Cardioprotective and Hepatoprotective Effects of Natural Products in Metabolic Syndrome

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In Loving Memory Of

My Grandfather (Mr. Pratap Narayan Sharma)

and

Grandmother (Mrs. Dhanwati Sharma)

Certification of Dissertation

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

Signature of Candidate — Date: July 5, 2012

Endorsement

Signature of Principal Supervisor — Date: 4th July 2012

Signature of Associate Supervisor — Date: 9th July 2012

Table of contents

Acknowledgements ..... **i**

Declaration ..... **iv**

Statement of contributions ..... **v**

List of publications included in this thesis ..... **xiii**

List of publications included as an appendix to this thesis ..... **xv**

Abstract ..... **xvi**

Chapter 1: Metabolic syndrome, cardiovascular remodelling and non-alcoholic fatty liver disease **1**

Chapter 2: Natural products

2.1 Potential health benefits of polyphenols in metabolic syndrome 12

2.2 Potential health benefits of Indian spices in the symptoms of the metabolic syndrome: A review 68

Chapter 3: A suitable animal model for metabolic syndrome studies

3.1 Rodent models for metabolic syndrome research 83

3.2 High-carbohydrate, high-fat diet-induced metabolic syndrome and cardiovascular remodeling in rats 97

Chapter 4: Flavonoids in rat model of human metabolic syndrome

4.1 Rutin attenuates metabolic changes, nonalcoholic steatohepatitis**,** and cardiovascular remodeling in high-carbohydrate, high-fat diet-fed rats 111

4.2 Quercetin ameliorates cardiovascular, hepatic**,** and metabolic changes in diet-induced metabolic syndrome in rats 121

Chapter 5: Ellagic acid and its complex derivatives (ellagitannins) in rat model of human metabolic syndrome

5.1 Cardioprotective and hepatoprotective effects of ellagitannins from European oak bark (*Quercus petraea* L.) extract in rats 130

5.2 Ellagic acid attenuates high-carbohydrate, high-fat diet-induced metabolic syndrome in rats 145

Chapter 6: Coffee and caffeine in metabolic syndrome

6.1 Coffee extract attenuates changes in cardiovascular and hepatic structure and function without decreasing obesity in high-carbohydrate, high-fat diet-fed male rats 155

6.2 Caffeine attenuates metabolic syndrome in diet-induced obese rats 166

Chapter 7: L-Carnitine attenuates symptoms of metabolic syndrome in high-carbohydrate, high-fat diet-fed rats 174

Chapter 8: Chronic ethanol consumption in rats fed a high-carbohydrate, high-fat diet 215

Chapter 9: Discussion and conclusion 239

Appendix A: Comparison of purple carrot juice and β-carotene in a high-carbohydrate, high-fat diet-fed rat model of the metabolic syndrome 251

Appendix B: Lipid redistribution by α-linolenic acid-rich chia seed inhibits stearoyl-CoA desaturase-1 and induces cardiac and hepatic protection in diet-induced obese rats 262

Appendix C: Omega-3 fatty acids and metabolic syndrome: Effects and emerging mechanisms of action 272

Appendix D: Chronic high-carbohydrate, high-fat feeding in rats induces reversible metabolic, cardiovascular, and liver changes 288

Appendix E: Conference presentations during the candidature 299

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Declaration

This thesis consists of my original work and does not contain any material that is previously published or written by another person, except where due reference has been made. I have clearly disclosed the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly indicated the contribution of others to my thesis as a whole, including help with study design, data analysis and technical assistance, required for the successful completion of my thesis. I declare that the content of my thesis is based on the result of experiments I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university. I have clearly stated wherever the substantial part has been used towards fulfilment of other degree.

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Statement of contributions by others to the thesis as a whole

No contributions by others.

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The following articles from my thesis have been used as part of PhD thesis by Mr. Abishek Iyer, The University of Queensland, awarded in December 2011, as these papers were co-authored.

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- **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;142:690-697.

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- **Panchal SK,** Brown L. Cardioprotective and hepatoprotective effects of ellagitannins from European oak bark (*Quercus petraea* L.) extract in rats. *European Journal of Nutrition* 2011; doi-10.1007/s00394-011-0277-1.

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- ✧ **Panchal SK**, Ward L, Brown L. Ellagic acid attenuates high-carbohydrate, high-fat diet-induced metabolic syndrome in rats. *European Journal of Nutrition* 2012; doi:10.1007/s00394-012-0358-9.

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Abstract

The prevalence of metabolic syndrome is increasing throughout the world. Metabolic syndrome is the clustering of risk factors for cardiovascular disease and type 2 diabetes. These include obesity, hypertension, impaired glucose tolerance, dyslipidaemia and insulin resistance, caused by oxidative stress and inflammation.

Nature has provided us with immense diversity in the form of plants and herbs, which contain a wide range of chemicals. Development of natural products as a potential treatment for metabolic syndrome requires a suitable rodent model. To induce metabolic syndrome, young male Wistar rats were fed with a high-carbohydrate, high-fat diet for 16 weeks with corn starch serving as control diet. Treatments were given as interventions in the final 8 weeks only as a reversal protocol. These interventions included rutin (1.6 g/kg food), quercetin (0.8 g/kg food), oak bark extract (0.5 ml/kg food), ellagic acid (0.8 g/kg food), coffee extract (5% in food), caffeine (0.5 g/kg food) and L-carnitine (1.2 % in food).

High-carbohydrate, high-fat diet induced hypertension, obesity, impaired glucose tolerance, dyslipidaemia, cardiovascular remodelling including ventricular dilatation, cardiomyocyte hypertrophy and cardiac fibrosis, reduced ventricular function, hepatic steatosis, hepatic inflammation and portal fibrosis and increased plasma markers of liver function.

Rutin and quercetin ameliorated these cardiovascular and hepatic changes and attenuated impairment in glucose tolerance and hypertension whereas only rutin improved obesity and dyslipidaemia. Ellagitannins from oak bark extract and ellagic acid attenuated cardiovascular remodelling and non-alcoholic fatty liver disease. Both ellagitannins from oak bark extract and ellagic acid reduced body weight and abdominal fat, however ellagic acid failed to reduce total body fat mass. Although coffee did not change body weight and abdominal fat, it improved the structure and function of the heart and the liver.

These effects differed from caffeine, which reduced total body fat, body weight and abdominal fat, along with the attenuation of symptoms of metabolic syndrome, cardiovascular remodelling and non-alcoholic steatohepatitis. L-Carnitine, a transporter of fatty acid across mitochondrial membrane, attenuated the metabolic syndrome by increasing β-oxidation and decreasing lipogenesis in both the heart and the liver.

In humans, fatty liver is caused by the excess consumption of diets rich in ethanol, animal fats, simple carbohydrates such as fructose or a combination of all three. An initiative was taken to mimic the combined effects of high-carbohydrate, high-fat diet and ethanol in rats as in human fatty liver disease. Ethanol synergistically aggravated hepatic steatosis induced by high-carbohydrate, high-fat diet; hepatic inflammation and fibrosis were unchanged compared to high-carbohydrate, high-fat fed rats. In contrast, ethanol feeding to high-carbohydrate, high-fat fed rats prevented cardiac fibrosis but caused minimal changes to cardiac function.

These studies clearly indicate the potential present in many natural products. The mechanisms of action of these natural products were also different as some of them reduced abdominal fat, possibly due to increased utilisation or removal of excess fat from the body through excretion, and others redistributed the fat to other areas such as subcutaneous fat, coffee being the exception.

Keywords

metabolic syndrome; obesity; non-alcoholic fatty liver disease; cardiovascular remodelling; natural products; high-carbohydrate, high-fat diet; polyphenols; dyslipidaemia; hypertension; impaired glucose tolerance.

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Chapter 1: Metabolic syndrome, cardiovascular remodelling and non-alcoholic fatty liver disease

Metabolic syndrome

Metabolic syndrome refers to the co-occurrence of hypertension, central obesity, dyslipidaemia, insulin resistance and impaired glucose tolerance as risk factors for the development of cardiovascular disease and type 2 diabetes [1]. These risk factors tend to cluster and thereby increase the overall risk [1]. Different diagnostic criteria have been provided [2-5]; with some of these definitions described in *Table 1*.

Metabolic syndrome is common in adult populations throughout the world. In Australia (1999-2000), the prevalence of metabolic syndrome ranged between 13.4% to 30.7%, depending on the definition used to define metabolic syndrome [6]. In the USA (1999-2002), 34% males and females were diagnosed with metabolic syndrome [7]. In Russia (2000), the overall prevalence of metabolic syndrome was 18%-19%. This prevalence was higher in women (23%-26%) compared to men (13%-15%) using various definitions [8]. Similarly, high prevalence rates of metabolic syndrome have been reported in the Indian subcontinent [9].

The presence of metabolic syndrome is associated with the development of cardiovascular disease and non-alcoholic fatty liver disease. Clinical, epidemiological and animal studies have proved the relationship between metabolic syndrome, especially obesity and dyslipidaemia, and the presence of non-alcoholic fatty liver disease [10-12]. Studies have also shown the increasing risk of development of non-alcoholic fatty liver disease in the presence of metabolic syndrome and *vice versa* [13,14]. Metabolic syndrome and non-alcoholic fatty liver disease are also associated with cardiovascular remodelling, including cardiac hypertrophy, ventricular dysfunction and endothelial dysfunction [15,16].

Cardiovascular remodelling

Cardiovascular remodelling is a process of change in the size, shape and function of the heart as a physiological response to metabolic or hormonal changes in the body [17,18]. These physiological responses lead to the development of molecular and cellular changes, including hypertrophy, necrosis and apoptosis of the myocyte, fibroblast proliferation and fibrosis in the interstitium [18]. These changes results in abnormalities in myocardial function including impaired contractility and relaxation, diminished cardiac pump function, dilatation and increased sphericity of the heart. U ltimately, these changes lead to systolic and diastolic cardiac dysfunction, which form the basis of heart failure and death [18].

Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease is a clinical condition that includes a wide spectrum of liver complications ranging from simple steatosis to steatohepatitis, advanced fibrosis and cirrhosis [19,20]. The liver is a metabolic workhorse that performs a range of biochemical functions including metabolism of lipids. Hepatic steatosis develops when there is excess deposition of triglycerides in the hepatocytes. This pathological condition can develop when the total input of fatty acids is more than the total output [21]. Sources of fatty acids in the liver include the hepatic free fatty acid uptake from blood and *de novo* lipogenesis. The output of fatty acids from the liver can be through fatty acid oxidation and fatty acid export within very low-density lipoproteins [21]. The disturbances in hepatic input and output of lipids can occur in the conditions of dyslipidaemia, obesity and insulin resistance [10,19]. Steatosis is characterized by excess fat storage and it can progress to steatohepatitis and finally leads to cirrhosis and structural and functional abnormalities of the liver. Some patients only develop steatosis whereas others develop steatohepatitis and fibrosis.

Table 1. Various definitions of metabolic syndrome [1]

	WHO (1998)	***EGIR (1999)***	***NCEP ATP III (2005 revision)***	***IDF (2005)***
Compulsory	Insulin resistance	Hyperinsulinaemia	None	Central obesity
Requirements	Insulin resistance or diabetes, plus any two of the five criteria	Hyperinsulinaemia, plus any two of the four criteria	Any three of the five criteria	Central obesity, plus any two of the four criteria
Central obesity	Waist:hip ratio (>0.90 in males, >0.85 in females) or BMI >30 kg/m^2	Waist circumference (≥94 cm in males, ≥80 cm in females)	Waist circumference (>40 inches in males, >35 inches in females)	Waist circumference (≥94 cm in males, ≥80 cm in females)
Hyperglycaemia	Impaired glucose tolerance, impaired fasting glucose, type 2 diabetes or other evidence of insulin resistance	Hyperinsulinaemia required (Plasma insulin >75th percentile)	Fasting glucose ≥100 mg/dl	Fasting glucose ≥100 mg/dl
Dyslipidaemia (Triglycerides)	Triglycerides ≥150 mg/dl or HDL-cholesterol (<35 mg/dl in males, <39 mg/dl in females)	Triglycerides ≥177 mg/dl or HDL-cholesterol (<39 mg/dl)	Triglycerides ≥150 mg/dl	Triglycerides ≥150 mg/dl
Dyslipidaemia (Cholesterol)			HDL-cholesterol (<40 mg/dl in males, <50 mg/dl in females)	HDL-cholesterol (<40 mg/dl in males, <50 mg/dl in females)
Hypertension	≥140/90 mmHg	≥140/90 mmHg	>130/85 mmHg	>130/85 mmHg
Other criteria	Microalbuminuria (Urinary albumin excretion of 20 μg/min or albumin-to-creatinine ratio of 30 mg/g)			

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

Oxidative stress

Oxidative stress is the cellular damage caused by an imbalance between the reactive oxygen or nitrogen species and the antioxidant system in the body. Reactive oxygen and nitrogen species are highly reactive, short-lived derivatives of oxygen or nitrogen, produced in all biological systems [22]. Due to the high reactivity of these chemical species, they react with surrounding molecules at or near the site of formation. Some of these species include the superoxide radical ($O_2^{\bullet -}$), the hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), nitric oxide (NO) and the peroxynitrite radical ($ONOO^{\bullet}$). These molecules play important roles in many physiological conditions including vascular reactivity [22]. Oxidative stress has been linked to the presence of obesity and metabolic syndrome [23,24]. It has now been proposed that the changes in metabolic syndrome are initiated by oxidative stress [25]. In metabolic syndrome, oxidative stress is responsible for the primary myocardial insult leading to cardiovascular remodelling [26,27]. Earlier studies have already shown that oxidative stress can lead to cardiac stiffness and fibrosis [28,29]. Similarly, oxidative stress may initiate the development of non-alcoholic steatohepatitis [30].

Inflammation

Inflammation is part of the non-specific immune response that occurs in reaction to any injury to the tissues. In some pathological conditions, the inflammatory process becomes chronic after initial activation subsequently leading to the development of long-term conditions such as obesity [31,32]. Initially, a link between obesity and inflammation was established through the expression of tumor necrosis factor-α (TNF-α) and this was further strengthen by the increased plasma concentrations of proinflammatory markers including cytokines and acute phase proteins such as C-reactive protein (CRP) in obese subjects [33-35]. CRP is now considered an independent risk factor for the development of cardiovascular disease [36]. Many inflammatory markers

present in the plasma of obese individuals originate from adipose tissue [34]. Thus, obesity is now defined as the state of chronic low-grade inflammation, which is initiated by the morphological changes in the adipose tissue [37]. Some of the proinflammatory cytokines from adipose tissue interfere with the signalling pathway for insulin. This ultimately leads to insulin resistance [38-42]. Adipocytes also secrete certain hormones (adipokines) in response to the increased fat deposition. These adipokines, including adiponectin, leptin and resistin, modulate metabolism in the body including food intake and energy expenditure [43]. Liver is the other tissue that is affected by excess adipose tissue and proinflammatory cytokines produced by adipose tissue. Chronic activation of nuclear factor-κB (NF-κB) by cytokines leads to the development of insulin resistance in liver [44,45]. The development of hepatic steatosis and non-alcoholic fatty liver disease in presence of insulin resistance has been established [46-49].

Conclusion

Metabolic syndrome is a combination of various risk factors for the development of cardiovascular disease and non-alcoholic fatty liver disease. These risk factors are responsible for the development of oxidative stress and inflammation in organs including the heart and liver. Oxidative damage and inflammation initiate the complications associated with the symptoms of metabolic syndrome, cardiovascular disease and non-alcoholic fatty liver disease. Thus, in this study, oxidative stress and inflammation have been targeted with antioxidants and anti-inflammatory agents for the treatment of metabolic syndrome and related disorders.

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Critical Reviews in Food Science and Nutrition

Chapter 2.1 Potential health benefits of polyphenols in metabolic syndrome

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Abstract

Natural products have been used for medicinal purposes for millennia. Ayurveda and Chinese medicine systems are based on us e of natural products from herbs and plant parts. One of the major classes of compounds found in the nature is polyphenol. Polyphenols are the plant secondary metabolites synthesized from shikimate pathway-derived products, with more than one phenolic ring without any nitrogen-based functional group. These polyphenols have been categorized into different classes including flavonoids. Metabolic syndrome, being the clustering of the risk factors for the development of cardiovascular disease and type 2 diabetes, is increasing in its prevalence throughout the world. This review has described the potential benefits associated with the dietary supplementation of polyphenols, in particular the wine polyphenols, resveratrol, rutin and quercetin, ellagtiannins and ellagic acid, catechins, and curcumin against the symptoms of metabolic syndrome. To characterize the effects of polyphenols, most studies have used biochemical methods, cell cultures, or animal models with few clinical trials in humans with metabolic syndrome or its components. Further clinical trials are required to confirm the effects in humans with multiple symptoms of metabolic syndrome. Also, the synergistic effects of these polyphenols should be considered with standard drug treatment of hypertension, diabetes, and obesity.

Keywords – Obesity, Resveratrol, Quercetin, Curcumin, Catechins.

Polyphenols: definition and classification

Polyphenols are compounds that are widely distributed in nature. The history of polyphenols goes back to the archaic period in Ancient Greece (800-500 BC) where they were called 'vegetable tannins' for use in the processing of leather (Quideau *et al.*, 2011). Polyphenols can be defined as the plant secondary metabolites that are derived exclusively from the shikimate-derived phenylpropanoid and/or the polyketide pathway(s), featuring more than one phenolic ring and being devoid of any nitrogen-based functional group in their most basic structural expressions (Quideau *et al.*, 2011). Thus, procyanidins, gallotannins, ellagitannins, flavonoids, flavanoids, lignans, ellagic acid, and curcumin are included as polyphenols.

Polyphenols can be categorized into different classes including flavonoids, phenolic acids (e.g. chlorogenic acid), lignans (e.g. seco-isolariciresinol), and stilbenes (e.g. resveratrol and pterostilbene). Flavonoids can further be classified as flavanols, flavanones (e.g. naringenin and hesperetin), flavonols (e.g. quercetin), anthocyanins (e.g. malvidin), isoflavones (e.g. genistein and daidzein), and chalcones (e.g. phloridzin) (Cook & Samman, 1996; Kim *et al.*, 2004). Flavanols include monomers (e.g. catechins and epicatechins) and polymers (condensed and hydrolysable tannins; e.g. ellagitannins) (***Figure 1***).

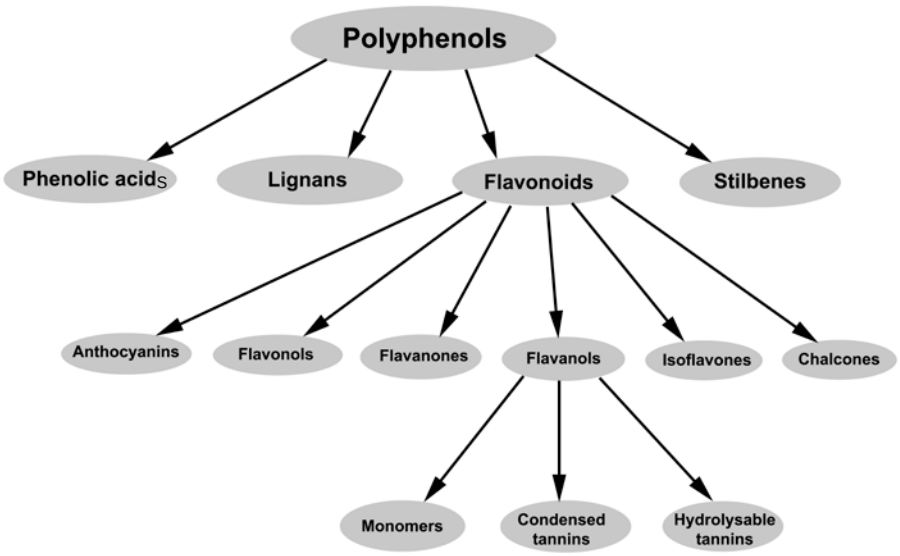

Figure 1: Classification of polyphenols

Biosynthesis of polyphenols: The shikimate pathway

Polyphenols are synthesized through shikimate-derived phenylpropanoid pathway or polyketide pathway. This pathway is used by plants and microorganisms for the biosynthesis of aromatic amino acids such as phenylalanine, tyrosine, and tryptophan (Herrmann, 1995b; Herrmann & Weaver, 1999). The seven steps of the shikimate pathway lead to the formation of chorismate which is the precursor of the three aromatic amino acids and several other aromatic compounds of primary metabolism (***Figure 2***). The intermediates of the shikimate pathway can also lead to the formation of many secondary metabolites including chlorogenic acid (Herrmann, 1995b; Herrmann, 1995a; Herrmann & Weaver, 1999). In addition, the three aromatic amino acids are precursors for many plant secondary metabolites including indole-3-acetic acid, indole glucosinolates,

ephedrine, taxine, lunarine, morphine, and dhurrin (Radwanski & Last, 1995; Celenza, 2001). Synthesis of polyphenols from chorismate-derived metabolites has been extensively reviewed (Dewick, 1998; Knaggs, 1999).

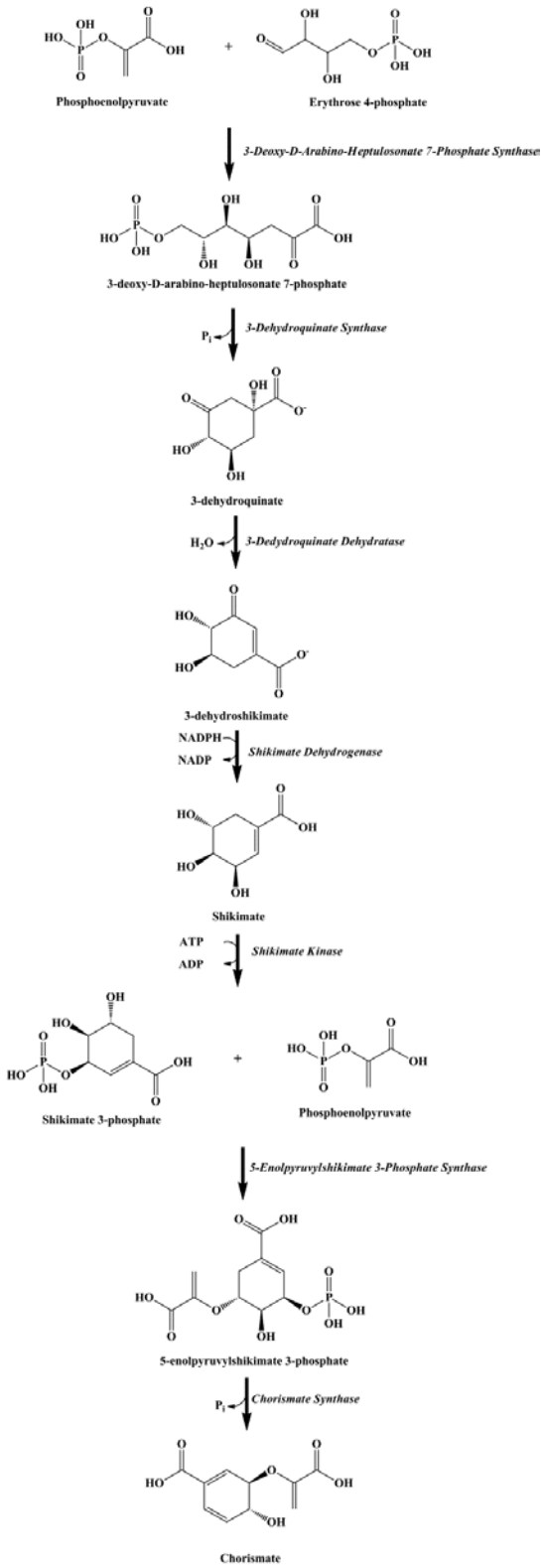

Figure 2: Shikimate pathway for biosynthesis of chorismate

Metabolic syndrome

Metabolic syndrome has become one of the major public health challenges worldwide (Lakka *et al.*, 2002; Cameron *et al.*, 2004; Day, 2007; Scholze *et al.*, 2010). It refers to the clustering of insulin resistance, hypertension, central obesity, impaired glucose tolerance, and dyslipidemia (Chew *et al.*, 2006) as risk factors for cardiovascular disease (Isomaa *et al.*, 2001; Lakka *et al.*, 2002), type 2 diabetes (Grundy, 2004), and nonalcoholic fatty liver disease (Abdelmalek & Diehl, 2007). Prevalence of metabolic syndrome is increasing throughout the world. In Australian population, the prevalence of metabolic syndrome ranged between 13.4% to 30.7%, depending on the definition used to define metabolic syndrome (Cameron *et al.*, 2007). This prevalence was 34% in males and females in the USA (Ford, 2005). In Russia, the overall prevalence of metabolic syndrome was approximately 18%-19% using various definitions (Sidorenkov *et al.*, 2010). Similarly, high prevalence rates of metabolic syndrome have been reported in the Indian subcontinent and European countries such as Germany, Italy, and Spain (Ramaraj & Chellappa, 2008; Scholze *et al.*, 2010). This high prevalence of metabolic syndrome throughout the world makes the finding of interventions that can reduce the risk to health an important exercise.

This review will focus on t he potentials of polyphenols as nutraceuticals to improve the clinical conditions of metabolic syndrome. Nutraceuticals are defined as alternative/complementary medicines derived from nutritional sources, such as plants; the study of their therapeutic possibilities is termed nutrapharmacology. Plants and microorganisms

produce non-nutritive products including polyphenols for many purposes, one example being the production of flavonoids as protection against fungal infection (Grayer & Harborne, 1994; Harborne, 1999; Galeotti *et al.*, 2008; Iwashina *et al.*, 2010; Hichri *et al.*, 2011). The relatively easy availability of polyphenols from herbs and plants increases their use in traditional medical systems and decreases their cost. As examples, both Ayurveda, the traditional medical system practiced in India (Mishra *et al.*, 2001), and traditional Chinese medicine (Kam & Liew, 2002) have used natural products containing polyphenols in the prevention and treatment of disease. There has been a push to combine anecdotal evidence with modern science to indicate the usefulness of natural products, including polyphenols, for therapeutic use outside these traditional systems.

Wine: rich source of polyphenols

Consumption of wine with food is a typical feature of the Mediterranean diet (Ursini & Sevanian, 2002). The low incidence of cardiovascular disease in France, despite high dietary intake of saturated fat, has been described as the *French Paradox*, and attributed to moderate regular red wine intake (Renaud & de Lorgeril, 1992). Wine contains many polyphenols including phenolic acids, stilbenes, flavonoids, and tannins with the antioxidant capacity of red wine being greater than white wine (Tubaro *et al.*, 1999; Waterhouse, 2002). Most of the polyphenols in wine come from grapes whereas tannins are extracted into the wine during maturation in oak barrels (Waterhouse, 2002). Red wine contains about 20 times more polyphenols than white wine, in particular flavonoids (e.g.,

quercetin, catechin, and epicatechin) and stilbenes (e.g., resveratrol) (Soleas *et al.*, 1997; Parks & Booyse, 2002; Waterhouse, 2002). However, white wine can also protect the heart from damage caused by ischemia-reperfusion injury by improving post-ischemic ventricular function and reducing myocardial infarction (Cui *et al.*, 2002). Red wine reduced body weight gain as well as adipocyte size in rats fed with normal diet (Monteiro *et al.*, 2009) and reduced epididymal fat content along with reduction in body weight in high-fat diet-fed rats (Vadillo *et al.*, 2006). In humans, red wine reduced total and LDL cholesterol and increased HDL cholesterol more than white wine; triglycerides were minimally affected with both wines (van Velden *et al.*, 2002). Plasma total antioxidant status was increased with red wine while it was decreased with white wine (van Velden *et al.*, 2002). These responses to red wine can be attributed to polyphenols, including phenolic acids, resveratrol, hydrolysable tannins, flavonols, and anthocyanins (Waterhouse, 2002).

Resveratrol

Resveratrol (***Figure 3A***) is extracted into the wine from the grape skin where it is synthesized as a response to fungal infection (Kroon *et al.*, 2010). Red wine (0.1-14.3 mg/L) contains more resveratrol than white wine (0.002-0.1 mg/L) (Romero-Pérez *et al.*, 1996; Baur & Sinclair, 2006). The physiological and biochemical responses of resveratrol in animal models, tissues, and cells have been extensively reviewed (Brown *et al.*, 2009; Kroon *et al.*, 2010; Mullin, 2011).

Both *in vivo* and *in vitro* studies have confirmed the effects of resveratrol in the components of metabolic syndrome (Kroon *et al.*, 2010). Resveratrol induced apoptosis and inhibited cell differentiation in 3T3-L1 preadipocytes by up-regulating expression of Sirt1 and inhibition of Akt (Rayalam *et al.*, 2008; Chen *et al.*, 2011a; Chen *et al.*, 2011b). Short-term incubation of freshly isolated rat hepatocytes with 25 μM resveratrol for 30 minutes decreased incorporation of acetate into triglycerides (Gnoni & Paglialonga, 2009). Resveratrol (10 mg/kg/day) improved dyslipidemia, hyperinsulinemia, and hypertension in obese Zucker rats (Rivera *et al.*, 2009). Resveratrol failed to inhibit the increase in body weight and body fat of high-fat diet-fed mice or rats (Baur *et al.*, 2006; Rivera *et al.*, 2009; Rocha *et al.*, 2009; Tauriainen *et al.*, 2011). In another study, resveratrol reduced body weight as well as body fat (Lagouge *et al.*, 2006). However, resveratrol inhibited the development of hepatic steatosis and hepatocyte ballooning in high-fat diet-fed mice (Tauriainen *et al.*, 2011). Pre-treatment with resveratrol (30 mg/kg/day for 1 week) in streptozotocin (STZ)-induced diabetic rats inhibited apoptosis in pancreatic β-cells along with the reduction in blood glucose concentrations and increase in serum insulin concentrations (Ku *et al.*, 2012). Resveratrol (1 mg/kg/day for 32 da ys) lowered systolic blood pressure and cardiac fibrosis, attenuated inflammation in the left ventricle, and improved ventricular and vascular function in deoxycorticosterone acetate-salt hypertensive rats (Chan *et al.*, 2011). Similarly, resveratrol (10 mg/kg/day) treatment reduced systolic blood pressure and cardiac hypertrophy in fructose-fed rats (Miatello *et al.*,

2005). These changes were accompanied by an increased expression of eNOS in mesenteric vascular bed and heart (Miatello *et al.*, 2005).

Daily 10 mg resveratrol was given for 3 months in a double-blind, placebo-controlled, randomized study, to patients with a history of myocardial infarction. Blood pressures and plasma concentrations of C-reactive protein, glycosylated haemoglobin, TNF-α, total cholesterol, triglyceride, and HDL-cholesterol were unchanged. Resveratrol increased flow-mediated dilation of the brachial artery and improved left ventricular function (increases in ejection fraction and E/A ratio) (Magyar *et al.*, 2012). In older adults with impaired glucose tolerance, resveratrol treatment for 4 weeks (1, 1.5, and 2 g/day) did not change fasting glucose concentrations, but peak post-meal glucose and 3-hour glucose area under the curve were lowered. Furthermore, insulin sensitivity (using the Matsuda index) was improved following treatment with resveratrol treatment, although body weight, percent body fat, blood pressure, fasting lipid profile, plasma high sensitivity C-reactive protein and adiponectin concentrations were unchanged (Crandall *et al.*, 2012). In obese humans, resveratrol (150 mg/day) treatment for 30 days decreased hepatic lipid content, plasma inflammation markers, glucose, and triglycerides, and alanine-aminotransferase activity; also, systolic blood pressure was lowered and HOMA index improved after resveratrol treatment (Timmers *et al.*, 2011). The responses to resveratrol have been widely studied in animal tissues *in vivo* and *in vitro*, but there is minimal large-scale evidence on chronic responses to resveratrol from human studies in conditions such as obesity (Chachay *et al.*, 2011).

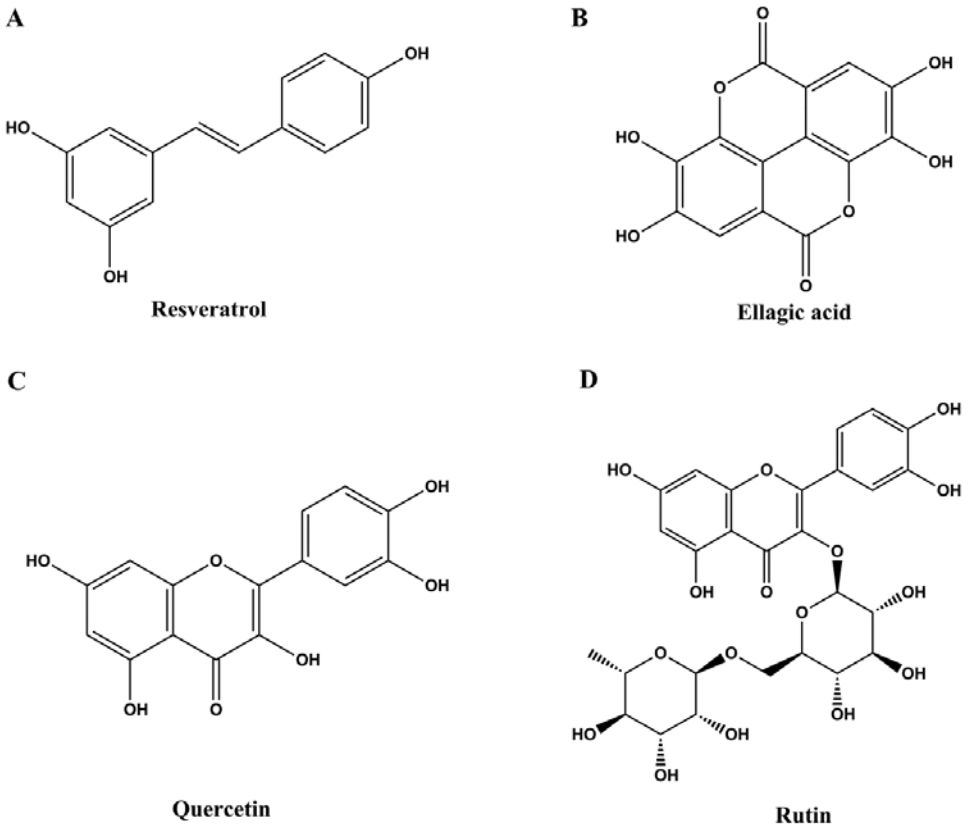

Figure 3: Polyphenols found in wine. (A) Resveratrol, (B) Ellagic acid, (C) Quercetin, and (D) Rutin

Ellagitannins and ellagic acid

Ellagitannins become constituents of red wine during its maturation in oak barrels (Saucier *et al.*, 2006; García-Estévez *et al.*, 2010). Different species of oak are used in the wine-making industry including American oak (*Quercus alba*) and European oak (*Quercus petraea and Quercus robur*). Ellagitannins are complex polyphenolic compounds found in oak as monomers or oligomers (Karonen *et al.*, 2010; Yoshida *et al.*, 2010). The major ellagitannins found in oak are vescalagin, castalagin, roburin and grandinin. Related ellagitannins are found in pomegranates, chestnuts, raspberries, strawberries, blackberries, and walnuts (Cerdá *et al.*, 2005; Seeram *et al.*, 2006; Coates *et al.*, 2007; Bakkalbaşi *et al.*, 2009).

Chemically, ellagitannins are hydrolysable tannins, which upon hydrolysis release ellagic acid. Ellagic acid (***Figure 3B***) is a polyphenol found in nuts as well as fruits such as raspberries, pomegranates, grapes, and blackcurrants (Talcott & Lee, 2002; Mari Kannan & Darlin Quine, 2012). The potential therapeutic role of the ellagitannins in red wine has been neglected, even though the presence of these polyphenols in wine and other dietary constituents has been known for a long time.

Ellagitannins from pomegranate juice attenuated isoproterenol-induced cardiac necrosis in rats (Jadeja *et al.*, 2010) and angiotensin-induced hypertension, glucosuria and proteinuria in STZ-induced diabetic rats (Mohan *et al.*, 2010). Pomegranate fruit extract containing punicalagin as the major ellagitannin improved vascular endothelial function without affecting the plasma lipid profile in obese Zucker rats (Seeram *et al.*, 2004; de Nigris *et al.*, 2007). In elderly hypertensive subjects, pomegranate juice consumption for 2 weeks reduced systolic blood pressure along with serum angiotensin converting enzyme activity (Aviram & Dornfeld, 2001). In type 2 diabetic subjects, concentrated pomegranate juice consumption (40 g/day) for 9 weeks reduced serum total cholesterol and LDL cholesterol (Esmaillzadeh *et al.*, 2004). In our recent study, we have shown that the ellagitannins from European oak (*Quercus petraea*) attenuated cardiovascular remodeling and nonalcoholic fatty liver, accompanied by reduction in systolic blood pressure, abdominal fat deposition, blood lipid concentrations, and improvements in glucose tolerance in high-carbohydrate, high-fat diet-fed rats, probably due to anti-oxidative and anti-inflammatory activities of ellagitannins (Panchal & Brown, 2011). Also,

ellagitannins from oak attenuated cardiovascular remodeling and lowered systolic blood pressure in Spontaneously Hypertensive Rats (Panchal & Brown, 2011). These studies have used rich but complex sources of ellagitannins including oak wood extract and pomegranate juice; studies with purified compound are justified.

Studies with purified ellagic acid in animals and humans with metabolic syndrome are limited (Larrosa *et al.*, 2010). Ellagic acid induced cardioprotection against isoproterenol-induced myocardial infarction (Kannan & Quine, 2011) and also showed anti-proliferative effects in cancerous cell lines (Losso *et al.*, 2004). Ellagic acid protected liver cells *in vitro* and *in vivo* in rat models of hepatic damage (Girish *et al.*, 2009; Hwang *et al.*, 2010). In high-carbohydrate, high-fat diet-fed rats, ellagic acid reduced body weight, abdominal fat content, basal blood glucose concentrations, plasma triglycerides, plasma total cholesterol, and plasma nonesterified fatty acid concentrations. In these rats, ellagic acid also improved the structure and function of the heart and the liver (Panchal *et al.*, 2012b).

Flavonoids

Flavonoids are naturally occurring low molecular weight polyphenolic compounds widely distributed in fruits and vegetables (Park *et al.*, 2008). More than 6000 flavonoids have been identified to date (Egert *et al.*, 2010). Anticancer, antimicrobial, antiviral, anti-inflammatory, immunomodulatory, and antithrombotic activities have been claimed for flavonoids (Havsteen, 1983; Kim *et al.*, 2004). Some flavonoids have shown

promising results in metabolic syndrome (Hu *et al.*, 2009; Mulvihill *et al.*, 2009). Effects of major flavonoids present in the diet have been described here.

Quercetin

Quercetin (3,3′,4′,5,7-pentahydroxy flavone; ***Figure 3C***) is a major flavonoid found in plants and plant products (Galisteo *et al.*, 2004), including red onions, apples, berries, citrus fruits, tea, and red wine (Ruiz *et al.*, 2009; Egert *et al.*, 2010). Quercetin has improved most aspects of the metabolic syndrome in experimental studies, but the doses/concentrations have differed markedly. Quercetin and its metabolites inhibited neutrophil-mediated modification of LDL *in vitro* through the inhibition of myeloperoxidase (Loke *et al.*, 2008). Quercetin protected human hepatocytes from ethanol-induced oxidative stress by activating Nrf2-mediated heme oxygenase-1 (Yao *et al.*, 2007). Quercetin reduced systolic blood pressure in hypertensive human patients as well as in animal models of hypertension (Egert *et al.*, 2009; Perez-Vizcaino *et al.*, 2009). Quercetin (0.5% in diet) reduced systolic blood pressure of high-fat, high-sucrose diet-fed rats after 4 weeks of treatment, possibly due to increased NO bioavailability through increased NOS activity (Yamamoto & Oue, 2006). Quercetin dilated isolated rat vascular smooth muscle, possibly by inhibiting protein kinase C (Duarte *et al.*, 1993). Quercetin (0.8% diet) increased the energy expenditure in high fat diet-fed mice without affecting the body weight and body composition (Stewart *et al.*, 2008). Quercetin (0.05 mg/kg/day) reduced serum triglyceride and cholesterol concentrations in

high-fat diet-fed rabbits after 12 weeks of treatment, whereas it did not affect the serum concentrations of triglyceride and cholesterol after 4 weeks of treatment (Juźwiak *et al.*, 2005). Quercetin (10 mg/kg/day) reduced body weight in obese Zucker rats without changing the average daily food intake (Rivera *et al.*, 2008). It also reduced plasma levels of triglycerides, free fatty acids, total cholesterol, and insulin in obese rats whereas adiponectin concentrations in plasma were increased. In this study, quercetin increased eNOS expression while decreasing iNOS expression in visceral adipose tissue of obese Zucker rats (Rivera *et al.*, 2008). In mice fed with a diet rich in fat, cholesterol, and sucrose, quercetin reduced visceral and liver fat accumulation and improved hyperglycemia, hyperinsulinemia, dyslipidemia, and plasma concentrations of adiponectin and TNF-α. Quercetin also reduced oxidative stress in these mice (Kobori *et al.*, 2011). In Spontaneously Hypertensive Rats, quercetin supplementation (1.5 g/kg in food) did not change blood pressure or left ventricular dimensions, ventricular functions, vascular morphology, vascular resistance, and conductance vessel reactivity (Carlstrom *et al.*, 2007). In high-carbohydrate, high-fat diet-fed rats, quercetin (~50 mg/kg/day) attenuated hypertension, impairment in glucose tolerance, and central obesity without reducing body weight (Panchal *et al.*, 2012a). These changes were accompanied by the improvements in structure and function of the heart and the liver (Panchal *et al.*, 2012a).

