of triglycerides and NEFA were higher in HL rats compared to H rats while plasma concentrations of total cholesterol were not different between H and HL groups. Leuprolide treatment had no effects on the lean or fat mass in HL rats compared to H rats. Bone mineral content decreased in HL rats compared to H rats whereas bone mineral density did not change with leuprolide treatment. Retroperitoneal fat increased in HL rats compared to H rats, while omental and epididymal fat did not change between H and HL rats (Table 1).

Cardiovascular and liver function

CL rats had increased LVIDd, LVIDs, diastolic and systolic volumes, and systolic wall stress along with decreased fractional shortening and ejection fraction compared to C rats (Table 2). Leuprolide induced inflammation in CL rats (Figure 2C) and cardiac fibrosis (Figure 2I) and reduced aortic responses to noradrenaline and acetylcholine while not changing the response to sodium nitroprusside in CL rats compared to C rats (Figure 3A, 3B, and 3C). Leuprolide did not induce inflammation or fat deposition in livers of CL rats (Figure 4C and 4I). No changes in liver weight or ALT and AST activities were observed between C and CL rats (Table 2).

HL rats had decreased heart rate and fractional shortening with increased LVIDd, LVIDs, and systolic volume than H rats (Table 2). HL rats showed inflammation and fibrosis in the heart (Figure 2F and 2L) and had similar aortic responses as in H and HOr rats (Figure 3A, 3B, and 3C). HL rats showed inflammation and fat deposition in liver (Figure 4F and 4L) and no changes in liver weight or ALT and AST activities were observed between H and HL rats (Table 2).

Omacor treatment

Dietary intake, body composition, and metabolic parameters

Plasma total testosterone concentrations were reduced in CLOm rats from 8 weeks after initial injection, as in CL rats, and there were no differences in plasma total testosterone concentrations between CL and CLOm rats at 16 weeks (Figure 1B). Omacor treatment in CLOm rats starting 8 weeks after the initial leuprolide injection decreased body weight even after increasing food and energy intakes. Omacor reduced the feed efficiency, abdominal circumference, basal blood glucose concentrations, and area under the curve in CLOm rats. Plasma concentrations of total cholesterol and NEFA were decreased while triglycerides did not change when compared to CL rats. Total body lean mass, total body fat mass, retroperitoneal fat, and epididymal fat were decreased in CLOm rats compared to CL rats (Table 3).

Omacor treatment did not change body weight and feed efficiency but it increased water intake (fructose-containing water) in HLOm rats compared to HL rats. Food and energy intakes did not change in HLOm rats compared to HL rats after 16 weeks (Table 1). Abdominal circumference did not change in HLOm rats compared to HL rats. The area under the curve for glucose load was higher in HLOm rats compared to HL rats while basal blood glucose concentrations were decreased in HLOm rats compared to HL rats (Table 1). Plasma concentrations of total cholesterol, triglycerides, and NEFA were reduced in HLOm rats compared to HL rats. In contrast to the plasma lipid profile, Omacor decreased lean mass in HLOm rats compared to HL rats while it increased fat mass in HLOm rats compared to HL rats. Bone mineral content was unchanged while bone mineral density increased in HLOm rats. Retroperitoneal fat, epididymal fat, and visceral adiposity index did not change in HL and HLOm rats while omental fat was increased in HLOm rats in comparison to HL rats (Table 3).

Cardiovascular and hepatic function

In CLOm rats, blood pressure and left ventricular diastolic stiffness constant decreased with Omacor treatment compared to CL rats. Omacor treatment reduced inflammation and fibrosis in hearts of CLOm rats (Figure 5B and 5F) compared to CL rats (Figure 5A and 5E). Omacor treatment did not improve aortic responses in CLOm rats compared to CL rats (Figure 6A, 6B, and 6C). Liver weights decreased and ALT activity increased in CLOm rats (Table 4). Omacor treatment attenuated inflammation in livers of CLOm rats (Figure 7B) compared to CL rats (Figure 7A).

In HLOm rats, LVIDs, diastolic volume, systolic volume, systolic wall stress, estimated left ventricular mass, right ventricular weight, and left ventricular weight were increased when compared to HL rats. LVPWs, fractional shortening, ejection fraction, systolic blood pressure, and left ventricular diastolic stiffness decreased in HLOm rats when compared with HL rats (Table 4). Omacor treatment reduced inflammation and fibrosis in hearts of HLOm rats (Figure 5D and 5H) compared to HL rats (Figure 5C and 5G). Omacor treatment did not improve aortic responses in HLOm rats compared to HL rats (Figure 6A, 6B, and 6C). Omacor treatment attenuated inflammation and fat deposition in livers of HLOm (Figure 7D and 7H) rats compared to HL rats (Figure 7C and 7G). No changes were observed in liver wet weight or plasma ALT and AST activities between HL and HLOm rats (Table 4).

Discussion

Prostate cancer is the second most commonly diagnosed cancer world-wide covering 14% of newly diagnosed cases of cancer and it is associated with about 6% total cancer deaths (Jemal*, et al.*, 2011). The association between metabolic syndrome and prostate cancer focused on the function of insulin, IGF-1, and their receptors as strategic factors in

downstream signaling pathways that stimulate tumor growth (Renehan*, et al.*, 2004). The GnRH agonist leuprolide decreased testosterone concentrations to lead to improved health status of prostate cancer patients by restricting the process of tumorigenesis in the prostate leading to tumor regression, easing of urinary symptoms and bone pain, and prolonged survival (You*, et al.*, 2010). However, patients on androgen deprivation therapy show detrimental changes in body composition such as weight gain, loss of muscle mass, increased fat mass, and decreased muscle strength, with increased fasting glucose, triglycerides, and cholesterol concentrations (Macleod*, et al.*, 2015).

In the present study, we firstly demonstrated that orchidectomy is associated with worse pathophysiological signs of metabolic syndrome in rats fed a high-carbohydrate, high-fat diet. These metabolic changes mimic the progression present in humans with metabolic syndrome (Panchal*, et al.*, 2011). Secondly, we demonstrated that rats with testosterone deficiency induced either by leuprolide or orchidectomy developed similar pathophysiological changes when fed with high-carbohydrate, high-fat diet. Noticeably, orchidectomy delivered more severe pathophysiological complications than leuprolide. When leuprolide is given, there is a period of testosterone flare which, in humans, lasts for around 1-2 weeks (Damber*, et al.*, 2012). This is probably the reason for the slower decrease in testosterone concentrations in the leuprolide-treated groups than in the orchidectomized groups. It also means that the exposure to testosterone deficiency in leuprolide-treated rats is shorter than in the orchidectomized groups. Further, testosterone deficiency induced cardiac inflammation and fibrosis with liver steatosis in the corn starch diet-fed rats. Thirdly, we demonstrated that a commercially-available mixture of ethyl esters of EPA and DHA (Omacor) reversed the cardiovascular complications such as decreased blood pressure and left ventricular wall stiffness, reduced hepatic damage such as decreased inflammation and fat deposition in liver and decreased plasma lipid concentrations in these leuprolide-treated

high-carbohydrate high-fat diet-fed rats. Thus, the positive changes against metabolic syndrome with the dietary supplementation have validated our hypothesis that this mixture of EPA and DHA ethyl esters has potential as a nutraceutical approach for reducing components of metabolic syndrome in prostate cancer patients treated long-term with leuprolide acetate.

Orchidectomy and treatment with GnRH agonists such as leuprolide are successful interventions for prostate cancer by initiating testosterone deficiency (Marberger*, et al.*, 2010), although orchidectomy is more effective for the initiation of glandular apoptosis and atrophy (Cakiroglu*, et al.*, 2016). Testosterone deficiency produces complications including increased central obesity, increased triglycerides concentrations and elevated fasting plasma glucose concentrations (Gandaglia*, et al.*, 2014; Goldenberg*, et al.*, 2011; Sidaway, 2015; Smith*, et al.*, 2002; Wang*, et al.*, 2011). Leuprolide acetate, although associated with these metabolic complications, has shown tolerability in routine clinical use (Tunn, 2011). Thus, it is important to treat complications associated with GnRH agonist therapy in order to provide benefits against prostate cancer while minimising the development of metabolic and cardiovascular complications.

This study compared the physiological changes produced by testosterone deficiency, either by orchidectomy or leuprolide treatment, and whether these changes worsen during a high-carbohydrate, high-fat diet. In this study, corn starch was used as the control diet as corn starch is slowly digestible, low-glycemic carbohydrate (Panchal*, et al.*, 2011). Unlike fructose in high-carbohydrate, high-fat diet, corn starch does not induce clinical signs of metabolic syndrome (Panchal*, et al.*, 2011; Patel*, et al.*, 2009; Thirunavukkarasu*, et al.*, 2004). High-carbohydrate, high-fat diet induced metabolic syndrome with obesity, dyslipidemia, hypertension and impaired glucose tolerance along with changes in liver and heart structure and function (Panchal*, et al.*, 2011). Orchidectomy reduced lean mass while increasing fat

mass to a greater extent than leuprolide, possibly due to the slower onset of testosterone deficiency with leuprolide. Both orchidectomy and leuprolide increased the deposition of retroperitoneal fat, a major component of the abdominal fat. Presence of abdominal obesity is the major metabolic complication in the initiation of inflammation, potentially increasing the risk of cardiovascular disease (Després, 2012). During the protocol, rats were provided with similar environment including cage size and environmental enrichment with no facility to increase physical activity. We have previously shown that exercise in our high-carbohydrate, high-fat diet-fed rats improved metabolic and cardiovascular function (Cameron*, et al.*, 2012). This indicates that the basal physical activity in this study is unlikely to change metabolic or cardiovascular function by itself.

Metabolic syndrome, characterized by the presence of central obesity, hypertension, and dyslipidemia as risk factors for type 2 diabetes and cardiovascular disease, is one of the major complications with leuprolide treatment (Dockery*, et al.*, 2003; Haffner*, et al.*, 1993; Simon*, et al.*, 1997; Stellato*, et al.*, 2000). Obesity as the major component of metabolic syndrome is still without an effective, non-invasive treatment free from adverse effects, with functional foods and nutraceuticals, including omega-3 fatty acids, proposed as effective treatment options (Brown*, et al.*, 2015). EPA and DHA have been reviewed extensively for their effectiveness against metabolic syndrome (Bennacer*, et al.*, 2017; Guo*, et al.*, 2017; Tortosa-Caparros*, et al.*, 2017). Omega-3 and omega-6 fatty acids play vital roles in regulating metabolism as well as state of inflammation (Poudyal*, et al.*, 2011; Simopoulos, 2016). A balance is required between pro-inflammatory omega-6 and anti-inflammatory omega-3 fatty acids for the body to maintain homeostasis (Simopoulos, 2016). With the increases in omega-6 fatty acid intake in the modern world, dietary supplementation of omega-3 fatty acids will reduce the imbalance between omega-3 and omega-6 intake and hence contribute in alleviating metabolic and inflammation-related complications

(Simopoulos, 2016). For omega-3 fatty acids, animal studies described anti-obesity effects, but human studies do not conclusively suggest this (Buckley & Howe, 2009). Further, conflicting results are available from the randomized trials of omega-3 fatty acids which may have resulted from differences in study design, dosage used, omega-6/omega-3 fatty acid ratio of the background diet, duration of omega-3 fatty acid supplementation, use of other supplements in addition to omega-3 fatty acids, and demographics of the study population (Simopoulos, 2016).

This study used a commercial mixture of the ethyl esters of EPA and DHA, Omacor, to identify the beneficial effects in the treatment of leuprolide-treated obese rats. Omacor treatment improved bone mineral density, plasma concentrations of total cholesterol, triglycerides, and NEFA, and basal blood glucose concentrations. Omega-3 fatty acids attenuated obesity and glucose intolerance and decreased plasma triglycerides in humans (Lorente-Cebrian*, et al.*, 2013; Rafiee*, et al.*, 2016; Skulas-Ray*, et al.*, 2011). Further, EPA and DHA suppressed the production of pro-inflammatory cytokines including IL-6, TNF-α, IL-1, and IL-2 (Das, 2006). Omega-3 fatty acids upregulated lipoprotein lipase and adipose triglyceride lipase, enzymes catalysing hydrolysis of triglycerides in skeletal muscle and adipose tissue, respectively (Zimmermann*, et al.*, 2004). Further, omega-3 fatty acids played crucial roles in lowering the rate of fatty acid synthesis and glucose metabolism through downregulation of fatty acid synthase in liver (Kim*, et al.*, 1999). Our study results also suggest that omega-3 fatty acid supplementation improved cardiovascular responses including reduced systolic blood pressure and left ventricular diastolic stiffness along with reduced inflammatory cell infiltration and collagen deposition. Further, Omacor reduced lipid accumulation and inflammatory cell infiltration without changing wet weight of liver and its enzyme activities.

The mechanism of action of omega-3 fatty acids is based on their anti-inflammatory responses (Mori & Beilin, 2004). Omega-3 fatty acids protected against metabolic syndrome through their anti-inflammatory and platelet activating properties that enhance endothelial function and normalize blood pressure by restricting the lipogenesis and activation of lipid oxidation (Carpentier*, et al.*, 2006). In our previous study, EPA and DHA individually improved metabolic syndrome in obese rats at the same dose as in this study (3% in food) (Poudyal*, et al.*, 2013). Colon-specific delivery of EPA or DHA increased the release of glucagon-like peptide 1 and insulin with subsequent reduction in glucose concentrations (Morishita*, et al.*, 2008). Increasing adiponectin is one mechanism by which omega-3 fatty acids can improve cardiometabolic profile in people with cardiovascular risk (Barbosa*, et al.*, 2017). Plasma adiponectin was associated with insulin sensitivity (Borges*, et al.*, 2017) and reduced plasma adiponectin was a marker of insulin resistance and increased risk of type 2 diabetes (Yamauchi*, et al.*, 2001). Both animal and human studies showed that omega-3 fatty acid supplements improved plasma adiponectin concentrations (Barbosa*, et al.*, 2017; Itoh*, et al.*, 2007; Mohammadi*, et al.*, 2012; Neschen*, et al.*, 2006). Thus, increasing both glucagon-like peptide 1 secretion and adiponectin production by omega-3 fatty acids could improve both insulin secretion and sensitivity (Bhaswant*, et al.*, 2015) resulting in improved recovery from insulin resistance and dyslipidemia, thus attenuating metabolic syndrome. Measuring glucagon-like peptide 1 and adiponectin before and after intervention with omega-3 fatty acids could support a plausible mechanism of action of EPA and DHA. However, this study was unable to measure these hormonal changes making it a limitation of our study.

Conclusions

We demonstrated that orchidectomy and treatment with a GnRH agonist produced similar worsening of metabolic syndrome symptoms and cardiovascular function. Omacor, a

combination of ethyl esters of EPA and DHA, delivered positive physiological and biochemical responses to reduce symptoms of metabolic syndrome. Further, this study is consistent with previous outcomes obtained for omega 3-fatty acids except for attenuating visceral obesity. Reduced systolic blood pressure and left ventricular stiffness reduction were the major cardiovascular outcomes from this study. Given these observations and the ease of administration, clinical trials of Omacor in men with prostate cancer being managed with androgen deprivation therapy are warranted.

Funding support

This study was supported by a grant from AbbVie to the University of Southern Queensland.

Acknowledgement

The authors would like to thank Dr Kate Kauter for plasma testosterone analyses. Authors also acknowledge Mr Jason Brightwell (The Prince Charles Hospital, Brisbane, QLD, Australia) and Mr Brian Bynon (School of Veterinary Sciences, The University of Queensland, Gatton, QLD, Australia) for echocardiography and plasma biochemical analyses.

Author contribution

J.M., L.B., and S.K.P. designed the research protocol. S.K.P. performed orchidectomy on rats. N.B., S.A.K., and S.K.P. conducted animal experiments and collected the data. N.B., S.A.K., and S.K.P. analysed the data and S.K.P. and L.B. interpreted the data. N.B. and S.K.P. wrote the manuscript with S.K.P. and L.B. having primary responsibility for final content. All authors read and approved the final manuscript.

Disclosures

Authors have nothing to disclose.

References

Barbosa MM, Melo AL & Damasceno NR. (2017) The benefits of omega-3 supplementation depend on adiponectin basal level and adiponectin increase after the supplementation: A randomized clinical trial. *Nutrition* 34, 7-13.

Bennacer AF, Haffaf E, Kacimi G, Oudjit B & Koceir EA. (2017) Association of polyunsaturated/saturated fatty acids to metabolic syndrome cardiovascular risk factors and lipoprotein (a) in hypertensive type 2 diabetic patients. *Ann Biol Clin (Paris)* 75, 293-304.

Bhaswant M, Poudyal H & Brown L. (2015) Mechanisms of enhanced insulin secretion and sensitivity with n-3 unsaturated fatty acids. *J Nutr Biochem* 26, 571-584.

Borges MC, Oliveira IO, Freitas DF, Horta BL, Ong KK, Gigante DP & Barros AJD. (2017) Obesity-induced hypoadiponectinaemia: the opposite influences of central and peripheral fat compartments. *Int J Epidemiol* 46, 2044-2055.

Bouwens M, van de Rest O, Dellschaft N, Bromhaar MG, de Groot LC, Geleijnse JM, Muller M & Afman LA. (2009) Fish-oil supplementation induces antiinflammatory gene expression profiles in human blood mononuclear cells. *Am J Clin Nutr* 90, 415-424.

Brown L, Poudyal H & Panchal SK. (2015) Functional foods as potential therapeutic options for metabolic syndrome. *Obes Rev* 16, 914-941.

Buckley JD & Howe PR. (2009) Anti-obesity effects of long-chain omega-3 polyunsaturated fatty acids. *Obes Rev* 10, 648-659.

Cakiroglu B, Hazar AI, Eyyupoglu SE, Can Balci MB, Sinanoglu O & Tuzlali P. (2016) The comparative evaluation of apoptosis produced by leuprolide or orchiectomy on rat prostate tissue. *Arch Ital Urol Androl* 87, 265-269.

Calder PC. (2013) Omega-3 polyunsaturated fatty acids and inflammatory processes: nutrition or pharmacology? *Br J Clin Pharmacol* 75, 645-662.

Cameron I, Alam MA, Wang J & Brown L. (2012) Endurance exercise in a rat model of metabolic syndrome. *Can J Physiol Pharmacol* 90, 1490-1497.

Carpentier YA, Portois L & Malaisse WJ. (2006) n−3 fatty acids and the metabolic syndrome. *Am J Clin Nutr* 83, S1499-S1504.

Damber JE, Tammela TL, Iversen P, Abrahamsson PA, Boccon-Gibod L, Olesen TK, van der Meulen E & Persson BE. (2012) The effect of baseline testosterone on the efficacy of degarelix and leuprolide: further insights from a 12-month, comparative, phase III study in prostate cancer patients. *Urology* 80, 174-180.

Das UN. (2006) Essential fatty acids: biochemistry, physiology and pathology. *Biotechnol J* 1, 420-439.

Dasilva G, Pazos M, García-Egido E, Pérez-Jiménez J, Torres JL, Giralt M, Nogués M-R & Medina I. (2016) Lipidomics to analyze the influence of diets with different EPA:DHA ratios in the progression of Metabolic Syndrome using SHROB rats as a model. *Food Chem* 205, 196-203.

Després J-P. (2012) Abdominal obesity and cardiovascular disease: Is inflammation the missing link? *Can J Cardiol* 28, 642-652.

Di Francesco S & Tenaglia RL. (2017) Metabolic syndrome and aggressive prostate cancer at initial diagnosis. *Horm Metab Res* 49, 507-509.