In a double-blind, randomized, placebo-controlled, crossover trial in obese subjects, quercetin supplementation (150 mg/day for 6 weeks) did not change body weight, waist circumference, fat mass, fat-free mass, serum

concentrations of total cholesterol, triacylglycerols, or glucose, although a decreased systolic blood pressure was observed (Egert *et al.*, 2010). Quercetin did not change serum concentrations of hs-CRP whereas serum concentrations of hs-TNF-α were decreased (Egert *et al.*, 2010). Quercetin supplementation (730 mg/day for 28 d ays) reduced systolic and diastolic blood pressure in a randomized, double-blind, placebo-controlled, crossover study in hypertensive subjects (Edwards *et al.*, 2007).

Rutin

Rutin (***Figure 3D***) is found abundantly in onions (200-300 mg/kg), apples (0.17% of dry weight), tea (10.18% in dry weight), and red wine (4-5 mg/L) (López *et al.*, 2001; Makris & Rossiter, 2001; Slimestad *et al.*, 2007; Atanassova & Bagdassarian, 2009). Rutin (1 mg/ml of medium) suppressed the differentiation of adiopocytes in pre-adipocyte 3T3-L1 cell lines (Choi *et al.*, 2006). Rutin (50 mg/kg/day) decreased serum glucose and lipid concentrations in STZ-induced type I diabetic rats (Fernandes *et al.*, 2010). In these diabetic rats, rutin decreased the serum activities of liver enzymes (alanine transaminase (ALT), aspartate transaminase (AST), and lactate dehydrogenase) (Fernandes *et al.*, 2010). Similarly, serum concentrations of insulin, total cholesterol, free fatty acid, and triglyceride were decreased with rutin treatment in STZ-diabetic rats (Fernandes *et al.*, 2010). Reduction in concentrations of total cholesterol may result from inhibition of HMG-CoA reductase (Fernandes *et al.*, 2010). In STZ-induced diabetic rats, rutin (100 and 300 mg/kg/day) inhibited the decrease in left ventricular function measured as fractional shortening and ejection fraction (Krishna *et al.*,

2005). High fat diet-fed C57BL/6 mice showed reduction in body weight and total cholesterol in blood with rutin treatment (25 and 50 mg/kg/day) whereas the treatment did not affect the triglyceride concentrations in blood (Choi *et al.*, 2006). Rutin (10 and 100 mg/kg/day) reduced concentrations of total cholesterol and LDL cholesterol along with plasma activities of AST and ALT in hypercholesterolemic rats after 4 weeks (Ziaee *et al.*, 2009). In rats fed a h igh-carbohydrate, high-fat diet, we have shown that rutin attenuated hepatic steatosis, cardiovascular remodeling, obesity, hypertension, dyslipidemia, hyperinsulinemia, and impaired glucose tolerance (Panchal *et al.*, 2011).

In a randomized controlled implementation trial in diabetic patients, rutin supplementation (500 mg/day) reduced blood pressure, body weight, and serum LDL cholesterol and increased serum HDL cholesterol but did not affect serum triglycerides (Sattanathan *et al.*, 2010). Withdrawal of rutin supplementation reversed these changes (Sattanathan *et al.*, 2010).

Anthocyanins

Anthocyanins are water-soluble natural pigments responsible for red-blue or purple colors in fruits and vegetables (He & Giusti, 2010). Cyanidin (***Figure 4A***), delphinidin (***Figure 4B***), malvidin (***Figure 4C***), pelargonidin, peonidin, and petunidin are the major dietary anthocyanins (Lamy *et al.*, 2006). Major dietary sources include fruits and vegetables such as berries (blueberry, bilberry, and chokeberry), purple carrot, black currant, red radish, purple maize, red cabbage, and purple sweet potato (He & Giusti, 2010; Tsuda, 2012).

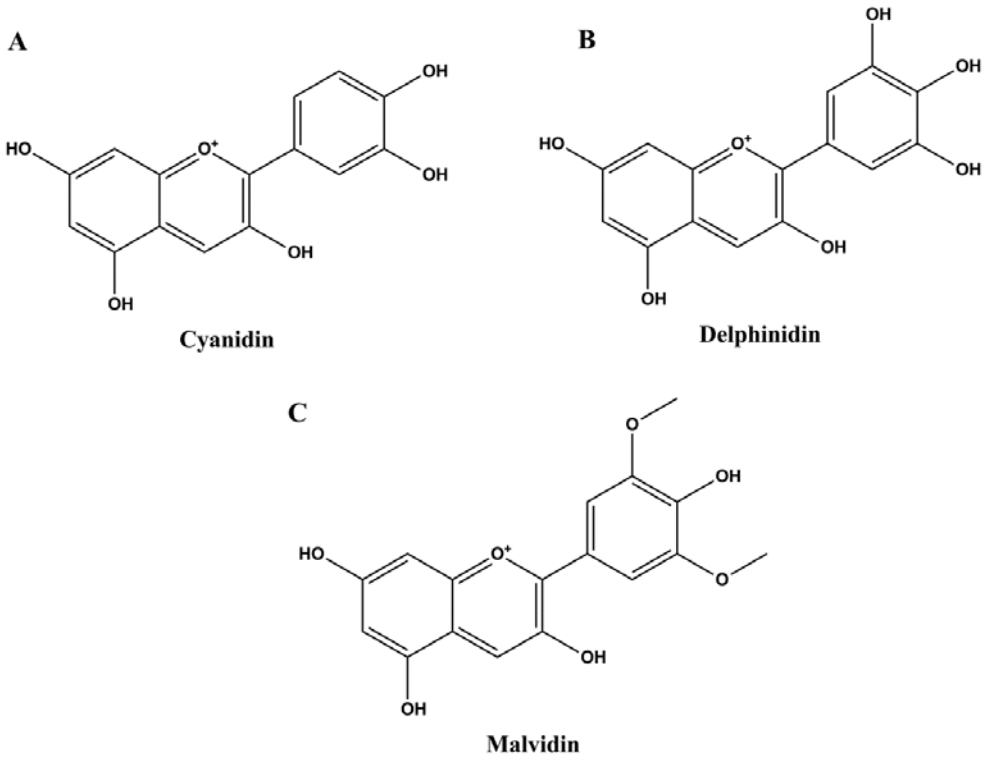

Figure 4: Anthocyanins found in fruits and vegetables. (A) Cyanidin, (B) Delphinidin, and (C) Malvidin

Epidemiological studies have shown that increased consumption of diets rich in anthocyanins reduced the risk of developing cardiovascular diseases (Wallace, 2011). Our studies have shown that purple carrot juice (5% of the diet) reduced abdominal obesity, systolic blood pressure, plasma lipids, hepatic steatosis, cardiac fibrosis, and inflammation along with improved glucose tolerance in high-carbohydrate, high-fat diet-fed rats (Poudyal *et al.*, 2010). Chokeberry fruit juice (5, 10, and 20 ml/kg body weight) for 30 days reduced total cholesterol, LDL cholesterol, and triglycerides in 4% cholesterol diet-fed rats (Valcheva-Kuzmanova *et al.*, 2007). Chokeberries (100 and 200 mg/kg/day) also reduced visceral adiposity, blood glucose, serum triglycerides, total cholesterol, and LDL cholesterol in fructose-fed rats (Qin & Anderson, 2011). In the same study,

anthocyanin supplementation increased plasma adiponectin levels, inhibited the plasma levels of pro-inflammatory cytokines such as TNF-α and IL-6, down-regulated adipogenic markers (Gsk3β, Fabp4, Fas, and Lpl) mRNA expression, and up-regulated important intermediates of the insulin signalling cascade (Irs1, Irs2, Pi3k, Glut1, Glut4, and Gys1) (Qin & Anderson, 2011). In diabetes induced by high-fructose diet and simultaneous single injection of STZ (20 mg/kg), dietary supplementation with chokeberry fruit extract (0.2%; ~400mg/g of anthocyanin glycosides in the extract) decreased antioxidant status of vital organs, total plasma cholesterol, and blood glucose concentrations (Jurgoński *et al.*, 2008). In Zucker rats fed a high-fat diet, supplementation with 2% dietary blueberry or 1% whole tart cherry powder reduced plasma triglycerides, fasting insulin, HOMA-IR, and abdominal fat mass and increased adipose and skeletal muscle PPAR-α and PPAR-γ activity along with reductions in TNF-α, IL-6, and NF-κB in the plasma and the adipose tissue, and improved glucose tolerance (Seymour *et al.*, 2009; Seymour *et al.*, 2011). At the same dosage after 90 days, tart cherries reduced fasting blood glucose, hyperlipidemia, hyperinsulinemia, and fatty liver with increased hepatic PPAR-α expression in salt-sensitive Dahl rats (Seymour *et al.*, 2008). Anthocyanins from black soybean seed coats (cyanidin-3-glucoside, delphinidin-3-glucoside, and petunidin-3-glucoside; 10-100 μg/mL) inhibited TNF-α-mediated VCAM-1 expression in human umbilical vein endothelial cells (Nizamutdinova *et al.*, 2009). In the same cell line, delphinidin inhibited oxidised LDL-induced cell viability loss primarily by up-regulating proteins involved in inhibiting apoptosis including Bcl-2 and

Bax proteins (Chen *et al.*, 2010). These results suggest that the responses to anthocyanins supplementation may be mediated by its anti-inflammatory and antiapoptotic properties and up-regulation of the insulin-signalling cascade.

Anthocyanins have direct protective effects on the heart. Hearts from male Wistar rats fed on a diet based on a nthocyanins containing maize kernels for 8 weeks were more resistant to regional ischemia and reperfusion insult induced in an isolated heart preparation (Toufektsian *et al.*, 2008). This diet also reduced the infarct size in coronary occlusion and reperfusion model (Toufektsian *et al.*, 2008). Anthocyanin extract from black rice (5 g/kg diet) lowered body weight gain, serum triglyceride, raised hepatic CPT-1 expression, and inhibited platelet hyperactivity suggested by decreased thromboxane A2, the thrombogenic ratio of thromboxane A_2 and prostacyclin, serum calmodulin, and soluble P-selectin expression in high-fat diet-fed rats (Yang *et al.*, 2011).

Human studies concur with the therapeutic responses produced in animal models to dietary anthocyanins. In patients with metabolic syndrome, chokeberries extract (3 x 100 mg/day) for two months decreased both systolic and diastolic blood pressure, endothelin-1, total cholesterol, LDL cholesterol, triglycerides, TBARS, catalase activity, and induced superoxide dismutase activity (Broncel *et al.*, 2010). In hypercholesterolemic patients, 320 mg/day of purified anthocyanins isolated from bilberries and blackcurrants increased brachial artery flow-mediated dilation, cGMP, and HDL cholesterol concentrations and decreased the

serum soluble vascular adhesion molecule-1 and LDL cholesterol concentrations (Zhu *et al.*, 2011). Anthocyanins also improved endothelial function in these patients as well as in isolated rat aortic rings; these effects were abolished by nitric oxide-cGMP inhibitors suggesting the role of nitric oxide-cGMP signalling pathway in anthocyanins-mediated vasodilation (Zhu *et al.*, 2011)

Tea polyphenols: Catechins

Tea is one of the most widely consumed beverages in the world. It is consumed in the form of green tea, oolong tea, or black tea. Green tea is prepared by drying the leaves of the plant *Camellia sinensis* using steam. This process prevents the oxidation of tea polyphenols by inactivating the enzymes in the leaves. Black tea is prepared by crushing the tea leaves to promote enzymatic oxidation which leads to condensation of tea polyphenols resulting in polymers (Chacko *et al.*, 2010). Green tea mainly contains catechins (***Figure 5A-D***), along with flavanols and polymeric flavonoids (Balentine *et al.*, 1997; Lakenbrink *et al.*, 2000). Epigallocatechin gallate is the major catechin found in green tea. Black tea mainly contains oligomeric and polymeric polyphenols such as thearubigin and theaflavins (Balentine *et al.*, 1997; Lakenbrink *et al.*, 2000)

Catechins are flavonoids often found as the gallate derivatives, usually catechin gallate (CG), epigallocatechin (EGC), epigallocatechin gallate (EGCG), epicatechin gallate (ECG), and epicatechin (EC). Tea is one of the major sources of catechins (Cabrera *et al.*, 2006); other sources

include wine, grape skins, grape seeds, and cacao beans (Yilmaz & Toledo, 2004; Auger *et al.*, 2005).

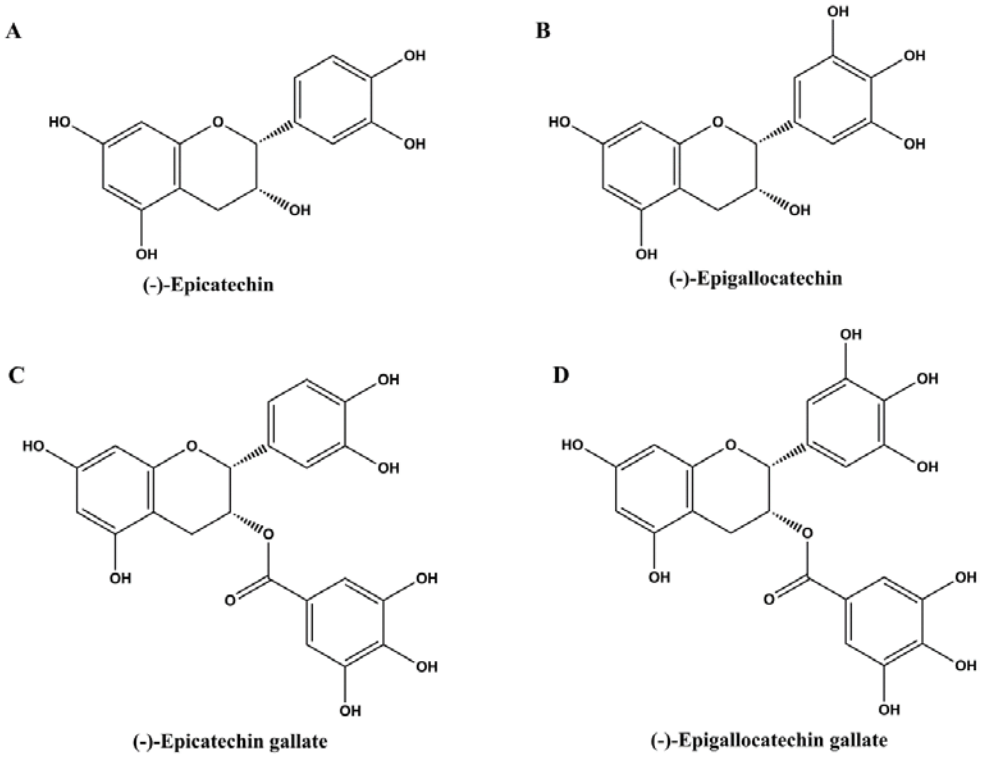

Figure 5: Catechins found in tea

CG, EGC, EGCG, and ECG prevented the differentiation of preadipocytes to adipocytes (Furuyashiki *et al.*, 2004). Administration of 0.5% tea catechins in drinking water to rats for 3 weeks reduced liver lipid content, serum total cholesterol concentration, and body weight in normal diet-fed rats, indicating that tea catechins can control lipid metabolism in obese as well as non-obese rodents (Ito *et al.*, 2008). Tea catechins showed increased excretion of carbohydrates, proteins, and lipids in the feces suggesting the reduction in digestibility of these macromolecules accompanied by the reduction in the body weight gain (Unno *et al.*, 2009). Tea catechins (1% of the diet) also showed body fat reduction along with

reduction in liver triglyceride content in rats after 23 days of feeding (Ikeda *et al.*, 2005).

EGCG reduced body weight, blood glucose, and plasma insulin concentrations and improved insulin sensitivity in high-fat diet-fed mice (Sae-Tan *et al.*, 2011). EGCG also reduced plasma ALT activity, hepatic steatosis, and liver weight in these mice (Sae-Tan *et al.*, 2011). In high-fat diet-fed mice, tea catechins reduced body weight gain, abdominal fat deposition, liver triglycerides, and plasma concentrations of insulin, leptin, and total cholesterol (Murase *et al.*, 2002). The anti-obesity effect of EGCG may involve mediating the balance between energy intake and energy expenditure (Moon *et al.*, 2007). In a pressure overload rat model induced by abdominal aortic constriction, EGCG inhibited cardiac hypertrophy and cardiac fibrosis with improved ventricular function without changing systolic blood pressure (Hao *et al.*, 2007). In stroke-prone Spontaneously Hypertensive Rats, green tea polyphenols reduced blood pressure and increased aortic expression of catalase (Negishi *et al.*, 2004). This study confirms the antioxidant effect of green tea polyphenols in reducing blood pressure.

The reported responses to catechins from tea have shown health benefits in humans (Higdon & Frei, 2003; Cabrera *et al.*, 2006; Feng, 2006; Hsu *et al.*, 2011; Yang *et al.*, 2012). In overweight human subjects, supplementation of green tea extract containing catechins reduced body weight, body mass index, total body fat mass, total body lean mass, systolic blood pressure, and blood glucose concentrations (Yang *et al.*, 2012). In the

Ohsaki National Health Insurance Cohort Study, green tea consumption reduced cardiovascular mortality (Kuriyama *et al.*, 2006). In a meta-analysis study, it was concluded that green tea consumption lowered serum total cholesterol and LDL cholesterol concentrations (Zheng *et al.*, 2011). In a prospective cohort study, it was concluded that the consumption of green tea substantially decreased serum concentrations of total cholesterol, LDL cholesterol, VLDL cholesterol, and triglycerides whereas increased serum HDL cholesterol concentration was found (Imai & Nakachi, 1995). An inverse association was found between green tea consumption and serum activity of AST (Imai & Nakachi, 1995). Results from this cross-sectional study indicated that the consumption of green tea might prevent cardiovascular and liver diseases.

Turmeric polyphenol: Curcumin

Turmeric (*Curcuma longa*) is an Indian spice used as a dry powder in food preparations all over the world. Turmeric has been used as a medicine in Ayurveda, traditional Chinese medicine, and in traditional household treatments. Curcumin (***Figure 6***) is the principal ingredient found in turmeric (Kiuchi *et al.*, 1993; Goel *et al.*, 2008b). Responses to curcumin have been extensively reviewed (Aggarwal *et al.*, 2007; Anand *et al.*, 2008; Goel *et al.*, 2008a; Aggarwal & Harikumar, 2009; Aggarwal & Sung, 2009).

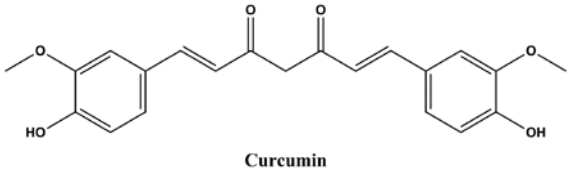

Figure 6: Structure of curcumin

Curcumin decreased the symptoms of metabolic syndrome in rodent models. In high-fat diet-fed rats, curcumin (80 mg/kg/day) reduced fasting plasma concentrations of glucose, insulin, total cholesterol, triglycerides, LDL cholesterol, free fatty acids, and TNF-α whereas it increased plasma HDL cholesterol concentrations after 60 days in a prevention protocol; these changes were accompanied by improved insulin sensitivity and glucose tolerance (El-Moselhy *et al.*, 2011). High-fat diet-fed mice (treated with 4 g curcumin/kg diet for 2 days/week for 28 weeks) showed reduction in body weight, epididymal fat content, total body fat content, fasting plasma insulin concentrations, and improved tolerance to glucose, pyruvate, and insulin with inhibition of hepatic steatosis and infiltration of macrophages in adipose tissue and liver (Shao *et al.*, 2012). Similar results were found with curcumin (3% in food) in *ob/ob* mice (Weisberg *et al.*, 2008). Curcumin inhibited adipokine-induced adipogenesis in 3T3-L1 adipocytes and preadipocyte differentiation (Ejaz *et al.*, 2009). Curcumin treatment (500 mg/kg/day) suppressed body weight gain, reduced total body fat, prevented hepatic steatosis, and lowered serum total cholesterol in high-fat diet-fed mice (Ejaz *et al.*, 2009). Curcumin treatment (100 mg/kg/day) lowered blood pressure in L-N^G-nitroarginine methyl ester-hypertensive rats (Hlavačková *et al.*, 2011). Curcumin (50 mg/kg/day) improved ventricular dimensions and functions in salt-sensitive Dahl rats and in surgically-induced myocardial infarction in rats by attenuating cardiac hypertrophy and fibrosis (Morimoto *et al.*, 2008). Blood pressure in these rats was unaltered with curcumin treatment (Morimoto *et al.*, 2008). Similar cardioprotective

roles of curcumin have been reviewed previously (Wongcharoen & Phrommintikul, 2009; Alappat & Awad, 2010).

A phase I clinical trial showed that 8 g/day curcumin orally for 3 months had no side-effects or toxicity in subjects (Cheng *et al.*, 2001). In healthy individuals, administration of curcumin (500 mg/day) decreased serum lipid peroxides and total cholesterol concentration while increasing serum HDL cholesterol concentrations (Soni & Kuttan, 1992). Similarly, curcumin (10 mg twice a day, for 30 days) reduced serum LDL cholesterol and increased serum HDL cholesterol in atherosclerosis patients (Ramírez-Boscá *et al.*, 2000).

Dosage and chronic intake of polyphenols

In vivo studies with polyphenols have shown that these compounds can be effective in treating the symptoms of metabolic syndrome. Although the doses of polyphenols used in animal studies have been effective, the doses cannot be directly applied to the humans due to various differences between rodents and human. There are various conversion equations that can be used to transform the animal doses to the human doses. Some of these are scaling equation based on metabolic rate difference between animals and humans (Bachmann *et al.*, 1996) and a conversion based on body surface area (Reagan-Shaw *et al.*, 2008). Also, the length of protocol in animal is very short and varies from one study to the other study. Similarly, the life span of animals used in the studies (for example, 27-30 months for a rat) is much shorter than the human life span (65-70 years on

an average). Thus, a relative period has to be considered in humans to see a consistent response with the polyphenols.

Toxicity

Hazards, safety, and risks related to polyphenol consumption have previously been considered (Schilter *et al.*, 2003; Mennen *et al.*, 2005; Lambert *et al.*, 2007; Martin & Appel, 2010). Most studies with polyphenols or natural products have focussed on characterizing their beneficial effects in various disease conditions and the toxicity related to polyphenols has been overlooked (Mennen *et al.*, 2005). Repeated oral doses of pomegranate husk extract (6% punicalagin-containing diet) to the rats for 37 days did not show any toxic effects (Cerdá *et al.*, 2003). In another long-term study, toxicity and carcinogenicity of quercetin were tested in male and female rats. Quercetin (0, 1000, 10,000, or 40,000 ppm) was given in the diet for 2 years at doses from ~40 to 1900 mg/kg/day. High dose groups showed reduction in body weight gain compared to controls during the 2nd year of the study. After 2 years, quercetin showed carcinogenic activity in the kidney of the male rat without inducing tumors at other sites (Dunnick & Hailey, 1992). Similarly, tea catechins (EGCG) have shown pro-oxidant activities in different studies (Yang *et al.*, 2000; Hong *et al.*, 2002; Weisburg *et al.*, 2004; Hou *et al.*, 2005). Another study reported the reduction in rat hepatocyte viability with EGCG (Galati *et al.*, 2006).

The use of polyphenols can be questioned, considering the differences between various cancers and other pathological conditions including cardiovascular disease, hypertension, and diabetes. To reduce cell

growth in cancer, compounds will cause toxicity to the cells whereas in metabolic diseases such as diabetes, cell growth needs to be maintained and its function should be improved. Thus, theoretically, anything that is active in cancer may not be effective in metabolic diseases; unless highly different doses are used. Thus, the toxicity of polyphenols in cancer can be used though application of higher doses whereas the potential benefits of polyphenols could be utilized by using a relatively lower but pharmacologically active dose.

Conclusion

Natural products are abundant in phytochemicals some of which belong to the class of polyphenols. These polyphenols are synthesized by the plants through shikimate pathway. Some of the polyphenols found in wine and tea have been studied for their pharmacological activities *in vitro*, *in vivo* in animal models of metabolic syndrome, and in humans. Red wine consists of a range of polyphenols including resveratrol, ellagitannins, quercetin and anthocyanins. These phytochemicals are effective in attenuating the symptoms of metabolic syndrome and associated complications. Similarly, tea catechins and curcumin from turmeric have been effective against the symptoms of metabolic syndrome. Some of the polyphenols such as ellagic acid have been studied to lesser extent for their activity against metabolic syndrome. With the effectiveness shown by polyphenols in metabolic syndrome, it can be proposed that these phytochemicals, either in extract form or purified form, can be used as a complimentary medicine in treatment of metabolic syndrome,

cardiovascular disease, type 2 diabetes, and non-alcoholic fatty liver disease.

Although most of the polyphenols have shown therapeutic effects in metabolic syndrome in animal models, there have been limited numbers of human trials to justify the data. Also, the subjects in the human trials were considered for limited parameters related to metabolic syndrome in a particular trial. Thus, the responses to polyphenols need to be explored in subjects with wide range of symptoms of metabolic syndrome. Also, the literature on the combinatorial effects of polyphenols with standard drugs in various symptoms of metabolic syndrome is missing. If not used alone, these phytochemicals may enhance the effectiveness of standard drugs in attenuating the symptoms of metabolic syndrome.

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Review

Chapter 2.2

Potential Health Benefits of Indian Spices in the Symptoms of the Metabolic Syndrome: A Review

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Spices used in Indian cooking have a long history of use as medicines to prevent and treat diseases. Many studies have confirmed that spices can be useful medicines, but the major challenge is now to provide scientific evidence and plausible mechanisms for their therapeutic responses. This review focuses on the therapeutic potential of Indian spices to treat multiple symptoms of the metabolic syndrome such as insulin resistance, diabetes, obesity, altered lipid profile and hypertension. The metabolic syndrome is prevalent and has become an important financial burden to the healthcare system in both developed and developing countries. Inflammation and oxidative stress have been proposed as initiators of the metabolic syndrome, especially of insulin resistance. Natural products with anti-inflammatory and anti-oxidant properties are found in spices. Adequate doses of these compounds may be effective in treating the metabolic syndrome. Testing these potential treatments requires adequate animal models, usually rodents, so the limitations of these models are important. Furthermore, this review highlights the need for adequate legislation and regulation to ensure the safety and success of evidence-based functional foods and nutraceuticals.

Keywords: Spices, Diabetes, Cardiovascular disease, Metabolic syndrome, Inflammation, Oxidative stress, Nutraceuticals, Food safety

Introduction

Dietary choice remains the basis for maintaining a healthy lifestyle and well-being, especially relating to cardiovascular disease (CVD), despite remarkable advances in medicine and pharmaceutical drug development[1,2]. Besides food being a lifestyle choice, age-old anecdotal reports from many cultures strongly suggest a role for diet as well as Indian spices in both preventive and therapeutic medicine[3,4]. However, the major challenge in the use of spices as preventive and therapeutic medicines is in demonstrating their health benefits by scientific means, comparable with the standards applied for pharmaceutical agents[3]. Further, unlike pharmaceutical agents which are administered in predetermined doses as pure and concentrated preparations, spices are consumed in combinations and in unmeasured and variable quantities in different cultural settings. Therefore, the major challenge is to provide scientific evidence to define these benefits as well as plausible mechanisms by which these products are effective in a disease setting.

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Around 60% of the world's population depends on herbal medicine, a broad term including spices, for primary healthcare[5]. Spices are pungent or aromatic substances from dried seeds, fruits, roots, bark or leaves used as additives to flavour, colour or preserve food; the differences with dried herbs used for flavouring is somewhat arbitrary. In India, many of these spices are part of everyday cooking and significant quantities may be consumed in a single meal. It is estimated that an adult in India can consume 80-200 mg/day of curcumin, the bioactive component of turmeric[3]. Some Indians have been reported to consume up to 50 g of garlic in a week[3,6]. These data suggest a realistic possibility to achieve therapeutic doses of the active ingredients in spices by dietary consumption alone. However, for many patients, treatment with functional foods or nutraceuticals with enhanced concentrations of the active ingredients of the spices may be necessary. There is a widespread research effort in India to define the potential health benefits of herbal medicines, including spices, and identify the active ingredients, especially compounds with anti-oxidant and anti-inflammatory properties[4,5].

This review investigates the potential of commonly used spices in India for treatment for the symptoms of metabolic syndrome in humans. To do this, we will define the metabolic syndrome and the likely mechanisms for the symptoms, discuss the choice of an appropriate rodent model for preclinical studies and present the available evidence for individual products. Two other important issues will then be presented: evaluation of the quality of the evidence as well as testing the safety and efficacy of these products.

Metabolic syndrome and its significance

The spices used in Indian cooking have potential for use in the treatment of the metabolic syndrome, a central preliminary pathological indication in the development of cardiovascular diseases, causing major public health challenges worldwide[7-9] and especially in the Indian sub-continent[10]. Metabolic syndrome refers to the clustering of several cardiovascular and metabolic risk factors, including dyslipidaemia, hyperglycaemia and increased blood pressure, where abdominal obesity and insulin resistance represent core parameters of this cluster[7,8,11]. There are several definitions of the metabolic syndrome (Table 1), of which the first functioning definition was provided by the World Health Organization (WHO), emphasising insulin resistance and hyperglycaemia as key symptoms with additional associated metabolic symptoms. More recently, the US National Cholesterol Education Program Adult Treatment Panel III, International Diabetes Federation and the American Heart Association and National Heart, Lung, and Blood

Table 1—Diagnostic criteria and definitions for the metabolic syndrome provided by major agencies around the world

Clinical measure	WHO* (1998)	EGIR^ (1999)	AACE% (2003)	ATP III #(2004)	IDF** (2005)	AHA/NHLBI^^ (2005)
Insulin resistance	Impaired glucose tolerance (IGT), impaired fasting glucose (IFG) or insulin resistance (IR) plus any 2 of the following:	Plasma insulin > 75th percentile plus any 2 of the following:	Impaired glucose tolerance, impaired fasting glucose plus any of the following:	NONE Any 3 of the following 5 features:		
Dyslipidaemia	Triglycerides (TG): ≥ 1.695 mmol/L and high-density lipoprotein cholesterol (HDL-C) ≤ 0.9 mmol/L (male), ≤ 1.0 mmol/L (female)	TG ≥ 2.0 mmol/L and/or HDL-C < 1.0 mmol/L or treated for dyslipidaemia	TG ≥ 1.69 mmol/L and HDL-C < 1.03 mmol/L	TG ≥ 1.695 mmol/L HDL-C < 40 mg/dL (male), < 50 mg/dL (female)	TG ≥ 1.7 mmol/L or on TG Rx, HDL-C < 1.03 mmol/L or on HDL-C Rx	TG ≥ 1.69 mmol/L or on TG Rx, HDL-C < 1.03 mmol/L or on HDL-C Rx
Blood pressure	≥ 140/90 mm Hg	≥ 140/90 mm Hg	≥ 130/85 mm Hg	≥ 130/85 mm Hg	≥ 130/85 mm Hg	≥ 130/85 mm Hg
Plasma glucose	≥ 7.0 mmol/l (Fasting)	≥ 6.1 mmol/l (Fasting)	IGT or IFG (but not diabetes)	> 5.6 mmol/L	≥ 5.6 mmol/L (includes diabetes)	≥ 5.6 mmol/L or on hypoglycemic
Central obesity	Waist: hip ratio > 0.90 (male); > 0.85 (female), and/or body mass index > 30 kg/m^2	Waist circumference ≥ 94 cm (male), ≥ 80 cm (female)	BMI ≥ 25 kg/m^2	Waist circumference ≥ 102 cm (male), ≥ 88 cm (female)	Waist circumference > 94 cm	Waist circumference ≥ 102 cm
Other	Urinary albumin excretion ratio ≥ 20 mg/min or albumin:creatinine ratio ≥ 30 mg/g					

*World Health Organization[22], ^The European Group for the Study of Insulin Resistance[23], %American Association of Clinical Endocrinologists[24], #The US National Cholesterol Education Program Adult Treatment Panel III[25], **International Diabetes Federation[26], ^^American Heart Association and National Heart, Lung, and Blood Institute[12]; Rx, medication; BMI, body mass index.

Institute have suggested that at least three of the following conditions must be met for a diagnosis of metabolic syndrome, although threshold values may differ: abdominal obesity, elevated triglyceride concentrations, reduced HDL-cholesterol concentrations, elevated blood pressure or fasting glucose concentrations[12] (Table 1).

The metabolic syndrome is common in adult populations throughout the world. In Australian adults, its prevalence ranges between 13.4 and 30.7%, depending on the definition used[13,14]. In the USA, its overall percentage in adults was 22.8% for men and 22.6% for women[15]. In the Japanese population, 51% of male and 53% of female subjects met the WHO criteria for the metabolic syndrome, whereas 45% of male and 38% of female subjects met the US National Cholesterol Education Program Adult Treatment Panel III criteria for the metabolic syndrome[16]. In Finland, metabolic syndrome was present in 38.8% of men and 22.2% of women[17].

This situation appears to be similar in the Indian sub-continent with recent data suggesting about one-fourth to one-third of the adult Indian population suffer from the metabolic syndrome[18,19]. Some communities such as the Punjabi Bhatia community in north India are more prone to be obese with type II diabetes and the symptoms of metabolic syndrome[18,19]. The prevalence of metabolic syndrome in the Sri Lankan population is high with 35% and 51% in males and females, respectively[18,19]. In adults aged 25 years and older from an urban population in Karachi (Pakistan), the prevalence of metabolic syndrome was 34.8 and 49%, according to the International Diabetes Federation and US National Cholesterol Education Program Adult Treatment Panel III definitions, respectively[20]. The overall prevalence in Pakistan has been reported as 18-46%, comparable to other South Asian countries[19,21]. Comparable data from Bangladesh and Nepal are not available, but the prevalence would be expected to be similar. Intrauterine and early postnatal under-nutrition has been suggested as an important cause of the relatively high incidence of cardiovascular disease and metabolic syndrome in Indian populations[10]. Thus, the metabolic syndrome provides many challenges to governments and healthcare providers from birth to death.

Aetiology of metabolic syndrome

The aetiology of metabolic syndrome involves many complex biochemical pathways. Different mechanisms linking the symptoms of metabolic syndrome have been postulated, all possibly hampering normal cardiovascular function. Increased levels of reactive oxygen species (ROS), non-esterified fatty acids, oxidized LDL and lipotoxicity may be related to insulin resistance[27]. Adipose tissue releases numerous bioactive mediators, including pro-inflammatory cytokines that not only influence body weight homeostasis, but also induce changes in cardiovascular structure and function, glucose metabolism, blood pressure, lipid metabolism, coagulation and inflammation, leading to endothelial dysfunction and atherosclerosis[27,28].

Inflammation has been proposed as the critical process initiating the symptoms of metabolic syndrome[29]. The pro-inflammatory state of obesity and metabolic syndrome is probably initiated by an excessive caloric intake in a high carbohydrate/high fat diet[29,30]. Increased oxidative stress in the adipose tissues of obese subjects is closely linked to enhanced inflammatory signals, adipokine dysregulation and insulin resistance[31-33]. Redox regulation of inflammatory signalling occurs at several levels, including direct effects of oxidants, modulation by antioxidants, alterations in the redox equilibrium (for example, ratio of reduced: oxidized glutathione and thioredoxin) and activation of oxidant- and redox-sensitive transcription cofactors such as NF-κB and AP-1[34,35].

Activation of the NF-κB pathway has been linked to a range of inflammatory disorders, including atherosclerosis, myocardial infarction and diabetes; this pathway can be interrupted by phytochemicals derived from the spices[36]. The pro-inflammatory/pro-oxidant state induces insulin resistance, leading to the clinical and biochemical symptoms of metabolic syndrome[27,32]. This resistance to insulin action promotes inflammation through an increase in free fatty acid concentrations and hinders the anti-inflammatory effects of insulin[29,30]. Both human and animal studies have shown that diets rich in carbohydrates and saturated fats contribute to insulin resistance, metabolic defects, excess body weight, lipid abnormalities, increased reactive oxygen/ nitrogen species, decreased anti-oxidant defences and the development of pre-diabetic or diabetic state[27]**.** The contributions towards this metabolic dysfunction from insulin signalling, oxidative stress and inflammation (Figure 1) have been difficult to separate[27].

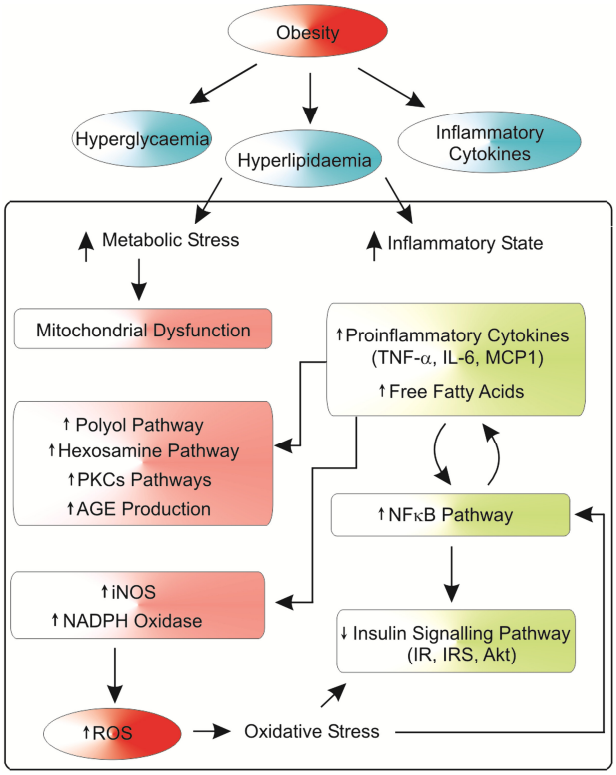


Fig. 1—Metabolic changes in obesity. Hyperglycaemia, hyperlipidaemia and elevated inflammatory cytokines are found in obese and diabetic conditions, which elevate both metabolic and inflammatory stress. Mitochondrial dysfunction, polyol pathway, hexosamine pathway, AGE pathway, and pro-oxidative genes such as iNOS and NADPH oxidases are associated with ROS generation and result in oxidative stress. Such stresses combine to activate PKCs and NF-κB signalling pathways, which cause insulin resistance by attenuating the insulin signalling pathway. *Abbreviations used*: TNF-α, tumour necrosis factor-α; IL6, interleukin 6; MCP, Monocyte chemoattractant protein; NFκB, nuclear factor kappa B; IR, insulin receptor; IRS, insulin receptor substrate; PKC, protein kinase C; AGE, advanced glycation end-products; iNOS, inducible nitric oxide synthase; NADPH, *n*icotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species; Akt, thymoma viral proto-oncogene designated as protein kinase B (PKB). Figure adapted from[33]

Table 2—Rodent models of the metabolic syndrome

Spontaneously diabetic rodents	
Otsuka Long-Evans Tokushima fatty rats (OLETF)[43,44]	Mild obesity, postprandial hyperglycaemia, insulin resistance, hyperinsulinaemia, hyperplasia with fibrosis followed by atrophy of the pancreatic islet, diabetic nephropathy, left ventricular fibrosis, endothelial dysfunction, hyperlipidemia
Goto-Kakizaki rats[45]	Non-obese, hyperglycaemia, hyperinsulinaemia, insulin resistance, endothelial dysfunction
Zucker diabetic fatty rats[46,47]	Non-functional leptin receptors, obese, dyslipidaemia, hyperinsulinaemia, insulin resistance, hyperleptinaemia, moderately elevated blood pressure
The JCR: LA-Cp rat[48,49]	Obese, insulin resistance, hyperinsulinaemia, impairment of endothelium-dependent vascular relaxation
Spontaneously hypertensive rat[50]	Insulin resistance, hypertension, cardiac hypertrophy, endothelial dysfunction
ob/ob mouse[51]	Obesity, hyperglycaemia, impaired glucose tolerance, dyslipidaemia
db/db mouse[52]	Hyperinsulinaemia, insulin resistance, hyperglycaemia, polyuria, glycosuria
Genetically engineered diabetic mice	
Insulin receptor-deficient mouse ($IR^{-/-}$)[53]	Fatty acid infiltration in the liver, increased ketone bodies production, hyperglycaemia, hyperinsulinaemia, increased serum triglycerides, death within a week
GLUT4 deficient mouse ($GLUT4^{-/-}$)[54]	Retarded growth, decreased longevity, cardiac hypertrophy, post-prandial hyperinsulinaemia, insulin resistance
IRS-1 deficient mice ($IRS\text{-}1^{-/-}$)[55]	Highly reduced intrauterine growth, impaired glucose tolerance, decreased insulin/IGF-1-stimulated glucose uptake
Artificially-induced diabetic rodents	
Fructose-fed rats[56,57]	Hyperinsulinaemia, impaired glucose tolerance, insulin resistance, hypertension, hypertriglyceridaemia, cardiac fibrosis, endothelial dysfunction
Sucrose-fed rats[58]	Insulin resistance, dyslipidaemia, obesity
High fat-diet model[59,60]	Obesity, dyslipidaemia, insulin resistance, hyperinsulinaemia, hyperglycaemia, impaired glucose tolerance, endothelial dysfunction, hypertension, hyperleptinaemia

Rodent models of metabolic syndrome

Rat and mouse models of human diseases such as obesity, diabetes and cardiovascular diseases have been widely used to investigate the progression of disease symptoms (Table 2)[37-39]. Understanding the influence of diet as a cause of the symptoms of metabolic syndrome requires adequate animal models with the symptoms relevant to human pathology, such as diet-induced obesity, diabetic and cardiovascular symptoms, to test proposed treatment options and dietary interventions. The most-used rat models of the complications of diabetes are induced by either streptozotocin[40] or a high fructose diet[41,42]. However, streptozotocin induces type I diabetes and a high fructose diet does not induce abdominal obesity[42]. Thus, results from these models may be relevant only to a small proportion of diabetic patients.