Dockery F, Bulpitt CJ, Agarwal S, Donaldson M & Rajkumar C. (2003) Testosterone suppression in men with prostate cancer leads to an increase in arterial stiffness and hyperinsulinaemia. *Clin Sci (Lond)* 104, 195-201.

Ellulu MS, Khaza'ai H, Patimah I, Rahmat A & Abed Y. (2016) Effect of long chain omega-3 polyunsaturated fatty acids on inflammation and metabolic markers in hypertensive and/or diabetic obese adults: a randomized controlled trial. *Food Nutr Res* 60, 29268.

Gacci M, Russo GI, De Nunzio C, Sebastianelli A, Salvi M, Vignozzi L, Tubaro A, Morgia G & Serni S. (2017) Meta-analysis of metabolic syndrome and prostate cancer. *Prostate Cancer Prostatic Dis* 20, 146-155.

Gandaglia G, Sun M, Popa I, Schiffmann J, Abdollah F, Trinh QD, Saad F, Graefen M, Briganti A, Montorsi F & Karakiewicz PI. (2014) The impact of androgen-deprivation therapy (ADT) on the risk of cardiovascular (CV) events in patients with non-metastatic prostate cancer: a population-based study. *BJU Int* 114, E82-E89.

Goldenberg SL, Koupparis A & Robinson ME. (2011) Differing levels of testosterone and the prostate: a physiological interplay. *Nat Rev Urol* 8, 365-377.

Guo XF, Li X, Shi M & Li D. (2017) n-3 polyunsaturated fatty acids and metabolic syndrome risk: A meta-analysis. *Nutrients* 9, 703.

Haffner SM, Mykkanen L, Valdez RA & Katz MS. (1993) Relationship of sex hormones to lipids and lipoproteins in nondiabetic men. *J Clin Endocrinol Metab* 77, 1610-1615.

Itoh M, Suganami T, Satoh N, Tanimoto-Koyama K, Yuan X, Tanaka M, Kawano H, Yano T, Aoe S, Takeya M, Shimatsu A, Kuzuya H, Kamei Y & Ogawa Y. (2007) Increased adiponectin secretion by highly purified eicosapentaenoic acid in rodent models of obesity and human obese subjects. *Arterioscler Thromb Vasc Biol* 27, 1918-1925.

Jemal A, Bray F, Center MM, Ferlay J, Ward E & Forman D. (2011) Global cancer statistics. *CA Cancer J Clin* 61, 69-90.

Kim HJ, Takahashi M & Ezaki O. (1999) Fish oil feeding decreases mature sterol regulatory element-binding protein 1 (SREBP-1) by down-regulation of SREBP-1c mRNA in mouse liver. A possible mechanism for down-regulation of lipogenic enzyme mRNAs. *J Biol Chem* 274, 25892-25898.

Lepor H & Shore ND. (2012) LHRH agonists for the treatment of prostate cancer: 2012. *Rev Urol* 14, 1-12.

Lorente-Cebrian S, Costa AG, Navas-Carretero S, Zabala M, Martinez JA & Moreno-Aliaga MJ. (2013) Role of omega-3 fatty acids in obesity, metabolic syndrome, and cardiovascular diseases: a review of the evidence. *J Physiol Biochem* 69, 633-651.

Macleod LC, Chery LJ, Hu EY, Zeliadt SB, Holt SK, Lin DW, Porter MP, Gore JL & Wright JL. (2015) Metabolic syndrome, dyslipidemia and prostate cancer recurrence after primary surgery or radiation in a veterans cohort. *Prostate Cancer Prostatic Dis* 18, 190-195.

Marberger M, Kaisary AV, Shore ND, Karlin GS, Savulsky C, Mis R, Leuratti C & Germa JR. (2010) Effectiveness, pharmacokinetics, and safety of a new sustained-release leuprolide acetate 3.75-mg depot formulation for testosterone suppression in patients with prostate cancer: a Phase III, open-label, international multicenter study. *Clin Ther* 32, 744-757.

Mohammadi E, Rafraf M, Farzadi L, Asghari-Jafarabadi M & Sabour S. (2012) Effects of omega-3 fatty acids supplementation on serum adiponectin levels and some metabolic risk factors in women with polycystic ovary syndrome. *Asia Pac J Clin Nutr* 21, 511-518.

Mori TA & Beilin LJ. (2004) Omega-3 fatty acids and inflammation. *Curr Atheroscler Rep* 6, 461-467.

Morishita M, Tanaka T, Shida T & Takayama K. (2008) Usefulness of colon targeted DHA and EPA as novel diabetes medications that promote intrinsic GLP-1 secretion. *J Control Release* 132, 99-104.

Neschen S, Morino K, Rossbacher JC, Pongratz RL, Cline GW, Sono S, Gillum M & Shulman GI. (2006) Fish oil regulates adiponectin secretion by a peroxisome proliferator-activated receptor-gamma-dependent mechanism in mice. *Diabetes* 55, 924-928.

O'Neill S & O'Driscoll L. (2015) Metabolic syndrome: a closer look at the growing epidemic and its associated pathologies. *Obes Rev* 16, 1-12.

Panchal SK, Poudyal H, Iyer A, Nazer R, Alam MA, Diwan V, Kauter K, Sernia C, Campbell F, Ward L, Gobe G, Fenning A & Brown L. (2011) High-carbohydrate, high-fat diet-induced metabolic syndrome and cardiovascular remodeling in rats. *J Cardiovasc Pharmacol* 57, 611-624.

Patel J, Iyer A & Brown L. (2009) Evaluation of the chronic complications of diabetes in a high fructose diet in rats. *Indian J Biochem Biophys* 46, 66-72.

Poudyal H, Panchal SK, Diwan V & Brown L. (2011) Omega-3 fatty acids and metabolic syndrome: effects and emerging mechanisms of action. *Prog Lipid Res* 50, 372-387.

Poudyal H, Panchal SK, Ward LC & Brown L. (2013) Effects of ALA, EPA and DHA in high-carbohydrate, high-fat diet-induced metabolic syndrome in rats. *J Nutr Biochem* 24, 1041-1052.

Rafiee M, Sotoudeh G, Djalali M, Alvandi E, Eshraghian M, Sojoudi F & Koohdani F. (2016) Dietary omega-3 polyunsaturated fatty acid intake modulates impact of Insertion/Deletion polymorphism of ApoB gene on obesity risk in type 2 diabetic patients. *Nutrition* 32, 1110-1115.

Renehan AG, Zwahlen M, Minder C, T O'Dwyer S, Shalet SM & Egger M. (2004) Insulin-like growth factor (IGF)-I, IGF binding protein-3, and cancer risk: systematic review and meta-regression analysis. *Lancet* 363, 1346-1353.

Sidaway P. (2015) Prostate cancer: Intermediate risk: RT or ADT? *Nat Rev Urol* 12, 300.

Simon D, Charles MA, Nahoul K, Orssaud G, Kremski J, Hully V, Joubert E, Papoz L & Eschwege E. (1997) Association between plasma total testosterone and cardiovascular risk factors in healthy adult men: The Telecom Study. *J Clin Endocrinol Metab* 82, 682-685.

Simopoulos AP. (2016) An increase in the omega-6/omega-3 fatty acid ratio increases the risk for obesity. *Nutrients* 8, 128.

Skulas-Ray AC, Kris-Etherton PM, Harris WS, Vanden Heuvel JP, Wagner PR & West SG. (2011) Dose-response effects of omega-3 fatty acids on triglycerides, inflammation, and endothelial function in healthy persons with moderate hypertriglyceridemia. *Am J Clin Nutr* 93, 243-252.

Smith MR, Finkelstein JS, McGovern FJ, Zietman AL, Fallon MA, Schoenfeld DA & Kantoff PW. (2002) Changes in body composition during androgen deprivation therapy for prostate cancer. *J Clin Endocrinol Metab* 87, 599-603.

Smith MR, O'Malley AJ & Keating NL. (2008) Gonadotrophin-releasing hormone agonists, diabetes and cardiovascular disease in men with prostate cancer: Which metabolic syndrome? *BJU Int* 101, 1335-1336.

Stellato RK, Feldman HA, Hamdy O, Horton ES & McKinlay JB. (2000) Testosterone, sex hormone-binding globulin, and the development of type 2 diabetes in middle-aged men: prospective results from the Massachusetts male aging study. *Diabetes Care* 23, 490-494.

Thirunavukkarasu V, Anitha Nandhini AT & Anuradha CV. (2004) Lipoic acid attenuates hypertension and improves insulin sensitivity, kallikrein activity and nitrite levels in high fructose-fed rats. *J Comp Physiol B* 174, 587-592.

Tortosa-Caparros E, Navas-Carrillo D, Marin F & Orenes-Pinero E. (2017) Anti-inflammatory effects of omega 3 and omega 6 polyunsaturated fatty acids in cardiovascular disease and metabolic syndrome. *Crit Rev Food Sci Nutr* 57, 3421-3429.

Tunn UW. (2011) A 6-month depot formulation of leuprolide acetate is safe and effective in daily clinical practice: a non-interventional prospective study in 1273 patients. *BMC Urol* 11, 15.

van Londen GJ, Levy ME, Perera S, Nelson JB & Greenspan SL. (2008) Body composition changes during androgen deprivation therapy for prostate cancer: A 2-year prospective study. *Crit Rev Oncol Hematol* 68, 172-177.

Wang C, Jackson G, Jones TH, Matsumoto AM, Nehra A, Perelman MA, Swerdloff RS, Traish A, Zitzmann M & Cunningham G. (2011) Low testosterone associated with obesity and the metabolic syndrome contributes to sexual dysfunction and cardiovascular disease risk in men with type 2 diabetes. *Diabetes Care* 34, 1669-1675.

Ward LC & Battersby KJ. (2009) Assessment of body composition of rats by bioimpedance spectroscopy: Validation against dual-energy x-ray absorptiometry. *Scand J Lab Anim Sci* 36, 9.

Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T, Murakami K, Tsuboyama-Kasaoka N, Ezaki O, Akanuma Y, Gavrilova O, Vinson C, Reitman ML, Kagechika H, Shudo K, Yoda M, Nakano Y, Tobe K, Nagai R, Kimura S, Tomita M, Froguel P & Kadowaki T. (2001) The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med* 7, 941-946.

You D, Jeong IG, Kim SW, Chung BH, Cho JS, Lee HM, Yun SC & Kim CS. (2010) Impacts of leuprolide acetate on quality of life in patients with prostate cancer: A prospective multicenter study. *Scand J Urol Nephrol* 44, 399-405.

Zhang T, Zhang H, Li Y, Li S, Fernandez C, Bazzano L, He J, Xue F & Chen W. (2017) Long-term impact of temporal sequence from childhood obesity to hyperinsulinemia on adult metabolic syndrome and diabetes: The Bogalusa heart study. *Sci Rep* 7, 43422.

Zimmermann R, Strauss JG, Haemmerle G, Schoiswohl G, Birner-Gruenberger R, Riederer M, Lass A, Neuberger G, Eisenhaber F, Hermetter A & Zechner R. (2004) Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. *Science* 306, 1383-1386.

Figure legends

Figure 1. Plasma total testosterone concentrations in C, COr, CL, H, HOr, and HL rats (A) and in CL, CLOm, HL, and HLOm rats (B). Data are shown as mean ± SEM, n = 6–10/group. End-point means without a common alphabet significantly differ, $P<0.05$.

Figure 2. Effects of orchidectomy and leuprolide on the structure of the heart. Top row represents hematoxylin and eosin staining of left ventricle showing inflammatory cell ("in") while the bottom row represents picrosirius red staining of left ventricle showing collagen deposition ("cd") from C (A, G), COr (B, H), CL (C, I), H (D, J), HOr (E, K), and HL (F, L) rats.

Figure 3. Cumulative concentration-response curves for noradrenaline (A), sodium nitroprusside (B), and acetylcholine (C) in thoracic aortic rings from C, COr, CL, H, HOr, and HL rats. Data are shown as mean ± SEM, n = 10–12/group. End-point means without a common alphabet significantly differ, $P<0.05$.

Figure 4. Effects of orchidectomy and leuprolide on the structure of the liver. Top row represents hematoxylin and eosin staining of liver showing inflammatory cell ("in") while the bottom row represents hematoxylin and eosin staining of liver showing fat deposition ("fd") from C (A, G), COr (B, H), CL (C, I), H (D, J), HOr (E, K), and HL (F, L) rats.

Figure 5. Effects of Omacor on leuprolide-induced changes in the structure of the heart. Top row represents hematoxylin and eosin staining of left ventricle showing inflammatory cell ("in") while the bottom row represents picrosirius red staining of left ventricle showing collagen deposition ("cd") from CL (A, E), CLOm (B, F), HL (C, G), and HLOm (D, H) rats.

Figure 6. Cumulative concentration-response curves for noradrenaline (A), sodium nitroprusside (B), and acetylcholine (C) in thoracic aortic rings from CL, CLOm, HL, and

HLOm rats. Data are shown as mean ± SEM, n = 10–12/group. End-point means without a common alphabet significantly differ, $P<0.05$.

Figure 7. Effects of Omacor on leuprolide-induced changes in the structure of the liver. Top row represents hematoxylin and eosin staining of liver showing inflammatory cell ("in") while the bottom row represents hematoxylin and eosin staining of liver showing fat deposition ("fd") from CL (A, E), CLOm (B, F), HL (C, G), and HLOm (D, H) rats.

Table 1. Effects of orchidectomy and leuprolide-induced changes in physiological and metabolic parameters

Variables	*C*	*COr*	*CL*	*H*	*HOr*	*HL*	*P* value		
							Diet	*Testosterone deficiency*	*Diet × testosterone deficiency*
Initial body weight (g)	337 ± 1	338 ± 1	337 ± 1	336 ± 1	339 ± 1	338 ± 1	0.68	0.14	0.52
Final body weight (g)	419 ± 5^{b}	429 ± 5^{b}	417 ± 8^{b}	520 ± 6^{a}	522 ± 14^{a}	539 ± 18^{a}	<0.0001	0.71	0.37
Water intake (mL/day)	31.0 ± 2.1^{a}	32.0 ± 2.4^{a}	26.4 ± 2.6^{ab}	19.8 ± 1.6^{bc}	19.8 ± 1.6^{bc}	17.5 ± 1.7^{c}	<0.0001	0.12	0.71
Food intake (g/day)	31.6 ± 2.0^{a}	31.9 ± 2.3^{a}	36.1 ± 2.2^{a}	20.8 ± 1.3^{b}	21.1 ± 1.8^{b}	24.3 ± 1.9^{b}	<0.0001	0.08	0.96
Energy intake (kJ/day)	346 ± 3^{d}	356 ± 3^{d}	401 ± 6^{c}	444 ± 3^{b}	447 ± 5^{b}	507 ± 12^{a}	<0.0001	<0.0001	0.54
Feed efficiency (g/kJ)	0.23 ± 0.01^{b}	0.25 ± 0.02^{b}	0.20 ± 0.02^{b}	0.41 ± 0.01^{a}	0.40 ± 0.03^{a}	0.40 ± 0.03^{a}	<0.0001	0.48	0.51
Abdominal circumference (cm)	18.4 ± 0.1^{d}	20.1 ± 0.4^{c}	20.1 ± 0.2^{c}	22.0 ± 0.2^{b}	23.2 ± 0.5^{a}	23.0 ± 0.3^{a}	<0.0001	<0.0001	0.52
Basal blood glucose concentrations (mmol/L)	4.1 ± 0.1^{d}	4.7 ± 0.1^{c}	4.8 ± 0.1^{c}	5.1 ± 0.1^{bc}	5.6 ± 0.1^{a}	5.4 ± 0.2^{ab}	<0.0001	<0.0001	0.24
Area under the curve (mmol/L×minutes)	632 ± 21^{c}	776 ± 19^{b}	781 ± 10^{b}	774 ± 16^{b}	854 ± 14^{a}	844 ± 24^{a}	<0.0001	<0.0001	0.07
Total cholesterol (mmol/L)	1.37 ± 0.05^{b}	1.71 ± 0.15^{ab}	1.65 ± 0.04^{ab}	1.88 ± 0.06^{a}	1.84 ± 0.17^{a}	1.90 ± 0.10^{a}	0.001	0.28	0.20
Triglyceride (mmol/L)	0.60 ± 0.03^{c}	0.56 ± 0.05^{c}	0.60 ± 0.06^{c}	1.42 ± 0.15^{b}	1.06 ± 0.22^{bc}	2.02 ± 0.32^{a}	<0.0001	0.019	0.032
NEFA (mmol/L)	1.74 ± 0.16^{c}	2.47 ± 0.17^{c}	2.38 ± 0.19^{c}	3.69 ± 0.37^{b}	3.41 ± 0.43^{b}	4.60 ± 0.35^{a}	<0.0001	0.034	0.09
Whole-body lean mass (g)	312 ± 11^{a}	271 ± 11^{b}	275 ± 5^{b}	314 ± 8^{a}	229 ± 14^{c}	308 ± 9^{a}	0.78	<0.0001	0.002
Whole-body fat mass (g)	114 ± 11^{c}	121 ± 10^{c}	112 ± 8^{c}	191 ± 8^{b}	259 ± 23^{a}	161 ± 15^{bc}	<0.0001	0.0007	0.006
Bone mineral content (g)	13.3 ± 0.5^{b}	12.4 ± 0.4^{bc}	11.9 ± 0.2^{bc}	13.3 ± 0.4^{b}	16.2 ± 0.6^{a}	11.3 ± 0.5^{c}	0.005	<0.0001	<0.0001
Bone mineral density (g/cm^2)	0.183 ± 0.003	0.173 ± 0.004	0.174 ± 0.003	0.183 ± 0.004	0.173 ± 0.003	0.177 ± 0.003	0.72	0.12	0.88
Retroperitoneal fat (mg/mm tibial length)	122 ± 8^{d}	231 ± 18^{c}	220 ± 18^{c}	366 ± 17^{b}	537 ± 26^{a}	479 ± 54^{a}	<0.0001	<0.0001	0.51
Epididymal fat (mg/mm tibial length)	101 ± 4^{b}	-	125 ± 11^{b}	224 ± 17^{a}	-	263 ± 26^{a}	<0.0001	0.07	0.65
Omental fat (mg/mm tibial length)	65 ± 6^{b}	90 ± 6^{b}	83 ± 6^{b}	132 ± 9^{a}	137 ± 13^{a}	147 ± 13^{a}	<0.0001	0.16	0.52

Value are means ± SEM, n = 10–12. Mean values in a row with unlike superscript letters are significantly different (P<0.05). C, corn starch diet-fed rats; COr, orchidectomized rats fed corn starch diet; CL, leuprolide-treated rats fed corn starch diet; H, high-carbohydrate high-fat diet fed rats; HOr, orchidectomized rats fed high-carbohydrate high-fat diet; HL, leuprolide-treated rats fed high-carbohydrate high-fat diet; NEFA, non-esterified fatty acids.