Diets including a significant component of animal-derived fats, such as lard or beef tallow, or plant oils such as corn or safflower oil may cause diabetes and obesity as well as cardiovascular changes[39]. Obesity and diabetes increase the risk of cardiovascular disease and its associated metabolic risk factors, probably by the releasing bioactive mediators from adipose tissue initiated by an excessive caloric intake in a high carbohydrate/high fat diet[27,29,30]. Thus, administering a high carbohydrate/high fat diet should induce the range of symptoms of metabolic syndrome rather than streptozotocin- or fructose-induced diabetes. These dietary interventions should serve as a more relevant model to investigate possible therapeutic interventions for the complications of metabolic syndrome.

Health benefits of Indian spices: what is the evidence?

The contribution of oxidative stress and inflammation in initiating the symptoms of metabolic syndrome is now well-known. Suitable therapeutic interventions targeting these oxidative and inflamematory processes may be effective in preventing and treating the metabolic syndrome. Several reviews have discussed the use of herbal medicines, including spices, in the treatment of the symptoms of metabolic syndrome such as diabetes[4,61], insulin resistance[62], hypertension and other cardiovascular diseases[63], and inflammation[64]. This section deals with the possible therapeutic benefits of the spices in the treatment of the symptoms of metabolic syndrome, in particular because of their anti-oxidant or anti-inflammatory effects.

Cardamon or Elaichi (*Elettaria cardamomum*)

Cardamon, a perennial herb indigenous to the Indian subcontinent, contains a wide variety of compounds, including α-terpineol, myrcene, subinene, limonene, cineol, α-phellandrene, menthone, α and β-pinene[65], *cis/trans*-linalol oxides, *trans*-nerolidol[66], β-sitostenone, γ-sitosterol, phytol, eugenyl acetate[67], bisabolene, borneol, citronellol, p-cymene, geraniol, geranyl acetate, stigmasterol and terpinene[68]. *In vitro* studies showed that cardamon inhibited platelet aggregation, when induced with agents such as ADP, epinephrine, collagen and calcium ionophore A 23187[69]. Cardamon reduced blood pressure in rats, probably by acting through cholinergic and calcium antagonist mechanisms[70].

Cinnamon or Dalchini *(Cinnamomum verum)*

Cinnamon is a small evergreen tree, approximately 10-15 m tall, native to Sri Lanka and Southern India[71]. Its bark has been widely used as a spice and flavouring agent for centuries. Cinnamon has been suggested to have many pharmacological properties, including antioxidant activity and antimicrobial effects[72,73]. The major active components of aqueous cinnamon extract appear to be doubly-linked procyanidin type-A polymers[74], cinnamaldehyde and esters such as ethyl cinnamate (Figure 2).

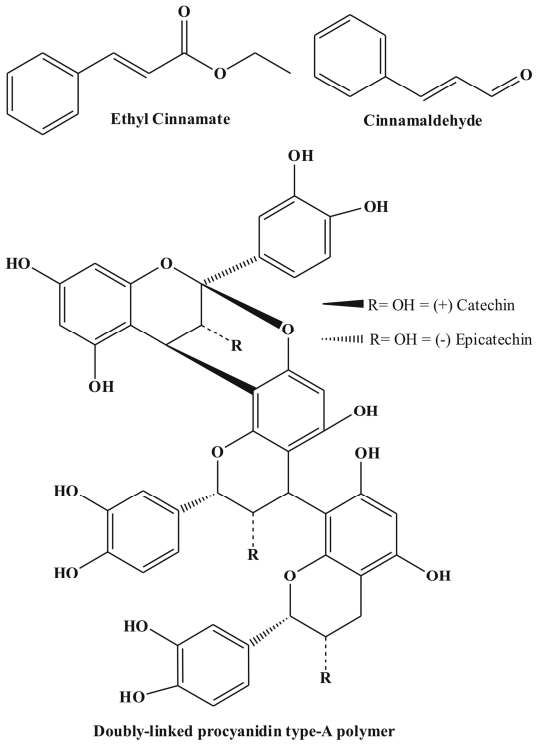


Fig. 2—Bioactive constituents in cinnamon

Pre-clinical and clinical data show that cinnamon attenuated the progression of type II diabetes[75,76]. Cinnamon (8% w/w in diet) prevented sucrose-induced increases in blood pressure in spontaneously hypertensive rats[77] and a cinnamon extract (300 mg/kg/day) decreased insulin resistance in fructose-fed diabetic rats, partly by enhancing insulin signalling and partly by activating the NO pathway in skeletal muscle[78]. An aqueous extract of cinnamon bark improved insulin resistance and prevented lipid abnormalities in fructose-fed rats[79]. In cholesterol-fed rats, cinnamate (0.1 g/100 g diet) inhibited hepatic HMGCoA reductase activity and suppressed lipid peroxidation in the liver[80]. Cinnamaldehyde (5-20 mg/kg/day) decreased plasma glucose, glycosylated haemoglobin, cholesterol and triglyceride concentrations, while increasing plasma insulin and HDL-cholesterol concentrations as well as hepatic glycogen in streptozotocin-induced diabetic rats[81]. Similar decreases were reported when poorly-controlled type 2 diabetic patients were given 1-6 g/day cinnamon for 40 days[75].

A related species, cassia cinnamon (*C. aromaticum*) also known as Chinese cinnamon, reduced fasting blood glucose concentrations, improved plasma lipid profiles in diabetic humans and reduced glucose and insulin responses in oral glucose tolerance testing[75,82,83]. In subjects diagnosed with the metabolic syndrome, an aqueous extract of Chinese cinnamon standardised to doubly-linked polyphenol type-A polymers as the bioactive component (500 mg/day) reduced systolic blood pressure, fasting blood glucose concentrations and also attenuated whole body fat deposition to body weight ratio[84]. The pre-clinical and clinical evidence for therapeutic usefulness of both common and cassia cinnamon has been reviewed[71].

Coriander or Dhania (*Coriandrum sativum*)

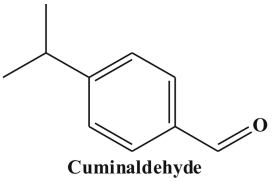
Coriandrum sativum is a well-known herb, native to Europe and Western Asia, used as fresh and dried fruit and leaves to flavour meals and as an ingredient in curry powder (http://www.botanical.com/botanical/mgmh/c/corian99.html). It is generally used in gastrointestinal complaints such as anorexia, dyspepsia, flatulence, diarrhoea, griping pain and vomiting[85]. The stems and leaves contain caffeic, chlorogenic, ferulic and gallic acids[86]. The seeds were effective as an antidiabetic agent administered as 6.25% in food in streptozotocin-diabetic mice[87], and as a hypolipidaemic agent as 10% in food in rats fed 15% coconut oil and 2% cholesterol[88]. Continuous intravenous infusion of the crude aqueous extract of coriander (40 and 100 mg/kg) induced dose-dependent diuresis, natriuresis, kaliuresis, increased chloride excretion and increased glomerular filtration rate in anaesthetized Wistar rats[89]. Alcoholic extract of coriander (200 mg/kg) decreased fasting serum glucose concentration and increased insulin release from pancreatic β-cells in streptozotocin-induced diabetic rats[90]. Oral intake of fruit powder (8% w/w of food) in cholesterol-fed rats decreased plasma total cholesterol, LDL-cholesterol and total lipid concentrations, while increasing the HDL-cholesterol[91].

Cumin seeds or Jeera *(Cuminum cyminum)*

Cumin seeds are a common dietary spice consumed in fairly large quantities in India. They are widely used in Ayurvedic medicine for treatment of dyspepsia, diarrhoea and jaundice[80]. Cuminaldehyde is suggested as the active ingredient in cumin seeds (Figure 3)[92]. An aqueous extract of cumin seeds prevented the accumulation of advanced glycation end-products due to fructose-mediated *in vitro* glycation of eye lens soluble proteins[92]. Hypoglycaemic effects of cumin seeds were also observed in normal rabbits[93]. Dietary cumin showed marked hypoglycaemic responses in streptozotocin-diabetic rats by reducing blood and urinary glucose concentrations[94]. An aqueous extract of seeds lowered blood glucose and plasma and tissue lipid concentrations in alloxan-induced diabetic rats[80].

Curry plant/leaves or Kadipatta *(Murraya koenigii)*

Curry leaves are used as a culinary spice and also as a traditional medicine. Several carbazole alkaloids were reported in the plant[95]. Recently, a 35 kDa antioxidant protein PII, purified from leaf powder, inhibited lipid peroxidation and lipoxygenase activity *in vitro* in human erythrocyte ghosts and also effectively scavenged ROS[96]. The leaf extract supplementation (80 mg/kg) decreased blood cholesterol and glucose concentrations in diabetic *ob/ob* mice with



Cuminaldehyde

Fig. 3—Cuminaldehyde, an active ingredient of cumin

reduction in body weight[97]. Extracts of leaves showed hypoglycaemic effect in both normal and alloxan-induced diabetic dogs and rabbits[98]. In alloxan-induced mild and streptozotocin-induced moderately diabetic rats, feeding of 15% curry leaf powder diet reduced blood glucose concentrations by 21.4% and 8.2% respectively[99]. In normal rats, 10% leaf administration in food increased hepatic glycogen concentration and glycogenesis by increased activity of glycogen synthetase, and decreased glycogenolysis and gluconeogenesis by decreased activity of glycogen phosphorylase and gluconeogenic enzymes[100]. Curry leaf supplementation lowered lipid peroxidation and also modulated the hepatic function to near normal level in rats fed with a high-fat diet[101]. Supplementation with 10% curry leaves in high fat-fed young male albino rats reduced total serum cholesterol, LDL and VLDL concentrations, increased HDL concentration, lowered release of lipoproteins into the circulation and increased the lecithin cholesterol acyltransferase (LCAT) activity[102].

Fenugreek or Methi (*Trigonella foenum*)

Fenugreek, a strongly scented annual herb, is recommended for the treatment of rheumatism in traditional medicine. Saponins, glycoside-D and trigofoenoside-A are major components in the seeds[103], while alkaloids, cardiac glycosides and phenols are found in the leaf extract[104]. The steroidal saponins present in the seeds as parent compounds for physiological steroid production could influence the local inflammatory response[105,106]. Galactomannan, a guar gum comprising approx. 50% of the seed weight is postulated as another active ingredient in fenugreek seeds[107]. In high sucrose-fed rats, galactomannan feeding reduced appetite, body weight gain, glycemic response, plasma insulin concentrations and plasma triglycerides and total cholesterol concentrations[108]. In human studies, galactomannan reduced post-prandial blood glucose concentrations[109,110] and improved insulin sensitivity in both non-diabetic[111] and diabetic subjects[112]. Feeding guar-galactomannan fibre reduced both total and LDL cholesterol concentrations in healthy and type 2 diabetic subjects[113].

Garlic or Lahsun (*Allium sativum*)

Garlic has been used as both an important dietary constituent and a medicine in different cultures around the world. A broad range of therapeutic responses to garlic has been reported, including decreases in blood pressure, blood lipid and glucose concentrations and the risk of atherosclerosis as well as antimicrobial, anticancer and hepatoprotective effects[114-116]. The responses with garlic have been attributed to its sulphur-containing antioxidants (Figure 4). Allicin (diallyl thiosulphinate) is the major bioactive thiosulphinate compound found in garlic homogenate and is liberated from alliin by the enzyme alliinase when garlic is crushed or bruised[117]. Other important sulphur-containing compounds include allyl methyl thiosulphonate, 1-propenyl allyl thiosulphonate and γ-L-glutamyl-S-allyl-L-cysteine[117].

In vitro animal and human tissue studies showed that garlic inhibited angiotensin II responses, possibly by inhibition of angiotensin converting enzyme to promote vasodilation[118-120]. In animal studies, dose-dependent antioxidant and organ-protective effects of raw garlic homogenate (125, 250 and 500 mg/kg doses) have been observed in various organs such as heart, liver and kidneys[121,122]. An aqueous extract of garlic neutralised free radicals and reduced oxidative damage in rabbit liver homogenates[123]. Furthermore, an aged garlic extract inhibited the development of thickened, lipid-filled lesions by approximately 50% in preformed neointimas produced following injury of the right carotid artery in cholesterol-fed rabbits, in addition to reducing the surface area of the thoracic aorta covered by fatty streaks (64% decrease)[114]. Two meta-analyses have demonstrated that garlic preparations reduced blood pressure in hypertensive

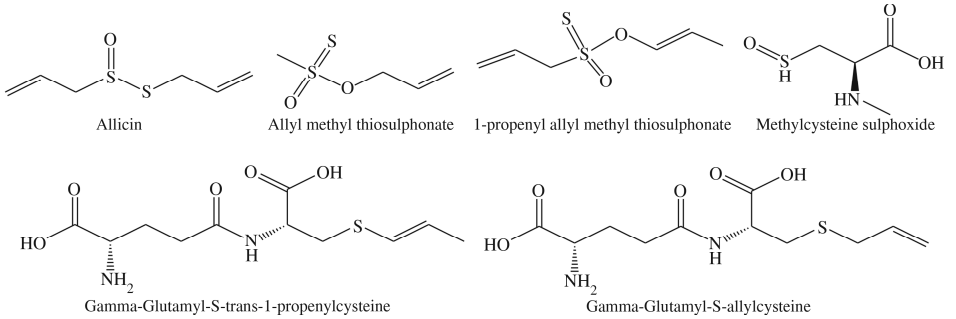


Fig. 4—Bioactive constituents in garlic

patients[124,125], although an earlier review concluded that the reported effects were too small to be clinically meaningful[126].

Ginger or Sonth (*Zingiber officinale*)

The therapeutic potential of ginger is recognised in a wide range of unrelated disease states including stomach aches, diarrhoea, nausea, asthma, respiratory disorders, toothache, gingivitis and arthritis[127,128]. Recent research has focussed on elucidating the anti-inflammatory properties of ginger. As with most herbal preparations, ginger extracts are complex with more than 400 chemical compounds already isolated and identified[129]. A subfraction containing the structurally related compounds gingerols, shogaols and paradols is likely to account for the anti-inflammatory properties of ginger (Figure 5)[129]. The anti-inflammatory effect of ginger was attributed to its ability to inhibit cycloxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX), the enzymes critical in prostaglandin and leukotriene synthesis, respectively[129]. Ginger lowered blood pressure through both stimulation of muscarinic receptors and blockade of calcium channels[128,130].

Malabar tamarind or Imli *(Garcinia cambogia)*

Fruits of *G. cambogia* are commonly known as gambooge or brindleberries. The active ingredient is hydroxycitric acid (Figure 6), a potent inhibitor of adenosine triphosphate (ATP) citrate lyase[131]. ATP citrate lyase is critical in catalyzing the cleavage of citrate to oxaloacetate and acetyl-coenzyme A. Inhibition of ATP citrate lyase can reduce the availability of acetyl-coenzyme A units for fatty acid synthesis and lipogenesis, thus modulating fat metabolism[131]. In human studies, oral intake of hydroxycitrate increased fat oxidation, when supplemented with moderate intensity exercise[132]. In animal studies, oral intake of *G. cambogia* extract effectively attenuated body weight gain, visceral fat accumulation, blood and hepatic lipid concentrations and plasma insulin and leptin concentrations in obese mice fed a high fat diet[133]. The extract ameliorated diet-induced obesity by modulating multiple genes associated with adipogenesis, such as aP2, SREBP1c, PPARγ2, and C/EBPα in the visceral fat tissue[133]. The calcium-potassium salt known as HCA-SX or Super citrimax, a derivative of hydroxycitric acid, reduced food intake and body weight gain in obese Zucker rats and also attenuated the increased inflammation, oxidative stress and insulin resistance in untreated Zucker rats[134]. In a human study, treatment with HCA-SX for 8 weeks decreased body weight and BMI by 5.4% and 5.2%, respectively[135].

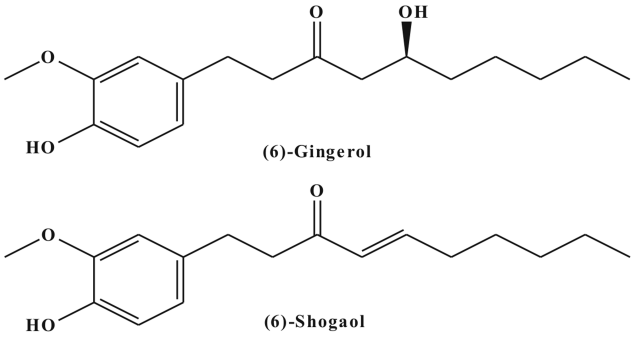


Fig. 5—Bioactive compounds in ginger; shogaols are formed upon drying.

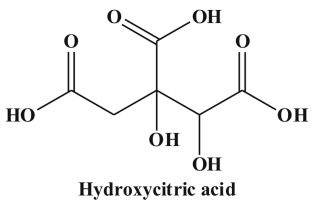


Fig. 6—Hydroxycitric acid, the active principle of Malabar tamarind

Turmeric or Haldi (*Curcuma longa*)

Turmeric is a perennial herb, yielding a rhizome widely used as a culinary ingredient. Both Ayurvedic and traditional Chinese medicines have used turmeric for the treatment of inflammatory and digestive disorders. Research has focussed the antioxidant, hepatoprotective, anti-inflammatory, anticarcinogenic and anti-microbial properties of turmeric, in addition to its use in cardiovascular disease and gastrointestinal disorders[136]. Curcuminoids constitute 5% of the turmeric rhizome and are suggested as the active ingredients, showing both antioxidant and anti-inflammatory effects. Both turmeric extract and curcumin (Figure 7), one of the major curcuminoids, have been widely examined for possible therapeutic effects in the symptoms of metabolic syndrome. In *in vitro* studies, turmeric prevented protein glycosylation and lipid peroxidation induced by high

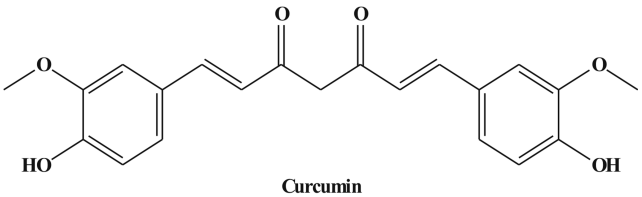


Fig. 7—Curcumin, the major bioactive ingredient of turmeric.

glucose concentrations[137]. Curcumin showed anti-differentiation effects, possibly through AMPKα-PPAR-γ in 3T3-L1 adipocytes[138]. It also inhibited NFκB-mediated cytokine expression in adipocytes[139]. In this study, curcumin inhibited TNF-α, IL-1β, IL-6 and COX-2 gene expression at an IC50 of 2 μM and also showed a reduction in secreted IL-6 and PGE2 at IC_{50} ~20 μM in adipocytes[139]. Dietary curcumin improved inflammation and diabetes associated with obesity[140]. In high-fat fed obese and leptin-deficient *ob/ob* mice, curcumin reduced macrophage infiltration of white adipose tissue, increased adipose tissue adiponectin production and decreased hepatic NF-κB activity, hepatomegaly and other markers of hepatic inflammation[140]. Curcumin and other curcuminoids prevented lipid accumulation in the liver and epididymal adipose tissues in high fat-fed Sprague-Dawley rats, possibly by altering fatty acid metabolism[141].

An extract of turmeric decreased LDL oxidation and lowered oxidation of erythrocyte and liver membranes in rabbits on a high saturated fat and cholesterol diet[142]. Interestingly, curcumin did not show any hypoglycaemic activity in streptozotocin-induced diabetic rats[61], suggesting it may have a therapeutic potential in type II but not type I diabetes. Early studies showed anti-thrombotic responses by curcumin in normal mice, possibly by suppressing thromboxane-2 production[143]. In humans, a longer-term study with 30 healthy subjects given turmeric extract equivalent to 20 mg curcumin for 60 days also showed decreased peroxidation of both HDL and LDL cholesterol[144]. The biological activity of curcumin in humans has been extensively reviewed, although evidence on the symptoms of metabolic syndrome is limited[145].

Black mustard or Rai (*Brassica nigra*), black pepper or Kali Mirchi (*Piper nigrum*) and nutmeg or Jaiphal (*Myristica fragrans*)

Black mustard, black pepper and nutmeg are some of the other spices sparingly examined for possible therapeutic effects in the symptoms of metabolic syndrome in experimental diseased animals. Mustard showed hypoglycaemic effect in rats in a study to test its effect on the enzymes of carbohydrate metabolism[61,100]. The mucilage (soluble fibre) of mustard at 5%, 10% and 15% in the food improved post-prandial glucose concentrations and insulinaemia in normal rats[61].

Black pepper supplementation (0.25 or 0.5 g/kg body weight) in high fat-fed rats lowered concentrations of thiobarbituric acid reactive substances and conjugated dienes, maintained superoxide dismutase, catalase, glutathione peroxidase and glutathione-S-transferase concentrations and reduced glutathione concentrations in the liver, heart, kidney, intestine and aorta compared to control rats[146]. Black peppers also showed potential in obesity by increasing thermogenesis and fat oxidation[147].

An *in vitro* study suggests that meso-dihydroguaiaretic acid isolated from the nutmeg plant may act as an enhancing agent in intracellular insulin signalling, possibly through the inhibition of PTP1B activity[148]. An extract of nutmeg seeds prevented lipid abnormalities and atherosclerosis in hypercholesterolaemic rabbits[149,150].

Health benefits of Indian spices: how strong is the evidence?

The quality of evidence for health benefits can be categorised into different levels. As an example, the evidence for cinnamon bark has been reported under the following levels[71]: A: very strong scientific evidence from systematic reviews or meta-analysis; B1: strong scientific evidence from one or more randomised controlled trials; B2: good scientific evidence from one or more randomised controlled trials of limited size or methodology; C: fair scientific evidence from one or more cohort studies or outcome studies or case control studies; D: weak scientific evidence from case series; E: indirect evidence from case reports or expert opinion or laboratory studies; and F: historical or traditional evidence.

This provides an evaluation on each study and possible clinical indication; for example, the randomised clinical trials of 60-79 patients using cinnamon for diabetes[75,82] have been both classified as level B1, a smaller clinical trial of 25 patients[151] is classified as level B2, while a case report of a chronic carrier of *Salmonella enteritidis* showing improvement after consumption of cinnamon bark is classified as level E[71]. A thorough evaluation of all evidence for therapeutic effectiveness of spices in the metabolic syndrome is not possible for this review. However, the evidence for individual spices would appear to be level B for cinnamon to lower blood glucose concentrations or insulin resistance, for turmeric to improve the lipid profile, for hydroxycitrate from tamarind to reduce obesity and for garlic to reduce blood pressure.

Similarly, the potential of therapeutic interventions to harm the patient can be categorised into levels as follows[71]: 1a: very strong scientific evidence from systematic reviews or randomised clinical trials; 1b: strong scientific evidence from outcome studies, cohort studies or case control studies; 1c: good scientific evidence from one or more case series; 2: fair scientific evidence from case reports; 3: *in vitro* scientific evidence from studies on animals, insects, micro-organisms or cell cultures; 4: indirect evidence based on scientific theory or expert opinion; and 5: no available evidence.

In general, spices are generally recognised as safe (GRAS) by the United States Food and Drug Administration, when used in therapeutic doses. Some studies have reported minor adverse effects, usually gastrointestinal complaints with spices used as therapeutic agents.

The development of more active nutraceuticals and purified products from herbal medicines raises the controversial issues of patents and protection of intellectual property[152]. This issue is very relevant to India, as shown by the discussions on the Traditional Knowledge Digital Library (http://www.tkdl.res.in/tkdl/langdefault/common/home.asp?GL=Eng), a searchable database of more than 230,000 formulations taken from ancient texts on Indian systems of medicine - Ayurveda, Unani, Siddha and Yoga - in Hindi, Sanskrit, Arabic, Persian and Urdu[153]. Since spices are a part of herbal medicines, these issues become relevant to this review.

Safety and toxicity

Herbal medicines, including spices, have the potential to produce adverse effects, especially when used in concentrated forms. Further, these products may interact with other herbal products as well as drugs[154]. Although spices are regularly consumed in the diet by many populations, their use as nutraceuticals in a concentrated form needs further investigation for efficacy and toxicity. Nutraceuticals may tend to show fewer adverse effects compared to prescription drugs or herbal remedies, but the lack of documentation does not necessarily mean they are safe. Other safety issues include variability in biologic potency in different crops, contamination and use of the incorrect plant species[155]. In addition, it is extremely difficult to guard against consumer fraud in this unregulated industry. Very limited regulation can lead to problems including unreliable herb quality, the marketing of secret formulas with unsubstantiated claims, the proliferation of unqualified practitioners and the possibility of deliberate adulteration of the product. Even with the ban imposed on claims that a food can cure a disease in the United States, European Union and Australia, consumers are still being lured into buying the products by "marketing strategies"[156]. Stronger regulation of this industry is essential to ensure that the same high standards of preparation, quality control and management are enforced as with conventional pharmaceutical products. Stricter regulations will bring modern scientific techniques and intellectual rigour to traditional herbal medicine.

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Chapter 3.1

Review Article

Rodent Models for Metabolic Syndrome Research

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Rodents are widely used to mimic human diseases to improve understanding of the causes and progression of disease symptoms and to test potential therapeutic interventions. Chronic diseases such as obesity, diabetes and hypertension, together known as the metabolic syndrome, are causing increasing morbidity and mortality. To control these diseases, research in rodent models that closely mimic the changes in humans is essential. This review will examine the adequacy of the many rodent models of metabolic syndrome to mimic the causes and progression of the disease in humans. The primary criterion will be whether a rodent model initiates all of the signs, especially obesity, diabetes, hypertension and dysfunction of the heart, blood vessels, liver and kidney, primarily by diet since these are the diet-induced signs in humans with metabolic syndrome. We conclude that the model that comes closest to fulfilling this criterion is the high carbohydrate, high fat-fed male rodent.

1. Introduction

Hypertension, diabetes and obesity are common but not independent in humans and the combination is referred to as metabolic syndrome [1, 2]. While the definition of the syndrome may help understanding causes and prognosis, there are continuing arguments on the clinical usefulness of defining the syndrome in humans. Human metabolic syndrome is accepted as a consequence of dietary imbalance rather than a genetically programmed disease. This syndrome includes central obesity, insulin resistance, elevated blood pressure, impaired glucose tolerance and dyslipidaemia [1, 2]; these are accepted risk factors that increase the incidence of cardiovascular disease and type 2 diabetes [3–5]. Metabolic syndrome is also associated with an increased risk of nonalcoholic fatty liver disease and kidney dysfunction [6, 7]. Similarly, there is solid evidence for correlations between metabolic syndrome and functional changes in the lungs, dementia and cancers of the breast, pancreas and bladder (Figure 1) [8–12]. Lifestyle and diet modulate metabolic syndrome [4, 13] and this induces pathophysiological changes throughout the body. Hence it is important to study the progression and treatment strategies for metabolic syndrome.

The number of adults with metabolic syndrome is substantial and the prevalence is increasing throughout the world [14]. The gender ratio was similar in the USA [15], Singapore and Australia showed increased rates in females [16, 17], while Japan showed increased rates in males [18]. In 2002, the prevalence of metabolic syndrome in the USA was 24% and 23.4% in males and females, respectively [19]. In 2005 and 2006, this prevalence had increased to 34% in both males and females [15, 20]. In the Australian population, 18.8% of males and 25.4% of females fulfilled the requirements for diagnosis with metabolic syndrome in 2000 [17]. In a Japanese study, 45% of males and 38% of females were diagnosed with metabolic syndrome [18]. Similar prevalence rates of metabolic syndrome have been reported in the Indian subcontinent [21].

The widespread occurrence of metabolic syndrome in humans means that there is an urgent need to study relevant causes and progression of the signs. These studies require viable animal models that adequately mimic all the aspects of the human disease, developing all major signs of metabolic syndrome, especially obesity, diabetes, dyslipidaemia, hypertension and possibly fatty liver disease and kidney dysfunction. Rodents have been used for many years as models of human disease, especially hypertension,

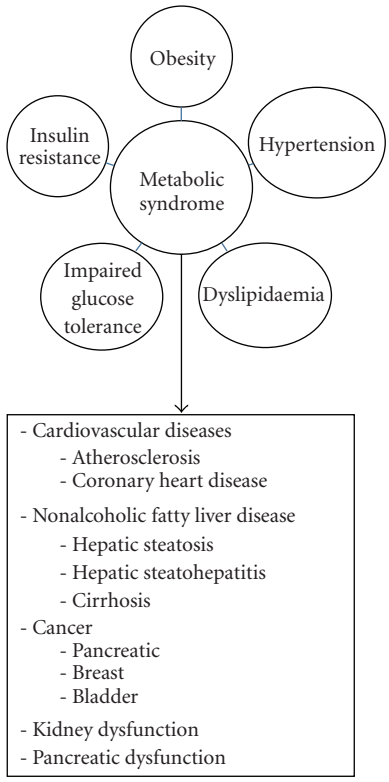

Figure 1: Metabolic syndrome and associated complications.

diabetes and obesity [22–25]. This review will examine whether the existing rodent models for components of metabolic syndrome mimic the range of changes in humans and are therefore suitable to evaluate potential treatments for human metabolic syndrome.

2. Genetic Models of Obesity and Type 2 Diabetes

Genetic models of obesity and diabetes include *db/db* mice, *ob/ob* mice, Zucker diabetic fatty rats and Otsuka Long-Evans Tokushima Fatty rats, while Goto-Kakizaki rats are diabetic but nonobese. These models are useful in evaluating specific molecular mechanisms that may be involved in development of obesity in rodents, but the metabolic syndrome in humans is not a monogenetic disorder. Therefore, the relevant questions are whether these genetic changes mimic those observed in humans and whether these models show the range of signs that characterise the metabolic syndrome. As an example, several of these models have mutations in the leptin gene or receptor (Figure 2), yet similar mutations are a very rare recessive genetic disorder in humans with only 4 mutations in 15 people reported up until 2009 [26]. Further, although cholecystokinin is important as a satiation signal [27], there are only a few reports of CCK-1 receptor mutations, as found in the Otsuka Long-Evans Tokushima fatty rats, inducing obesity in humans [28, 29].

3. *ob/ob* (C57BL/6J-*ob/ob*) Mice

This was one of the first genetic models used for the study of diabetes [30]. These mice inherited a monogenetic autosomal recessive mutation in the leptin gene on chromosome 6 [31, 32] and developed obesity, hyperinsulinaemia and hyperglycaemia after 4 weeks of age [33]. They showed an increased body weight compared to their lean littermates at all ages [33, 34]. The presence of impaired glucose tolerance was found after 12 weeks of age [35]. These mice developed left ventricular hypertrophy with decreased cardiac function at 24 weeks of age [36], cardiac fibrosis after 20 weeks of age [37] and hepatic steatosis and inflammation at 12 weeks of age [38, 39]. Unlike humans with metabolic syndrome, these mice showed reduced blood pressure [34] and did not develop dyslipidaemia even after the age of 36 weeks [35].

4. *db/db* (C57BL/KsJ-*db/db*) Mice

These mice have inherited an autosomal recessive mutation in the leptin receptor gene present on chromosome 4 [40] leading to higher body weights than their lean littermates after 6 weeks of age [41]. Fasting blood glucose concentrations were higher after 8 weeks of age and these mice showed increased plasma concentrations of triglycerides, total cholesterol and nonesterified fatty acids along with reduced HDL/LDL cholesterol ratio after 13 weeks of age [42]. Hyperinsulinaemia and impaired glucose tolerance were observed after 12 weeks of age [41, 43]. In the heart, both infiltration with inflammatory cells and fibrosis were present after 12 weeks of age, although blood pressure was unchanged [41]. These mice showed vascular endothelial dysfunction at 12 weeks of age [41] and developed hepatic steatosis after 20 weeks of age [44]. *db/db* mice failed to show hepatic inflammation and fibrosis [45].

5. Zucker Diabetic Fatty Rats (*fa/fa*)

Diabetic Zucker fatty rats (ZDF), a model of early onset obesity, have a mutation in the leptin receptor gene [46]. ZDF rats became hyperglycaemic after 13–15 weeks of age [47] with hyperinsulinaemia and hypertriglyceridaemia after 12–14 weeks of age along with diastolic and systolic dysfunction [48]. Serum cholesterol concentrations were slightly increased in ZDF rats compared to lean Zucker rats at 10 weeks of age whereas the serum concentration of cholesterol was ~2.5 times higher compared to lean Zucker rats at 20 weeks of age [49]. These rats also developed endothelial dysfunction after 12 weeks of age [50]. ZDF rats showed only moderate increases in systolic blood pressure by 15 weeks of age [51]. Albuminuria was present at the age of 31 weeks [52] with thickening of basal membrane and glomerular fibrosis after 47 weeks [52]. Increased hepatic triglyceride deposition was observed after 20 weeks of age in ZDF rats [53]. ZDF rats also showed increased serum markers of inflammation such as TNF-α and IL-1β after 26 weeks of age [54].

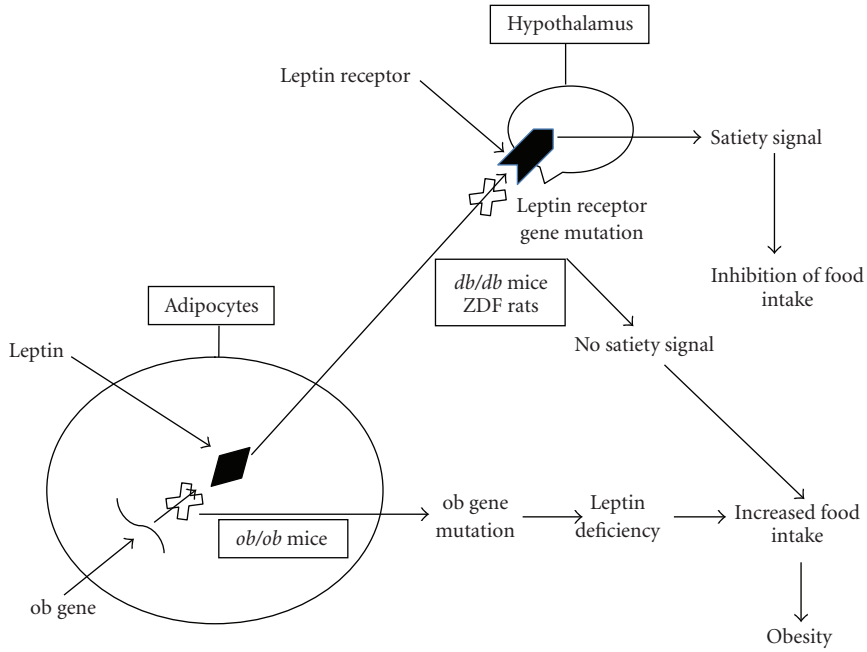

Figure 2: Mechanism of the actions of leptin including the effects of leptin deficiency or leptin receptor deficiency.

6. Otsuka Long-Evans Tokushima Fatty Rats

Otsuka Long-Evans Tokushima Fatty (OLETF) rats have been used as a rat model of human diabetes and obesity [55]. Pancreatic acini cells in OLETF rats were insensitive to the actions of cholecystokinin (CCK), which controls food intake [56], due to the absence of CCK-1 receptors [57]. Male and female OLETF rats were similar in body weight to lean Long-Evans Tokushima rats at the time of weaning but they became 30–40% heavier than age-matched lean Long-Evans Tokushima Otsuka rats after 20 weeks [58]. Due to the lack of CCK-1 receptors, the average meal size and overall food intake were higher in OLETF rats [57]. OLETF rats presented with high blood glucose concentrations after 18 weeks of age but they showed impaired glucose tolerance starting at 24 weeks of age [58]. Plasma triglyceride concentrations in OLETF rats started increasing from 8 weeks of age but cholesterol concentrations were only slightly higher even after 40 weeks of age [58]. After week 40 of age, OLETF rats showed diffuse glomerulosclerosis [58]. Hearts from OLETF rats showed cardiac hypertrophy with left ventricular systolic and diastolic dysfunction [59]. OLETF rats showed higher blood pressure compared to lean Long-Evans Tokushima Otsuka rats after 14 weeks of age [60]. After 34 weeks of age, OLETF rats showed 5 times higher triglyceride deposition in liver compared to the lean Long-Evans Tokushima Otsuka rats [61].

7. Goto-Kakizaki Rats

Goto-Kakizaki (GK) rats are nonobese and spontaneously diabetic [62]. The occurrence of diabetes in these rats is an interaction of several events including presence of susceptibility loci for some diabetic traits, gestational impairment inducing decreased β-cell neogenesis and proliferation and loss of β-cell differentiation [63]. These inbred rats were hyperglycaemic after 4 weeks of age with impaired glucose tolerance but they were lighter than the age-matched Wistar rats [64]. These rats developed cardiac hypertrophy and decreased systolic function at 20 weeks of age [65]. There was no change in blood pressure even after 14 months of age [66]. Plasma and liver lipid concentrations were higher in Goto-Kakizaki rats after 8 weeks of age compared to age-matched Wistar rats [67]. Goto-Kakizaki rats had higher urinary excretion of albumin and decreased creatinine clearance after 14 months of age along with increases in glomerular volume, basement membrane thickness and kidney weight [66].

These genetic models consistently develop obesity and non-insulin-dependent diabetes, but metabolic syndrome is a much broader constellation of pathophysiological changes, especially including hypertension. Thus, these rodent models, although used in obesity research, replicate neither the causes nor the changes that occur in human metabolic syndrome (summarised in Table 1).

8. Genetically Engineered Diabetic Mice

In recent years, genetically engineered mice models, either transgenic or knockout, have been developed to study the normal and abnormal effects of a particular protein or a set of proteins. Different proteins, signalling molecules and hormones, important in development of diabetes and obesity, can be removed by changes in the genome of the mice. Some of the important proteins that have been deleted from the mice for obesity and diabetes research include

Table 1: Different rodent models with the signs of metabolic syndrome.

Rodent model	Age (weeks)	Signs of metabolic syndrome shown by rodents							References
		Obesity	Hypertension	Dyslipidaemia	Cardiovascular dysfunction	Impaired glucose tolerance	Fatty liver	Kidney dysfunction	
ob/ob mice	4	✓	×	×	×	×	×	U	[33–39]
	12	✓	×	×	×	✓	✓	U	
	24	✓	×	×	✓	✓	✓	U	
db/db mice	6	✓	×	×	×	×	×	U	[41–44]
	12-13	✓	×	✓	✓	✓	×	U	
	20	✓	×	✓	✓	✓	✓	U	
ZDF rat	12–15	✓	✓	✓	✓	×	×	×	[47–53]
	20	✓	✓	✓	✓	×	✓	×	
	31–47	✓	✓	✓	✓	×	✓	✓	
OLETF rats	8	×	×	✓	×	×	×	×	[58–61]
	14	×	✓	✓	×	×	×	×	
	20	✓	✓	✓	×	×	×	×	
	24	✓	✓	✓	×	✓	×	×	
	34	✓	✓	✓	×	✓	✓	×	
	40	✓	✓	✓	×	✓	✓	✓	
	60–66	✓	✓	✓	✓	✓	✓	✓	
Goto-Kakizaki rats	4	×	×	×	×	✓	×	×	[64–67]
	8	×	×	✓	×	✓	✓	×	
	20	×	×	✓	✓	✓	✓	×	
	60	×	×	✓	✓	✓	✓	✓	

This table represents the signs of metabolic syndrome at different ages. The symbols ✓ and × indicate the presence and absence of these signs of metabolic syndrome at that age, respectively, whereas U indicates unavailability of the data. The table indicates that age is an important parameter since some of the signs are developed in very young rodents whereas others take much longer to develop.

insulin receptor, GLUT4, IRS-1 and IRS-2. Insulin receptor-null mice do not survive for more than 72 hours as they develop severe ketoacidosis [68] with hyperglycaemia and hyperinsulinaemia [69]. Thus they cannot be used in long-term studies as adults. Further, the insulin receptor knockout mice are unlikely to mimic human conditions as this receptor loss is very rare in humans [68, 70]. Other models lacking GLUT4, IRS-1 and IRS-2 may give useful information about the roles of each protein [71–75], but they do not mimic the cause of human metabolic syndrome.