Table 2. Effects of orchidectomy and leuprolide-induced changes in cardiovascular and hepatic function

Variables	*C*	*COr*	*CL*	*H*	*HOr*	*HL*	*P* value		
							Diet	*Testosterone deficiency*	*Diet × testosterone deficiency*
Heart rate (bpm)	253 ± 23^{bc}	227 ± 17^{c}	238 ± 11^{bc}	350 ± 10^{a}	282 ± 15^{bc}	290 ± 15^{b}	<0.0001	0.01	0.29
LVIDd (mm)	6.64 ± 0.28^{c}	6.63 ± 0.30^{c}	7.70 ± 0.15^{ab}	7.09 ± 0.22^{bc}	7.23 ± 0.20^{bc}	8.24 ± 0.22^{a}	0.007	<0.0001	0.95
LVIDs (mm)	3.11 ± 0.19^{c}	3.68 ± 0.18^{bc}	4.52 ± 0.19^{a}	3.22 ± 0.23^{c}	3.48 ± 0.25^{bc}	4.14 ± 0.18^{ab}	0.35	<0.0001	0.49
IVSd (mm)	1.91 ± 0.12	1.96 ± 0.10	1.77 ± 0.06	2.05 ± 0.08	2.05 ± 0.17	1.82 ± 0.06	0.28	0.10	0.91
IVSs (mm)	3.09 ± 0.26^{ab}	3.13 ± 0.20^{ab}	2.80 ± 0.08^{b}	3.65 ± 0.09^{a}	3.43 ± 0.20^{ab}	3.12 ± 0.11^{ab}	0.006	0.047	0.70
LVPWd (mm)	1.70 ± 0.09	1.91 ± 0.12	1.79 ± 0.03	2.04 ± 0.06	2.08 ± 0.15	1.75 ± 0.05	0.05	0.06	0.14
LVPWs (mm)	2.73 ± 0.16^{b}	2.65 ± 0.14^{b}	2.58 ± 0.15^{b}	3.27 ± 0.14^{a}	3.04 ± 0.13^{ab}	2.95 ± 0.10^{ab}	0.0003	0.23	0.80
Diastolic volume (μL)	317 ± 41^{c}	318 ± 38^{c}	481 ± 26^{ab}	427 ± 40^{abc}	356 ± 30^{bc}	531 ± 47^{a}	0.036	0.0001	0.59
Systolic volume (μL)	34.2 ± 6.1^{c}	55.0 ± 8.2^{bc}	100.2 ± 12.8^{a}	38.4 ± 7.0^{c}	49.2 ± 10.5^{bc}	77.1 ± 8.6^{ab}	0.28	<0.0001	0.33
Stroke volume (μL)	283 ± 38^{b}	263 ± 33^{b}	381 ± 22^{ab}	388 ± 35^{ab}	306 ± 24^{b}	454 ± 43^{a}	0.009	0.0007	0.65
SBP:LVIDs	42.4 ± 2.9^{ab}	40.9 ± 1.8^{abc}	32.1 ± 1.5^{c}	48.3 ± 3.8^{a}	48.0 ± 3.0^{a}	36.9 ± 2.4^{bc}	0.009	0.0001	0.91
SBP:systolic volume	4826 ± 956^{ab}	3016 ± 418^{ab}	1599 ± 198^{b}	5562 ± 1462^{a}	4436 ± 840^{ab}	2291 ± 479^{ab}	0.17	0.001	0.89
ESS:LVIDs	2.40 ± 0.10^{b}	2.44 ± 0.08^{ab}	2.84 ± 0.16^{a}	2.30 ± 0.09^{b}	2.69 ± 0.09^{ab}	2.56 ± 0.11^{ab}	0.63	0.007	0.05
Cardiac output (mL/min)	70.1 ± 9.2^{b}	56.3 ± 7.9^{b}	90.6 ± 7.3^{b}	135.2 ± 10.9^{a}	84.8 ± 5.6^{b}	130.9 ± 12.9^{a}	<0.0001	0.0001	0.14
Relative wall thickness	0.56 ± 0.05	0.58 ± 0.05	0.47 ± 0.02	0.56 ± 0.02	0.60 ± 0.05	0.45 ± 0.02	1.00	0.003	0.87
Systolic wall stress	75.7 ± 7.0^{b}	104.4 ± 8.9^{ab}	129.9 ± 12.2^{a}	74.8 ± 7.1^{b}	93.3 ± 6.6^{b}	106.4 ± 7.3^{ab}	0.09	<0.0001	0.41
Estimated LV mass (g)	0.81 ± 0.03^{b}	0.89 ± 0.05^{ab}	1.01 ± 0.04^{ab}	1.16 ± 0.07^{a}	1.08 ± 0.12^{ab}	1.07 ± 0.06^{ab}	0.006	0.65	0.11
Fractional shortening (%)	53.1 ± 2.3^{ab}	47.1 ± 2.1^{bc}	41.2 ± 2.1^{c}	56.6 ± 2.5^{a}	49.6 ± 2.7^{abc}	47.7 ± 2.3^{bc}	0.033	0.0002	0.68
Ejection fraction (%)	89.1 ± 1.6^{a}	84.0 ± 2.0^{ab}	79.3 ± 2.0^{b}	91.2 ± 1.5^{a}	86.6 ± 2.1^{a}	85.2 ± 1.7^{ab}	0.021	0.0002	0.53
Systolic blood pressure (mmHg)	126 ± 2^{d}	146 ± 1^{bc}	144 ± 2^{c}	152 ± 2^{b}	161 ± 2^{a}	150 ± 2^{bc}	<0.0001	<0.0001	<0.0001
Right ventricular wet weight (mg/mm tibial length)	2.37 ± 0.19	3.39 ± 0.64	2.76 ± 0.16	4.21 ± 1.14	2.88 ± 0.12	3.19 ± 0.15	0.20	0.85	0.11
Left ventricular + septum wet weight (mg/mm tibial length)	17.6 ± 0.5^{b}	18.1 ± 0.7^{ab}	18.1 ± 0.5^{ab}	19.5 ± 0.5^{ab}	19.9 ± 0.2^{a}	19.1 ± 0.5^{ab}	0.003	0.62	0.62
Left ventricular diastolic stiffness constant, κ	23.8 ± 0.7^{b}	29.4 ± 0.6^{a}	27.3 ± 0.5^{a}	29.1 ± 0.7^{a}	29.1 ± 1.1^{a}	27.9 ± 0.7^{a}	0.003	0.002	0.0006
Liver wet weight (mg/mm tibial length)	241 ± 7^{b}	239 ± 7^{b}	250 ± 15^{b}	333 ± 8^{a}	351 ± 11^{a}	325 ± 19^{a}	<0.0001	0.76	0.31
ALT activity (U/L)	25.9 ± 1.6^{b}	29.0 ± 2.6^{b}	27.6 ± 1.9^{b}	41.8 ± 2.0^{a}	39.6 ± 1.6^{a}	40.4 ± 5.9^{a}	<0.0001	0.99	0.68
AST activity (U/L)	64.3 ± 2.9^{b}	78.3 ± 5.5^{ab}	64.1 ± 3.5^{b}	88.3 ± 3.6^{a}	95.1 ± 4.3^{a}	86.7 ± 9.4^{a}	<0.0001	0.07	0.78

Value are means ± SEM, n = 10–12. Mean values in a row with unlike superscript letters are significantly different (P<0.05). C, corn starch diet-fed rats; COr, orchidectomized rats fed corn starch diet; CL, leuprolide-treated rats fed corn starch diet; H, high-carbohydrate high-fat diet fed rats; HOr, orchidectomized rats fed high-carbohydrate high-fat diet; HL, leuprolide-treated rats fed high-carbohydrate high-fat diet; LVIDd, left ventricular internal diameter during diastole; LVIDs, left ventricular internal diameter during systole; IVSd, interventricular septal thickness during diastole; IVSs, interventricular septal thickness during systole; LVPWd, left ventricular

posterior wall thickness during diastole; LVPWs. left ventricular posterior wall thickness during systole; ESS, end-systolic stress; AST, aspartate transaminase; ALT, alanine transaminase.

Table 3. Effects of Omacor on leuprolide-induced changes in physiological and metabolic parameters

Variables	*CL*	*CLOm*	*HL*	*HLOm*	*P* value		
					Diet + leuprolide	*Omacor*	*(Diet + leuprolide) × Omacor*
Initial body weight (g)	337 ± 1	342 ± 2	338 ± 1	339 ± 3	0.61	0.13	0.31
Final body weight (g)	417 ± 8^{b}	319 ± 9^{c}	539 ± 18^{a}	536 ± 19^{a}	<0.0001	0.001	0.002
Water intake (mL/day)	26.4 ± 2.6^{b}	33.2 ± 2.6^{a}	17.5 ± 1.7^{c}	24.5 ± 1.1^{b}	0.0001	0.002	0.96
Food intake (g/day)	36.1 ± 2.2^{a}	38.3 ± 2.8^{a}	24.3 ± 1.9^{b}	26.1 ± 1.1^{b}	<0.0001	0.34	0.93
Energy intake (kJ/day)	401 ± 6^{c}	487 ± 34^{b}	507 ± 12^{ab}	560 ± 21^{a}	0.0001	0.002	0.44
Feed efficiency (g/kJ)	0.20 ± 0.02^{b}	-0.03 ± 0.01^{c}	0.40 ± 0.03^{a}	0.35 ± 0.03^{a}	<0.0001	<0.0001	0.0005
Abdominal circumference (cm)	20.1 ± 0.2^{b}	17.0 ± 0.2^{c}	23.0 ± 0.4^{a}	22.0 ± 0.4^{a}	<0.0001	<0.0001	0.002
Basal blood glucose concentrations (mmol/L)	4.8 ± 0.1^{b}	3.5 ± 0.1^{c}	5.4 ± 0.2^{a}	4.5 ± 0.3^{b}	0.0002	<0.0001	0.31
Area under the curve (mmol/L×minutes)	781 ± 10^{b}	599 ± 16^{c}	844 ± 24^{b}	933 ± 40^{a}	<0.0001	0.07	<0.0001
Total cholesterol (mmol/L)	1.65 ± 0.05^{b}	1.04 ± 0.03^{d}	1.89 ± 0.11^{a}	1.41 ± 0.07^{c}	0.0001	<0.0001	0.37
Triglyceride (mmol/L)	0.60 ± 0.06^{b}	0.27 ± 0.02^{b}	1.98 ± 0.31^{a}	0.33 ± 0.04^{b}	<0.0001	<0.0001	0.0004
NEFA (mmol/L)	2.38 ± 0.19^{b}	0.46 ± 0.03^{c}	4.63 ± 0.43^{a}	0.99 ± 0.18^{c}	<0.0001	<0.0001	0.0016
Whole-body lean mass (g)	314 ± 8^{a}	261 ± 10^{b}	308 ± 9^{a}	251 ± 13^{b}	0.44	<0.0001	0.85
Whole-body fat mass (g)	191 ± 8^{b}	59 ± 8^{c}	161 ± 15^{b}	248 ± 27^{a}	<0.0001	0.18	<0.0001
Bone mineral content (g)	0.173 ± 0.003	0.179 ± 0.003	0.177 ± 0.004	0.180 ± 0.001	0.40	0.14	0.62
Bone mineral density (g/cm^{2})	12.0 ± 0.2^{b}	10.6 ± 0.5^{b}	11.3 ± 0.5^{b}	16.8 ± 0.9^{a}	<0.0001	0.001	<0.0001
Retroperitoneal fat (mg/mm tibial length)	220 ± 18^{b}	95 ± 14^{c}	479 ± 54^{a}	406 ± 50^{a}	<0.0001	0.015	0.50
Epididymal fat (mg/mm tibial length)	125 ± 11^{b}	67 ± 7^{c}	263 ± 26^{a}	226 ± 26^{a}	<0.0001	0.02	0.59
Omental fat (mg/mm tibial length)	83 ± 6^{c}	79 ± 10^{c}	147 ± 13^{b}	249 ± 20^{a}	<0.0001	0.0007	0.0003