9. Chemically Induced Rodent Models of Diabetes

Alloxan and streptozotocin are structural analogues of glucose that enter pancreatic beta cells via the GLUT2 transporter [86]. Single injections of alloxan or streptozotocin induce selective necrosis of pancreatic β cells in rats, mice and rabbits [86–91] as a model of type 1 diabetes. Chemically induced diabetic rodents show fatty liver and inflammation [92] along with decreased ventricular contractility and function [93]. In contrast to patients with metabolic syndrome, alloxan- and streptozotocin-induced diabetic rats are hypoinsulinaemic [94], do not gain weight and are usually hypotensive. Thus, chemically induced type 1 diabetic rodents do not show the diverse characteristics of the metabolic syndrome and therefore they are not a suitable model for this syndrome in humans.

Type 2 diabetes may be induced by low-dose streptozotocin given neonatally, for example, at a dose of 70 mg/kg on day 5 of life, producing moderate hyperglycaemia in adult rats with decreased HDL-cholesterol concentrations but no other lipid abnormalities or oxidative enzyme changes [95]. Insulin resistance and an approximate doubling of plasma C-reactive peptide and TNF-α were produced in 14-week-old rats treated on day 2 of life with streptozotocin (90 mg/kg) [96]. However, these changes following neonatal streptozotocin are insufficient to define the signs of the metabolic syndrome. A better option may be treatment with low-dose streptozotocin in a nutritional model of type 2 diabetes induced by an increased energy diet. In 8-week-old rats, the combination of streptozotocin (25 mg/kg) and a high-fructose, high-fat diet for 6 weeks increased plasma glucose, insulin and triglyceride concentrations, decreased left ventricular contractile function and reduced myocardial metabolic efficiency [97]. A similar protocol with a high-energy diet for 5 weeks followed by streptozotocin administration (40 mg/kg) produced metabolic abnormalities with insulin resistance that could be decreased by administration

Table 2: Effects of some treatment strategies on rodent models of metabolic syndrome.

Rodent model	Interventions	Reversal or prevention of signs of metabolic syndrome and associated complications	Signs of metabolic syndrome not affected by drug treatment
ob/ob mice	Temocapril (ACE inhibitor) and olmesartan (AT_1 receptor blocker) [37]	Reduced blood pressure and ventricular fibrosis	No change in body weight and blood glucose concentrations
	Resveratrol [76]	Reduced blood glucose, plasma insulin, adiponectin concentrations, improved glucose tolerance	No change in body weight and blood lipid concentrations
db/db mice	Aliskiren (renin inhibitor) [41]	Reduced blood pressure, cardiac fibrosis, macrophage infiltration in heart and coronary remodelling, improved endothelial function and glucose tolerance, increased pancreatic insulin content and beta cell mass, reduced pancreatic fibrosis	No change in body weight, visceral fat and liver weight
ZDF rats	Sitagliptin (DPP-4 inhibitor) [54]	Reduced body weight and blood pressure, reduced blood glucose, plasma triglyceride, plasma insulin and serum inflammatory markers, reduced pancreatic fibrosis and inflammation	No change in total cholesterol concentration
OLETF rats	Rosiglitazone (PPARγ agonist) [77]	Reduced blood glucose, plasma insulin and serum inflammatory markers	No change in body weight
GK rats	Levosimendan (calcium sensitiser) [78]	Reduced cardiac fibrosis and cardiac hypertrophy, improved ventricular function	No change in blood pressure
	Hesperidin [67]	Reduced serum insulin and blood glucose, serum triglyceride, serum total cholesterol concentrations, increased serum HDL-cholesterol and adiponectin concentrations	—
Alloxan	*Cucurbita pepo* peel extract [79]	Reduced blood glucose, plasma total cholesterol, HDL-cholesterol, triglycerides, LDL-cholesterol and VLDL-cholesterol, increased plasma insulin concentrations	—
Streptozotocin	Quercetin [80]	Increase in body weight, reduced serum glucose concentrations and increased plasma insulin concentrations, pancreatic beta cell protection	—
Fructose-induced metabolic syndrome	Lipoic acid [81]	Reduced blood pressure, blood glucose and plasma insulin concentrations, improved renal function	—
Sucrose-induced metabolic syndrome	*Hippophae rhamnoides* (sea buckthorn) seed extract [82]	Reduced blood pressure, reduced plasma concentrations of triglycerides, total cholesterol and free fatty acids, increased plasma HDL-cholesterol concentrations	No change in body weight, blood glucose and plasma insulin concentrations
High fat-induced metabolic syndrome	Enalapril (ACE inhibitor) [83]	Reduced body weight, epididymal fat pads and plasma insulin concentrations, increased plasma leptin and cholesterol concentrations, improved vascular relaxation	No change in blood glucose, plasma triglyceride and plasma free fatty acids concentrations, glucose tolerance
High fructose, high fat-induced metabolic syndrome	Purple carrot juice [84]	Reduced body weight gain, improved glucose tolerance, reduced plasma triglycerides, total cholesterol, free fatty acids concentrations, reduced plasma inflammatory marker, improved ventricular function, reduced cardiac fibrosis and stiffness, reduced blood pressure, improved vascular relaxation, attenuation of fatty liver	—
High sucrose, high fat-induced metabolic syndrome	Piperine [85]	Reduced body weight, reduced abdominal fat pads	No change in blood glucose, plasma triglyceride, plasma total cholesterol and free fatty acid concentrations

ACE - Angiotensin converting enzyme, AT - Angiotensin, ZDF - Zucker diabetic fatty, DPP-4 - dipeptidyl peptidase-4, OLETF - Otsuka Long-Evans Tokushima Fatty, PPAR - peroxisome proliferator-activated receptor, GK - Goto-Kakizaki.

of chitooligosaccharides for 8 weeks [98]. While these models may be suitable for studies in type 2 diabetes [99], the key signs of hypertension and obesity necessary for the metabolic syndrome were not reported.

10. Diet-Induced Metabolic Syndrome

Diet plays an important role in growth and development as a source of nutrition, but the composition of the diet decides its nutritional status. The modern diet, especially in Western countries, is rich in carbohydrates such as fructose and sucrose as well as saturated fat. This increased calorific intake has been associated with many diet-induced complications including metabolic syndrome, cardiovascular diseases and nonalcoholic fatty liver disease [100, 101]. Combinations of carbohydrate and fat-rich dietary components have been used in rodents to mimic these signs and symptoms of human metabolic syndrome.

11. Fructose-Induced Metabolic Syndrome

Fructose has become an important and pervasive ingredient in Western diets [102, 103]. The world average per capita daily fructose intake increased by 16% between 1986 and 2007 [103]. Together with the increase in consumption of fructose in the diet over the last fifty years, there has been a proportionate increase in the incidence of obesity [104]. The main sources of fructose in the diet are sucrose, high-fructose corn syrup, fruits and honey. Unlike glucose, high-fructose feeding to rodents induced the development of symptoms of metabolic syndrome including high blood pressure, insulin resistance, impaired glucose tolerance and dyslipidemia [102, 105]. Fructose feeding induced ventricular dilatation, ventricular hypertrophy, decreased ventricular contractile function, infiltration of inflammatory cells in heart and hepatic steatosis [106, 107]. In the liver, fructose feeding induced both microvesicular and macrovesicular steatosis with periportal fibrosis and lobular inflammation [108]. Fructose has been reported to induce obesity [109] but this was not confirmed [106]. Fructose feeding in rats caused renal tubular injury, collagen deposition in interstitium and increased macrophage infiltration along with proliferation and hyperplasia of renal proximal tubules [110] as well as leptin resistance without changes in body weight and adiposity [111]. Increases in plasma uric acid and plasma triglyceride concentrations have been reported without changes in plasma cholesterol concentrations [112, 113].

Fructose, unlike glucose, did not elicit insulin secretion from pancreatic β-cells, possibly due to the absence of the fructose transporter (GLUT5) on pancreatic β-cells [104]. Fructose also lacks the ability to stimulate the secretion of leptin [104] whereas it has the ability to activate *de novo* lipogenesis in the liver (Figure 3) [114]. During metabolism, fructose bypasses the rate-limiting step, the reaction catalysed by phosphofructokinase, leading to uncontrolled supply of carbon skeleton for lipogenesis in liver [115].

12. Sucrose-Induced Metabolic Syndrome

Sucrose is a dietary source of fructose [103], thus sucrose feeding has been used to mimic human metabolic syndrome in animal models. Similar to fructose, sucrose feeding has shown variable results, especially with obesity [116, 117]. As with fructose, sucrose induced lipogenesis in rats along with increased plasma concentrations of insulin, leptin, triglycerides, glucose and free fatty acids, and impaired glucose tolerance [118, 119]. Sucrose feeding in rats led to an insulin-resistant state with no change in fasting plasma insulin and glucose concentrations, but higher postprandial plasma concentrations of insulin and glucose [117]. Sucrose feeding increased systolic blood pressure in rats with increased left ventricular mass but without cardiac fibrosis [120] and caused development of hepatic steatosis [121]. No changes were seen in kidneys of rats fed with high-sucrose diet [122].

13. High Fat-Induced Metabolic Syndrome

High-fat diets have been used to model obesity, dyslipidaemia and insulin resistance in rodents for many decades. The complications developed by high-fat diets resemble the human metabolic syndrome and these complications may extend to cardiac hypertrophy, cardiac fibrosis, myocardial necrosis and hepatic steatosis [123–126]. High-fat diet feeding in mice increased systolic blood pressure and induced endothelial dysfunction [126]. High-fat diet-fed mice also showed albuminuria, increased glomerular tuft area, mesangial expansion, renal lipid accumulation, collagen deposition in glomeruli and increased infiltration of macrophages in renal medulla [127]. Different types of high-fat diets have been used with fat fractions ranging between 20% and 60% energy as fat as either animal-derived fats, such as lard or beef tallow, or plant oils such as olive or coconut oil [125]. Long-term feeding of rats (60% of energy) and mice (35% fat wt/wt) with high-fat diet increased body weight compared to standard chow-fed controls [128, 129]. Although the increase in body weight was significant after as little as 2 weeks, the diet-induced phenotype became apparent after more than 4 weeks of high-fat diet feeding [128]. Long-term feeding with both animal and plant fat-enriched diets eventually led to moderate hyperglycaemia and impaired glucose tolerance in most rat and mouse strains [130, 131].

Lard, coconut oil and olive oil (42% of energy content) increased body weight, deposition of liver triglycerides, plasma triglyceride and free fatty acid concentrations and plasma insulin concentrations and decreased plasma adiponectin concentrations [125]. Lard and olive oil but not coconut oil decreased insulin sensitivity [125]. Lard, coconut oil and olive oil caused hepatic steatosis with no signs of inflammation and fibrosis in any of the groups [125]. Beef tallow when used as fat source (40% of energy) increased plasma insulin and leptin concentrations with increased plasma lipid concentrations and hepatic steatosis [132]. Although high-fat diet induces most of the symptoms of human metabolic syndrome in rodents, it does not resemble the diet causing metabolic syndrome and associated

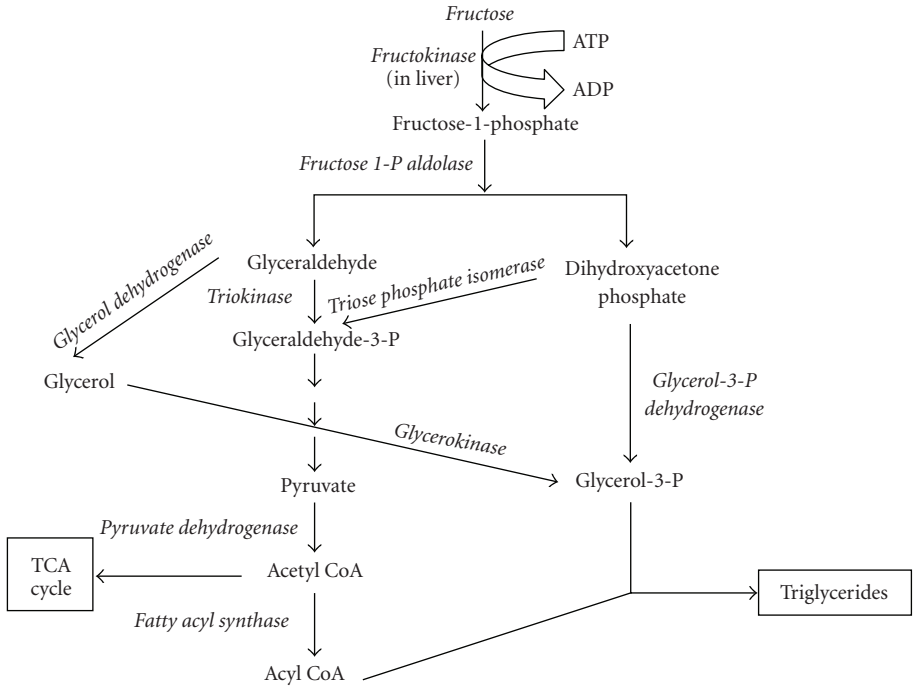

Figure 3: Metabolism of fructose.

complications, as the human diet is more complex than a high-fat diet.

14. High Carbohydrate-, High Fat-Induced Metabolic Syndrome

A diet high in carbohydrates together with fat, either of animal or plant origin, mimics the human diet more closely. This combined diet should induce metabolic syndrome in rodents (Figure 4). Different combinations and amounts of carbohydrates and fats have been used in different studies [133–136]. The common carbohydrates used are fructose and sucrose whereas the source of fat varies in different studies.

Different combinations of sucrose and fat have been used to induce signs of metabolic syndrome. Sucrose content varied between 10% and 30% whereas fat content in this diet group varied between 20% and 40% [137–139]. Rodents fed on high-sucrose, high-fat diet had increased body weight, abdominal fat deposition, hyperinsulinaemia, hyperglycaemia and hyperleptinaemia [137, 138]. Sucrose and fat in combination also caused hepatic steatosis and increased hepatic lipogenic enzymes [139].

Fructose and fat have been used in combination to induce metabolic syndrome. The fructose content varies between 10% and 60%, either in the diet or drinking water or both, whereas the fat content varies between 20% and 60% [133, 140–143]. Fructose and fat feeding increased body weight and the plasma concentrations of triglycerides, cholesterol, free fatty acids and leptin [133, 140]. The combination of fructose and fat also caused hyperinsulinaemia, insulin resistance, impaired glucose tolerance, increased abdominal fat deposition, hepatic steatosis and inflammation [133, 140]. The rats fed with the high-fructose, high-fat diet showed cardiac hypertrophy, increased ventricular stiffness, ventricular dilatation, cardiac inflammation and fibrosis, hypertension, decreased cardiac function and endothelial dysfunction along with mild renal damage and increased pancreatic islet mass [133].

Since high-carbohydrate, high-fat diet-fed rodents develop all the complications present in human metabolic syndrome and the diet is similar to human diets (sometimes called a "cafeteria diet"), this model is probably the best model to study the human metabolic syndrome. Pharmaceutical and nutraceutical preparations can be tested for treatment of diet-induced human metabolic syndrome in this high-carbohydrate, high-fat diet-fed model.

15. Obesity-Resistant Rat Strain

The interaction of genes with the diet is crucial for the induction of obesity in rodents and humans as shown by the studies with diet-induced obese (DIO) and diet-resistant (DR) rats [144, 145]. DR rats, even when fed with high-fat diet, did not produce the signs of metabolic syndrome, whereas DIO rats clearly showed those signs [144, 145]. The signs shown by DIO rats and not shown by DR rats included increases in body weight and body fat, impairment of glucose tolerance, dyslipidaemia, hyperinsulinaemia and hyperleptinaemia [144, 145]. However, these signs are similar to many control rats and mice fed standard rodent food that are sedentary, obese and develop impaired glucose tolerance, described as "metabolically morbid" [146].

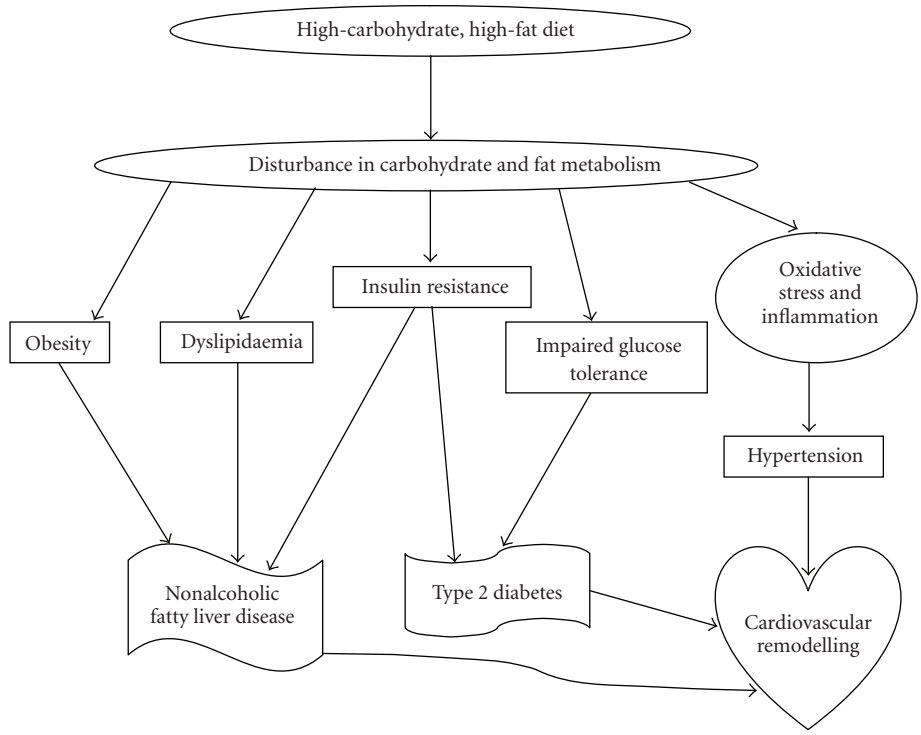

Figure 4: High-carbohydrate, high-fat diet-induced metabolic syndrome.

16. Fatty Liver Disease

Nonalcoholic steatohepatitis is now recognized as a complication of metabolic syndrome [147]. The most important model of nonalcoholic steatohepatitis is the methionine- and choline-deficient diet-fed rat. This special diet produced hepatic steatosis and fibrosis, increased hepatic triglycerides, increased serum activities of transaminases and alkaline phosphatase and increased serum concentrations of total bilirubin [148, 149]. Methionine- and choline-deficient diet-fed rats showed extreme reduction in body weight and liver weight along with decreased serum triglyceride and total protein concentrations [148, 149]. Although these rats develop nonalcoholic steatohepatitis, they do not show the other signs of metabolic syndrome.

17. High-Fat Diet-Fed Spontaneously Hypertensive Rats

Spontaneously hypertensive rats (SHRs) are the most widely used genetic model of human hypertension [22]. High-fat feeding to SHRs led to an increased body weight compared to SHRs fed on normal chow diet [150]. High-fat-fed SHRs also showed renal inflammation and albuminuria but did not show changes in plasma concentrations of total cholesterol, triglycerides and insulin, although plasma concentrations of free fatty acids were higher in high-fat-fed SHRs compared to normal diet-fed SHRs [150]. There was no change in systolic blood pressure with high-fat feeding in SHRs [151]. High-fat-fed SHRs also showed impaired glucose tolerance [152]. Although high-fat-fed SHRs show some symptoms of metabolic syndrome, they have genetically induced rather than diet-induced hypertension. Since human hypertension is not monogenetic, this model should not be considered appropriate as a model of the metabolic syndrome.

18. Nile Grass Rats

Apart from laboratory animals, wild rodents have been tested for the development of diabetes and obesity with laboratory diets. The Nile rat (African grass rat; *Arvicanthis niloticus*) and sand rat (*Psammomys obesus*) are two examples. These rats do not develop diabetes in the wild, but diabetes was induced when these rats were kept under laboratory conditions on chow diet [153]. These rats show hyperglycaemia and dyslipidaemia after 1 year of age [154]. They also develop liver steatosis, abdominal fat deposition, hypertension and hyperinsulinaemia [153, 154]. These rats show promise for metabolic syndrome research, even though these signs develop when fed on normal diet rather than the high-carbohydrate, high-fat diet in humans. This is similar to the concept of metabolically morbid rodents fed a normal diet [146].

19. Useful Treatment Strategies in Metabolic Syndrome Research

These rodent models have been used to characterize responses to many interventions. Success has been variable but some treatments have attenuated most of the signs

of the metabolic syndrome. These treatment strategies clearly indicate that it is possible to inhibit the progression of metabolic syndrome and associated complications and maybe to reverse them. Some of the responses to treatments in different rodent models have been described in Table 2.

20. Conclusion

Pharmaceutical and nutraceutical preparations are required to decrease morbidity and mortality in chronic diseases such as metabolic syndrome. These preparations need to be tested for efficacy in an appropriate rodent model. Thus, different animal models have been developed for this purpose. While many rodent models display some of the signs of the metabolic syndrome, few models can adequately mimic the range of signs that characterise this syndrome in humans. In particular, the presence of inflammation has often not been tested or defined. Further, many models rely on genetic changes to induce symptoms even though the human disease is usually diet induced. It is our opinion that chronic consumption of a high-carbohydrate, high-fat diet by normal rodents provides an adequate rodent model to mimic the human metabolic syndrome and for testing potential therapeutic interventions.

Conflict of Interests

The authors declare no conflict of interests.

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Chapter 3.2

High-carbohydrate, High-fat Diet–induced Metabolic Syndrome and Cardiovascular Remodeling in Rats

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Abstract: The prevalence of metabolic syndrome including central obesity, insulin resistance, impaired glucose tolerance, hypertension, and dyslipidemia is increasing. Development of adequate therapy for metabolic syndrome requires an animal model that mimics the human disease state. Therefore, we have characterized the metabolic, cardiovascular, hepatic, renal, and pancreatic changes in male Wistar rats (8–9 weeks old) fed on a high-carbohydrate, high-fat diet including condensed milk (39.5%), beef tallow (20%), and fructose (17.5%) together with 25% fructose in drinking water; control rats were fed a cornstarch diet. During 16 weeks on this diet, rats showed progressive increases in body weight, energy intake, abdominal fat deposition, and abdominal circumference along with impaired glucose tolerance, dyslipidemia, hyperinsulinemia, and increased plasma leptin and malondialdehyde concentrations. Cardiovascular signs included increased systolic blood pressure and endothelial dysfunction together with inflammation, fibrosis, hypertrophy, increased stiffness, and delayed repolarization in the left ventricle of the heart. The liver showed increased wet weight, fat deposition, inflammation, and fibrosis with increased plasma activity of liver enzymes. The kidneys showed inflammation and fibrosis, whereas the pancreas showed increased islet size. In comparison with other models of diabetes and obesity, this diet-induced model more closely mimics the changes observed in human metabolic syndrome.

Key Words: metabolic syndrome, obesity, cardiovascular disease, hypertension, dyslipidemia, high-carbohydrate, high-fat diet

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INTRODUCTION

Cardiovascular disease and type 2 diabetes remain major public health challenges.[1] Metabolic syndrome as the clustering of risk factors for cardiovascular disease and type 2 diabetes increases cardiovascular mortality.[2–5] These risk factors include central obesity, elevated blood pressure, impaired glucose tolerance, insulin resistance, and dyslipidemia.[6,7] The initiation of symptoms of metabolic syndrome has been linked to high-carbohydrate, high-fat diet–induced oxidative stress.[8] Oxidative stress along with chronic low-grade inflammation, induced by sugars and lipids,[9] may initiate changes in cardiovascular structure and function such as endothelial dysfunction, cardiac hypertrophy, cardiac fibrosis, and ventricular contractile dysfunction.[10–13]

Understanding the causes and progression of metabolic syndrome leading to cardiovascular disease is the foundation for the development of better pharmacological interventions for metabolic syndrome and cardiovascular disease. Appropriate animal models, mimicking the human pathogenesis of metabolic syndrome leading to cardiovascular disease, are therefore necessary to investigate the causes and progression of disease and potential pharmacological interventions.[14,15] There are several spontaneous (genetic) rodent models of metabolic syndrome with cardiovascular complications, including Zucker diabetic fatty rats,[16,17] Goto-Kakizaki rats,[18] spontaneously hypertensive rats,[19] and Otsuka Long Evans Tokushima fatty rats.[20] The most commonly used nongenetic rodent models of diabetes are those induced by death of pancreatic β cells by streptozotocin or alloxan.[21] Although these models may provide useful information on the causes and treatment of some aspects of metabolic syndrome, they are unlikely to be relevant to the diet-induced human metabolic syndrome.[22]

Rats fed on high-carbohydrate diets, for example, with sucrose[23] or fructose,[24] developed hypertension, dyslipidemia, and impaired glucose tolerance, but failed to develop central obesity while the structural and functional changes in the cardiovascular system were not addressed. In contrast, high-fat diets in rats induced marked central obesity and dyslipidemia,[25] but human diets causing metabolic syndrome are much more complex than a high-fat diet. Thus, a combination of high carbohydrate with high fat in the diet may be more relevant to mimic the diet responsible for human metabolic syndrome and cardiovascular complications as a basis to

investigate potential interventions. High-carbohydrate, high-fat diets (HCHF) in rats induced the symptoms of metabolic syndrome such as hypertension, dyslipidemia, impaired glucose tolerance, excess fat deposition, increased proinflammatory markers, and decreased antioxidant defenses.[8,26,27] Although these studies have defined the biochemical changes, the structural and functional changes in the cardiovascular system, liver, kidneys, and pancreas are yet to be thoroughly addressed. Therefore, the aim of this study was to define the structural and functional changes in heart, thoracic aorta, liver, kidneys, and pancreas in presence of metabolic syndrome.

This study has characterized structural changes in the heart by echocardiography and histopathology, while heart function was assessed in vivo using echocardiography and ex vivo in isolated perfused hearts. Single-cell microelectrode studies were performed on isolated left ventricular papillary muscles to quantify the changes in electrical conductance. Isolated thoracic aortic rings were used to measure vascular reactivity. Histological and biochemical parameters were assessed to define structural and functional changes in the liver, kidneys, and pancreas.

MATERIALS AND METHODS

Diet-induced Metabolic Syndrome in Rats

All experimental protocols were approved by The University of Queensland Animal Experimentation Ethics Committee, under the guidelines of the National Health and Medical Research Council of Australia. Male Wistar rats (8–9 weeks old, weighing 331 ± 6 g, n = 77) were obtained from The University of Queensland Biological Resources facility. The rats were randomly divided into 3 experimental groups and were fed with chow diet (n = 21), cornstarch diet (CS; n = 21) or HCHF diet (n = 35). Thirteen rats from HCHF group were euthanized after 8 weeks of feeding to assess the progression of pathophysiological changes and the remaining rats continued with the HCHF diet for a further 8 weeks; total feeding time was 16 weeks. The CS group was fed for 16 weeks. The CS diet contained 570 g of cornstarch, 155 g of powdered rat food (Specialty Feeds, Glen Forest, Western Australia, Australia; all nutritional parameters of this diet meet or exceed the National Research Council, Canada, guidelines for rats and mice), 25 g of Hubble, Mendel, and Wakeman salt mixture,[28] and 250 g of water per kilogram of diet. HCHF diet consisted of 175 g of fructose, 395 g of sweetened condensed milk, 200 g of beef tallow, 155 g of powdered rat food, 25 g of Hubble, Mendel and Wakeman salt mixture, and 50 g of water per kilogram of diet. In addition, the drinking water for the HCHF group was supplemented with 25% fructose. Chow-fed rats were fed powdered rat food (Specialty Feeds; all nutritional parameters of this diet meet or exceed the National Research Council, Canada, guidelines for rats and mice). Rats were given *ad libitum* access to food and water and were individually housed in a temperature-controlled 12-hour light–dark conditions. Energy intake was calculated from the following values in kilojoules per gram: fructose, 15.40; CS, 15.94; condensed milk, 13.80; beef tallow, 37.70; and powdered rat food, 13.80. The energy densities of the CS diet and the HCHF diet were 11.23 kJ/g and 17.83 kJ/g of food, respectively, and an additional 3.85 kJ/mL in the drinking water for the HCHF diet–fed rats.

Physiological Parameters

Body weight and food and water intakes were measured daily. Oral glucose tolerance tests were performed every fourth week after determining overnight fasting blood glucose concentrations in tail vein blood using Medisense Precision Q.I.D. glucose meters (Abbott Laboratories, Bedford, MA). For overnight fasting, rats were deprived of all types of diets for 12 hours. Fructose-supplemented drinking water in HCHF 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. Abdominal circumference and body length (nose to anus) were measured every fourth week using a standard measuring tape under light anesthesia with Zoletil [tiletamine 10 mg/kg, zolazepam 10 mg/kg, intraperitoneal (IP); Virbac, Peakhurst, New South Wales, Australia]. Body mass index (BMI) was calculated as body weight (in grams)/[body length (in centimeters)]^2.[29] Feed efficiency was calculated as [mean body weight gain (in grams)/daily energy intake (in kilojoules)].[29] Rectal temperature was measured using a digital thermometer lubricated with a smear of Xylocaine jelly (lidocaine; AstraZeneca Pty Ltd, New South Wales, Australia).

Body Composition Measurements

Dual-energy X-ray absorptiometric (DXA) measurements were performed on the rats after 8 and 16 weeks of feeding (2 days before rats were euthanized for pathophysiological assessments) using a Norland XR36 DXA instrument (Norland Corp, Fort Atkinson, WI). DXA scans were analyzed using the manufacturer's recommended software for use in laboratory animals (Small Subject Analysis Software, version 2.5.3/1.3.1; Norland Corp) as previously described.[30] The precision error of lean mass for replicate measurements, with repositioning, was 3.2%.

Experimental Protocol

Ten rats from each group were used for isolated Langendorff preparations and vascular reactivity studies, and 3 rats per group were taken exclusively for histopathological analysis. Eight chow-fed rats, 8 rats from the CS group, and 9 rats from the HCHF group (all 16 weeks) were used for single-cell microelectrode studies. For terminal experiments, rats were euthanized with Lethabarb (pentobarbitone sodium, 100 mg/kg, IP; Virbac). After euthanasia, heparin (200 IU; Sigma-Aldrich Australia, Sydney, New South Wales, Australia) was injected through the right femoral vein. The abdomen was then opened and blood (~6 mL) was withdrawn from the abdominal aorta and collected into heparinized tubes. One milliliter of blood was stored at 4°C, and the remaining blood was centrifuged at 5,000*g* for 15 minutes to obtain plasma. Plasma was stored at −20°C for further characterization. Hearts were removed from rats for isolated

Langendorff preparation, and thoracic aorta was used for vascular reactivity studies as described below. Liver, kidney, and fat pads were removed from these rats and weighed. After perfusion studies, right ventricles from the hearts were removed and weighed, whereas the left ventricle (LV) was weighed with septum. Weights of these organs were normalized relative to the tibial length at the time of removal (expressed as tissue weight in milligram per millimeter tibial length). Heart, liver, kidney, and pancreas from the rats used for histopathological analysis were removed and fixed in appropriate solutions.

Cardiovascular Structure and Function

Systolic blood pressure of rats was measured every fourth week under light sedation with Zoletil (tiletamine 10 mg/kg, zolazepam 10 mg/kg, IP), using an MLT1010 Piezo-Electric Pulse Transducer (ADInstruments, Sydney, New South Wales, Australia) and inflatable tail-cuff connected to an MLT844 Physiological Pressure Transducer (ADInstruments) and PowerLab data acquisition unit (ADInstruments).

Echocardiographic examination (Phillips iE33, 12-MHz transducer) was performed as previously described[31] in all rats after 8 and 16 weeks of feeding. Briefly, rats were anesthetized using Zoletil (tiletamine 25 mg/kg and zolazepam 25 mg/kg, IP) and Ilium Xylazil (xylazine 15 mg/kg, IP; Troy Laboratories, Smithfield, New South Wales, Australia) and positioned in dorsal recumbency. Electrodes attached to the skin overlying the elbows and right stifle facilitated the simultaneous recording of a lead II electrocardiogram (ECG).

A short-axis view of the LV at the level of the papillary muscles was obtained and used to direct acquisition of M-mode images of the LV for measurement of interventricular septum in diastole, diastolic posterior wall thickness (LVPWd), internal systolic dimensions, and end-diastolic dimension (LVIDd). Measurements were taken in accordance with the guidelines of the American Society of Echocardiography using the leading-edge method.[32] For these parameters, diastole was defined by the beginning of the QRS complex on the simultaneously recorded ECG and systole identified as the nadir of systolic anterior wall motion independent of the ECG complex.

Pulsed-wave Doppler velocity profiles of mitral inflow were obtained from the left apical 4-chamber view for measurement of early (E_M) and late (A_M) mitral inflow velocity, mitral inflow E-wave deceleration time, and time from mitral valve closure to opening. A pulsed-wave Doppler velocity profile of the ascending aorta was obtained from a suprasternal view for measurement of ejection time and the diameter of the ascending and descending aorta at the point of transition to the transverse aorta.

Derived indices of LV systolic function (fractional shortening and ejection fraction) were calculated using well-established formulas.[31] Left ventricular mass was estimated using the standard cube equation as modified by Litwin et al[33]: Left ventricular mass (in grams) = $[1.04\ (\text{IVSDd} + \text{LVIDd} + \text{LVPWd})^3 - (\text{LVIDd})^3] \times 0.8 + 0.14$, where 1.04 is the specific gravity of muscle, IVSDd is interventricular septum diameter in diastole (in centimeters), LVIDd is left ventricular internal diameter in diastole (in centimeters), and LVPWd is left ventricular posterior wall thickness in diastole (in centimeters). Multiplication factor of 0.8 and the addition factor of 0.14 are constants similar to those used in the anatomically validated Devereux correction for American Society for Echocardiography dimensions in humans, which allow more accurate prediction of LV mass in rats.[33]

The isolated Langendorff heart preparation was used to assess left ventricular function of the rats in all the groups as in previous studies.[34–36] Hearts isolated from euthanized rats were perfused with modified Krebs–Henseleit bicarbonate buffer, containing (in millimolar): NaCl, 119.1; KCl, 4.75; $MgSO_4$, 1.19; KH_2PO_4, 1.19; $NaHCO_3$, 25.0; glucose, 11.0; and $CaCl_2$, 2.16. Buffer was bubbled with 95% O_2–5% CO_2 and maintained at 35°C. Isovolumetric ventricular function was measured by inserting a latex balloon catheter into the LV connected to a Capto SP844 MLT844 physiological pressure transducer and Chart software on a Maclab system (ADInstruments). 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 up to 30 mmHg for calculation of diastolic stiffness constant (κ, dimensionless) as described in previous studies.[34–36]

Microelectrode studies were performed on isolated left ventricular papillary muscles quickly dissected in cold Tyrode physiological salt solution (in millimolar: NaCl, 136.9; KCl, 5.4; $MgCl_2 \cdot H_2O$, 1.0; $NaH_2PO_4 \cdot 2H_2O$, 0.4; $NaHCO_3$, 22.6; $CaCl_2 \cdot 2H_2O$, 1.8; glucose, 5.5; ascorbic acid, 0.3; and ethylenediaminetetraacetic acid, 0.05) bubbled with 95% O_2–5% CO_2. Action potential duration (APD) at 20%, 50%, and 90% of repolarization, action potential amplitude, resting membrane potential, and force of contraction were measured as in previous studies.[35,36]

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 and maintained at 35°C and allowed to stabilize at a resting tension of 10 mN.[35,36] Cumulative concentration–response curves (contraction) were obtained for norepinephrine (Sigma-Aldrich Australia), and cumulative concentration–response curves (relaxation) were obtained for acetylcholine (Sigma-Aldrich Australia) and sodium nitroprusside (Sigma-Aldrich Australia) after submaximal (70%) contraction to norepinephrine.[35,36]

Hearts were processed by 2 different procedures for histopathological studies. For picrosirius red staining, hearts were initially fixed for 3 days in Telly fixative (85 mL of 70% ethanol, 5 mL of glacial acetic acid, and 10 mL of 40% formaldehyde) and then transferred into modified Bouin fluid (85 mL of saturated picric acid, 5 mL of glacial acetic acid, and 10 mL of 40% formaldehyde) for 2 days. The samples were then dehydrated and embedded in paraffin wax. Collagen deposition in the LV was observed after staining with picrosirius red (magnification ×40) and analyzed by laser confocal microscopy (Zeiss LSM 510 upright Confocal Microscope).[35] Color intensity was quantified using NIH-imageJ software (National Institutes of Health, Bethesda, MD) to determine the extent of collagen deposition in selected tissue sections. Results are presented as mean ± standard error of the mean (SEM) of the area of view. For other staining, hearts were fixed in 10% neutral

buffered formalin for 3 days. The samples were then dehydrated and embedded in paraffin wax. Thin sections (5 μm) of LV were cut and stained with hematoxylin and eosin to study infiltration of inflammatory cells and with toluidine blue to study mast cells. After staining, pictures were taken with a Zeiss Microscope (magnification ×40).

Oxidative Stress Markers

Erythrocyte Reactive Oxygen Species Production

Heparinized blood stored at 4°C was used 2 days after withdrawal to measure the production of reactive oxygen species by erythrocytes using a fluorometric assay based on the oxidation of the fluorochrome 2′,7′-dichloroflourescein-diacetate (DCFH-DA; Sigma-Aldrich Australia).[37] Plasma was removed from blood by washing with 1:9 phosphate-buffered saline, followed by centrifugation at 1500*g* for 5 minutes. The supernatant was removed, and the packed erythrocytes were diluted with phosphate-buffered saline to a final content of 1% erythrocytes. Aliquots of 50 μL of this erythrocyte suspension were added to a microtitre plate followed immediately by 50 μL of 100 μM DCFH-DA. The rate at which DCFH-DA was oxidized by intracellular reactive oxygen species to 2′,7′-dichlorofluorescein was determined fluorometrically by reading the microtitre plate with a SPECTRAMax M2 spectrofluorometer set at Ex484/Em535. Fluorescence was measured at 0, 30, 60, 120, and 180 minutes. The data were expressed in arbitrary fluorescence units.

Plasma Marker of Oxidative Stress

Plasma concentrations of malondialdehyde were determined by high-performance liquid chromatography (Shimadzu, Kyoto, Japan).[38]

Metabolic Parameters and Plasma Inflammatory Marker

Activities of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and creatine kinase and concentrations of total cholesterol, triglycerides, nonesterified fatty acids (NEFA), uric acid, creatinine, urea, total bilirubin, albumin:-globulin ratio, sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), and chloride (Cl^-) in plasma were measured. Plasma enzymatic activities and analyte concentrations were determined using kits and controls supplied by Olympus using an Olympus analyzer (AU 400; Tokyo, Japan): LDH, Olympus OSR6127 kinetic UV test; ALT, Olympus OSR6107 kinetic UV test; AST, Olympus OSR6109 kinetic UV test; total plasma cholesterol, Olympus OSR6516 enzymatic color test; plasma triglycerides, Olympus OSR6133 enzymatic color test; urea, Olympus OSR6134 kinetic UV test; uric acid, Olympus OSR6098 enzymatic color test; albumin, Olympus OSR 6101 photometric color test; and total bilirubin, Olympus OSR 6111 photometric color test. Globulin was calculated as total protein − albumin; NEFA were determined using a commercial kit (Wako, Osaka, Japan). Plasma C-reactive protein (CRP) concentrations were estimated using commercial kits (Kamiya Biomedical, Thousand Oaks, CA) according to manufacturer-provided standards and protocols using a Cobas-Mira automated analyzer (Roche Diagnostics, Basel, Switzerland). Plasma insulin (Laboratory Diagnostics, Kurnell, New South Wales, Australia) and leptin and corticosterone (both Quantum Scientific, Murarrie, Queensland, Australia) were measured using commercial kits according to manufacturer-provided standards and protocols using Titertek Multiskan MCC/340 spectrophotometer (Flow Laboratories, Irvine, Scotland).

Structural Changes in Liver, Kidney, and Pancreas

Liver, kidneys, and pancreas were fixed with 10% neutral buffered formalin for 3 days. These tissue samples were dehydrated and then embedded in paraffin wax. Thin sections (5 μm) of these tissues were cut and stained with hematoxylin and eosin for determining inflammatory cell infiltration and for determining the fat vacuoles in liver. Liver sections were also stained with Milligan's stain to determine collagen deposition and with oil red "O" to determine fat droplets.

Fixed kidney tissues were embedded in paraffin, and 4 μm sections were cut onto glass slides. Routine histological stains used were hematoxylin and eosin for general histology and Masson's trichrome for assessment of collagen deposition. For quantification of collagen, Masson's trichrome–stained slides were visualized using a Nikon Eclipse 50*i* microscope (Kanagawa, Japan) fitted with a DSF*i*1 camera, and images were captured directly digitally (magnification ×200). NIS Elements software was used for morphometrical analysis. All color parameters were kept constant for each section. The NIS Elements software automatically calculates the area of blue-stained color in each of the areas selected, which were all 284,023 μm^2. For each kidney section, 10 randomly placed fields in the cortex were analyzed separately. Positive areas around large vessels were removed digitally from the morphometric assessment. Results are presented as mean ± SEM of the Masson's trichrome-positive percentage of the area of view.

Pancreatic sections were stained with aldehyde fuchsin staining after pretreatment with potassium permanganate (0.5%) and hematoxylin and eosin staining to determine infiltration of inflammatory cells. After staining, tissues were mounted and pictures were taken with a Zeiss Microscope (magnification ×40 for hematoxylin and eosin and ×20 for aldehyde fuchsin, oil red "O," and Milligan's stain). Islet area was quantified using NIH-imageJ software (National Institutes of Health). Results are presented as mean ± SEM of the area of view. The α and β cells in 12 islets were counted in each rat using NIH-imageJ software. Numbers of these cells are presented as mean ± SEM per islet.

Statistical Analysis

All data are presented as mean ± SEM. Differences between the groups were determined by 1-way analysis of variance. Statistically significant variables were treated with Neumann–Keuls post hoc test to compare all the groups of animals. All statistical analyses were performed using GraphPad Prism version 5 for Windows (San Diego, CA). *P* value of <0.05 was considered as statistically significant.

RESULTS

Food Intake, Water Intake, Body Weight, and Compositional Changes

Although mean daily food and water intakes were reduced (Table 1), HCHF diet–fed rats showed higher body weight (Fig. 1A), mean daily energy intake, and feed efficiency (Table 1) compared with CS diet–fed rats after 16 weeks. Abdominal circumference (Fig. 1B), nose to anus length, and BMI (Table 1) were greater for HCHF diet–fed rats than for the CS diet–fed rats after 16 weeks. Abdominal fat fads (Table 1) were unchanged and doubled after 8 and 16 weeks, respectively, in HCHF diet–fed rats compared with CS diet–fed rats. Whole-body fat mass (Fig. 1C) was increased by 126% ± 7% in HCHF diet–fed rats compared with CS diet–fed rats after 16 weeks, whereas the whole-body lean mass (Fig. 1D) was increased by only 11.6% ± 0.6% in HCHF diet–fed rats. Rectal temperature was unchanged between the groups (Table 1). Values from age-matched rats fed standard rat chow showed values intermediate between CS diet– and HCHF diet–fed rats with a greater increase in total and lean body mass (Table 1, Fig. 1).

Cardiovascular Structure and Function

Echocardiographic assessment of HCHF diet–fed rats showed ventricular dilation (increased left ventricular end-diastolic dimensions), increased systolic volume, and increased estimated left ventricular mass. However, indices of left ventricular function, mainly fractional shortening and ejection fraction, were decreased in HCHF diet–fed rats when compared with CS diet– or chow-fed rats after 16 weeks (Table 2).

Many inflammatory cells were observed in the LV of HCHF diet–fed rats, whereas the number of inflammatory cells in LV of CS diet– or chow-fed rats was very low (Fig. 2). Increased numbers of mast cells were found in the LV of HCHF diet–fed rats with an increased number of degranulated mast cells (Fig. 2). Both perivascular and interstitial collagen contents in LV were increased in HCHF diet–fed rats compared with CS diet– or chow-fed rats (Fig. 2). HCHF diet–fed rats also showed hypertrophy of cardiomyocytes (Fig. 2). The isolated Langendorff heart preparation showed increased diastolic stiffness in HCHF diet–fed rats at both 8 and 16 weeks compared with CS diet– or chow-fed rats (Table 2). Systolic blood pressure in HCHF diet–fed rats was increased at all measurement times up to 16 weeks compared with CS diet–fed or chow-fed rats (Table 2).

Single-cell microelectrode studies in isolated left ventricular papillary muscles of HCHF diet–fed rat hearts showed an increased APD at 50% and 90% of repolarization when compared with CS diet– or chow-fed rat hearts (Table 3). The resting membrane potential was decreased in HCHF diet–fed rats when compared with CS diet (Table 3) with the action potential amplitude and force of contraction being unaffected (Table 3).

Thoracic aorta isolated from HCHF diet– or chow-fed rats showed reduced acetylcholine-induced vascular relaxation compared with thoracic aorta from CS diet–fed rats, suggesting endothelial dysfunction with HCHF diet or chow feeding (Fig. 3A). The responses to norepinephrine and sodium nitroprusside were decreased in HCHF diet–fed rats compared with CS diet– or chow-fed rats, suggesting smooth muscle dysfunction (Figs. 3B, C).

Oxidative Stress and Plasma Inflammatory Marker

In HCHF diet–fed rats, the production of reactive oxygen species by erythrocytes over 180 minutes was almost double that of the CS diet– or chow-fed rats (Fig. 4A). Plasma malondialdehyde concentrations were increased in HCHF diet–fed rats in comparison with CS diet– or chow-fed rats (Table 4). Plasma concentrations of CRP were higher with HCHF diet after 16 weeks compared with CS diet– or chow-fed rats (Table 4).

Metabolic Changes

Fasting blood glucose concentrations were not different between the 2 groups after 8 weeks and 16 weeks of feeding (Table 4). After glucose loading, blood glucose concentrations in CS diet– or chow-fed rats decreased rapidly and returned to fasting glucose concentrations 2 hours after glucose loading. In HCHF (16 weeks) diet–fed rats, blood glucose concentrations were higher than the fasting blood glucose concentration after 2 hours of glucose loading (Fig. 4B). Plasma concentrations of insulin were unchanged with HCHF diet after 8 weeks but increased after 16 weeks in comparison with CS diet– or chow-fed rats (Table 4). Plasma leptin concentrations were increased with HCHF diet compared with CS diet or chow feeding as early as 8 weeks. There was no

TABLE 1. Physiological Parameters in Chow-fed (16 Weeks), CS (16 Weeks), HCHF (8 Weeks), and HCHF (16 Weeks) Groups

Variables	Chow-fed (16 wk)	CS (16 wk)	HCHF (8 wk)	HCHF (16 wk)
Food intake, g/d	27.9 ± 0.6^{b}	31.9 ± 0.6^{a}	19.8 ± 0.5^{c}	20.9 ± 0.3^{c}
Water intake, mL/d	45.5 ± 1.7^{a}	31.8 ± 0.5^{b}	17.9 ± 0.6^{c}	18.3 ± 0.3^{c}
Energy intake, kJ/d	385 ± 8^{b}	364 ± 9^{b}	425 ± 11^{a}	443 ± 7^{a}
Feed efficiency, g/kJ	0.64 ± 0.04^{a}	0.21 ± 0.02^{c}	0.23 ± 0.02^{c}	0.40 ± 0.02^{b}
Nose to anus length, cm	26.7 ± 0.7^{a}	24.1 ± 0.2^{c}	24.9 ± 0.3^{b}	25.7 ± 0.2^{ab}
BMI, g/cm^2	0.79 ± 0.01^{a}	0.66 ± 0.01^{c}	0.69 ± 0.01^{b}	0.78 ± 0.01^{a}
Abdominal fat pads, mg/mm	464 ± 34^{b}	395 ± 30^{b}	501 ± 46^{b}	799 ± 57^{a}
Rectal temperature, °C	38.6 ± 0.2	38.7 ± 0.1	38.4 ± 0.2	38.7 ± 0.1

All data are presented as mean ± SEM and n = 10 for each group. Mean values within a row with unlike superscript letters are significantly different ($P < 0.05$).

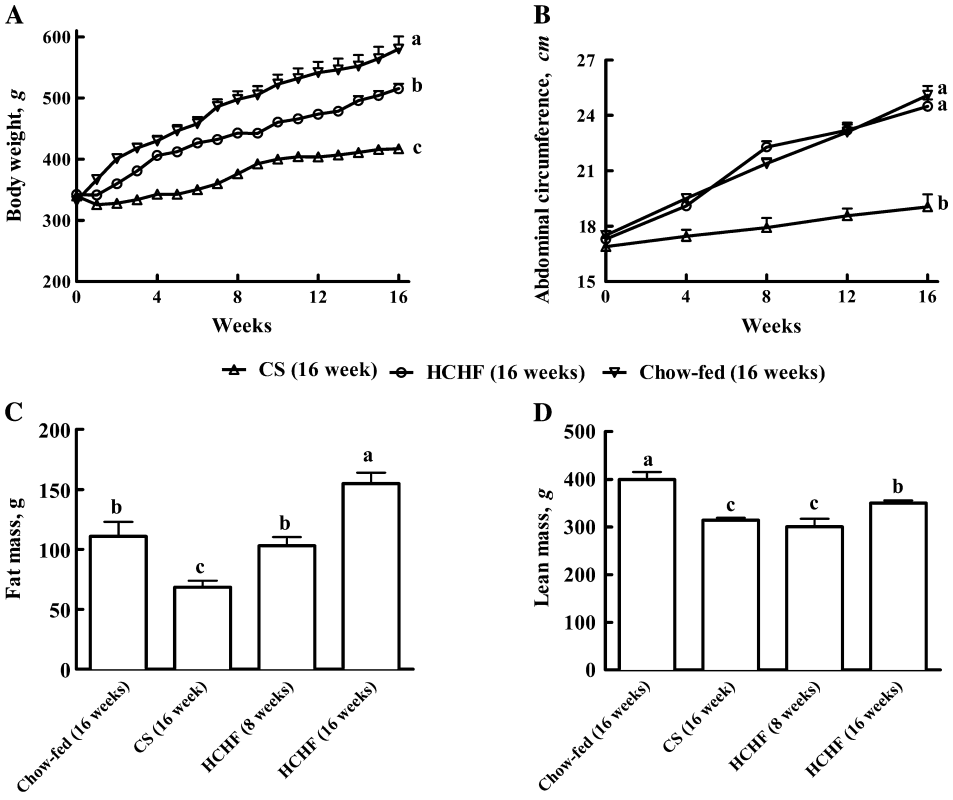

FIGURE 1. Changes in body weight (A), abdominal circumference (B), whole-body fat mass (C), and whole-body lean mass (D) in chow-fed rats (16 weeks), CS (16 weeks), HCHF (8 weeks), and HCHF (16 weeks) rats. Mean values with an unlike superscript letter are significantly different ($P < 0.05$).

TABLE 2. Cardiovascular Structure and Function in Chow-fed (16 Weeks), CS (16 Weeks), HCHF (8 Weeks), and HCHF (16 Weeks) Groups

Variables	Chow-fed (16 wk)	CS (16 wk)	HCHF (8 wk)	HCHF (16 wk)
LVIDd, mm	7.01 ± 0.16^{b}	6.61 ± 0.12^{b}	6.79 ± 0.10^{b}	7.46 ± 0.11^{a}
LVIDs, mm	3.52 ± 0.13^{c}	3.31 ± 0.18^{c}	4.04 ± 0.11^{b}	4.60 ± 0.09^{a}
LVPWd, mm	2.81 ± 0.16	2.69 ± 0.14	2.72 ± 0.13	2.85 ± 0.15
Systolic volume, mL	0.08 ± 0.01^{b}	0.06 ± 0.00^{b}	0.07 ± 0.00^{b}	0.10 ± 0.00^{a}
Relative wall thickness	0.50 ± 0.02	0.48 ± 0.01	0.52 ± 0.01	0.48 ± 0.02
Ascending aorta diameter, mm	0.84 ± 0.05	0.82 ± 0.03	0.87 ± 0.04	0.87 ± 0.06
Descending aorta diameter, mm	0.73 ± 0.04	0.73 ± 0.05	0.78 ± 0.03	0.77 ± 0.05
Fractional shortening, %	56.4 ± 2.0^{a}	52.6 ± 1.3^{a}	44.9 ± 2.2^{b}	38.3 ± 1.4^{c}
Ejection fraction, %	84.1 ± 2.2^{ab}	86.9 ± 1.6^{a}	79.7 ± 0.7^{b}	72.1 ± 1.3^{c}
Ejection time, ms	90.7 ± 2.3	91.1 ± 1.9	92.9 ± 2.9	87.9 ± 2.6
Deceleration time, ms	53.6 ± 2.2	52.1 ± 2.0	47.7 ± 1.5	55.5 ± 2.9
E_M, m/s	0.68 ± 0.02	0.69 ± 0.03	0.69 ± 0.03	0.69 ± 0.02
A_M, m/s	0.41 ± 0.03	0.38 ± 0.03	0.47 ± 0.03	0.44 ± 0.03
MCMO, ms	113 ± 3	114 ± 4	111 ± 3	114 ± 4
Estimated LV mass, g	0.71 ± 0.04^{c}	0.67 ± 0.02^{c}	0.82 ± 0.03^{b}	0.97 ± 0.04^{a}
LV + septum wet weight, mg/mm	21.3 ± 0.9	18.9 ± 0.9	19.4 ± 0.7	21.8 ± 1.2
Right ventricular wet weight, mg/mm	4.59 ± 0.30	4.14 ± 0.27	4.49 ± 0.29	4.48 ± 0.37
Interstitial LV fibrosis, % surface area	5.2 ± 0.9^{c}	4.8 ± 0.5^{c}	14.6 ± 1.4^{b}	19.9 ± 1.2^{a}
Perivascular LV fibrosis, % surface area	20.7 ± 3.6^{b}	21.2 ± 1.6^{b}	31.2 ± 2.6^{a}	35.3 ± 3.0^{a}
LV diastolic stiffness constant, κ	24.6 ± 1.8^{b}	20.8 ± 1.4^{b}	26.3 ± 2.2^{b}	27.7 ± 1.4^{a}
Systolic blood pressure, mmHg	125 ± 7^{b}	120 ± 4^{b}	144 ± 4^{a}	153 ± 5^{a}

All data are presented as mean ± SEM and n = 10 for each group. Mean values within a row with unlike superscript letters are significantly different ($P < 0.05$). LVIDs, left ventricular internal systolic dimension; MCMO, time from mitral valve closure to opening; ms, milliseconds.

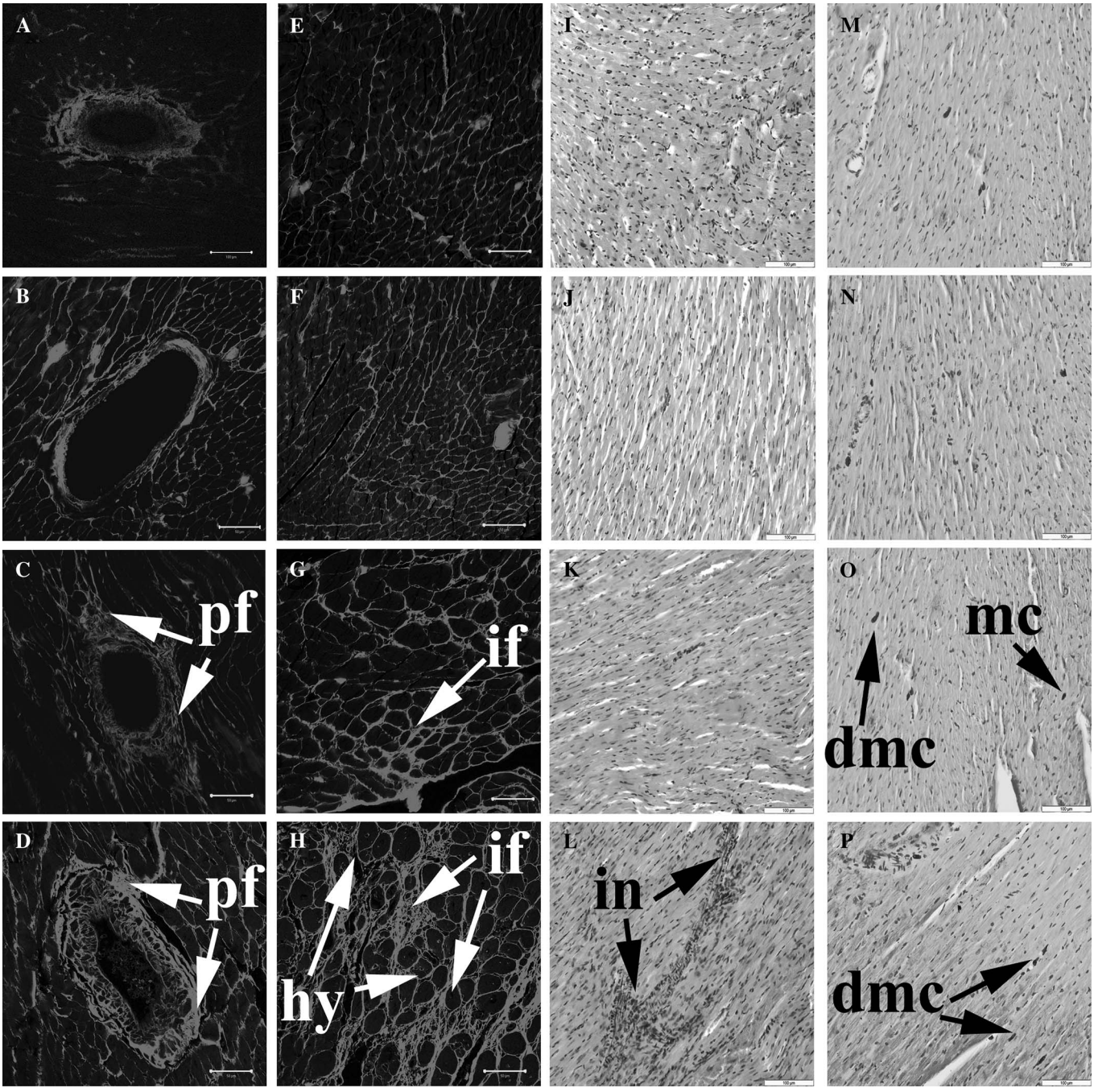

FIGURE 2. Inflammation and fibrosis in the heart. Picrosirius red staining of left ventricular perivascular and interstitial collagen deposition (×40) in chow-fed rats (16 weeks) (A, E), CS (16 weeks) (B, F), HCHF (8 weeks) (C, G), HCHF (16 weeks) (D, H); perivascular fibrosis is marked as ''pf,'' interstitial fibrosis is marked as ''if'' and hypertrophied cardiomyocytes are marked as ''hy.'' Hematoxylin and eosin staining of LV of the heart (×40) showing infiltration of inflammatory cells (''in'' represents inflammatory cells) as dark spots surrounding the myocytes; chow-fed rats (16 weeks) (I), CS (16 weeks) (J), HCHF (8 weeks) (K), HCHF (16 weeks) (L). Toluidine blue staining (×40) of LV showing presence of mast cells in chow-fed rats (16 weeks) (M), CS (16 weeks) (N), HCHF (8 weeks) (O), HCHF (16 weeks) (P); mast cells are marked as ''mc'' and degranulated mast cells are marked as ''dmc.''

difference in plasma leptin concentrations between HCHF diet groups fed for 8 and 16 weeks (Table 4). Plasma corticosterone concentrations were unchanged between the groups (Table 4). Plasma concentrations of triglycerides, total cholesterol, and NEFA were higher in HCHF diet–fed group compared with CS diet–fed group (Table 4). Plasma concentrations of uric acid were higher in HCHF diet–fed rats compared with CS diet– or chow-fed rats, whereas no change was observed in the plasma

TABLE 3. Single-cell Microelectrode Studies on Left Ventricular Papillary Muscles Isolated from Chow-fed (16 Weeks), CS (16 Weeks), and HCHF (16 Weeks) Groups

Variables	Chow-fed (16 wk)	CS (16 wk)	HCHF (16 wk)
Resting membrane potential, mV	-69.1 ± 2.5^{ab}	-73.0 ± 2.7^{b}	-62.4 ± 1.9^{a}
Action potential amplitude, mV	72.4 ± 5.5	69.5 ± 4.4	63.4 ± 2.6
APD at 20% repolarization, ms	10.9 ± 0.9^{b}	15.0 ± 1.2^{a}	15.7 ± 0.8^{a}
APD at 50% repolarization, ms	19.5 ± 1.4^{b}	23.9 ± 1.9^{b}	28.9 ± 1.7^{a}
APD at 90% repolarization, ms	45.7 ± 3.2^{b}	55.5 ± 4.0^{b}	80.9 ± 8.5^{a}
Force of contraction, mN	2.4 ± 0.4	4.2 ± 1.2	2.9 ± 0.8

All data are presented as mean ± SEM and n = 8 for chow-fed and CS group and n = 9 for HCHF group. Mean values within a row with unlike superscript letters are significantly different ($P < 0.05$); ms, milliseconds.

albumin:globulin ratio between the groups and plasma concentrations of bilirubin were lowered in chow-fed rats (Table 4).

Hepatic Structure and Function

Livers from HCHF diet– and chow-fed rats were higher in wet weight compared with CS diet–fed rats (Table 5). In the liver as in the LV, HCHF diet–fed rats showed increased infiltration of inflammatory cells and increased deposition of collagen around the blood vessels compared with CS diet– or chow-fed rats (Fig. 5). HCHF diet–fed rats showed deposition of fat droplets in liver, which were rarely visible in livers from CS diet–fed rats (Fig. 5). Also, the size of fat vacuoles was larger in HCHF diet–fed rats (Fig. 5). Similarly, HCHF diet resulted in increased plasma activity of AST, ALT, ALP, and LDH compared with CS diet–fed rats, although plasma AST was higher in chow-fed rats (Table 5).

Renal and Pancreatic Structure and Function

HCHF diet– and chow-fed rats showed increased wet weight of kidneys in comparison with CS diet–fed rats (Table 5). There were no anomalies in the kidneys of the CS diet–fed rats. The Masson's trichrome-positive tissue in these sections was 2.27% ± 0.64%. Kidney sections from HCHF diet–fed rats showed glomerular and tubular damage, including lysis of the glomerular tuft that was seen in more than 50% of the glomeruli, and tubular lesions consisted of hyaline and/or vacuolar degeneration and single-cell necrosis. There was also increased Masson's trichrome-positive tissue (10.13% ± 2.76%), mainly in an expanded interstitial space and in the tubular and glomerular basement membranes (Fig. 6). There were no changes in the plasma concentrations of creatinine, Na^+, and Cl^- between the 2 groups, but plasma K^+ concentrations were increased and urea concentrations were decreased in HCHF diet–fed rats compared with CS diet– or chow-fed rats (Table 5). Pancreas from HCHF diet–fed rats showed an increased number of inflammatory cells (Fig. 7) with increased size of islets of Langerhans (15.3% ± 1.5% area) compared with CS diet– or chow-fed rats (6.2% ± 1.4% area) (Fig. 7). Numbers of α and β cells were also increased in HCHF diet–fed rats compared with CS diet–fed rats (CS: 16.9 ± 0.5 and 72.3 ± 2.8 per islet; HCHF: 39.3 ± 1.2 and 124.7 ± 7.2 per islet, respectively).

DISCUSSION

Long-term metabolic syndrome leads to multiorgan dysfunction, especially cardiovascular disease, a major cause of mortality in modern society.[3,4,39] Metabolic syndrome also damages the structure and function of liver, kidneys, and

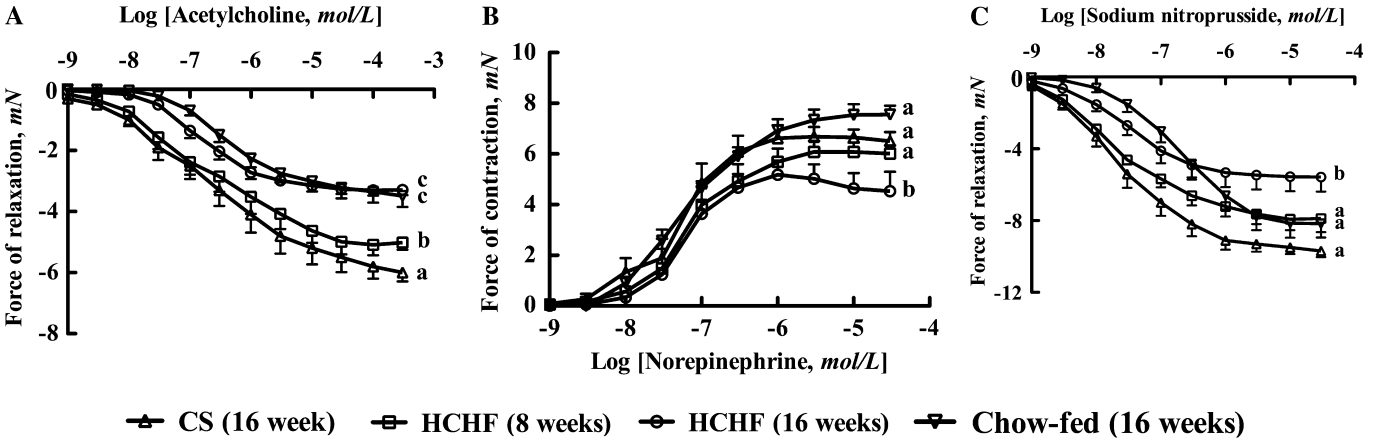

FIGURE 3. Thoracic aortic responses to various drugs. Thoracic aortic preparations developing acetylcholine-induced relaxation (A), norepinephrine-induced contraction (B), and sodium nitroprusside–induced relaxation (C). Mean values with an unlike superscript letter are significantly different ($P < 0.05$).

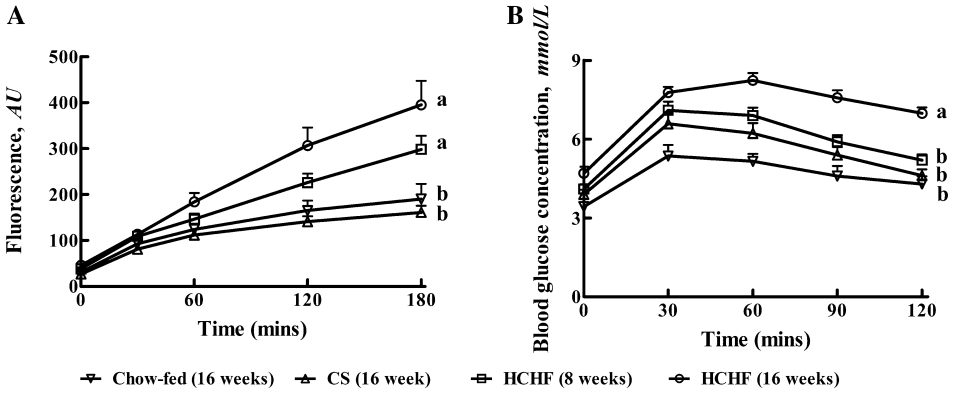

FIGURE 4. Changes in superoxide production by erythrocyte (A) and 120-minute oral glucose tolerance (B) in chow-fed rats (16 weeks), CS (16 weeks), HCHF (8 weeks), and HCHF (16 weeks) rats. Mean values with an unlike superscript letter are significantly different ($P < 0.05$).

pancreas.[40] A suitable animal model is required that mimics all these symptoms of human metabolic syndrome to test potential pharmacological interventions to reverse organ dysfunction. The primary focus of this study was to characterize organ damage with this HCHF diet, especially to the heart and vascular system, liver, kidneys, and pancreas.

CS was used as control diet in this study. CS is a slowly digestible glycemic carbohydrate[41] and served as a control for the HCHF diet in this study where the key carbohydrate is fructose. Unlike fructose, CS does not increase blood glucose concentrations, systolic blood pressure, abdominal fat deposition, plasma insulin, or plasma lipid concentrations.[42,43] CS diet is the appropriate control diet for the HCHF diet because the only differences are the replacement of the carbohydrates (CS replacing fructose and condensed milk) and addition of the high fat as beef tallow in the HCHF diet. Rats fed a standard chow for 16 weeks showed evidence of "metabolic morbidity,"[44] showing that standard chow is not an appropriate control in our model of obesity-induced changes.

In this model, fat and sugars present in the diet provided more energy than required by the animals. This excess energy is stored in the adipocyte and led to hypertrophy and hyperplasia of adipocytes.[45,46] Thus, HCHF diet–fed rats displayed increased body weight, feed efficiency, BMI, abdominal fat deposition, and abdominal circumference. HCHF diet–fed rats also showed increased plasma leptin concentrations as early as 8 weeks. Increases in leptin concentrations have been linked with high-fat feeding, leading to increased body weight gain, energy intake, and increased adiposity.[47] Fructose and fat feeding induced dyslipidemia in laboratory animals,[48] which was also found in our model. Dyslipidemia in these rats was characterized by the increase in plasma concentrations of triglycerides, total cholesterol, and NEFA. Decreased rates of glucose clearance from the blood was also observed with HCHF diet feeding, clearly indicating impaired glucose tolerance.

Oxidative stress is associated with obesity and metabolic syndrome[10,49] and initiates the changes occurring in the metabolic syndrome.[8] One of the hallmarks of metabolic syndrome is oxidative stress–induced primary myocardial insult resulting in cardiovascular remodeling.[50,51] Previous studies have ascertained the direct relationship between increased reactive oxygen species production, diastolic stiffness, and cardiac fibrosis.[36,52] Overproduction of reactive oxygen species seems to be the first and key event in the activation of signaling pathways including extracellular signal-regulated kinase (ERK)1/2, c-Jun amino terminal kinase 1/2, thymoma viral proto-oncogene (AKT; designated as protein kinase B), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and Smad, leading to cardiac hypertrophy, inflammation, and fibrosis.[53–55] Other effects of increased reactive oxygen species production on the cardiovascular system included alteration of ion channel flux and membrane ion pump function in a biologically significant manner in heart muscle[56] and the vascular smooth muscle.[57] Reactive oxygen species targeted L-type calcium channels on the sarcolemma and suppressed the Ca^{2+} current.[58] Reactive oxygen species increased the cytosolic Ca^{2+} concentration through mobilization of intracellular Ca^{2+} stores and/or through the influx of extracellular Ca^{2+}.[57,59,60] Both direct modulation of Ca^{2+} channels by reactive oxygen species and reactive oxygen species–dependent increases in vascular intracellular Ca^{2+} primarily via extracellular Ca^{2+} influx have been conclusively demonstrated.[57,61] The metabolic syndrome diet seems to result in a less negative resting membrane potential in rat papillary muscles relating to changes in Na^{+}/K^{+}ATPase function or reduced cardiac cell repolarization.

Our finding of ventricular dilation, increased left ventricular mass, and increased relative wall thickness in the LV is consistent with subtle eccentric left ventricular hypertrophy[62] induced by HCHF diet feeding. Hypertrophied cardiomyocytes were also observed histologically. The left ventricular hypertrophy may have been produced in response to systemic hypertension caused by HCHF diet feeding.[62] Pressure overload may induce hypertrophy of the left ventricular walls to compensate the stress on the walls.[63,64] Consistent with these observations, HCHF diet feeding increased left ventricular diastolic stiffness and fibrosis and decreased function compared with the CS diet–fed rats. Myocardial fibrosis, the major mechanism in the development of left ventricular dysfunction, results from disproportionate collagen deposition accompanied by reduced degradation of extracellular matrix thus leading to increased tissue stiffness.[65] HCHF diet–fed rats showed increased systolic blood pressure, possibly due to the increased production of reactive oxygen

TABLE 4. Metabolic Parameters, Plasma Oxidative Stress, and Inflammatory Markers in Chow-fed (16 Weeks), CS (16 Weeks), HCHF (8 Weeks), and HCHF (16 Weeks) Groups

Variables	Chow-fed (16 wk)	CS (16 wk)	HCHF (8 wk)	HCHF (16 wk)
Fasting blood glucose, mmol/L	4.1 ± 0.3	3.9 ± 0.2	4.1 ± 0.4	4.7 ± 0.3
Plasma insulin, μg/L	3.04 ± 0.44^{b}	2.28 ± 0.36^{c}	1.49 ± 0.19^{c}	4.15 ± 0.34^{a}
Plasma leptin, μg/L	6.62 ± 0.80^{b}	6.80 ± 0.86^{b}	8.65 ± 0.81^{a}	8.63 ± 1.01^{a}
Plasma corticosterone, μg/L	143 ± 17	133 ± 9	114 ± 13	126 ± 15
Plasma triglyceride, mmol/L	0.54 ± 0.07^{b}	0.42 ± 0.06^{b}	0.49 ± 0.05^{b}	0.79 ± 0.09^{a}
Plasma total cholesterol, mmol/L	1.69 ± 0.09^{b}	1.43 ± 0.11^{b}	1.48 ± 0.05^{b}	1.99 ± 0.07^{a}
Plasma NEFA, mmol/L	1.77 ± 0.26^{b}	1.15 ± 0.14^{b}	1.35 ± 0.12^{b}	3.53 ± 0.21^{a}
Plasma uric acid, μmol/L	48 ± 4.3^{b}	32.1 ± 2.3^{c}	47.6 ± 1.9^{b}	60.2 ± 3.1^{a}
Plasma total bilirubin, μmol/L	1.56 ± 0.16^{b}	2.42 ± 0.09^{a}	2.20 ± 0.08^{a}	2.30 ± 0.06^{a}
Albumin:globulin ratio	0.96 ± 0.01	0.97 ± 0.03	0.99 ± 0.02	0.97 ± 0.02
Plasma malondialdehyde, μmol/L	24.2 ± 1^{d}	26.9 ± 0.7^{c}	29.4 ± 0.5^{b}	32.1 ± 1.0^{a}
Plasma CRP concentration, μmol/L	61.2 ± 6.4^{b}	59.3 ± 7.4^{b}	58.1 ± 7.4^{b}	102.3 ± 4.6^{a}

All data are presented as mean ± SEM and n = 10 for each group. Mean values within a row with unlike superscript letters are significantly different ($P < 0.05$).

species as shown in the erythrocytes thereby reducing the bioavailability of nitric oxide.[66] HCHF diet impaired endothelial function, defined by a decrease in acetylcholine-induced relaxation, which also adds to the increase in blood pressure. In obesity, adipocytes also expressed angiotensinogen, leading to an angiotensin II–induced increase in blood pressure.[50]

HCHF diet–fed rats showed prolonged APD at 50% and 90% of repolarization, which is consistent with the other rat models of cardiovascular disease such as the deoxycorticosterone acetate salt hypertensive rats,[36] spontaneously hypertensive rat,[67] and streptozotocin-induced diabetic rat model.[68] These hypertensive and diabetic rat models induce aggressive changes in LV structure and function with prolonged action potentials and elevated levels of reactive oxygen species.[36,68] The remodeled ventricle shows changes in Ito and Ik channel densities and function that are thought to lengthen the action potential and predispose the ventricle to arrhythmias.[69,70] In rat heart, the Ito current is active throughout the entire action potential repolarization[71] with the Ik inward rectifier current driving the later phase of repolarization.[72] It would seem from these results that the HCHF diet–induced metabolic syndrome causes changes in the later phase of repolarization and hence might alter Ik channel function more significantly than Ito. Although these changes are less marked than those found in streptozotocin diabetic or deoxycorticosterone acetate-salt hypertensive rat hearts, it is significant that the HCHF diet can induce systemic reactive oxygen species production and inflammation that remodels the ventricle and potentiates arrhythmias. These findings have implications for human patients who are obese, showing early changes in electrophysiological remodeling of the heart, which could potentiate later fatal arrhythmias.

HCHF diet feeding increased plasma concentrations of malondialdehyde, the main product of polyunsaturated fatty acid peroxidation,[73] and increased time-dependent production of reactive oxygen species in erythrocytes (Table 1). These markers of oxidative stress were elevated as early as 8 weeks of

TABLE 5. Hepatic and Renal Function in Chow-fed (16 Weeks), CS (16 Weeks), HCHF (8 Weeks), and HCHF (16 Weeks) Groups

Variables	Chow-fed (16 wk)	CS (16 wk)	HCHF (8 wk)	HCHF (16 wk)
Liver wet weight, mg/mm	279 ± 10^{ab}	234 ± 14^{b}	255 ± 17^{ab}	287 ± 12^{a}
Plasma ALT activity, U/L	42.4 ± 0.9^{c}	37.0 ± 0.6^{d}	50.4 ± 0.5^{b}	59.6 ± 0.3^{a}
Plasma AST activity, U/L	106.0 ± 6.9^{a}	73.2 ± 5.6^{b}	88.1 ± 6.2^{ab}	105.3 ± 9.2^{a}
Plasma ALP activity, U/L	180 ± 23^{b}	174 ± 19^{b}	219 ± 16^{ab}	251 ± 21^{a}
Plasma LDH activity, U/L	302 ± 37^{b}	204 ± 23^{c}	282 ± 20^{b}	497 ± 14^{a}
Kidney wet weight, mg/mm	53.6 ± 1.0	49.8 ± 1.4	50.8 ± 2.3	53.9 ± 1.5
Plasma creatinine, μmol/L	41.0 ± 1.6^{b}	46.6 ± 1.3^{ab}	47.6 ± 2.1^{ab}	49.4 ± 2.5^{a}
Plasma urea, mmol/L	6.05 ± 0.21^{ab}	6.45 ± 0.16^{a}	5.80 ± 0.11^{b}	2.69 ± 0.11^{c}
Plasma Na^{+}, mmol/L	142 ± 2	145 ± 1	144 ± 0	144 ± 0
Plasma Cl^{-}, mmol/L	100 ± 2	102 ± 1	101 ± 1	101 ± 1
Plasma K^{+}, mmol/L	3.92 ± 0.17^{bc}	3.67 ± 0.11^{c}	4.29 ± 0.14^{b}	4.89 ± 0.18^{a}
Plasma Ca^{2+}, mmol/L	2.17 ± 0.03^{b}	2.38 ± 0.02^{a}	2.41 ± 0.02^{a}	2.39 ± 0.03^{a}

All data are presented as mean ± SEM and n = 10 for each group. Mean values within a row with unlike superscript letters are significantly different ($P < 0.05$).

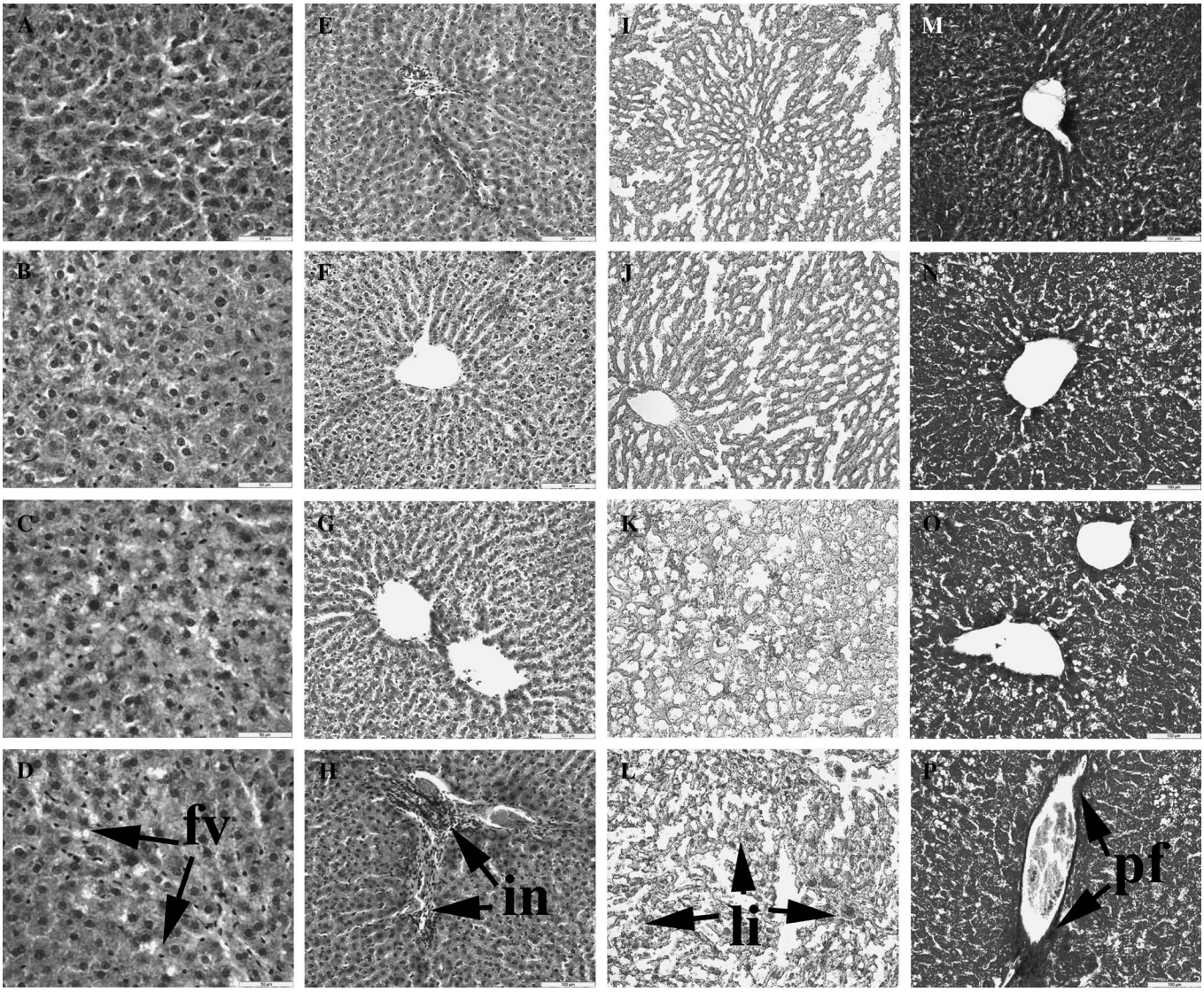

FIGURE 5. Fat deposition, inflammation, and fibrosis in liver showing nonalcoholic steatohepatitis. Hematoxylin and eosin staining of hepatocytes (×40) showing hepatocytes with enlarged fat vacuoles (marked as ''fv'') and inflammatory cells (marked as ''in'') (×20) from chow-fed rats (16 weeks) (A, E), CS (16 weeks) (B, F), HCHF (8 weeks) (C, G), and HCHF (16 weeks) (D, H). Oil red ''O'' staining showing lipid deposition in liver (marked as ''li''); chow-fed rats (16 weeks) (I), CS (16 weeks) (J), HCHF (8 weeks) (K), and HCHF (16 weeks) (L). Milligan's trichrome staining of the hepatic portal regions showing collagen (marked as ''pf'') (×20) in chow-fed rats (16 weeks) (M), CS (16 weeks) (N), HCHF (8 weeks) (O), and HCHF (16 weeks) (P).