Value are means ± SEM, n = 10–12. Mean values in a row with unlike superscript letters are significantly different (P<0.05). CL, leuprolide-treated rats fed corn starch diet; CLOm, leuprolide-treated rats fed corn starch diet supplemented with Omacor; HL, leuprolide-treated rats fed high-carbohydrate high-fat diet; HLOm, leuprolide-treated rats fed high-carbohydrate, high-fat diet supplemented with Omacor; NEFA, non-esterified fatty acids.

Table 4. Effects of Omacor on leuprolide-induced changes in cardiovascular and hepatic function

Variables	*CL*	*CLOm*	*HL*	*HLOm*	*P* value		
					Diet + leuprolide	*Omacor*	*(Diet + leuprolide) × Omacor*
Heart rate	238 ± 11	255 ± 22	290 ± 15	283 ± 11	0.08	0.75	0.44
LVIDd (mm)	7.70 ± 0.15^{b}	7.64 ± 0.17^{b}	8.24 ± 0.22^{ab}	8.54 ± 0.16^{a}	0.0002	0.50	0.32
LVIDs (mm)	4.52 ± 0.19^{b}	4.62 ± 0.13^{b}	4.14 ± 0.18^{b}	5.33 ± 0.09^{a}	0.29	0.0001	0.0009
IVSd (mm)	1.77 ± 0.06^{ab}	1.68 ± 0.05^{b}	1.82 ± 0.06^{ab}	1.91 ± 0.02^{a}	0.008	1.0000	0.08
IVSs (mm)	2.80 ± 0.08^{b}	2.72 ± 0.07^{b}	3.12 ± 0.11^{a}	2.92 ± 0.06^{ab}	0.0028	0.10	0.47
LVPWd (mm)	1.79 ± 0.03^{ab}	1.67 ± 0.03^{b}	1.75 ± 0.05^{ab}	1.84 ± 0.04^{a}	0.10	0.70	0.009
LVPWs (mm)	2.58 ± 0.15^{b}	2.39 ± 0.05^{b}	2.95 ± 0.10^{a}	2.65 ± 0.06^{b}	0.003	0.017	0.58
Diastolic volume (μL)	481 ± 26^{b}	473 ± 34^{b}	531 ± 47^{b}	658 ± 36^{a}	0.002	0.11	0.07
Systolic volume (μL)	100.0 ± 12.8^{b}	104.9 ± 8.9^{b}	77.0 ± 8.6^{b}	159.2 ± 7.8^{a}	0.12	<0.0001	0.0003
Stroke volume (μL)	381 ± 22^{b}	368 ± 25^{b}	454 ± 43^{ab}	499 ± 31^{a}	0.002	0.61	0.36
SBP:LVIDs	32.1 ± 1.5^{b}	26.5 ± 0.9^{c}	36.9 ± 2.4^{a}	25.9 ± 0.7^{c}	0.18	<0.0001	0.08
SBP:systolic volume	1599 ± 198^{ab}	1216 ± 101^{b}	2291 ± 479^{a}	882 ± 48^{b}	0.50	0.002	0.06
ESS:LVIDs	2.84 ± 0.16	2.55 ± 0.07	2.56 ± 0.11	2.61 ± 0.08	0.33	0.28	0.13
Cardiac output (mL)	90.6 ± 7.3^{b}	94.5 ± 10.8^{b}	130.9 ± 12.9^{a}	141.2 ± 10.0^{a}	0.0001	0.50	0.76
Relative wall thickness	0.47 ± 0.02	0.44 ± 0.01	0.45 ± 0.02	0.44 ± 0.01	0.53	0.21	0.53
Systolic wall stress	129.9 ± 12.2^{ab}	117.8 ± 4.0^{ab}	106.4 ± 7.3^{b}	139.0 ± 4.0^{a}	0.88	0.19	0.006
Estimated LV mass (g)	1.01 ± 0.04^{b}	0.92 ± 0.05^{b}	1.07 ± 0.06^{b}	1.29 ± 0.05^{a}	0.0001	0.21	0.004
Fractional shortening (%)	41.2 ± 2.1^{b}	39.6 ± 0.7^{b}	47.7 ± 2.3^{a}	37.6 ± 0.8^{b}	0.18	0.0009	0.013
Ejection fraction (%)	79.3 ± 2.0^{b}	77.9 ± 0.7^{b}	85.2 ± 1.7^{a}	75.6 ± 0.9^{b}	0.22	0.0004	0.006
Systolic blood pressure (mmHg)	144 ± 2^{b}	122 ± 1^{d}	150 ± 2^{a}	137 ± 2^{c}	<0.0001	<0.0001	0.016
Right ventricular wet weight (mg/mm tibial length)	2.76 ± 0.16^{c}	3.59 ± 0.29^{b}	3.19 ± 0.15^{bc}	4.71 ± 0.22^{a}	0.0008	<0.0001	0.11
Left ventricular + septum wet weight (mg/mm tibial length)	18.1 ± 0.5^{b}	17.2 ± 0.8^{b}	19.1 ± 0.5^{b}	22.9 ± 0.9^{a}	<0.0001	0.044	0.002
Left ventricular diastolic stiffness constant, κ	27.3 ± 0.5^{a}	22.8 ± 1.0^{bc}	27.9 ± 0.7^{a}	21.3 ± 1.0^{c}	0.59	<0.0001	0.21
Liver wet weight (mg/mm tibial length)	250 ± 15^{b}	161 ± 7^{c}	325 ± 19^{a}	294 ± 14^{a}	<0.0001	0.0001	0.05
ALT activity (U/L)	27.6 ± 1.9^{b}	41.4 ± 4.4^{a}	34.9 ± 2.4^{ab}	39.7 ± 2.8^{a}	0.36	0.004	0.15
AST activity (U/L)	64.1 ± 3.5^{b}	72.5 ± 3.1^{ab}	86.7 ± 9.4^{a}	70.0 ± 4.2^{ab}	0.08	0.47	0.032

Value are means ± SEM, n = 10–12. Mean values in a row with unlike superscript letters are significantly different (P<0.05). CL, leuprolide-treated rats fed corn starch diet; CLOm, leuprolide-treated rats fed corn starch diet supplemented with Omacor; HL, leuprolide-treated rats fed high-carbohydrate high-fat diet; HLOm, leuprolide-treated rats fed high-carbohydrate, high-fat diet supplemented with Omacor; LVIDd, left ventricular internal diameter during diastole; LVIDs, left ventricular internal diameter during systole; IVSd, interventricular septal thickness during diastole; IVSs, interventricular septal thickness during systole; LVPWd, left ventricular posterior wall thickness during diastole; LVPWs. left ventricular posterior wall thickness during systole; ESS, end-systolic stress; AST, aspartate transaminase; ALT, alanine transaminase.

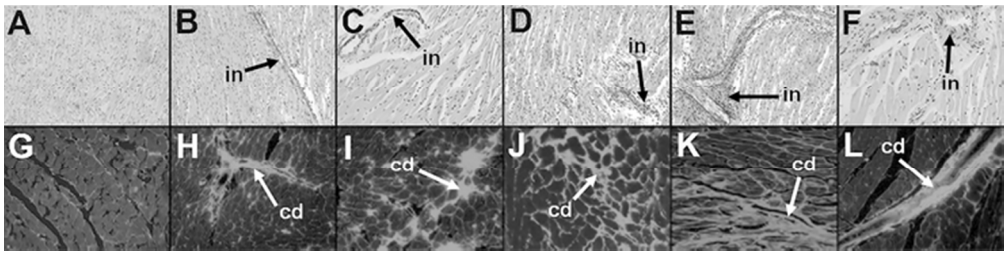


Figure 2. Effects of orchidectomy and leuprolide on the structure of the heart. Top row represents hematoxylin and eosin staining of left ventricle showing inflammatory cell ("in") while the bottom row represents picrosirius red staining of left ventricle showing collagen deposition ("cd") from C (A, G), COr (B, H), CL (C, I), H (D, J), HOr (E, K), and HL (F, L) rats.

62x15mm (300 x 300 DPI)

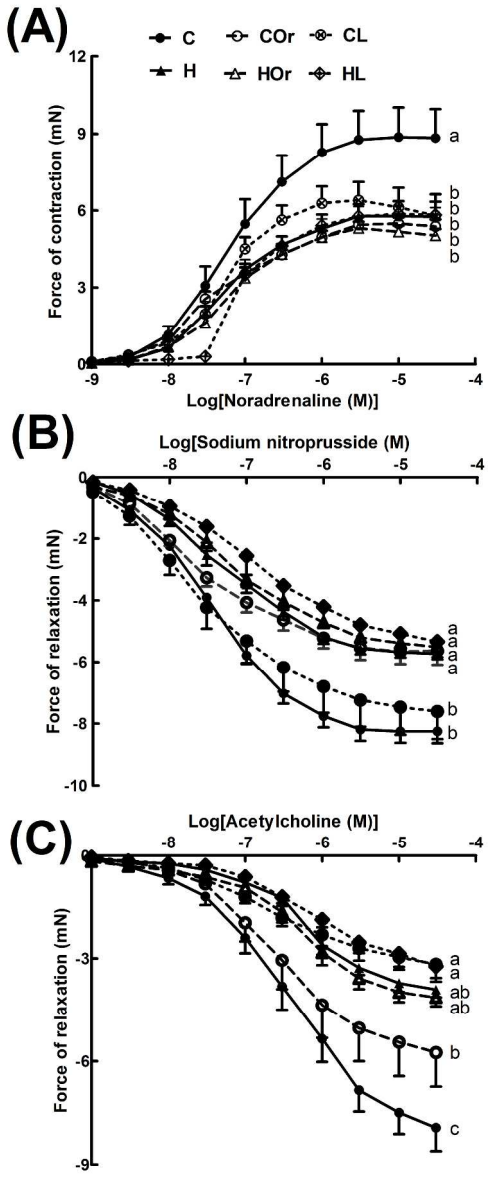


Figure 3. Cumulative concentration-response curves for noradrenaline (A), sodium nitroprusside (B), and acetylcholine (C) in thoracic aortic rings from C, COr, CL, H, HOr, and HL rats. Data are shown as mean ± SEM, n = 10–12/group. End-point means without a common alphabet significantly differ, P<0.05.

285x625mm (300 x 300 DPI)

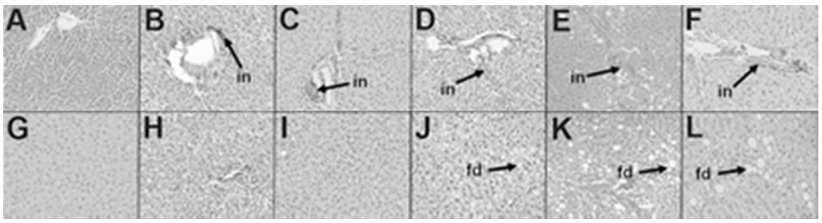


Figure 4. Effects of orchidectomy and leuprolide on the structure of the liver. Top row represents hematoxylin and eosin staining of liver showing inflammatory cell ("in") while the bottom row represents hematoxylin and eosin staining of liver showing fat deposition ("fd") from C (A, G), COr (B, H), CL (C, I), H (D, J), HOr (E, K), and HL (F, L) rats.

34x8mm (300 x 300 DPI)

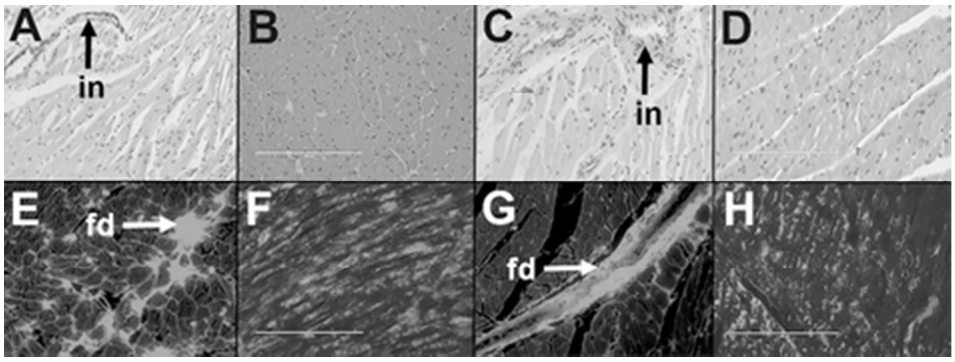


Figure 5. Effects of Omacor on leuprolide-induced changes in the structure of the heart. Top row represents hematoxylin and eosin staining of left ventricle showing inflammatory cell ("in") while the bottom row represents picrosirius red staining of left ventricle showing collagen deposition ("cd") from CL (A, E), CLOm (B, F), HL (C, G), and HLOm (D, H) rats.

40x14mm (300 x 300 DPI)

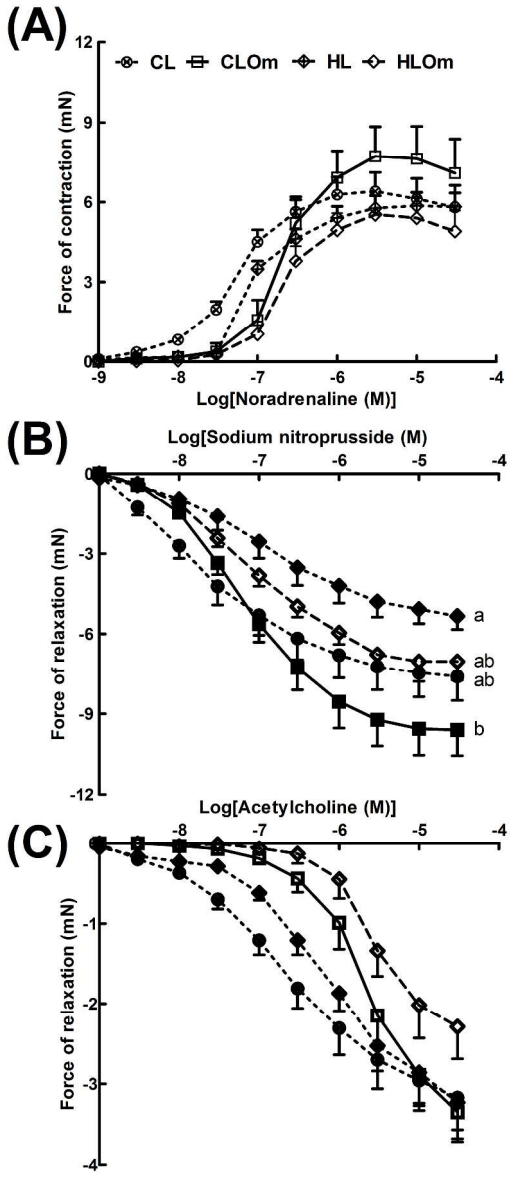


Figure 6. Cumulative concentration-response curves for noradrenaline (A), sodium nitroprusside (B), and acetylcholine (C) in thoracic aortic rings from CL, CLOm, HL, and HLOm rats. Data are shown as mean ± SEM, n = 10–12/group. End-point means without a common alphabet significantly differ, P<0.05.

293x617mm (300 x 300 DPI)

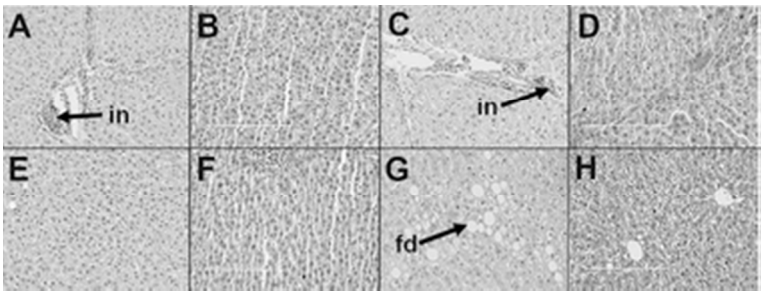


Figure 7. Effects of Omacor on leuprolide-induced changes in the structure of the liver. Top row represents hematoxylin and eosin staining of liver showing inflammatory cell ("in") while the bottom row represents hematoxylin and eosin staining of liver showing fat deposition ("fd") from CL (A, E), CLOm (B, F), HL (C, G), and HLOm (D, H) rats.

32x12mm (300 x 300 DPI)

Chapter 7. Discussion and conclusions

Summary of results: This thesis has emphasised preclinical studies with an appropriate and characterised diet-induced rat model that mimics metabolic syndrome in humans to demonstrate that foods are sources of potentially effective treatments. *Chapter 1* describes metabolic syndrome and its components, metabolic syndrome-associated cardiovascular complications and role of coffee and its components against the metabolic syndrome. Prevalence of metabolic syndrome is increasing worldwide [1]. The constellation of metabolic disorders such as obesity, elevated blood pressure, dyslipidaemia and insulin resistance or impaired glucose tolerance escalates the risk of cardiovascular diseases, hepatic disorders and type 2 diabetes, which can lead to death. Oxidative stress and chronic inflammation have crucial roles in the development of these metabolic consequences. Since coffee and its components show antioxidant and anti-inflammatory properties, it is logical to examine possible beneficial effects of coffee components against metabolic syndrome.

Chapter 2 describes the metabolic, cardiovascular and liver remodeling induced by high-calorie rich diet in rats and development of metabolic syndrome parameters such as abdominal obesity, hypertension, impaired glucose tolerance and dyslipidaemia. Previous studies from our research group in diet-induced obese rats with Colombian coffee extract containing caffeine, chlorogenic acid, trigonelline and diterpenoids showed reversal of the cardiovascular and liver remodelling to reduce hypertension and cardiac stiffness as well as non-alcoholic steatohepatitis, decreased impaired glucose tolerance and hypertension without affecting plasma lipids or abdominal obesity [2]. Further, caffeine produced different responses, especially with pronounced decreases in body weight [3]. My project extended these studies, initially by defining responses to green (unroasted) coffee and decaffeinated green coffee in rats with diet-induced metabolic syndrome. Both coffee products were provided as an extract incorporated in the food. Green coffee reduced body weight, abdominal fats and body mass index without altering plasma lipid contents indicating fat redistribution. Decaffeinated green coffee reduced body weight and retroperitoneal fat without causing change plasma content of lipid. Both decreased infiltration of inflammatory cells in the heart and the liver, and decreased collagen accumulation in the heart wall and fibrosis in the liver. The similarity of the responses suggests that caffeine is not the major bioactive compound in green coffee

reducing metabolic syndrome. Other compounds in green coffee that could have biological effects include chlorogenic acid, trigonelline, diterpenes such as cafestol and kahweol, and phenolic acids. The doses of chlorogenic acid are similar to literature studies which decreased some of the physiological changes in metabolic syndrome [4, 5], so this compound was then investigated.