HCHF diet feeding. However, minimal symptoms of subclinical inflammation were observed at 8 weeks, although histological analysis revealed increased infiltration by inflammatory cells into cardiac and hepatic tissues and elevated plasma CRP concentrations at 16 weeks. These results suggest that oxidative stress as a result of metabolic disturbances in HCHF diet–fed rats causes cellular insult thereby eliciting a local inflammatory response. Increased adiposity is associated with the activation and migration of inflammatory cells into the adipose tissue.[46] These inflammatory cells then secrete increased amounts of inflammatory cytokines and finally lead to low-grade chronic inflammation.[46] Fructose feeding is associated with the induction of oxidative stress[74] and increased production of proinflammatory factors such as tumor necrosis factor α and c-Jun amino terminal kinase.[75] Apart from this, fructose induced hepatic de novo lipogenesis, leading to hepatic steatosis,[76] which is evident in this study as well.

Nonalcoholic fatty liver disease is considered as the hepatic manifestation of metabolic syndrome.[77] The increased activities of ALT, AST, ALP, and LDH in plasma are indicative of hepatic injury and along with inflammation, steatosis, and fibrosis in the liver, HCHF diet–fed rats clearly show the presence of nonalcoholic steatohepatitis. Metabolic syndrome is a characteristic of chronic kidney disease in humans.[78] The

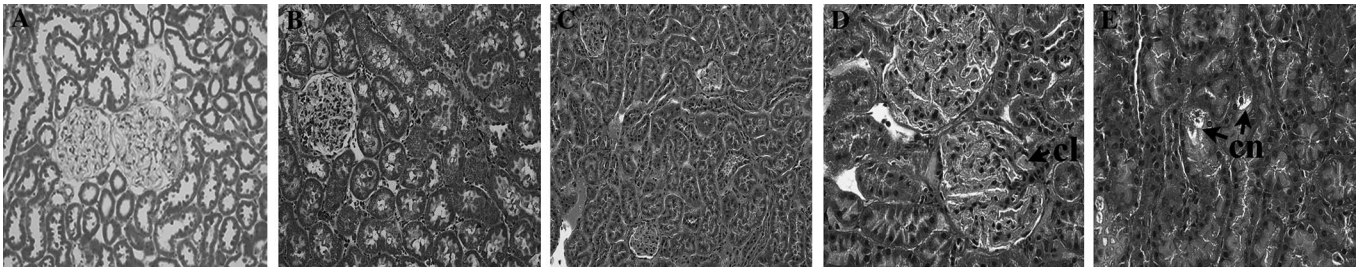

FIGURE 6. Masson's trichrome staining of chow-fed (16 weeks) (A), CS (16 weeks) (B), and HCHF (16 weeks) (C–E) rat kidneys. There were no histological anomalies in kidneys from the chow-fed (A) and CS diet–fed rats (B) and very little Masson trichrome–positive tissue (A, B). In comparison, HCHF rat kidneys (stained with Masson trichrome) showed increased Masson trichrome–positive tissue. The expanded interstitial space contains collagen (stained blue in the histopathology sections) and is evident between the nephron structures (C). Lysis of the glomerular tuft (D, an example of single-cell lysis arrowed and marked as ''cl'') and vacuolar degeneration and single-cell necrosis of tubular epithelium (E, examples arrows and marked ''cn'') demonstrate some subtle but consistent degenerative changes in the HCHF rat kidneys (A-C: magnification, ×200; D, E: magnification, ×400).

glomerular and tubular damage and fibrosis in the kidney of HCHF diet–fed rats shows that kidney damage has been initiated.

Along with the moderate impairment in glucose tolerance, HCHF diet–fed rats showed increased islet mass and increased number of α and β cells within the islets of Langerhans in the pancreas, suggesting hyperplasia of these cell types. These changes were accompanied by increases in plasma insulin concentration. This may suggest that the rats have reduced sensitivity to insulin and to compensate for the reduced sensitivity, islet mass and number of β cells increased to produce and secrete more insulin. Earlier studies have correlated increased islet mass with presence of insulin resistance.[79] Although there is increased plasma insulin concentration with HCHF diet, there is only moderate impairment of glucose tolerance. This indicates that the rats are prediabetic and may progress to hyperglycemia with severe glucose intolerance after further feeding of the HCHF diet.

CONCLUSIONS

HCHF diet feeding in rats induces metabolic syndrome and cardiovascular remodeling, especially hypertension, hypertrophy, ventricular fibrosis, conduction changes, and endothelial dysfunction, along with nonalcoholic steatohepatitis, degeneration of renal structure, and increased mass of islets of pancreas. The presence of all these changes in this model makes it a viable model to test pharmacological and other interventions for the prevention and reversal of metabolic syndrome, cardiovascular disease, and nonalcoholic fatty liver disease.

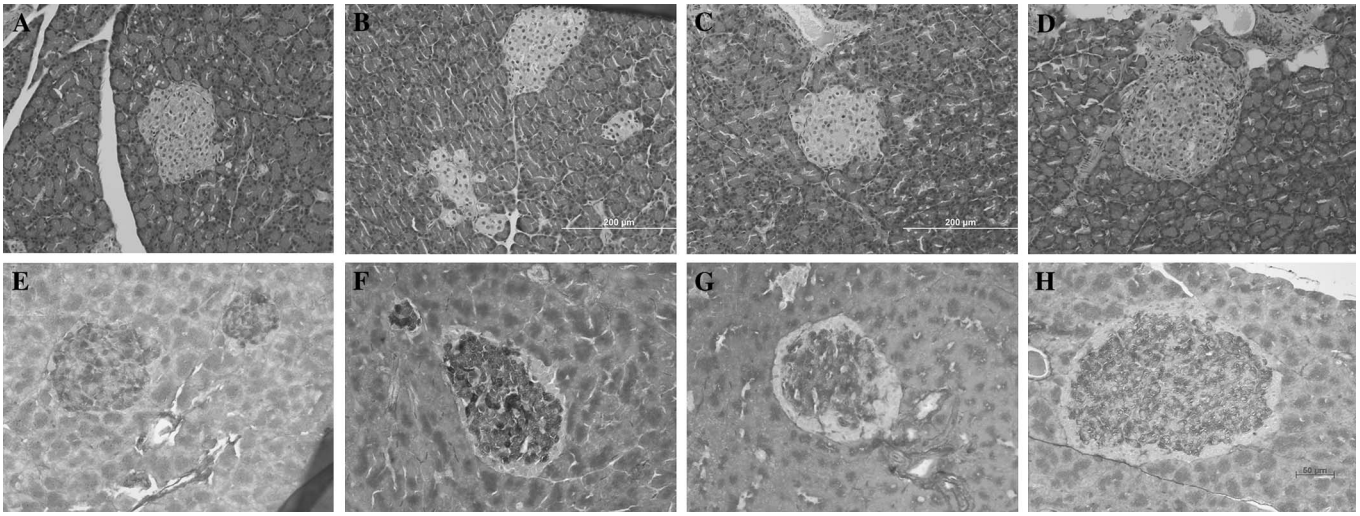

FIGURE 7. Inflammation and increased islet size in pancreas. Hematoxylin and eosin staining (×20) of pancreas showing infiltration of inflammatory cells; chow-fed rats (16 weeks) (A), CS (16 weeks) (B), HCHF (8 weeks) (C), and HCHF (16 weeks) (D). Aldehyde fuchsin staining (×20) of pancreas showing increased size of islet; chow-fed rats (16 weeks) (E), CS (16 weeks) (F), HCHF (8 weeks) (G), and HCHF (16 weeks) (H).

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Chapter 4: Flavonoids in rat model of human metabolic syndrome

Chapter 4.1

Rutin Attenuates Metabolic Changes, Nonalcoholic Steatohepatitis, and Cardiovascular Remodeling in High-Carbohydrate, High-Fat Diet-Fed Rats[1–3]

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Abstract

Metabolic syndrome (obesity, diabetes, and hypertension) increases hepatic and cardiovascular damage. This study investigated preventive or reversal responses to rutin in high-carbohydrate, high-fat diet-fed rats as a model of metabolic syndrome. Rats were divided into 6 groups: 2 groups were fed a corn starch-rich diet for 8 or 16 wk, 2 groups were fed a high-carbohydrate, high-fat diet for 8 or 16 wk, and 2 groups received rutin (1.6 g/kg diet) in either diet for the last 8 wk only of the 16-wk protocol. Metabolic changes and hepatic and cardiovascular structure and function were then evaluated in these rats. The corn starch-rich diet contained 68% carbohydrate (mainly cornstarch) and 0.7% fat, whereas the high-carbohydrate, high-fat diet contained 50% carbohydrate (mainly fructose) and 24% fat (mainly beef tallow) along with 25% fructose in drinking water (total 68% carbohydrate using mean food and water intakes). The high-carbohydrate, high-fat diet produced obesity, dyslipidemia, hypertension, impaired glucose tolerance, hepatic steatosis, infiltration of inflammatory cells in the liver and the heart, higher cardiac stiffness, endothelial dysfunction, and higher plasma markers of oxidative stress with lower expression of markers for oxidative stress and apoptosis in the liver. Rutin reversed or prevented metabolic changes such as abdominal fat pads and glucose tolerance, reversed or prevented changes in hepatic and cardiovascular structure and function, reversed oxidative stress and inflammation in the liver and heart, and normalized expression of liver markers. These results suggest a non-nutritive role for rutin to attenuate chronic changes in metabolic syndrome. J. Nutr. 141: 1062–1069, 2011.

Introduction

Polyphenolic compounds including flavonoids are widely distributed in fruits and vegetables (1,2). Flavonoids such as quercetin and naringenin are effective in the treatment of cancer, hypertension, obesity, and dyslipidemia in both animals and humans (3–5). Rutin (**Supplemental Fig. 1***A*), a glycoside of quercetin (Supplemental Fig. 1*B*), is a non-nutritive component of many foods such as onions, apples, tea, and red wine (6).

Metabolic syndrome is the constellation of central obesity, dyslipidemia, hypertension, impaired glucose tolerance, and insulin resistance (7). Clinical, epidemiological, and animal studies have shown the relationship between metabolic syndrome, especially obesity and dyslipidemia, and the presence of nonalcoholic fatty liver disease (NAFLD)[6] (8–10), with metabolic syndrome increasing the risk of development of NAFLD and vice versa (11,12). Metabolic syndrome and NAFLD are also associated with cardiovascular remodeling, including cardiac hypertrophy, ventricular dysfunction, and endothelial dysfunction (13,14).

NAFLD refers to a wide spectrum of liver damage, ranging from simple steatosis to steatohepatitis and cirrhosis (8,15). Steatosis represents the deposition of fat in hepatocytes, whereas steatohepatitis is the combination of steatosis with hepatic inflammation and fibrosis (16). Development of steatosis involves impaired lipid metabolism in the liver (17). Insulin resistance, hyperinsulinemia, and oxidative stress are important factors in the development of nonalcoholic steatohepatitis (NASH) (18,19).

Rutin has shown health-improving effects in different animal studies. In high-fat diet-fed rats, rutin reduced the gains in body weight, liver weight, and blood cholesterol concentrations without changing blood TG concentrations (20). Rutin suppressed adipocyte differentiation of 3T3-L1 cells (20) and suppressed

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[3] Supplemental Figures 1 and 2 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at jn.nutrition.org.
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hyperglycemia, increased plasma concentrations of insulin, and decreased oxidative stress in streptozotocin-induced diabetic rats (21,22). Rutin was cardioprotective in streptozotocin-induced diabetic rats (23) and isoproterenol-induced myocardial infarction (24) and hepatoprotective in paracetamol- and carbon tetrachloride-induced hepatotoxicity (25).

In this study, we characterized the metabolic, hepatic, and cardiovascular responses to rutin in a rat model of high-carbohydrate, high-fat diet-induced metabolic syndrome with obesity, mild steatohepatitis, and cardiovascular remodeling (10). In addition, Western blotting was used to study changes in the hepatic expression pattern of caspase-3, extracellular signal-regulated kinase (Erk), and heat shock protein (Hsp)70.

Materials and Methods

Rats and diets

All experimental protocols were approved by The University of Queensland Animal Experimentation Ethics Committee under the guidelines of the National Health and Medical Research Council of Australia. Male Wistar rats (8–9 wk old, 332 ± 7 g, n = 72) were obtained from The University of Queensland Biological Resources facility. Rats were individually housed in a temperature-controlled room under 12-h-light/-dark conditions and consumed ad libitum food and water.

Rats were randomly divided into 6 experimental groups (n = 12 each group). Four groups were fed either a corn starch-rich diet (C) or a high-carbohydrate, high-fat diet (H) for 8 wk (C8, H8) or 16 wk (C16, H16); 2 further groups, 1 fed the C diet and the other fed the H diet, were administered rutin (1.6 g/kg food) for the last 8 wk of the 16-wk protocol (CR, HR). The C8 and H8 groups were used to study the pathophysiology before rutin treatment began and to determine whether rutin reversed, prevented, or did not affect the measured variables.

The composition of the C and H diets was described in detail in our previous study (26). The energy densities of C and H were 11.2 and 17.8 kJ/g of food, respectively, with an additional 3.85 MJ/L in the drinking water for the H8, H16, and HR rats (26).

Physiological and metabolic variables

All the rats were monitored daily for body weight, food, and water intakes. Abdominal circumference was measured at the end of the feeding period using a standard measuring tape under light anesthesia with Zoletil (tiletamine 10 mg/kg, zolazepam 10 mg/kg, i.p.; Virbac, Peakhurst). BMI and feed efficiency were calculated as previously described (10).

At the end of feeding period, rats were feed deprived for 12 h and oral glucose tolerance tests were performed as described in our previous studies (10,26,27). During feed deprivation, H rats (H8, H16, and HR) were given drinking water without fructose supplementation. Plasma concentrations of total cholesterol and TG were determined using kits and controls supplied by Olympus using an Olympus analyzer (AU 400) (10,26,27). Nonesterified fatty acids (NEFA) in plasma were determined using a commercial kit (Wako). Plasma insulin concentrations (Laboratory Diagnostics) were measured using commercial kits according to the manufacturer-provided standards and protocols using a Titertek Multiskan MCC/340 spectrophotometer (Flow Laboratories) as in a previous study (10).

Terminal experiments

Rats were killed with Lethabarb (pentobarbitone sodium, 100 mg/kg i.p.; Virbac). After euthanasia, 200 IU heparin (Sigma-Aldrich Australia) was injected through the right femoral vein. The abdomen was then opened and blood (~6 mL) was withdrawn from the abdominal aorta and collected into heparinized tubes. One milliliter of blood was stored at 4°C and used for erythrocyte reactive oxygen species measurements, whereas the remaining blood was centrifuged at 5000 × g for 15 min to obtain plasma, which was then stored at −20°C for biochemical analyses.

Assessment of oxidative stress variables

Erythrocyte reactive oxygen species production. Heparinized blood stored at 4°C was used to measure the production of erythrocyte reactive oxygen species by a fluorometric assay based on the oxidation of the fluorochrome 2′,7′-dichloroflourescein diacetate (Sigma-Aldrich Australia) as described in our previous study (10).

Other plasma markers of oxidative stress. Plasma concentrations of malondialdehyde were determined by HPLC (Shimadzu) as previously described (28). Plasma glutathione peroxidase activity and total antioxidant capacity were measured using an automated spectrophotometer (Cobas Mira) as previously described (29).

Assessment of hepatic structure and function

Histology of liver. Livers (n = 9 from each group) were isolated after euthanasia and weighed. Liver portions were isolated (n = 3) and fixed in 10% neutral buffered formalin for 3 d. These tissue samples were dehydrated and then embedded in paraffin wax. Thin sections (5 μm) of these tissues were cut and stained with hematoxylin and eosin for determination of inflammatory cell infiltration (magnification ×20) and fat vacuoles in liver (magnification ×40). Liver sections were also stained with Milligan's Trichrome stain to determine fibrosis (magnification ×20).

Liver enzymes in plasma. Plasma activity of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and the concentrations of albumin, total bilirubin, urea, and uric acid were determined using kits and controls supplied by Olympus using an Olympus analyzer (AU 400) as previously described (10,26,27).

Western-blot analysis. Liver samples isolated from rats (n = 3 from each group) were stored at −80°C. Liver samples were thawed and sonicated after adding cell lysis buffer. These samples were then ultracentrifuged at 100,000 × g for 30 min at 4°C. Supernatants were used to measure the protein concentration in each sample by the bicinchoninic acid method (Thermo Scientific). The expression of Erk, Hsp70, and caspase-3 was studied in supernatants at equal protein concentrations by Western-blot analysis.

Assessment of cardiovascular structure and function

Systolic blood pressure measurements. The systolic blood pressure of rats was measured at the end of feeding period under light sedation with Zoletil by using an MLT1010 Piezo-Electric Pulse Transducer (ADInstruments) and inflatable tail-cuff connected to a MLT844 Physiological Pressure Transducer (ADInstruments) and PowerLab data acquisition unit (ADInstruments).

Echocardiography. Echocardiographic examination (Phillips iE33, 12-MHz transducer) was performed to assess the cardiovascular structure and function in all the rats. The examination was performed as previously described (10,26,27) after 8 wk for C8 and H8 and after 16 wk for the C16, CR, H16, and HR groups.

Left ventricular function. Isolated Langendorff heart preparations (n = 9) were used to assess left ventricular function of the rats in each group as in previous studies (10,26,27). After performing Langendorff heart perfusion studies, the heart was separated into right ventricle and left ventricle (with septum) for weighing.

Vascular reactivity. Thoracic aortic rings (4 mm in length; n = 9 from each group) were suspended in an organ bath maintained at 35°C and filled with Tyrode physiological salt solution bubbled with 95% O_2-5% CO_2 and allowed to stabilize at a resting tension of 10 mN. Cumulative concentration-response curves (contraction) were obtained for noradrenaline (Sigma-Aldrich Australia) and cumulative concentration-response curves (relaxation) were obtained for acetylcholine (Sigma-Aldrich Australia) and sodium nitroprusside (Sigma-Aldrich Australia) following submaximal (70%) contraction to noradrenaline (10,26,27).

Histology of the heart. Hearts (n = 3 from each group) were fixed in 10% neutral buffered formalin for 3 d. These tissues were used to study collagen deposition and infiltration of inflammatory cells as previously described (26).

Statistical analysis

All data are presented as mean ± SEM. Results were tested for homogenous variance using Bartlett's test and variables that were not normally distributed were transformed (using log 10 function) prior to statistical analyses. C16, CR, H16, and HR groups were tested for effects of diet, treatment, and their interactions by 2-way ANOVA. When the interaction and/or the main effects were significant, means were compared using Newman-Keuls multiple comparison post test. The pairs of 8 wk and treatment groups (C8 and CR; H8 and HR) were compared by using Student's t test to determine whether rutin reverses, prevents, or does not alter the signs of metabolic syndrome. Rutin intakes in CR and HR rats were compared using Student's t test. $P < 0.05$ was considered significant. All statistical analyses were performed using GraphPad Prism version 5 for Windows.

Results

Physiological and metabolic variables. Body weight and energy intakes were significantly higher in H rats than in C rats both after 8 and 16 wk. Body weight was significantly attenuated by rutin in HR rats without affecting energy intake (**Table 1**). H16 rats consumed significantly less food and water than C16 rats. Rutin treatment significantly lowered the intake of water in HR rats. Although there was no difference in feed intake between the H16 and HR groups, it was significantly higher in the HR rats than in the H8 rats. This indicates that rutin treatment significantly increased feed intake in HR rats without changing feed intake in CR rats (Table 1). BMI and feed efficiency were significantly higher in H16 rats and significantly lower in HR rats, but these variables were not different in C and CR rats (Table 1). Abdominal circumference and abdominal fat pads were significantly higher in H16 rats than in the C16 rats. Rutin prevented the increase in abdominal fat pads and reversed the increase in abdominal circumference in HR rats and also prevented the increase in abdominal circumference in CR rats (Table 1).

Increased basal blood glucose concentrations with H feeding were normalized by rutin treatment in HR rats (Table 1). Although the increase in plasma concentrations of insulin was not prevented by rutin, plasma insulin concentrations were significantly lower in HR rats than in H16 rats (Table 1). H16 rats had impaired glucose tolerance and this impairment was reversed by rutin treatment in HR rats (**Fig. 1***A*). C16 and CR rats did not differ in glucose tolerance, basal blood glucose concentrations, or plasma insulin concentrations (Fig. 1*A*; Table 1). Plasma concentrations of TG, total cholesterol, and NEFA were significantly higher in H16 rats compared with C16 rats. Rutin treatment prevented the increase in plasma concentrations of these lipids at 16 wk (Table 1).

Rutin intake was greater in CR (107 ± 5 mg/kg body weight) than in HR (89 ± 6 mg/kg body weight) rats ($P < 0.05$).

Oxidative stress. Erythrocytes from H16 rats produced significantly more superoxide than those from C16 rats (Fig. 1*B*). This production of superoxide by erythrocytes was significantly lower in rutin-supplemented HR rats (Fig. 1*B*). H16 rats had higher plasma concentrations of malondialdehyde and lower plasma activities of glutathione peroxidase than C16 rats. HR rats had lower plasma concentrations of malondialdehyde and higher plasma activities of glutathione peroxidase than H16 rats (Table 1). The

TABLE 1 Effects of rutin on physiological, metabolic, and oxidative stress variables in C8, C16, CR, H8, H16, and HR rats[1]

					P-value				
Variables	C16	CR	H16	HR	Diet	Rutin	Diet × rutin	C8	H8
Physiological variables									
Initial body weight, *g*	338 ± 4	339 ± 7	342 ± 4	334 ± 7	>0.05	>0.05	>0.05	336 ± 6	338 ± 8
Final body weight, *g*	420 ± 9^{c}	433 ± 9^{bc}	525 ± 10^{a}	456 ± 12^{b}	<0.001	<0.001	<0.001	378 ± 8*	421 ± 9$^{\#}$
Water intake, *mL/d*	32.3 ± 1.6^{a}	31.2 ± 1.3^{a}	18.6 ± 0.9^{b}	13.4 ± 0.8^{c}	<0.001	<0.05	>0.05	34.4 ± 1.5	15.3 ± 1.5
Food intake, *g/d*	31.8 ± 0.6^{a}	30.4 ± 0.5^{a}	23.0 ± 0.7^{b}	24.4 ± 0.5^{b}	<0.001	>0.05	<0.05	30.8 ± 0.6	22.4 ± 0.6$^{\#}$
Energy intake, *kJ/d*	357 ± 15^{b}	341 ± 12^{b}	481 ± 14^{a}	487 ± 12^{a}	<0.001	>0.05	>0.05	346 ± 11	458 ± 14
BMI, *g/cm*^{2}	0.67 ± 0.00^{b}	0.69 ± 0.01^{b}	0.78 ± 0.01^{a}	0.69 ± 0.01^{b}	<0.001	<0.001	<0.001	0.67 ± 0.01	0.65 ± 0.01$^{\#}$
Feed efficiency, *g/kJ*	0.23 ± 0.01^{b}	0.28 ± 0.02^{b}	0.39 ± 0.01^{a}	0.28 ± 0.02^{b}	<0.001	>0.05	<0.001	0.12 ± 0.01*	0.18 ± 0.01$^{\#}$
Abdominal circumference, *cm*	21.0 ± 0.2^{b}	19.8 ± 0.2^{c}	24.1 ± 0.3^{a}	20.3 ± 0.2^{c}	<0.001	<0.001	<0.001	19.2 ± 0.2*	21.7 ± 0.3$^{\#}$
Abdominal fat pads, *mg/mm tibia*	401 ± 56^{b}	295 ± 36^{b}	798 ± 56^{a}	466 ± 77^{b}	<0.001	<0.001	>0.05	305 ± 44	514 ± 47
Metabolic variables									
Basal blood glucose, *mmol/L*	4.0 ± 0.2^{b}	3.8 ± 0.2^{b}	5.2 ± 0.3^{a}	3.8 ± 0.2^{b}	<0.05	<0.01	<0.05	3.7 ± 0.3	4.2 ± 0.3
AUC,[2] *mmol/L·min*	684 ± 11^{b}	630 ± 13^{c}	764 ± 10^{a}	626 ± 14^{c}	<0.01	<0.001	<0.01	656 ± 10	724 ± 9$^{\#}$
Plasma insulin, *pmol/L*	0.39 ± 0.06^{b}	0.46 ± 0.06^{b}	0.74 ± 0.06^{a}	0.56 ± 0.05^{b}	<0.001	>0.05	<0.05	0.48 ± 0.05	0.26 ± 0.04$^{\#}$
Plasma total cholesterol, *mmol/L*	1.4 ± 0.1^{b}	1.5 ± 0.1^{b}	2.0 ± 0.1^{a}	1.6 ± 0.1^{b}	<0.01	>0.05	<0.05	1.1 ± 0.1*	1.5 ± 0.1
Plasma triglycerides, *mmol/L*	0.4 ± 0.1^{b}	0.4 ± 0.1^{b}	0.9 ± 0.1^{a}	0.6 ± 0.1^{b}	<0.01	>0.05	>0.05	0.4 ± 0.1	0.5 ± 0.1
Plasma NEFA, *mmol/L*	1.2 ± 0.1^{b}	1.2 ± 0.1^{b}	2.9 ± 0.3^{a}	1.8 ± 0.2^{b}	<0.001	<0.01	<0.01	1.1 ± 0.1	1.4 ± 0.1
Oxidative stress variables									
Plasma malondialdehyde, *μmol/L*	27.4 ± 1.2^{b}	27.0 ± 1.1^{b}	32.2 ± 1.2^{a}	28.0 ± 1.0^{b}	<0.05	>0.05	>0.05	26.8 ± 1.4	28.9 ± 1.2
Plasma glutathione peroxidase activity, *U/L*	1340 ± 60^{a}	1372 ± 43^{a}	847 ± 43^{b}	1253 ± 38^{a}	<0.001	<0.001	<0.001	1361 ± 40	1119 ± 48$^{\#}$
Plasma total antioxidant capacity, *mmol 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid equivalents/L*	1.85 ± 0.02^{a}	1.81 ± 0.02^{ab}	1.78 ± 0.02^{b}	1.87 ± 0.02^{a}	>0.05	>0.05	<0.01	1.87 ± 0.02	1.80 ± 0.02$^{\#}$

[1] Values are mean ± SEM, n = 9–12. Means without a common letter differ, * vs. CR and $^{\#}$ vs. HR, $P < 0.05$.
[2] AUC were calculated using the x-axis as baseline.

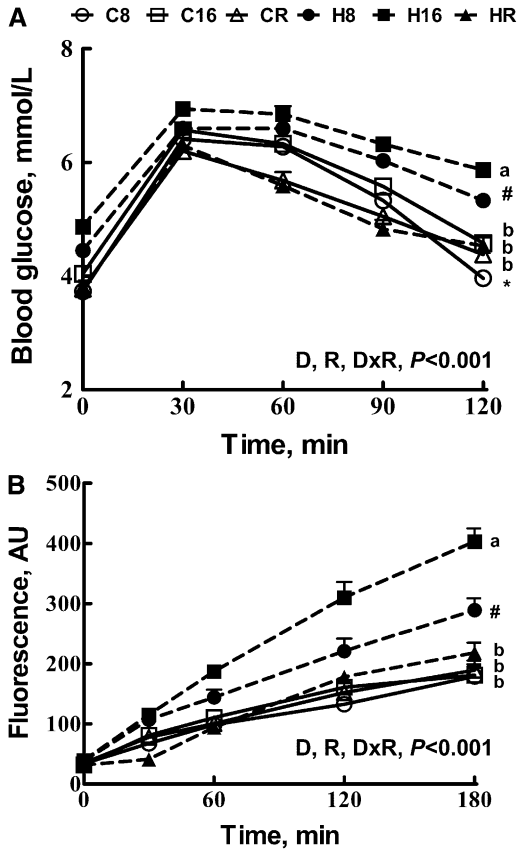

FIGURE 1 Effects of rutin on oral glucose tolerance (*A*) and superoxide production by erythrocytes (*B*) in C8, C16, CR, H8, H16, and HR rats. Values are mean ± SEM, $n = 12$. End-point means without a common letter differ, * vs. CR and # vs. HR, $P < 0.05$. D, R, and D×R represent effects of diet, rutin, and interaction of diet and rutin.

total plasma antioxidant capacity of H16 rats was lower than C16 rats and this was reversed in HR rats (Table 1).

Hepatic structure and function. Compared with C16 rats (**Fig. 2***B*,*H*,*N*), H16 rats had a higher wet weight of liver (**Table 2**) with steatosis (Fig. 2*E*) and higher infiltration of inflammatory cells (Fig. 2*K*) and fibrosis (Fig. 2*Q*) in the hepatic portal region; these changes were attenuated in HR rats (Fig. 2*F*,*L*,*R*; Table 2).

Plasma activities of ALT, AST, ALP, and LDH were significantly higher in H16 rats than in C16 rats (Table 2). Plasma concentrations of urea were significantly lower, whereas the plasma concentrations of total bilirubin and uric acid were significantly higher in H16 rats compared with C16 rats (Table 2). Plasma concentrations of albumin were unchanged between the groups (Table 2). Rutin treatment in HR rats reversed the increase in plasma ALT activity, whereas it prevented the increase in plasma activities of AST, ALP, and LDH (Table 2). Rutin treatment inhibited the increase in plasma concentrations of bilirubin in both CR and HR rats (Table 2) and also inhibited the increase in plasma uric acid concentrations in HR rats (Table 2). The plasma urea concentrations of HR rats were significantly higher than H16 rats but less than C16 rats (Table 2).

H16 rats had lower expression of caspase-3, Hsp70, and Erk1/2 in liver compared with C16 rats; rutin treatment reversed these changes in HR rats (**Supplemental Fig. 2**).

Cardiovascular structure and function. H rats had significantly higher systolic blood pressure as early as 8 wk (**Table 3**). Left ventricular internal diameter during diastole, left ventricular posterior wall thickness, and systolic volume were significantly higher and fractional shortening and ejection fraction were significantly lower in H16 rats compared with C16 rats (Table 3). Rutin treatment prevented increases in blood pressure and left ventricular internal diameter during diastole, whereas it reversed the increase in systolic volume without affecting left ventricular posterior wall thickness during diastole (Table 3). Rutin treatment reversed the decreases in fractional shortening and ejection fraction in HR rats (Table 3).

Greater left ventricular masses (both estimated as well as the actual wet weight) were observed in H16 rats than in C16 rats and these increases were prevented by rutin treatment in HR rats (Table 3). There were no differences in right ventricular wet weights among the groups (Table 3). Compared with C8 and C16 rats (**Fig. 3***G*,*H*), inflammatory cells were rare in the left ventricle of H8 rats (Fig. 3*J*) but more numerous in H16 rats (Fig. 3*K*), whereas they were markedly lower in HR rats (Fig. 3*L*). These changes were accompanied by higher collagen deposition in H16 rats (Fig. 3*E*) and this was reversed by rutin in HR rats (Fig. 3*F*). Left ventricles of H16 rats had significantly greater stiffness than C16 rats; rutin treatment prevented the increase in left ventricular stiffness in HR rats (Table 3). Vascular contraction with noradrenaline (**Fig. 4***A*) and vascular relaxation with acetylcholine and sodium nitroprusside (Fig. 4*B*,*C*) were significantly lower in isolated thoracic aortic rings from H16 rats than from C16 rats. Rutin treatment in HR rats prevented the impairment of vascular contraction and relaxation (Fig. 4*A*–*C*).

Discussion

One of the accepted causes for developing metabolic syndrome is the consumption of a high-carbohydrate, high-fat diet. Metabolic syndrome increases the risk of cardiovascular disease. NAFLD also increases the risk of developing cardiovascular diseases (30), which in turn is linked with the mortality associated with metabolic syndrome and NAFLD (31–33). The initiating factors linking these pathological conditions are oxidative stress and inflammation (34–36). Thus, reducing overall oxidative stress and inflammation can be a treatment strategy for metabolic syndrome, NAFLD, and cardiovascular disease. We have shown that rutin is a possible treatment for diet-induced metabolic, hepatic, and cardiovascular changes in a rat model of diet-induced metabolic syndrome. The dose used in this study would equal ~7 g/d in a 70-kg human based on body weight calculations or 1.1 g/d based on body surface area comparisons (37). The average human dietary intake of rutin is not known, but the intake of polyphenols is probably ~1 g/d, with two-thirds being flavonoids, including rutin (38). Although rutin is not used for therapeutic purposes, the closely related *O*-(β-hydroxyethyl)-rutosides have been used for venous insufficiency and leg edema (39) at doses of 1.5–2 g/d for up to 5 y (40), as an i.v. dosage of 1.5 g twice daily for 3–4 wk for severe leg ischemia (41), and for pharmacokinetic studies using up to 4 g as a single dose (42). In these studies, minimal adverse effects, if any, were reported, including headaches, gut disturbances, rashes, and flushing.

In this study, the H diet was used to induce metabolic syndrome, NAFLD, and cardiovascular remodeling in rats as previously described (10,26,27). Both the C and H diets contained the same amount of carbohydrates, but the carbohydrate components were very different in their actions. The C diet contained cornstarch, which is a slowly digestible starch and

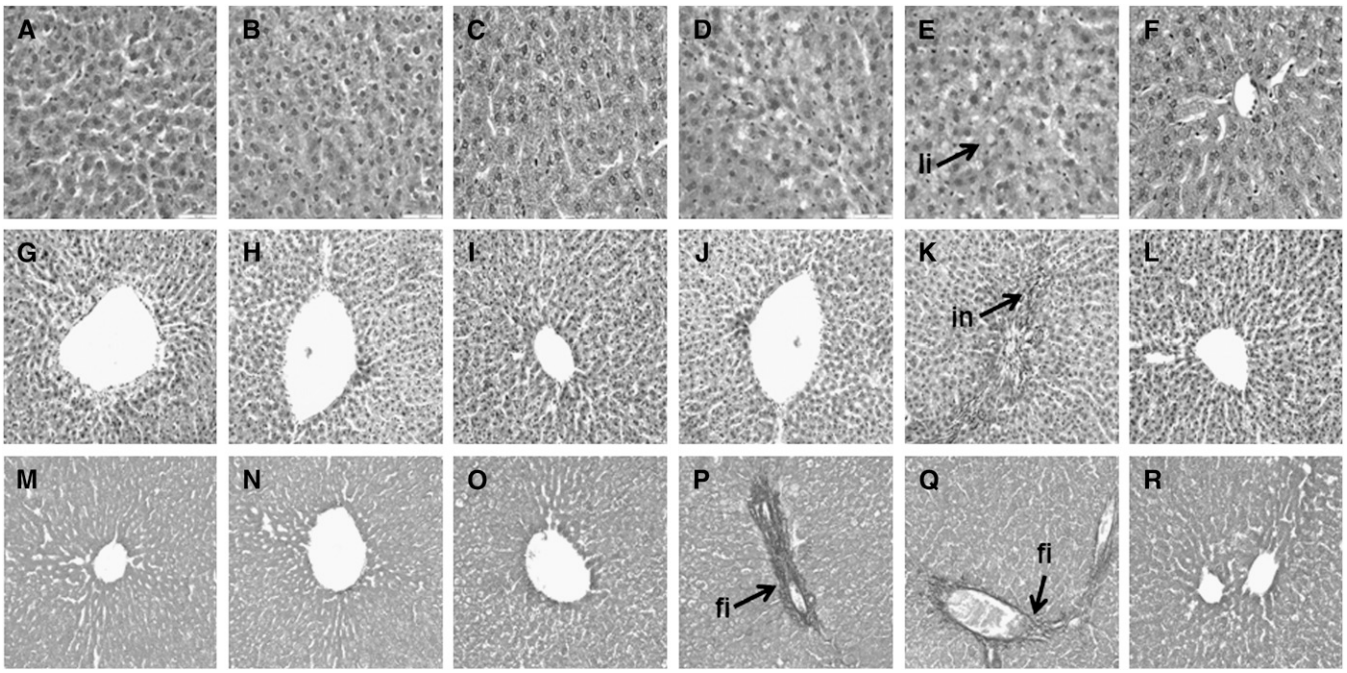

FIGURE 2 Effects of rutin on fat deposition, inflammation, and fibrosis in the liver of C8, C16, CR, H8, H16, and HR rats. Hematoxylin and eosin staining of liver showing enlarged lipid vacuoles (*A–F,* marked as "li") (×40) and inflammatory cells (*G–L*, marked as "in") (×20) from C8 (*A,G*), C16 (*B,H*), CR (*C,I*), H8 (*D,J*), H16 (*E,K*), and HR (*F,L*) rats. Milligan's Trichrome staining of hepatic portal regions showing collagen deposition (*M–R,* marked as "fi") (×20) from C8 (*M*), C16 (*N*), CR (*O*), H8 (*P*), H16 (*Q*), and HR (*R*) rats.

does not produce the signs of metabolic syndrome (10,26, 27,43,44). However, fructose, present in the H diet together with sucrose, produces signs of metabolic syndrome in both humans and rats (10,45–47). Thus, cornstarch in the C diet was replaced with fructose and sucrose in the H diet to provide a similar intake of carbohydrate in both diet groups.

Rats fed the H diet for 16 wk showed signs of oxidative stress, including higher plasma concentrations of malondialdehyde, lower plasma glutathione peroxidase activity, and higher superoxide production by erythrocytes. Along with oxidative stress, H-fed rats had signs of metabolic syndrome, including abdominal obesity, impaired glucose tolerance, higher systolic blood pressure, and dyslipidemia. Livers from these rats had steatosis, inflammation, and fibrosis, indicating the presence of NASH, and the hearts showed inflammation, fibrosis, and higher diastolic stiffness.

High energy-containing diets can cause steatosis or steatohepatitis and cardiovascular remodeling (48,49). Hyperinsulinemia, higher plasma NEFA concentrations, and obesity are additional risk factors for the development of steatohepatitis and cardiovascular remodeling (17,50). Oxidative stress in the liver of H-fed rats was indicated by the lower expression of Hsp70 and impaired apoptotic process was shown by lower expression of caspase-3. This also supports the higher wet weight of liver in H-fed rats. Further liver findings included higher activities of aminotransferases and ALP in plasma, higher plasma concentrations of total bilirubin and uric acid, lower urea concentration, and lower expression of Erk in liver. Cardiovascular remodeling was shown by inflammation, fibrosis, and higher stiffness in the heart of H-fed rats.

Rutin is effective as an antioxidant and reduced oxidative stress in plasma (21) and in the liver, kidney, and brain (6). Rutin produced antiinflammatory effects in adjuvant-induced arthritis in rats by inhibiting the expressions of proinflammatory cytokines (51,52). In this study, rutin administered to H-fed rats attenuated the diet-induced metabolic syndrome, NASH, and cardiovascular abnormalities.

TABLE 2 Effects of rutin treatment on hepatic function in C8, C16, CR, H8, H16, and HR rats[1]

					P-value				
Variables	C16	CR	H16	HR	Diet	Rutin	Diet × Rutin	C8	H8
Liver wet weight, *mg/mm tibia*	257 ± 11^{b}	262 ± 9^{b}	299 ± 8^{a}	266 ± 7^{b}	<0.05	>0.05	<0.05	238 ± 7	276 ± 9
Plasma ALT, *U/L*	35 ± 2^{c}	40 ± 2^{bc}	57 ± 2^{a}	42 ± 2^{b}	<0.001	<0.05	<0.001	31 ± 2*	51 ± 2$^{\#}$
Plasma AST, *U/L*	79 ± 3^{b}	70 ± 2^{c}	105 ± 3^{a}	80 ± 3^{b}	<0.001	<0.001	<0.01	65 ± 2	88 ± 3
Plasma ALP, *U/L*	173 ± 10^{c}	154 ± 8^{c}	251 ± 13^{a}	209 ± 11^{b}	<0.001	<0.05	>0.05	165 ± 11	219 ± 9
Plasma LDH, *U/L*	241 ± 24^{b}	260 ± 22^{b}	497 ± 32^{a}	308 ± 25^{b}	<0.001	<0.01	<0.001	219 ± 22	283 ± 26
Plasma albumin, *g/L*	28.0 ± 0.5	27.9 ± 0.4	28.6 ± 0.3	27.6 ± 0.3	>0.05	>0.05	>0.05	27.9 ± 0.3	27.8 ± 0.3
Plasma total bilirubin, μ*mol/L*	2.2 ± 0.1^{b}	1.8 ± 0.1^{c}	2.5 ± 0.1^{a}	1.9 ± 0.1^{c}	>0.05	<0.001	>0.05	2.0 ± 0.1	2.0 ± 0.1
Plasma urea, *mmol/L*	5.8 ± 0.2^{a}	5.6 ± 0.3^{a}	3.2 ± 0.3^{c}	4.0 ± 0.2^{b}	<0.001	>0.05	>0.05	6.2 ± 0.2	5.7 ± 0.2$^{\#}$
Plasma uric acid, μ*mol/L*	37 ± 2^{b}	34 ± 3^{b}	58 ± 3^{a}	43 ± 3^{b}	<0.001	<0.01	<0.05	32 ± 2	49 ± 3

[1] Values are mean ± SEM, n = 9 for each group. Means without a common letter differ, * vs. CR and $^{\#}$ vs. HR, $P < 0.05$.

TABLE 3 Effects of rutin treatment on cardiovascular structure and function in C8, C16, CR, H8, H16, and HR rats[1]

Variables	C16	CR	H16	HR	*P*-value			C8	H8
					Diet	Rutin	Diet × rutin		
Systolic blood pressure, *mm Hg*	129 ± 4^{b}	127 ± 5^{b}	151 ± 3^{a}	132 ± 4^{b}	<0.01	<0.05	<0.05	123 ± 3	143 ± 4
Left ventricular internal diameter during diastole, *mm*	6.6 ± 0.2^{b}	6.6 ± 0.2^{b}	7.5 ± 0.2^{a}	6.7 ± 0.2^{b}	<0.05	>0.05	>0.05	6.4 ± 0.2	6.8 ± 0.2
Left ventricular posterior wall thickness during diastole, *mm*	1.58 ± 0.03^{c}	1.53 ± 0.05^{c}	1.81 ± 0.05^{a}	1.69 ± 0.04^{b}	<0.001	<0.05	>0.05	1.51 ± 0.03	1.75 ± 0.04
Systolic volume, μL	43 ± 6^{b}	59 ± 6^{b}	122 ± 4^{a}	46 ± 6^{b}	<0.001	<0.001	<0.001	49 ± 4	71 ± 4$^{\#}$
Relative wall thickness	0.48 ± 0.01	0.51 ± 0.02	0.48 ± 0.01	0.49 ± 0.02	>0.05	>0.05	>0.05	0.48 ± 0.01	0.52 ± 0.01
Fractional shortening, *%*	53 ± 1^{a}	49 ± 2^{a}	39 ± 2^{b}	52 ± 2^{a}	<0.01	<0.05	<0.001	54 ± 2	46 ± 2$^{\#}$
Ejection fraction, *%*	87 ± 1^{a}	82 ± 2^{a}	72 ± 1^{b}	87 ± 2^{a}	<0.01	<0.01	<0.001	82 ± 1	78 ± 1$^{\#}$
Time from mitral valve closure to opening, *ms*	114 ± 3	117 ± 4	114 ± 3	116 ± 3	>0.05	>0.05	>0.05	114 ± 4	110 ± 3
Estimated left ventricular mass, *g*	0.68 ± 0.02^{b}	0.71 ± 0.02^{b}	0.91 ± 0.03^{a}	0.73 ± 0.03^{b}	<0.001	<0.01	<0.001	0.63 ± 0.02*	0.79 ± 0.02
Left ventricle + septum wet weight,[2] *mg/mm tibia*	19.5 ± 0.8^{b}	19.4 ± 0.8^{b}	22.8 ± 0.7^{a}	19.8 ± 0.9^{b}	<0.05	>0.05	>0.05	19.1 ± 0.7	20.7 ± 1.0
Right ventricular wet weight,[2] *mg/mm tibia*	4.2 ± 0.3	4.3 ± 0.3	4.6 ± 0.4	4.4 ± 0.3	>0.05	>0.05	>0.05	4.2 ± 0.4	4.5 ± 0.3
Left ventricular diastolic stiffness constant[2] (κ)	18.1 ± 0.9^{c}	19.6 ± 1.0^{c}	28.8 ± 1.2^{a}	24.0 ± 0.9^{b}	<0.001	>0.05	<0.01	18.3 ± 0.8	25.8 ± 0.9

[1] Values are mean ± SEM, n = 12 unless otherwise noted. Means without a common letter differ, * vs. CR and $^{\#}$ vs. HR, $P < 0.05$.
[2] n = 9.

Rutin lowered the lipid components in the serum of hypercholesterolemic rats, probably by reducing the activity of HMG-CoA reductase (53). In our study, rutin-treated rats had lower plasma concentrations of total cholesterol, TG, NEFA, and insulin. Apart from lower lipid concentrations in plasma, rutin-treated HR rats had lower abdominal fat deposition. Because there was no change in the energy intake with rutin treatment, we suggest that the rutin-treated rats had higher energy expenditure, thereby reducing the fat deposition, similar to the effect shown by the aglycone of rutin, quercetin (54). Rutin ameliorated NASH by attenuation of causative factors. Rutin also upregulated the expression of caspase-3 in the liver, suggesting higher apoptosis, probably of fat-containing hepatocytes, as indicated by the absence of fat vacuoles in the liver and lower wet weight of liver. Rutin prevented the infiltration of inflammatory cells in the liver through its antiinflammatory activity, as shown in other studies (55,56). Liver from rutin-treated rats also showed upregulation of Hsp70, indicating lower oxidative stress. Rutin-treated rats probably had lower injury and damage to hepatocytes caused by chronic H feeding, as shown by lower aminotransferase activities in plasma and lower plasma concentrations of total bilirubin and uric acid.

Rutin also improved cardiovascular structure and function in H rats. The eccentric hypertrophy, inflammation, and fibrosis caused by the H diet were prevented by rutin. Rutin-treated rats also had lower systolic blood pressure and improved endothelial function. High blood pressure and endothelial dysfunction have been shown to be correlated with oxidative stress and subsequently reduced NO bioavailability (57–59). Rutin, by scavenging free radicals, should increase the bioavailability of NO and thereby reduce systolic blood pressure and improve endothelial function as shown by our results.

In conclusion, a high-carbohydrate, high-fat diet induced symptoms of metabolic syndrome in rats, including obesity, dyslipidemia, hypertension, and impaired glucose tolerance along with NASH and cardiovascular remodeling. Increased markers of oxidative stress confirmed that oxidative stress plays a role in the development of these pathological conditions. Rutin, a flavonoid from plants, prevented or reversed these changes induced by H feeding in rats. Thus, rutin was effective in attenuating these diet-induced changes, probably by reducing oxidative stress and inflammation in the liver and heart. Thus, rutin should be considered as a treatment strategy for these conditions in humans.

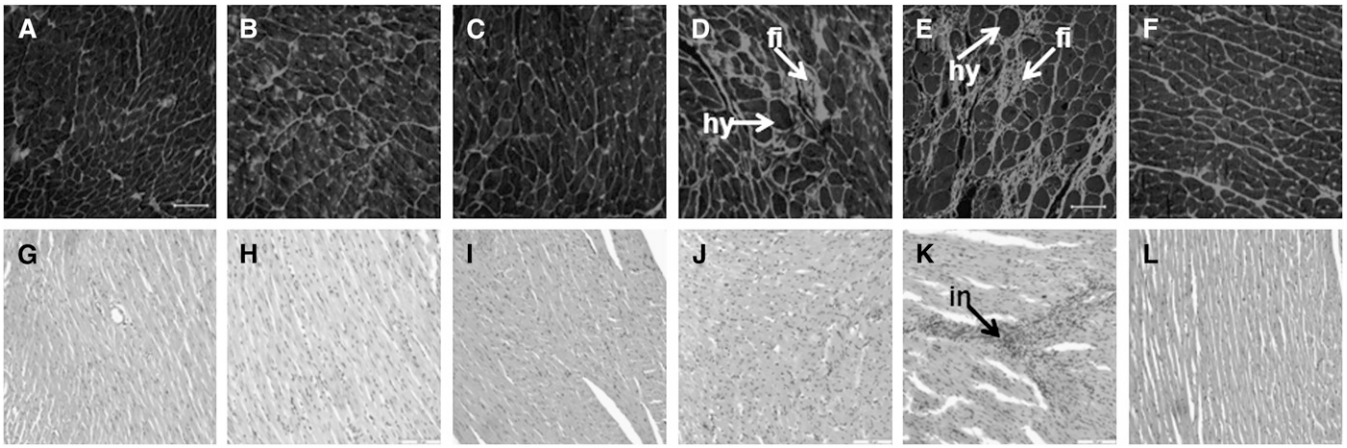

FIGURE 3 Effects of rutin on inflammation and fibrosis in the heart of H-fed rats. Picrosirius red staining of left ventricle showing collagen deposition (*A–F*, fibrosis marked as "fi" and hypertrophied cardiomyocytes as "hy") (×40) from C8 (*A*), C16 (*B*), CR (*C*), H8 (*D*), H16 (*E*), and HR (*F*) rats. Hematoxylin and eosin staining of left ventricle showing infiltration of inflammatory cells (*G–L*, inflammatory cells as dark spots surrounding the myocytes marked as "in") (×40) from C8 (*G*), C16 (*H*), CR (*I*), H8 (*J*), H16 (*K*), and HR (*L*) rats.

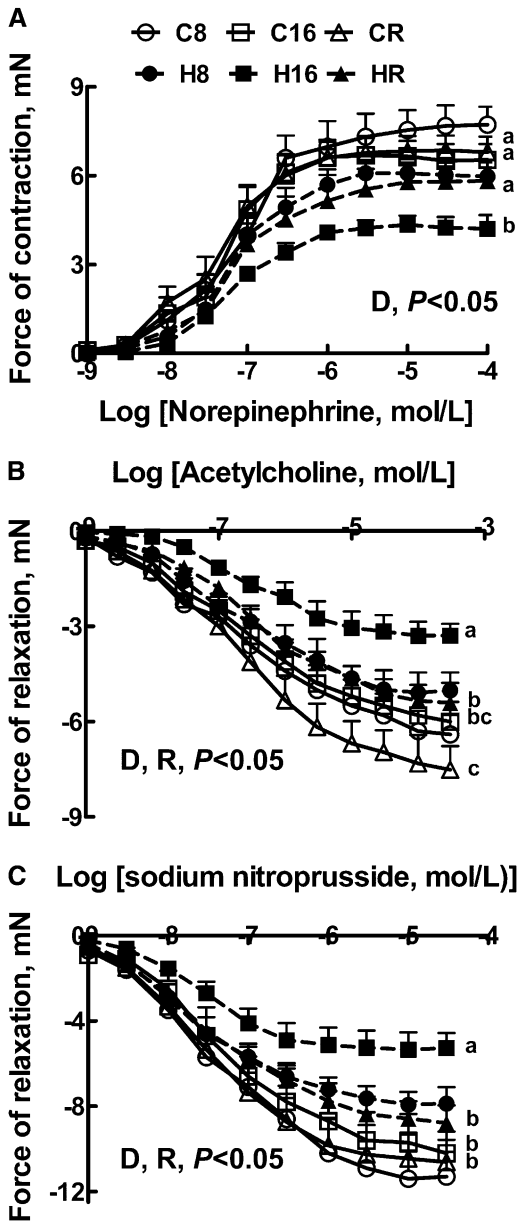

FIGURE 4 Effects of rutin on noradrenaline-induced contraction (*A*), acetylcholine-induced relaxation (*B*), and sodium nitroprusside-induced relaxation (*C*) in thoracic aortic preparations from C8, C16, CR, H8, H16, and HR rats. Values are mean ± SEM, $n = 9$. End-point means without a common letter differ, $P < 0.05$, * vs. CR and # vs. HR, $P < 0.05$. D and R represent effects of diet and rutin.

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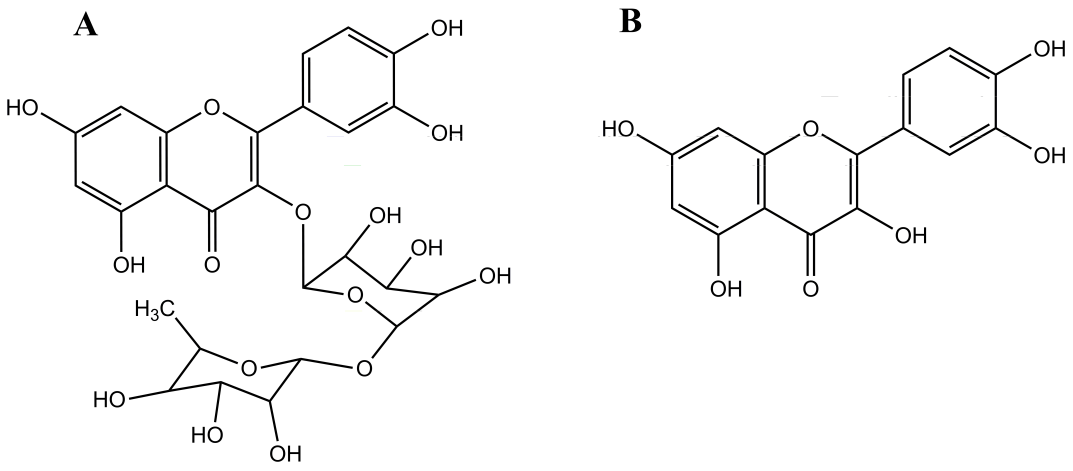
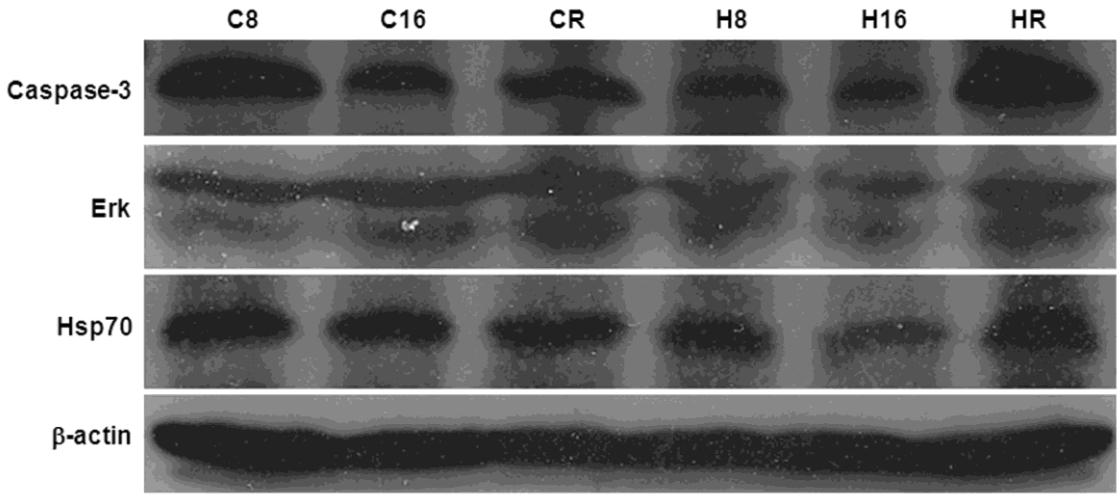
Online Supplemental Material



Supplemental Figure 1. Chemical structures of rutin (A) and quercetin (B).

Online Supplemental Material



Supplemental Figure 2. Effects of rutin on expression of Erk, Hsp70, and, caspase-3 in C8, C16, CR, H8, H16, and HR rat livers.

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Chapter 4.2

Quercetin Ameliorates Cardiovascular, Hepatic, and Metabolic Changes in Diet-Induced Metabolic Syndrome in Rats[1–3]

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Abstract

Metabolic syndrome is a risk factor for cardiovascular disease and nonalcoholic fatty liver disease (NAFLD). We investigated the responses to the flavonol, quercetin, in male Wistar rats (8–9 wk old) divided into 4 groups. Two groups were given either a corn starch–rich (C) or high-carbohydrate, high-fat (H) diet for 16 wk; the remaining 2 groups were given either a C or H diet for 8 wk followed by supplementation with 0.8 g/kg quercetin in the food for the following 8 wk (CQ and HQ, respectively). The H diet contained ~68% carbohydrates, mainly as fructose and sucrose, and ~24% fat from beef tallow; the C diet contained ~68% carbohydrates as polysaccharides and ~0.7% fat. Compared with the C rats, the H rats had greater body weight and abdominal obesity, dyslipidemia, higher systolic blood pressure, impaired glucose tolerance, cardiovascular remodeling, and NAFLD. The H rats had lower protein expressions of nuclear factor (erythroid-derived 2)-related factor-2 (Nrf2), heme oxygenase-1 (HO-1), and carnitine palmitoyltransferase 1 (CPT1) with greater expression of NF-κB in both the heart and the liver and less expression of caspase-3 in the liver than in C rats. HQ rats had higher expression of Nrf2, HO-1, and CPT1 and lower expression of NF-κB than H rats in both the heart and the liver. HQ rats had less abdominal fat and lower systolic blood pressure along with attenuation of changes in structure and function of the heart and the liver compared with H rats, although body weight and dyslipidemia did not differ between the H and HQ rats. Thus, quercetin treatment attenuated most of the symptoms of metabolic syndrome, including abdominal obesity, cardiovascular remodeling, and NAFLD, with the most likely mechanisms being decreases in oxidative stress and inflammation. J. Nutr. 142: 1026–1032, 2012.

Introduction

Metabolic syndrome refers to the clustering of insulin resistance, hypertension, central obesity, impaired glucose tolerance, and dyslipidemia (1). Metabolic syndrome increases the risk of cardiovascular disease, nonalcoholic fatty liver disease (NAFLD)[6], and diabetes (2–4). This increased prevalence of cardiovascular disease and NAFLD associated with metabolic syndrome necessitates the discovery of appropriate interventions for these complications. One of the major causes of obesity and NAFLD in Western society is a diet rich in both carbohydrates such as fructose or sucrose and saturated fats from animal sources (5,6). Excess consumption of fat and fructose in the diet leads to disturbances in fatty acid and carbohydrate metabolism (7,8). Excess fructose consumption also leads to increased lipid biosynthesis, because fructose is a lipogenic carbohydrate (9). This is accompanied by reduced fatty acid oxidation and increased storage of fat in the visceral area. Impairment of fatty acid metabolism in the liver leads to hepatic steatosis followed by NAFLD (10).

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is an important dietary flavonoid found in red onions, apples, berries, citrus fruits, tea, and red wine (11). Quercetin reduced systolic blood pressure in hypertensive human participants and in animal models of hypertension (12–14), reduced serum TG and cholesterol concentrations in high-fat diet-fed rabbits after 12 wk of treatment (15), and reduced body weight in obese Zucker rats without changing the mean daily food intake, also reducing plasma concentrations of TG, nonesterified fatty acids (NEFA) total

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[3] Supplemental Figures 1 and 2 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
[6] Abbreviations used: ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; C, corn starch–rich diet-fed rats; CPT1, carnitine palmitoyltransferase 1; CQ, corn starch–rich diet-fed rats treated with quercetin; H, high-carbohydrate, high-fat diet-fed rats; HO-1, heme oxygenase-1; HQ, high-carbohydrate, high-fat diet-fed rats treated with quercetin; LDH, lactate dehydrogenase; LV, left ventricle; NAFLD, nonalcoholic fatty liver disease; NEFA, nonesterified fatty acids; Nrf2, nuclear factor (erythroid-derived 2)-related factor-2.
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cholesterol, and insulin (16). High-fat, high-cholesterol, and high-sucrose diet-fed mice treated with quercetin had lower body weight, visceral fat, blood glucose, plasma insulin, plasma total cholesterol, plasma TG, plasma NEFA, and plasma TNFα concentrations with higher plasma adiponectin concentrations. These mice also had suppressed liver lipid accumulation (17). In high-fat diet-fed mice, quercetin increased energy expenditure and reduced plasma concentrations of inflammatory markers without any changes in food consumption, physical activity, body weight, or body composition (18). Recent studies have also shown the protective effects of quercetin in thioacetamide- and acrylonitrile-induced hepatotoxicity (19,20).

Thus, we characterized the effects of quercetin as a dietary intervention in a diet-induced rat model of NAFLD and cardiovascular remodeling as part of metabolic syndrome induced in rats by feeding a high-carbohydrate, high-fat diet for 16 wk (21). After treatment with quercetin, the structure and function of the cardiovascular system were characterized with echocardiography, isolated Langendorff heart preparation, vascular reactivity studies, and histopathological analysis. The structure and function of the liver were characterized with histopathological analysis and measurement of biochemical variables. Variables for obesity, dyslipidemia, and glucose tolerance were also measured. The possible mechanisms involved in the action of quercetin were characterized by the expression of proteins involved in cellular metabolism and stress regulation.

Methods

Rats, diets, and treatment with quercetin

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 wk old, 333 ± 2 g, n = 40) were obtained from The University of Queensland Biological Resources facility. Rats were randomly divided into 4 groups: corn starch–rich diet-fed rats (C; n = 10), corn starch–rich diet-fed rats treated with quercetin (CQ; 0.8 g/kg food; n = 10; MP Biomedicals), high-carbohydrate, high-fat diet-fed rats (H; n = 10), and high-carbohydrate, high-fat diet-fed rats treated with quercetin (HQ; 0.8 g/kg food; n = 10). The compositions of the diets were previously described in detail (21–23). C and H rats were fed with corn starch–rich and high-carbohydrate, high-fat diets, respectively, for 16 wk. CQ and HQ rats were fed with corn starch–rich and high-carbohydrate, high-fat diets, respectively, for the first 8 wk and the respective diets were supplemented with quercetin (0.8 g/kg food) for a further 8 wk. All the rats were individually housed under temperature-controlled, 12-h-light/-dark conditions and consumed food and water ad libitum.

Physiological and metabolic variables

All rats were monitored daily for body weight and food and water intakes. Abdominal circumference and body length were measured every 4 wk using a standard measuring tape under light anesthesia with Zoletil (10 mg/kg tiletamine, 10 mg/kg zolazepam, i.p.; Virbac) (21). BMI and energy efficiency were calculated as in a previous study (21).

Oral glucose tolerance tests were performed on rats following a 12-h food deprivation as described in a previous study (21). AUC was calculated as in a previous study (24) and plasma concentrations of total cholesterol, TG, and NEFA were also measured as in a previous study (21). At the end of the protocol, abdominal fat pads (including retroperitoneal, epididymal, and omental) were separately removed, weighed, and expressed as mg/mm of tibial length.

Assessment of cardiovascular structure and function

Systolic blood pressure measurements. Systolic blood pressure was measured every 4 wk under light sedation with Zoletil (10 mg/kg tiletamine, 10 mg/kg zolazepam, i.p.; Virbac) as previously described (21).

Echocardiography. Echocardiographic examinations (Phillips iE33, 12MHz transducer) were performed to assess cardiovascular structure and function in all groups. The examination was performed at the end of the protocol as previously described (21,22).

Isolated Langendorff heart preparation. Following terminal anesthesia and heparin injection, plasma was collected for biochemical analyses and isolated rat hearts were perfused for measurement of left ventricular diastolic stiffness as previously described (21).

Vascular reactivity. Thoracic aortic rings (~4 mm in length; 3–4 rings from 10 rats/group) were used to obtain cumulative concentration-response curves for noradrenaline (contraction), sodium nitroprusside (relaxation), and acetylcholine (relaxation) as in a previous study (21).

Histology of the heart. Two rats from each group were exclusively used for histology. Hearts were fixed, cut, and stained as previously reported (21).

Assessment of hepatic structure and function

Histology of liver. Livers (n = 8/group) were isolated and weighed. Two rats from each group were exclusively used for histology. Liver portions were isolated from these rats and fixed, cut, and stained as in a previous study (21).

Liver enzymes in plasma. Plasma activities of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) and the plasma concentrations of albumin, total bilirubin, urea, and uric acid were determined as previously described (21).

Western-blot analysis

After perfusion experiments, the heart samples were weighed and immediately stored at −80°C for protein extraction (n = 4/group). Similarly, the liver samples were immediately isolated after weighing the liver (n = 4/group) and were stored at −80°C for protein extraction. These samples were homogenized and sonicated after adding cell lysis buffer, followed by centrifugation at 15,000 × g for 30 min at 4°C. Supernatants were used to measure the protein concentration in each sample by the bicinconinic acid method (Thermo Scientific). Supernatants in equal concentrations from each group were used in Western-blot analyses to study the expression of carnitine palmitoyltransferase 1 (CPT1), nuclear factor (erythroid-derived 2)-related factor-2 (Nrf2), heme oxygenase-1 (HO-1) (antibodies from Santa Cruz Biotechnology), NF-κB, caspase-3 (antibodies from Cell Signaling Technology), and β-actin (antibody from Sigma-Aldrich) in the liver and heart. For quantitative analysis, the expression of proteins was normalized to the expression of β-actin.

Statistical analysis

Values 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 function) prior to statistical analyses. All the groups were tested for effects of diet, treatment, and their interaction by 2-way ANOVA. When the interaction and/or the main effects were significant, means were compared using the Newman-Keuls multiple comparison post test. Mean daily quercetin intakes in CQ and HQ groups were compared with Student's t test. $P < 0.05$ was considered significant. All statistical analyses were performed using GraphPad Prism version 5.00 for Windows.

Results

Physiological variables. Body weight was higher in the H rats than in the C rats at 16 wk. Although body weight was higher in CQ than in C rats at 16 wk, it did not differ between the H and HQ rats at 16 wk (**Table 1**). H rats consumed less food and

TABLE 1 Physiological and metabolic variables in rats fed C or H diets for 8 wk and those diets or CQ or HQ diets for an additional 8 wk[1]

Variables	C	CQ	H	HQ	*P* value D	Q	D×Q
Physiological variables							
Initial body weight, *g*	334 ± 1	332 ± 1	334 ± 1	331 ± 2	0.71	0.07	0.71
Final body weight, *g*	406 ± 5^{c}	441 ± 12^{b}	499 ± 9^{a}	498 ± 13^{a}	<0.0001	0.11	0.09
Water intake, *mL/d*	31.4 ± 1.1^{a}	24.8 ± 1.0^{b}	19.6 ± 0.8^{c}	23.4 ± 0.7^{b}	<0.0001	0.13	<0.0001
Food intake, *g/d*	30.4 ± 0.7^{b}	34.0 ± 0.4^{a}	22.1 ± 0.5^{d}	28.2 ± 0.5^{c}	<0.0001	<0.0001	0.026
Energy intake, *kJ/d*	349 ± 10^{d}	382 ± 6^{c}	462 ± 9^{b}	592 ± 11^{a}	<0.0001	<0.0001	<0.0001
Energy efficiency, *kJ/g*	0.19 ± 0.01^{c}	0.28 ± 0.03^{b}	0.36 ± 0.02^{a}	0.28 ± 0.02^{b}	0.0003	0.82	0.0003
BMI, *g/cm*^{2}	0.65 ± 0.01^{b}	0.66 ± 0.01^{b}	0.74 ± 0.01^{a}	0.75 ± 0.01^{a}	<0.0001	0.32	1.00
Abdominal circumference, *cm*	19.6 ± 0.4^{c}	19.0 ± 0.4^{c}	23.3 ± 0.4^{a}	20.9 ± 0.2^{b}	<0.0001	0.0002	0.017
Metabolic variables							
Basal blood glucose, *mmol/L*	4.0 ± 0.1^{b}	3.9 ± 0.1^{b}	5.0 ± 0.1^{a}	4.2 ± 0.2^{b}	<0.0001	0.002	0.012
Blood glucose AUC, *mmol/L · min*	680 ± 13^{b}	656 ± 8^{b}	771 ± 10^{a}	641 ± 24^{b}	0.016	<0.0001	0.001
Plasma total cholesterol, *mmol/L*	1.4 ± 0.1^{b}	1.4 ± 0.1^{b}	2.0 ± 0.1^{a}	1.8 ± 0.1^{a}	<0.0001	0.32	0.32
Plasma TG, *mmol/L*	0.4 ± 0.1^{c}	1.0 ± 0.1^{ab}	0.8 ± 0.1^{b}	1.3 ± 0.2^{a}	0.012	0.0002	0.71
Plasma NEFA, *mmol/L*	1.2 ± 0.3^{b}	2.9 ± 0.5^{a}	3.6 ± 0.7^{a}	3.4 ± 0.7^{a}	0.016	0.20	0.11
Retroperitoneal fat, *mg/mm tibial length*	213 ± 9^{b}	135 ± 7^{c}	357 ± 21^{a}	220 ± 11^{b}	<0.0001	<0.0001	0.031
Epididymal fat, *mg/mm tibial length*	129 ± 11^{b}	87 ± 3^{c}	225 ± 14^{a}	141 ± 4^{b}	<0.0001	<0.0001	0.029
Omental fat, *mg/mm tibial length*	93 ± 6^{b}	68 ± 4^{c}	194 ± 12^{a}	103 ± 4^{b}	<0.0001	<0.0001	<0.0001
Total abdominal fat, *mg/mm tibial length*	435 ± 24^{b}	290 ± 9^{c}	775 ± 46^{a}	465 ± 14^{b}	<0.0001	<0.0001	0.005

[1] Values are mean ± SEM, $n = 10$. Means in a row with superscripts without a common letter differ, $P < 0.05$. C, corn starch–rich diet-fed rats; CQ, corn starch–rich diet-fed rats treated with quercetin; D, effects of diet; D×Q, interaction between the effects of diet and quercetin; H, high-carbohydrate, high-fat diet-fed rats; HQ, high-carbohydrate, high-fat diet-fed rats treated with quercetin; NEFA, nonesterified fatty acids; Q, effects of quercetin.

water compared with C rats. However, total energy intake was higher in the H rats than in the C rats. HQ rats consumed more food and water than H rats, whereas CQ rats consumed less water and more food compared with C rats. CQ and HQ rats had higher energy intakes compared with C and H rats, respectively (Table 1). Energy efficiency and BMI were higher in the H rats than in the C rats. CQ rats had higher whereas HQ rats had lower energy efficiency compared with C and H rats, respectively. BMI in CQ and HQ rats did not differ from C and H rats, respectively (Table 1). Abdominal circumference was higher in H rats than in C rats. Abdominal circumference was lower in HQ rats compared with H rats, whereas it did not differ between the C and CQ rats (Table 1). Relative to body weight, the daily intake of quercetin was greater in CQ rats (64.3 ± 1.6 mg/kg body weight) compared with HQ rats (48.5 ± 1.1 mg/kg body weight) due to greater food intake ($P < 0.0001$).

Metabolic variables. Higher basal blood glucose concentrations and AUC in the H rats compared with C rats were normalized in HQ rats and there was no effect of quercetin in CQ rats on basal blood glucose concentrations and AUC (Table 1). Plasma concentrations of total cholesterol, TG, and NEFA were higher in the H rats than in C rats. CQ rats did not differ in plasma total cholesterol concentrations from C rats, whereas plasma concentrations of TG and NEFA were higher in the CQ rats than in the C rats (Table 1). H and HQ rats did not differ in plasma concentrations of total cholesterol and NEFA, whereas plasma TG concentrations were higher in HQ rats than in H rats (Table 1). Abdominal fat pad weights (retroperitoneal, epididymal, and omental) were higher in H rats compared with C rats and were normalized in HQ rats, but lower in the CQ rats than in the C rats (Table 1).

Cardiovascular structure and function. The LV (left ventricle) of the heart from H rats had more infiltration of inflammatory cells (**Supplemental Fig. 1***C*) along with hypertrophy and more collagen deposition (Supplemental Fig. 1*G*) than C rats (Supplemental Fig. 1*A*,*E*). These changes were attenuated in the LV of the HQ rats (Supplemental Fig. 1*D*,*H*). Systolic blood pressure was higher in H rats compared with C rats at 16 wk. It was normalized in HQ rats, whereas it was lower in CQ rats than in C rats (**Table 2**). The left ventricular internal diameter during systole and diastole and the systolic volume were higher in H rats than in C rats, whereas these variables were normalized in HQ rats and did not differ between the C and CQ rats (Table 2). Left ventricular posterior wall thickness during diastole was higher in H rats compared with C rats and it did not differ in the CQ and HQ rats compared with the C and H rats, respectively. Relative wall thickness did not differ between the C and H rats, whereas it was higher in both CQ and HQ rats compared with C and H rats, respectively (Table 2). Indicators of ventricular function (fractional shortening, ejection fraction, and the ratio of early mitral inflow velocity to late mitral inflow velocity) were lower in H rats than in C rats, indicating impaired ventricular function (Table 2). These indicators of ventricular function were normalized with quercetin supplementation in HQ rats (Table 2). The estimated LV mass was higher in H rats compared with C rats and did not differ between H and HQ rats, whereas it was higher in CQ rats than in C rats. The actual LV wet weight (with septum) did not differ between the groups. The right ventricular wet weight did not differ between the C and H rats, was higher in the HQ rats than in the H rats, but did not differ between the C and CQ rats (Table 2). The left ventricular diastolic stiffness constant was higher in H rats compared with C rats and it was lower in HQ rats than in H rats, whereas it did not differ between the C and CQ rats (Table 2). Vascular responses, including noradrenaline-induced contraction and sodium nitroprusside- and acetylcholine-induced relaxation, were impaired in H rats compared with C rats (**Fig. 1***A–C*). Noradrenaline-

TABLE 2 Cardiovascular structure and function in rats fed C or H diets for 8 wk and those diets or CQ or HQ diets for an additional 8 wk[1]

Variables	C	CQ	H	HQ	*P* value D	Q	D×Q
Systolic blood pressure, *mm Hg*	129 ± 1[b]	123 ± 1[c]	145 ± 1[a]	132 ± 2[b]	<0.0001	0.0001	0.012
LVIDs, *mm*	3.38 ± 0.15[b]	3.62 ± 0.25[b]	4.88 ± 0.14[a]	3.37 ± 0.22[b]	0.003	0.003	<0.0001
LVIDd, *mm*	6.64 ± 0.10[b]	6.60 ± 0.10[b]	7.47 ± 0.09[a]	6.70 ± 0.16[b]	0.0003	0.001	0.003
LVPWd, *mm*	1.58 ± 0.02[b]	1.76 ± 0.05[ab]	1.81 ± 0.09[a]	1.85 ± 0.08[a]	0.020	0.10	0.30
Systolic volume, *μL*	43 ± 6[b]	55 ± 9[b]	122 ± 11[a]	44 ± 9[b]	0.0005	0.0007	<0.0001
Relative wall thickness	0.48 ± 0.01[b]	0.55 ± 0.02[a]	0.48 ± 0.01[b]	0.56 ± 0.03[a]	0.88	0.0006	0.88
Fractional shortening, *%*	53 ± 1[a]	58 ± 3[a]	39 ± 1[b]	57 ± 2[a]	0.0004	<0.0001	0.002
Ejection fraction, *%*	86 ± 1[a]	82 ± 3[a]	72 ± 1[b]	86 ± 3[a]	0.032	0.032	0.0003
E:A ratio	1.88 ± 0.06[a]	1.75 ± 0.05[ab]	1.57 ± 0.09[b]	1.83 ± 0.07[a]	0.11	0.35	0.008
Estimated LV mass, *g*	0.68 ± 0.02[c]	0.79 ± 0.02[b]	0.91 ± 0.03[a]	0.85 ± 0.05[ab]	<0.0001	0.45	0.013
LV + septum wet weight, *mg/mm tibial length*	20.3 ± 0.7	21.1 ± 0.7	21.8 ± 1.1	21.7 ± 0.7	0.21	0.67	0.59
Right ventricular wet weight, *mg/mm tibial length*	4.8 ± 0.2[b]	4.4 ± 0.4[b]	4.5 ± 0.3[b]	6.1 ± 0.4[a]	0.044	0.08	0.005
LV diastolic stiffness constant (*κ*)	19.8 ± 0.8[c]	20.1 ± 1.0[c]	27.5 ± 1.1[a]	23.4 ± 1.1[b]	<0.0001	0.07	0.038

[1] Values are mean ± SEM, $n = 8$–10. Means in a row with superscripts without a common letter differ, $P < 0.05$. C, corn starch–rich diet-fed rats; CQ, corn starch–rich diet-fed rats treated with quercetin; D, effects of diet; D×Q, interaction between the effects of diet and quercetin; E:A, ratio of early mitral inflow velocity to late mitral inflow velocity; H, high-carbohydrate, high-fat diet-fed rats; HQ, high-carbohydrate, high-fat diet-fed rats treated with quercetin; LV, left ventricle; LVIDs, left ventricular internal diameter during systole; LVIDd, left ventricular internal diameter during diastole; LVPWd, left ventricular posterior wall thickness during diastole; Q, effects of quercetin.

induced contraction and acetylcholine-induced relaxation were normalized in HQ rats, whereas these responses did not differ between the C and CQ rats (Fig. 1*A*,*C*). The HQ rats had a greater sodium nitroprusside-induced relaxation response compared with H rats, whereas CQ rats had a lower response to sodium nitroprusside compared with C rats (Fig. 1*B*).

Hepatic structure and function. Liver from H rats had more infiltration of inflammatory cells (**Supplemental Fig. 2***C*) than C rats (Supplemental Fig. 2*A*) along with presence of fat vacuoles (Supplemental Fig. 2*G*) and portal fibrosis (Supplemental Fig. 2*K*), which were absent in the liver from C rats (Supplemental Fig. 2*E*,*I*). The wet weight of the liver was higher in H rats than in C rats and normalized in HQ rats, whereas it was lower in CQ rats than in C rats (**Table 3**). Plasma activities of ALT, AST, ALP, and LDH were higher in H rats compared with C rats. Plasma activities of ALT and ALP were normalized in HQ rats, whereas they were lower in CQ rats than in C rats. Plasma AST and LDH activities did not differ between C and CQ rats. Plasma AST activities did not differ between the H and HQ rats, whereas plasma LDH activity was normalized in HQ rats (Table 3). Although plasma total bilirubin concentrations did not differ between the C and H rats, they were lower in HQ rats compared with H rats. Plasma urea and plasma uric acid concentrations were lower and higher, respectively, in H rats than in C rats. The HQ rats had higher plasma urea concentrations compared with H rats, although not normalized, whereas plasma uric acid concentrations were normalized in HQ rats (Table 3).

Expression of regulatory proteins in the liver and the heart. In the liver from H rats, the protein expression of Nrf2, HO-1, CPT1, and caspase-3 was lower whereas NF-*κ*B expression was higher compared with C rats. In HQ rats, the expression of Nrf2, CPT1, and caspase-3 in the liver was normalized, whereas the expression of HO-1 was higher compared with H rats. NF-*κ*B expression in the liver was lower in both CQ and HQ rats compared with C and H rats, respectively. Hepatic expression of Nrf2 and CPT1 did not differ between C and CQ rats, whereas hepatic expression of HO-1 and caspase-3 were higher in CQ rats than in C rats. Hepatic expression of NF-*κ*B was lower in the CQ rats than in C rats (**Fig. 2***A*,*C*). In the heart from H rats, expression of Nrf2, HO-1, and CPT1 was lower, whereas expression of NF-*κ*B was higher compared with C rats. HQ rats had higher expression of Nrf2, HO-1, and CPT1, whereas the expression of NF-*κ*B was lower in the heart compared with H rats. Cardiac expression of Nrf2, HO-1, and CPT1 did not differ between the C and CQ rats, whereas cardiac expression of NF-*κ*B was lower in CQ rats than in C rats. Caspase-3 expression in the heart did not differ between the groups (Fig. 2*B*,*D*).