Chapter 3 describes the responses to chlorogenic acid as an intervention in rats with diet-induced metabolic syndrome. There are several isomers of chlorogenic acid in nature, and my study investigated the most common, 5-O-caffeoylquinic acid. Chlorogenic acid decreased abdominal fat pads and body weight and improved cardiovascular and liver structure and function. This strongly suggests that chlorogenic acid produces at least some of the biological responses to green coffee. The roles of other compounds, especially trigonelline, the diterpenes present in coffee such as cafestol and kahweol, and phenolic acids, need further investigation.

Chapter 4 examines whether coffee waste could become a functional food. In the coffee production process, coffee waste and by-products cause problems of waste management and environmental contamination. High consumption of coffee requires the processing of a large number of coffee berries which generates coffee pulp as the waste from processing. Coffee pulp accounts for ~29% (w/w) of the dry weight of the whole berry. The dry matter of coffee pulp contains carbohydrates (15.7%), proteins (17.4%), crude fibre (14.1%), cellulose (20.7%), hemicellulose (3.6%), lignin (14.3%) [6] and minerals (especially potassium), along with tannins, polyphenols such as chlorogenic acid, and caffeine [7]. Hence, the use of coffee pulp may be considered as a potential approach for management of metabolic complications associated with obesity as there are many reports of health benefits from these individual components of coffee pulp. My study showed that coffee pulp intervention attenuated obesity, hypertension, improved glucose tolerance, cardiovascular and hepatic dysfunction while reducing blood lipid concentrations without affecting plasma total cholesterol concentrations and vascular reactivity.

Chapter 5 investigated the potential therapeutic effects of another major waste product in the production of coffee as a beverage, the spent coffee. This product contains

at least 1000 compounds, including the melanoidins produced during roasting of the beans by caramelisation and Maillard reaction. When given as a dietary intervention in high-carbohydrate, high-fat diet-fed rats, spent coffee reversed the metabolic, cardiovascular and liver abnormalities. Addition of spent coffee normalised blood glucose concentrations and delayed intestinal glucose absorption, reduced the elevated triglycerides and non-esterified fatty acids in plasma, and reversed abdominal obesity. Further, spent coffee reduced systolic blood pressure, cardiac wall stiffness and inflammation in the heart and liver. My study identified that gut microbiota played a major role in improving metabolic syndrome with spent coffee treatment.

Prostate cancer is one of the commonly diagnosed cancers in men and is treated by chemical or surgical induction of testosterone deficiency [8]. However, testosterone deficiency is associated with metabolic disorders such as central obesity, dyslipidaemia, impaired glucose tolerance, hepatic and cardiovascular remodelling [9]. *Chapter 6* has investigated whether omega-3 unsaturated fatty acids are effective as functional foods in testosterone deficiency in rats. Testosterone deficiency produced by castration or treatment with GnRH agonist increased the metabolic disturbances measured in rats fed a high-carbohydrate, high-fat diet. Addition of omega-3 unsaturated fatty acids to the diet decreased systolic blood pressure and left ventricular diastolic stiffness, reduced left ventricular infiltration of inflammatory cells and collagen deposition, reduced liver lipid accumulation and inflammatory cell infiltration without improving liver function. These results suggest that increased omega-3 unsaturated fatty acid supplementation could attenuate metabolic complications in prostate cancer patients with induced testosterone deprivation.

Conclusions: My results from these studies with coffee, coffee waste and fish oil demonstrated the functional food properties where coffee and fish oil-intervention markedly reduced cardiovascular and hepatic disorder. Furthermore, positive outcomes from coffee waste studies introduced an innovation for coffee waste management and has shown the unique concept to apply them as dietary supplements in humans for managing obesity-associated health complications. These studies have shown that these components can improve several parameters of metabolic syndrome, particularly reversing

cardiovascular and adipocytes changes. Moreover, other fruits such as pineapple, apple, potato, strawberry and tomato are also sources of chlorogenic acid. Hence, their by-products such as pineapple waste, apple pomace, potato peel, berry-juice processing waste could hold the relevant functional food properties against metabolic syndrome parameters. Human clinical trial could be the future prospective of coffee component and their wastes to describe their potential advantage against metabolic syndrome for human health. Overall, functional foods such as coffee and fish oil can be therapeutic supplement if taken in regular and proper amount as per individual needs.

Similar to coffee (green or roasted), many fruits and vegetables possess a range of compounds [10]. Some of the components from such fruits and vegetables have shown health benefits, including flavonoids, anthocyanins, ellagic acid and stilbenes [11]. As I have done in my thesis for coffee, many other foods still require extensive research to confirm whether they have sufficient bioactive compounds or whether we are discarding the most important components in food waste. Further, when considering the role of various components from a food, the food matrix should be considered in characterising its health effects [12]. Once health effects are defined for certain foods, food industries need to design the food products, functional foods or nutraceuticals that can attract compliance from the general community.

My thesis has defined responses to green coffee, coffee components and coffee waste products in a validated rat model of metabolic syndrome and obesity. As these interventions share antioxidant and anti-inflammatory activities, these responses may well be extended to other chronic diseases that are associated with oxidative stress and chronic inflammation, for example, inflammatory bowel disease, chronic kidney disease, non-alcoholic fatty liver disease and osteoarthritis. Previous and current studies from our laboratory have characterised rat models of these chronic disease [13-15] (inflammatory bowel disease model – under review).

Another aspect to be explored from my thesis is the advantage of food waste such as coffee pulp and spent coffee. Other food waste such as red grape marc from the wine industry, citrus waste from juice industry and spent cereals from beer brewing are some of the major food wastes from food processing. These wastes generate greenhouse gases

[16] and have an immense environmental footprint. Thus, identifying foods wastes that can provide nutraceuticals will not only provide a cheaper source of bioactive compounds for human health improvement but also reduce the environmental footprint. Once these health effects are defined, it will be important to identify the commercial viability of these products and then develop food products that can be accepted by the general community. For defining health benefits and developing food products, human intervention trials will need to be carried out to define the extent of the responses produced in humans. Success of these trials will help in convincing the community to consume these foods and hence will generate a viable market for food industries.

Although this study has provided evidence for effects of green coffee, coffee components and coffee wastes against diet-induced obesity, this study was unable to confirm molecular mechanisms involved in improvements of obesity-associated metabolic changes. Further, measuring a range of metabolic hormones such as insulin, leptin, adiponectin and resistin could supplement the data provided in this thesis. Further, identifying the role of gut microbiota for each intervention would further support the role of gut microbiota in improving metabolic complications in obesity. Further, sufficient and cost-effective availability of pure trigonelline, cafestol and kahweol would have allowed the study of the effects of these individual components of coffee. This would have further enabled me to extend my study outcomes to individual components.

References

1. Vanlancker T, Schaubroeck E, Vyncke K, Cadenas-Sanchez C, Breidenassel C, Gonzalez-Gross M, et al. Comparison of definitions for the metabolic syndrome in adolescents. The HELENA study. European Journal of Pediatrics 2017; 176(2): 241-52.
2. 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.
3. Panchal SK, Wong WY, Kauter K, Ward LC, Brown L. Caffeine attenuates metabolic syndrome in diet-induced obese rats. Nutrition 2012; 28(10): 1055-62.

4. H.V S, K V, Patel D, K S. Biomechanism of chlorogenic acid complex mediated plasma free fatty acid metabolism in rat liver. BMC Complementary and Alternative Medicine 2016; 16: 274.

5. Ali N, Rashid S, Nafees S, Hasan SK, Shahid A, Majed F, et al. Protective effect of chlorogenic acid against methotrexate induced oxidative stress, inflammation and apoptosis in rat liver: An experimental approach. Chemico-Biological Interactions 2017; 272: 80-91.

6. Dias M, Melo M, Schwan R, Silva C. A new alternative use for coffee pulp from semi-dry process to β-glucosidase production by *Bacillus subtilis*. Letters in Applied Microbiology 2015; 61(6): 588-95.

7. Murthy PS, Naidu MM. Sustainable management of coffee industry by-products and value addition-A review. Resources Conservation and Recycling 2012; 66: 45-58.

8. Rezaei MM, Rezaei MM, Ghoreifi A, Kerigh BF. Metabolic syndrome in patients with prostate cancer undergoing intermittent androgen-deprivation therapy. Canadian Urologic Association Journal 2016; 10(9-10): E300-5.

9. Tzortzis V, Samarinas M, Zachos I, Oeconomou A, Pisters LL, Bargiota A. Adverse effects of androgen deprivation therapy in patients with prostate cancer: focus on metabolic complications. Hormones 2017; 16(2): 115-23.

10. Shashirekha MN, Mallikarjuna SE, Rajarathnam S. Status of bioactive compounds in foods, with focus on fruits and vegetables. Critical Reviews in Food Science and Nutrion 2015; 55(10): 1324-39.

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

12. He X, Lu S, Cheng LW, Rasooly R, Carter JM. Effect of food matrices on the biological activity of ricin. Journal of Food Protection 2008; 71(10): 2053-8.

13. Diwan V, Small D, Kauter K, Gobe GC, Brown L. Gender differences in adenine-induced chronic kidney disease and cardiovascular complications in rats. American Journal of Physiology - Renal Physiology 2014; 307(11): F1169-78.

14. Sekar S, Shafie SR, Prasadam I, Crawford R, Panchal SK, Brown L, et al. Saturated fatty acids induce development of both metabolic syndrome and osteoarthritis in rats. Scientific Reports 2017; 7: 46457.

15. Sun AR, Panchal SK, Friis T, Sekar S, Crawford R, Brown L, et al. Obesity-associated metabolic syndrome spontaneously induces infiltration of pro-inflammatory macrophage in synovium and promotes osteoarthritis. PLoS One 2017; 12(8): e0183693.
16. Yi S, Yoo K. Greenhouse gas emissions and cost analyses for the treatment options of food waste and human excrement. Journal of Environmental Protection 2014; 5: 597-607.