Discussion

Flavonoids are secondary plant metabolites that are useful, e.g., for protection of plants against fungal infection (25–27); quercetin is one of the most common flavonoids in the human diet. Because quercetin is abundant in plant-based products in the diet, it is important to determine whether quercetin can reduce human health challenges such as obesity, metabolic syndrome, and NAFLD. Hence, we have characterized the effects of quercetin in an appropriate animal model of diet-induced metabolic syndrome and associated complications (21,28). This rodent model mimics most of the complications associated with human metabolic syndrome (21).

Obesity is a chronic condition characterized by excess fat deposition in the abdomen, including retroperitoneal, epididymal, and omental fat pads. Excess fat deposition increases morbidity and mortality through health complications, including oxidative stress, chronic low-grade inflammation, dyslipidemia, type 2 diabetes, cardiovascular disease, NAFLD, and some cancers (29–36). In this study, we have targeted NAFLD, obesity, and cardiovascular disease with quercetin using an appropriate rat model of metabolic syndrome (21). Using the same model, we showed that rutin, a glycoside of quercetin,

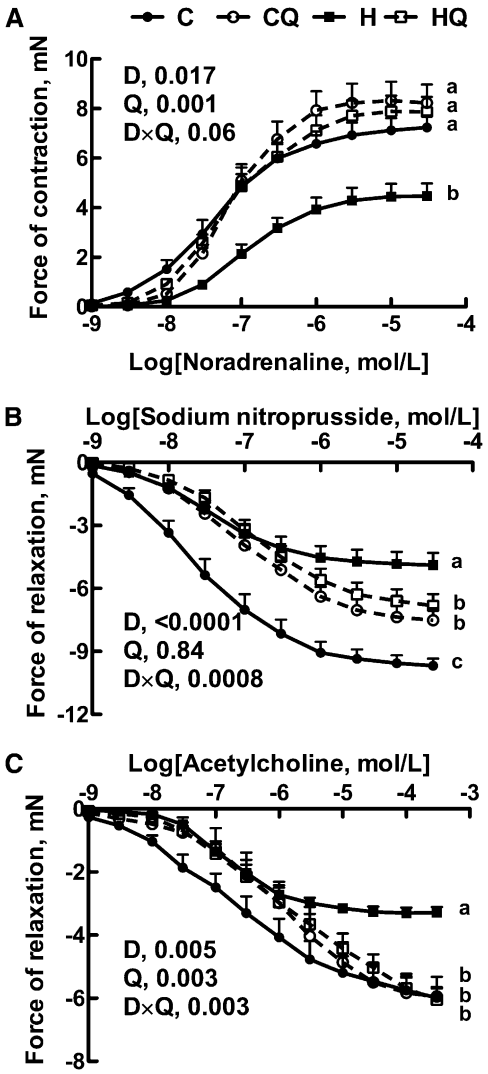

FIGURE 1 Noradrenaline-induced contraction (*A*), sodium nitroprusside-induced relaxation (*B*), and acetylcholine-induced relaxation (*C*) in thoracic aortic preparations from rats fed C or H diets for 8 wk and those diets or CQ or HQ diets for an additional 8 wk. Values are mean ± SEM, $n = 10$. Means at the highest concentration without a common letter differ, $P < 0.05$. C, corn starch–rich diet-fed rats; CQ, corn starch–rich diet-fed rats treated with quercetin; D, effects of diet; D×Q, interaction between the effects of diet and quercetin; H, high-carbohydrate, high-fat diet-fed rats; HQ, high-carbohydrate, high-fat diet-fed rats treated with quercetin; Q, effects of quercetin.

reduced abdominal fat and hence NAFLD and cardiovascular remodeling (24). Rutin reduced body weight as well as the abdominal fat content (24), indicating that it may enhance the utilization of fat and the lipogenic carbohydrate, fructose, or it may inhibit further absorption and deposition of fat, or both. In this study, quercetin did not reduce body weight, but it reduced the abdominal fat deposition. Previously, a 10-fold higher dose of quercetin (0.8% in food) compared with our study (0.08% in food) increased the energy expenditure in high-fat diet-fed mice without affecting the body weight and body composition (18). These results suggest that quercetin may cause lipid trafficking away from the abdomen and hence reduce the associated complications, mainly NAFLD and cardiovascular remodeling. Our recent study with chia seeds has demonstrated a link between lipid trafficking away from abdomen and attenuation of changes in the structure and function of the heart and the liver (23). This study also showed that the lipid components in plasma were higher with quercetin treatment consistent with the trafficking of fat by the circulation. The lack of difference in the body weights of quercetin-treated and untreated rats may suggest that the abdominal fat has been moved to other fat storage areas or has been converted to muscle mass.

Although dyslipidemia was not attenuated with quercetin, the other symptoms of metabolic syndrome were attenuated, including systolic blood pressure, glucose tolerance, and visceral obesity. Quercetin abolished hepatic steatosis, prevented the infiltration of inflammatory cells in the liver, and reduced the portal fibrosis along with improvements in liver function. Along with these changes were cardioprotective effects, including reduced collagen deposition, less infiltration of inflammatory cells, inhibition of cardiomyocyte hypertrophy, reduced ventricular stiffness, lower ventricular dimensions, and a return toward normal ventricular function.

The presence of inflammation and oxidative stress leads to cellular injury leading to organ dysfunction (37,38). One of the major defense systems against stress-related injury is the Nrf2 system (39). Nrf2 is the transcription factor present in inactive forms in the cell. Once activated, Nrf2 translocates to the nucleus and activates the antioxidant response elements (39). This, in turn, gives rise to proteins and enzymes, such as HO-1, which reduce the cellular stress (39). This suggests that the activators of Nrf2 system can protect organ systems. Similarly, the role of NF-κB has been established in the activation of inflammation (40).

In obesity, oxidative stress and inflammation induce organ dysfunction (31). Our results showed that the high-carbohydrate, high-fat diet upregulated the hepatic and cardiac expression of NF-κB, whereas the hepatic and cardiac expression of Nrf2 was downregulated, clearly indicating the presence of inflammation and oxidative stress in both the liver and the heart. The hepatic and cardiac expression of NF-κB was downregulated by quercetin, confirming its antiinflammatory role. Similarly, quercetin supplementation upregulated the expression of Nrf2, resulting in activation of antioxidant response elements, followed by upregulation of HO-1, and hence the reduction of oxidative stress. Thus, attenuation of hepatic and cardiac changes by quercetin could be mediated through its antioxidative and antiinflammatory actions.

Quercetin also upregulated the expression of CPT1, a regulator of fatty acid oxidation, in the liver and the heart. This change could attenuate NAFLD, thereby leading to attenuation of steatosis through higher fatty acid oxidation in the liver. Caspase-3 expression was also higher in the liver, but not in the heart, with quercetin as with rutin (24). Greater expression of caspase-3 indicates higher levels of apoptosis, possibly leading to the removal of steatotic cells from the liver. These results explain the role of quercetin in the attenuation of hepatic changes in metabolic syndrome.

In this study, 0.8 g/kg food of quercetin was used to provide a daily dose of ~50 mg/kg body weight. This dose corresponds to ~1 g/d quercetin in a 70-kg human based on scaling equation (41) or ~0.6 g/d based on body surface area comparisons between rats and humans (42). Although the mean daily human intake of quercetin is not known, the total intake of polyphenols is ~1 g/d, with two-thirds being flavonoids, including quercetin and rutin (43). This suggests that the dose of quercetin used in this study is realistic in humans.

In conclusion, quercetin was effective against the symptoms of metabolic syndrome in a diet-induced rat model. The trafficking of fat away from the abdomen did not lower body

TABLE 3 Hepatic structure and function in rats fed C or H diets for 8 wk and those diets or CQ or HQ diets for an additional 8 wk[1]

Variables	C	CQ	H	HQ	*P* value D	Q	D×Q
Liver wet weight, *mg/mm tibial length*	267 ± 13[b]	229 ± 8[c]	298 ± 11[a]	261 ± 8[b]	0.004	0.0008	0.96
Plasma ALT activity, *U/L*	35 ± 3[b]	22 ± 2[c]	58 ± 4[a]	42 ± 3[b]	<0.0001	<0.0001	0.63
Plasma AST activity, *U/L*	79 ± 6[b]	72 ± 5[b]	109 ± 9[a]	129 ± 11[a]	<0.0001	0.43	0.11
Plasma ALP activity, *U/L*	172 ± 12[b]	116 ± 9[c]	247 ± 19[a]	158 ± 16[b]	0.0003	<0.0001	0.26
Plasma LDH activity, *U/L*	252 ± 27[b]	211 ± 25[b]	459 ± 37[a]	283 ± 18[b]	<0.0001	0.0004	0.020
Plasma albumin, *g/L*	28.0 ± 0.6	28.1 ± 0.4	28.7 ± 0.3	29.0 ± 0.5	0.09	0.67	0.83
Plasma total bilirubin, *μmol/L*	2.2 ± 0.1[ab]	1.9 ± 0.1[b]	2.5 ± 0.1[a]	1.5 ± 0.2[c]	0.71	<0.0001	0.012
Plasma urea, *mmol/L*	5.8 ± 0.2[a]	5.1 ± 0.2[b]	3.0 ± 0.3[d]	3.8 ± 0.2[c]	<0.0001	0.83	0.002
Plasma uric acid, *μmol/L*	37 ± 3[b]	38 ± 3[b]	60 ± 9[a]	37 ± 4[b]	0.048	0.048	0.032

[1] Values are mean ± SEM, n = 8–10. Means in a row with superscripts without a common letter differ, $P < 0.05$. ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; C, corn starch–rich diet-fed rats; CQ, corn starch–rich diet-fed rats treated with quercetin; D, effects of diet; D×Q, interaction between the effects of diet and quercetin; H, high-carbohydrate, high-fat diet-fed rats; HQ, high-carbohydrate, high-fat diet-fed rats treated with quercetin; LDH, lactate dehydrogenase; Q, effects of quercetin.

weight and blood lipids, while the cardiovascular and liver complications of metabolic syndrome were attenuated. Quercetin supplementation attenuated the changes in expression of markers for oxidative stress and inflammation in the liver and the heart such as Nrf2, HO-1, and NF-κB along with higher fatty acid oxidation. Livers had greater expression of caspase-3, an apoptotic marker, indicating the attenuation of steatosis. Thus, quercetin can be considered as a nutraceutical with potential for the treatment of metabolic syndrome; clinical trials of this relatively safe natural compound should be undertaken.

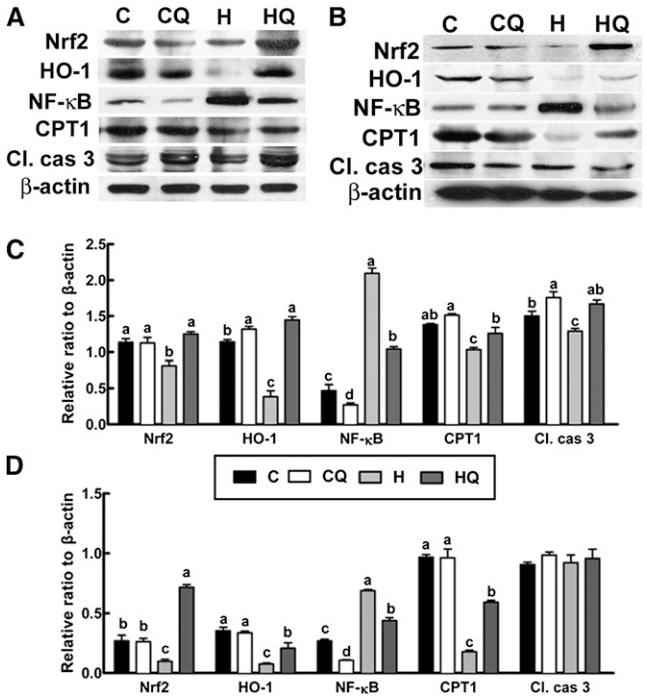

FIGURE 2 Expression of Nrf2, HO-1, NF-κB, CPT1, and cleaved caspase-3 (Cl. cas 3) in the liver (*A*,*C*) and the heart (*B*,*D*) from rats fed C or H diets for 8 wk and those diets or CQ or HQ diets for an additional 8 wk. Values are mean ± SEM, n = 4. Means without a common letter differ, $P < 0.05$. C, corn starch–rich diet-fed rats; CPT1, carnitine palmitoyltransferase 1; CQ, corn starch–rich diet-fed rats treated with quercetin; H, high-carbohydrate, high-fat diet-fed rats; HO-1, heme oxygenase-1; HQ, high-carbohydrate, high-fat diet-fed rats treated with quercetin; Nrf2, nuclear factor (erythroid-derived 2)-related factor-2.

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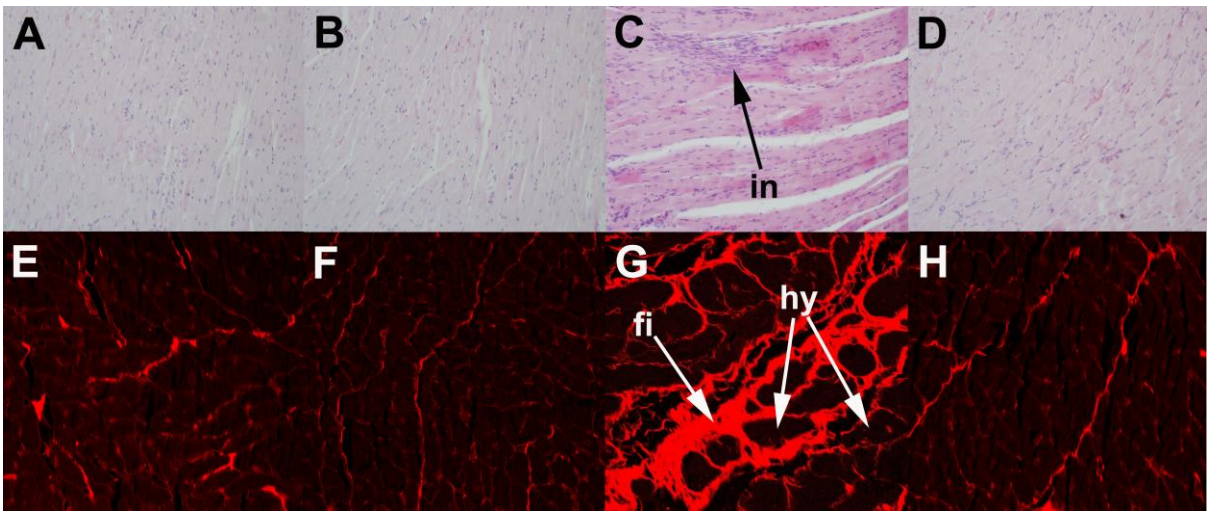
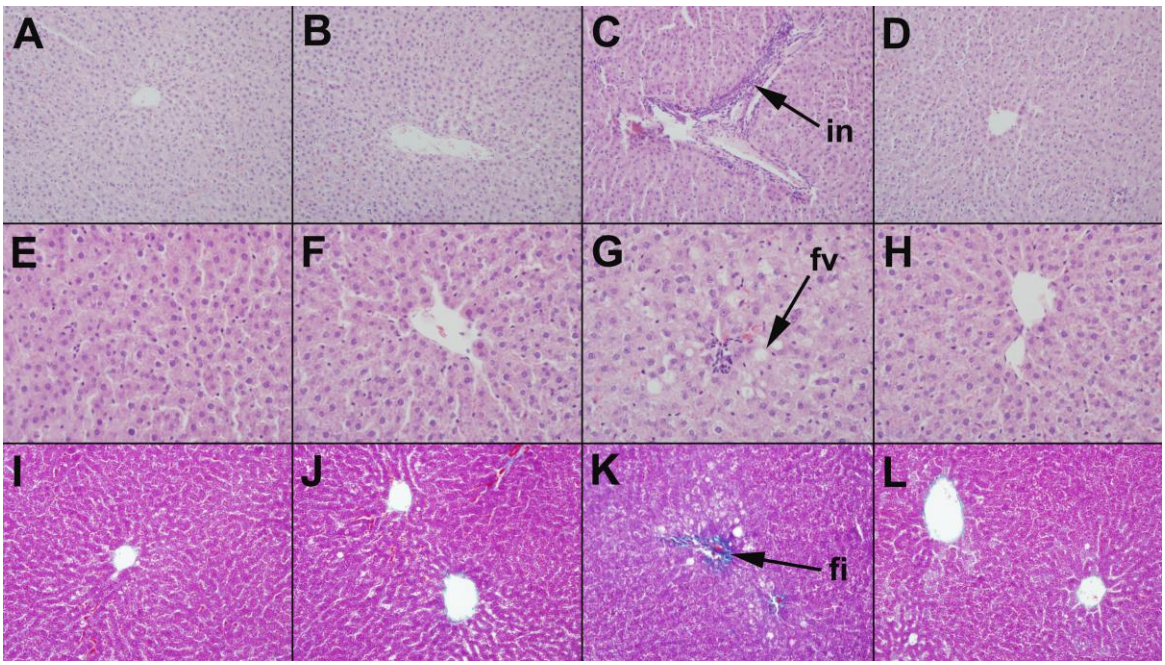


Supplemental Figure 1. Inflammation and fibrosis in the heart from rats fed corn starch-rich or high-carbohydrate, high-fat diets for 8 wk and those diets or with supplemental quercetin for an additional 8 wk. Hematoxylin and eosin staining of LV showing infiltration of inflammatory cells (A-D, inflammatory cells marked as "in"; ×20) from C (*A*), CQ (*B*), H (*C*), and HQ (*D*) rats. Picrosirius red staining of LV showing collagen deposition and hypertrophy (E-H, fibrosis marked as "fi" and hypertrophied cardiomyocytes as "hy"; ×40) from C (*E*), CQ (*F*), H (*G*), HQ (*H*) rats.

C, corn starch-rich diet-fed rats; CQ, corn starch-rich diet-fed rats treated with quercetin; H, high-carbohydrate, high-fat diet-fed rats; HQ, high-carbohydrate, high-fat diet-fed rats treated with quercetin.



Supplemental Figure 2. Inflammation, fat deposition, and fibrosis in the liver from rats fed corn starch-rich or high-carbohydrate, high-fat diets for 8 wk and those diets or with supplemental quercetin for an additional 8 wk. Hematoxylin and eosin staining of the liver showing inflammatory cells (A-D, marked as "in"; ×20) and enlarged fat vacuoles (E-H, marked as "fv"; ×40) from C (*A*,*E*), CQ (*B*,*F*), H (*C*,*G*), and HQ (*D*,*H*) rats. Milligan's trichrome staining of the liver showing collagen deposition in the hepatic portal region (I-L, marked as "fi"; ×20) from C (*I*), CQ (*J*), H (*K*), and HQ (*L*) rats.

C, corn starch-rich diet-fed rats; CQ, corn starch-rich diet-fed rats treated with quercetin; H, high-carbohydrate, high-fat diet-fed rats; HQ, high-carbohydrate, high-fat diet-fed rats treated with quercetin.

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ORIGINAL CONTRIBUTION

Chapter 5.1

Cardioprotective and hepatoprotective effects of ellagitannins from European oak bark (*Quercus petraea* L.) extract in rats

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Abstract
Background Red wine contains many potentially bioactive polyphenols including resveratrol, catechins, anthocyanins and flavonoids as well as tannins derived from oak during maturation. This study examined the effects of a mixture of ellagitannins from oak bark (*Quercus petraea* L.) on cardiovascular, metabolic and liver changes in high-carbohydrate, high-fat diet–fed rats and in Spontaneously Hypertensive Rats (SHR).
Methods First, 8-week-old male Wistar rats were divided into four groups and given either cornstarch diet, cornstarch diet + oak bark extract (0.5 mL/kg food), high-carbohydrate, high-fat diet or high-carbohydrate, high-fat diet + oak bark extract (0.5 mL/kg food) for 16 weeks. Oak bark extract was added to the diets for last 8 weeks of the feeding period. Secondly, SHR aged 42 weeks fed on standard chow diet were divided into two groups with and without oak bark extract treatment for 12 weeks (0.5 mL/kg food).
Results The high-carbohydrate, high-fat diet induced signs of metabolic syndrome along with cardiovascular remodelling and non-alcoholic steatohepatitis. Oak bark extract attenuated the signs of metabolic syndrome in high-carbohydrate, high-fat diet–fed rats and improved the structure and function of the heart and the liver. SHR after oak bark extract treatment for 12 weeks showed lower systolic blood pressure, lower cardiac fibrosis and cardiac stiffness and improved vascular reactivity.
Conclusions Oak bark extract containing ellagitannins improved cardiovascular, metabolic and liver parameters in these rat models of human disease, suggesting that part of the benefits attributed to red wine may be produced by these ellagitannins.

Keywords Obesity · Cardiovascular disease · Dyslipidaemia · Ellagitannins · Hypertension

Introduction

Oak has been used in winemaking for more than two millennia, starting in Europe during the Roman Empire. It was later discovered that wine stored in oak barrels had improved characteristics including better taste. The ellagitannins, extracted from oak barrels into the wine during this maturation process [1, 2], are complex naturally occurring polyphenolic compounds present as monomers or oligomers [3, 4]. These polyphenols are hexahydroxyldiphenoyl esters of carbohydrates with more than 500 naturally occurring tannins having been identified [4, 5]. Oak is one of the major sources of ellagitannins [6] with the European oak (*Querces petraea* L.) containing vescalagin, castalagin, grandinin and roburin E as the major ellagitannins [7]. The ellagitannins, although present in red wine, have received little attention as potential bioactive compounds. In contrast, the mechanisms for the cardiovascular benefits from other polyphenols in red wine such as resveratrol have been extensively studied [8].

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Other dietary sources of ellagitannins include pomegranates, chestnuts, raspberries, strawberries, blackberries and walnuts [5, 6, 9, 10]. Ellagitannins from pomegranates have been studied for their cardioprotective effects in laboratory animals [11]. Pomegranate juice attenuated isoproterenol-induced cardiac necrosis [12] and reduced angiotensin-induced hypertension, glucosuria and proteinuria in streptozotocin-induced diabetic rats [13]. Pomegranate fruit extract containing punicalagin as the major ellagitannin improved vascular endothelial function without affecting the plasma lipid profile in obese Zucker rats [14, 15].

Cardiovascular disease, including hypertension, remains a major cause of morbidity and mortality around the world [16]. Together with obesity and insulin resistance, hypertension is a key criterion for diagnosis of the metabolic syndrome, a major risk factor for cardiovascular disease and type 2 diabetes [17] as well as the development of non-alcoholic fatty liver disease [18]. Rats are widely used to mimic human disease states, especially cardiovascular and endocrine diseases [19–22]. Signs of human metabolic syndrome can be induced by high-carbohydrate, high-fat feeding in male Wistar rats [20]. The Spontaneously Hypertensive Rat (SHR) is the most common rat model of human hypertension [19].

This study has characterised the cardioprotective and hepatoprotective effects of oak bark extract (OBE) containing ellagitannins. Studies were performed on high-carbohydrate, high-fat diet–fed male Wistar rats (H) or the control cornstarch diet–fed rats (C). Subgroups of H and C rats were treated with OBE for 8 weeks (HO and CO rats, respectively). Further, adult male SHR with stable and high systolic blood pressure (S rats) were treated with OBE for 12 weeks (SO rats). Cardiovascular, hepatic and metabolic parameters were studied to evaluate whether OBE was effective in attenuating the complications observed in these rat models of human disease.

Materials and methods

Extract preparation and characterisation

European oak bark (*Querces petraea* L.) was converted into chips and extracted for 6 months at room temperature in brandy containing 70% alcohol. OBE was filtered to remove the solid material and de-alcoholised before being analysed by liquid chromatography/mass spectrometry (LC/MS) employing electrospray mass spectrometry and ultraviolet detection. The analysis was performed on a Micromass Quattro micro tandem quadrupole mass spectrometer (Waters, Manchester, UK). LC separation was provided by a Waters liquid chromatograph (Waters, Milford, USA), consisting of a 2,695 separation module and 2,487 dual-wavelength ultraviolet detector. Data were acquired by the Masslynx data system for both the MS and ultraviolet data. For LC, a flow rate of 1 mL/min was used with 0.1% aqueous formic acid and methanol as solvent and injection volume of 20 μL. For UV detection, 254 and 280 nm wavelengths were used. Castalagin,[1] vescalagin (see footnote 1), grandinin (see footnote 1), roburin E (see footnote 1) and ellagic acid (MP Biomedicals, Seven Hills, NSW, Australia) were used as standards in this procedure. There were 20% uncertainties in the measurements of ellagitannins derived from the contributions of the uncertainties in the preparation and analysis of the standards and samples.

Rats, diets and treatment with OBE

All experimental protocols were approved by The University of Queensland Animal Experimentation Ethics Committee, under the guidelines of the National Health and Medical Research Council of Australia. Male Wistar rats (8 weeks old, weighing 328 ± 2 g, $n = 40$) and male SHR (42 weeks old, weighing 422 ± 6 g, $n = 20$) were supplied by The University of Queensland Biological Resources facility and The Prince Charles Hospital, Brisbane, respectively.

Male Wistar rats were randomly divided into four experimental groups and were fed with either cornstarch diet (C; $n = 10$), cornstarch diet + OBE (0.5 mL/kg food; CO; $n = 10$), high-carbohydrate, high-fat diet (H; $n = 10$) or high-carbohydrate, high-fat diet + OBE (0.5 mL/kg food; HO; $n = 10$) for 16 weeks. CO and HO rats were fed with respective diets for the first 8 weeks without OBE; OBE was supplemented in the diets of CO and HO rats for the last 8 weeks of the protocol. Compositions of H and C diets used in this study have been described previously [20, 23]. Male adult SHR with high and stable systolic blood pressure were divided into two groups of 10 rats each, one without treatment (S) and one with OBE treatment in diet (SO; 0.5 mL/kg in food) for 12 weeks. All SHR were fed on standard powdered chow diet (Specialty Feeds, Glen Forest, WA, Australia). All rats were given ad libitum access to food and water and were individually housed in temperature-controlled 12-h light–dark conditions. Energy intakes were calculated as described previously [20, 23].

[1] Pure castalagin, vescalagin, grandinin and roburin E for use as standards were kindly provided by Professor Stéphane Quideau, European Institute of Chemistry and Biology, University of Bordeaux I, France.

Physiological and metabolic parameters

All rats were monitored daily for body weight, food intake and water intake. Abdominal circumference and body length of rats were measured at the end of protocol using a standard measuring tape under light anaesthesia with Zoletil (tiletamine, 10 mg/kg; zolazepam, 10 mg/kg, i.p.; Virbac, Peakhurst, NSW, Australia). Body mass index (BMI) and feed efficiency were calculated as previously described [20].

At the end of the protocol, rats were food-deprived for 12 h and oral glucose tolerance tests were performed as previously described [20, 23]. Blood glucose concentrations obtained from oral glucose tolerance tests were used to calculate area under the curve (AUC). Plasma concentrations of total cholesterol and triglycerides were determined using kits and controls supplied by Olympus using an Olympus analyser (AU 400, Tokyo, Japan) [20, 23]. Non-esterified fatty acids (NEFA) in plasma were determined using a commercial kit (Wako, Osaka, Japan) [20, 23]. During terminal experiments, abdominal fat pads were removed, weighed and normalised to tibial length at the time of fat removal.

Assessment of cardiovascular structure and function

Systolic blood pressure measurements

Systolic blood pressure of rats was measured under light anaesthesia with Zoletil, using an MLT1010 Piezo-Electric Pulse Transducer (ADInstruments, Sydney, Australia), an inflatable tail-cuff connected to a MLT844 Physiological Pressure Transducer (ADInstruments, Sydney, Australia) and PowerLab data acquisition unit (ADInstruments, Sydney, Australia) [20, 23]. These measurements were taken every fourth week for C, CO, H and HO groups starting at initiation of feeding period and every second week for S and SO groups starting 2 weeks before the initiation of protocol.

Echocardiography

Echocardiographic examinations (Phillips iE33, 12 MHz transducer) were performed to assess the cardiovascular structure and function in all the rats at the end of protocol as previously described [20].

Isolated Langendorff heart preparation

Rats were killed by injection with Lethabarb (pentobarbitone sodium, 100 mg/kg, i.p.; Virbac, Peakhurst, NSW, Australia), and heparin (200 IU; Sigma-Aldrich Australia, Sydney, Australia) was injected through the right femoral vein. The abdomen was then opened and blood ($\sim$5 mL) was withdrawn from the abdominal aorta, collected into heparinised tubes and centrifuged at 5,000$\times g$ for 15 min to obtain plasma. Plasma was stored at -20 °C before further biochemical analysis. Hearts were removed and used as isolated Langendorff heart preparations to assess left ventricular function ($n = 8$ in each group) as previously described [20, 23]. End-diastolic pressures were obtained for the calculation of diastolic stiffness constant (κ, dimensionless). After performing Langendorff heart perfusion studies, the heart was separated into right ventricle and left ventricle (with septum) and weighed.

Vascular reactivity

Thoracic aortic rings ($\sim$4 mm in length) from rats ($n = 10$ from each group) 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 approximately 10 mN. Cumulative concentration–response curves (contraction) were obtained for noradrenaline (Sigma-Aldrich Australia, Sydney, Australia) and cumulative concentration–response curves (relaxation) were obtained for acetylcholine (Sigma-Aldrich Australia, Sydney, Australia) and sodium nitroprusside (Sigma-Aldrich Australia, Sydney, Australia) following submaximal (70%) contraction to noradrenaline [20, 23].

Histology of the heart

Hearts were removed from the rats ($n = 2$ from each group) soon after death and were processed for histological assessments for inflammatory cells and collagen deposition [20].

Assessment of hepatic structure and function

Livers ($n = 8$ from each group) from C, CO, H and HO rats were isolated and weighed. Liver portions were isolated ($n = 2$ from each group) and fixed in 10% neutral buffered formalin for three days. These tissue samples were dehydrated and then embedded in paraffin wax. Thin sections (5 μm) of these tissues were cut and stained with haematoxylin and eosin for the determination of inflammatory cell infiltration (20×) and for determining the presence of fat vacuoles (40×) in the liver. Liver sections were also stained with Milligan's Trichrome stain to determine portal fibrosis (20×) [20, 23].

Plasma activities of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) and plasma concentrations of albumin, total bilirubin, urea and uric acid were

determined using kits and controls supplied by Olympus using an Olympus analyser (AU 400, Tokyo, Japan) in C, CO, H and HO rats [20, 23].

Oxidative stress and inflammatory markers

Plasma concentrations of malondialdehyde were determined by high-performance liquid chromatography (Shimadzu, Kyoto, Japan) as previously described [24]. Plasma glutathione peroxidase activity was measured using an automated spectrophotometer (Cobas Mira) as previously described [25]. Plasma C-reactive protein concentrations were estimated using commercial kits (BD Bioscience, Franklin Lakes, NJ) according to manufacturer-provided standards and protocols. Heart and liver samples isolated from rats ($n = 3$ from each group) were stored at −80 °C. These samples were thawed and sonicated after adding cell lysis buffer. These samples were then ultracentrifuged at 100,000×g for 30 min at 4 °C. Supernatants were used to measure the protein concentration in each sample by the bicinchoninic acid method (Thermo Scientific). The expression of Nrf2 and NF-κB was studied in these supernatants at equal protein amounts (40 µg) by Western blot analysis.

Statistical analysis

All data are mean ± SEM. Four groups of Wistar rats (C, CO, H and HO) were tested for homogenous variance using Bartlett's test, and variables that were not normally distributed were transformed (using log 10 function) prior to statistical analyses. These four groups were tested for effects of diet, treatment and their interactions by two-way ANOVA. When interaction and/or the main effects were significant, means were compared using Newman–Keuls multiple comparison post-test. Two SHR groups (S and SO) were compared using Student's t test. $P < 0.05$ was considered significant. All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (San Diego, CA, USA).

Results

Chemical profile of OBE and daily intake of ellagitannins

OBE contained vescalagin (1.2 mg/mL), castalagin (0.8 mg/mL), roburin E (0.8 mg/mL) and grandinin (2 mg/mL) as major ellagitannins as well as ellagic acid (0.1 mg/mL). Daily intakes of individual ellagitannins have been calculated from these values for CO, HO and SO rats (Table 1).

Effects of OBE on H-induced metabolic syndrome

Physiological and metabolic parameters

Body weights of H rats were higher than those of C rats after 16 weeks and HO rats had lower body weight (Table 2). Water intake was lower in H rats than in C rats. HO rats had lower water intake when compared to H rats (Table 2). H rats consumed less food than C rats, although the energy intake was higher for H rats. OBE did not change food intake or energy intake in CO and HO rats (Table 2). H rats showed abdominal obesity measured as higher abdominal circumferences and increased abdominal fat pads than C rats. These signs of obesity were attenuated with OBE treatment in HO rats (Table 2). BMI and feed efficiency were higher in H rats than in C rats, and both parameters were lower in CO and HO rats when compared to C rats and H rats, respectively (Table 2). H rats showed higher basal blood glucose concentrations when compared to C rats after 16 weeks, whereas HO rats had lower basal blood glucose concentrations (Table 2). H feeding impaired oral glucose tolerance, while OBE treatment improved it (Fig. 1a). Plasma concentrations of triglycerides, total cholesterol and NEFA were higher in H rats than in C rats. HO rats had lower plasma concentrations of these lipid components (Table 2). H rats showed lower plasma urea concentrations and higher plasma uric acid concentrations than C rats. Plasma urea concentrations were higher and plasma uric

Table 1 Daily intake of ellagitannins in OBE-treated rats

Components from OBE	CO	HO	SO
OBE (µL)	16.41 ± 0.37	10.66 ± 0.48	15. 21 ± 0.23
Vescalagin (µg)	19.70 ± 0.44	12.79 ± 0.57	18.25 ± 0.28
Castalagin (µg)	13.13 ± 0.30	8.52 ± 0.38	12.17 ± 0.19
Grandinin (µg)	32.83 ± 0.74	21.31 ± 0.95	30.42 ± 0.46
Roburin E (µg)	13.13 ± 0.30	8.52 ± 0.38	12.17 ± 0.19
Total ellagitannin intake (µg)	78.79 ± 1.77	51.15 ± 2.29	73.00 ± 1.11
Ellagic acid (µg)	1.64 ± 0.04	1.07 ± 0.05	1.52 ± 0.02

Values are mean ± SEM and $n = 10$ for each group. Daily intake of OBE and individual ellagitannins from extract was calculated based on the daily intake of food

CO cornstarch diet + OBE–fed rats, *HO* high-carbohydrate, high-fat diet + OBE–fed rats, *SO* SHR supplemented with OBE

Table 2 Effects of OBE on H-induced physiological, metabolic and oxidative stress variables in C, CO, H and HO rats

Variables	C	CO	H	HO	*P* value		
					Diet	OBE	Diet × OBE
Physiological variables							
Initial body weight (g)	334 ± 7	329 ± 8	332 ± 9	336 ± 6	>0.05	>0.05	>0.05
Final body weight (g)	424 ± 11^{bc}	401 ± 6^{c}	510 ± 10^{a}	440 ± 8^{b}	<0.001	<0.001	<0.05
Water intake (mL/day)	30.2 ± 0.6^{a}	31.5 ± 0.4^{a}	20.1 ± 0.4^{b}	18.6 ± 0.5^{c}	<0.001	>0.05	<0.01
Food intake (g/day)	30.7 ± 0.7^{a}	32.8 ± 0.8^{a}	22.5 ± 0.8^{b}	22.3 ± 1.0^{b}	<0.001	>0.05	>0.05
Energy intake (kJ/day)	345 ± 10^{b}	365 ± 11^{b}	479 ± 15^{a}	476 ± 16^{a}	<0.001	>0.05	>0.05
BMI (g/cm^2)	0.70 ± 0.01^{b}	0.65 ± 0.01^{c}	0.78 ± 0.01^{a}	0.69 ± 0.01^{b}	<0.001	<0.001	>0.05
Feed efficiency (g/kJ)	0.26 ± 0.02^{b}	0.20 ± 0.01^{c}	0.37 ± 0.02^{a}	0.22 ± 0.01^{bc}	<0.001	<0.001	<0.05
Abdominal circumference (cm)	21.4 ± 0.3^{b}	19.6 ± 0.2^{c}	23.8 ± 0.3^{a}	20.0 ± 0.2^{c}	<0.001	<0.001	<0.001
Abdominal fat pads (mg/mm tibial length)	407 ± 48^{b}	206 ± 31^{c}	805 ± 53^{a}	415 ± 47^{b}	<0.001	<0.001	<0.05
Metabolic variables							
Basal blood glucose (mmol/L)	4.2 ± 0.2^{b}	3.8 ± 0.2^{bc}	4.9 ± 0.2^{a}	3.3 ± 0.2^{c}	>0.05	<0.001	<0.01
AUC (mmol/L·min)	688 ± 13^{b}	626 ± 18^{c}	768 ± 12^{a}	664 ± 16^{bc}	<0.001	<0.001	>0.05
Plasma total cholesterol (mmol/L)	1.5 ± 0.1^{b}	1.3 ± 0.1^{b}	2.0 ± 0.1^{a}	1.4 ± 0.1^{b}	<0.01	<0.001	>0.05
Plasma triglycerides (mmol/L)	0.4 ± 0.1^{b}	0.4 ± 0.1^{b}	0.9 ± 0.1^{a}	0.5 ± 0.1^{b}	<0.01	>0.05	>0.05
Plasma non-esterified fatty acids (mmol/L)	1.3 ± 0.1^{b}	1.1 ± 0.1^{b}	2.6 ± 0.2^{a}	1.4 ± 0.1^{b}	<0.001	<0.001	<0.001
Plasma urea (mmol/L)	5.5 ± 0.3^{ab}	6.1 ± 0.3^{a}	3.4 ± 0.2^{c}	4.8 ± 0.3^{b}	<0.001	<0.01	>0.05
Plasma uric acid (μmol/L)	35.2 ± 1.8^{b}	37.2 ± 2.1^{b}	52.6 ± 2.6^{a}	33.1 ± 1.9^{b}	<0.01	<0.001	<0.001
Oxidative stress and inflammatory markers							
Plasma malondialdehyde (μmol/L)	27.1 ± 1.1^{b}	27.5 ± 1.0^{b}	31.5 ± 1.2^{a}	28.0 ± 1.2^{b}	<0.05	>0.05	>0.05
Plasma glutathione peroxidase activity (U/L)	1,165 ± 39^{a}	1,135 ± 26^{a}	880 ± 27^{b}	1,063 ± 27^{a}	<0.001	<0.05	<0.01
Plasma C-reactive protein (μmol/L)	2.60 ± 0.13^{bc}	3.27 ± 0.17^{a}	2.76 ± 0.08^{b}	2.36 ± 0.10^{c}	<0.01	>0.05	<0.001

Values are mean ± SEM and $n = 8$–10 for each group. Means without a common letter differ, $P < 0.05$

C cornstarch diet–fed rats, *CO* cornstarch diet + OBE–fed rats, *H* high-carbohydrate, high-fat diet–fed rats, *HO* high-carbohydrate, high-fat diet + OBE–fed rats

acid concentrations were lower in HO rats when compared to H rats (Table 2).

Cardiovascular structure and function

Systolic blood pressure was higher in H rats than in C rats (Fig. 1b). Left ventricular internal diameter during diastole (LVIDd), left ventricular wall thickness during diastole (LVPWd) and systolic volume were higher, whereas fractional shortening and ejection fraction were lower in H rats than in C rats after 16 weeks (Table 3). HO rats had lower systolic blood pressure, LVIDd and systolic volume without any change in LVPWd (Fig. 1b and Table 3). HO rats had higher fractional shortening and ejection fraction than H rats (Table 3). Left ventricular masses were higher in H rats than C rats, whereas HO rats showed lower left ventricular masses (Table 3). Infiltration of inflammatory cells was observed in the hearts of H rats after 16 weeks (Fig. 2c); this was not observed in HO rats (Fig. 2d). Left ventricular diastolic stiffness (Table 3) and fibrosis (Fig. 2g) were higher in H rats than in C rats (Table 3 and Fig. 2e) after 16 weeks. HO rats had lower left ventricular diastolic stiffness (Table 3) and fibrosis (Fig. 2h). Impairment in vascular contraction with noradrenaline (Fig. 3a) and vascular relaxation with acetylcholine and sodium nitroprusside (Fig. 3b and c) induced by H feeding were attenuated in HO rats.

Hepatic structure and function

Livers from H rats were higher in wet weight than those from C rats after 16 weeks, whereas liver wet weights were lower in HO rats (Table 3). H feeding for 16 weeks caused hepatic steatosis (Fig. 4c) and fibrosis (Fig. 4k) with infiltration of inflammatory cells in liver (Fig. 4g). These changes in the liver were attenuated in HO rats (Fig. 4d, l, and h). H rats showed higher plasma activities of ALT, AST, ALP and LDH when compared to C rats. HO rats had

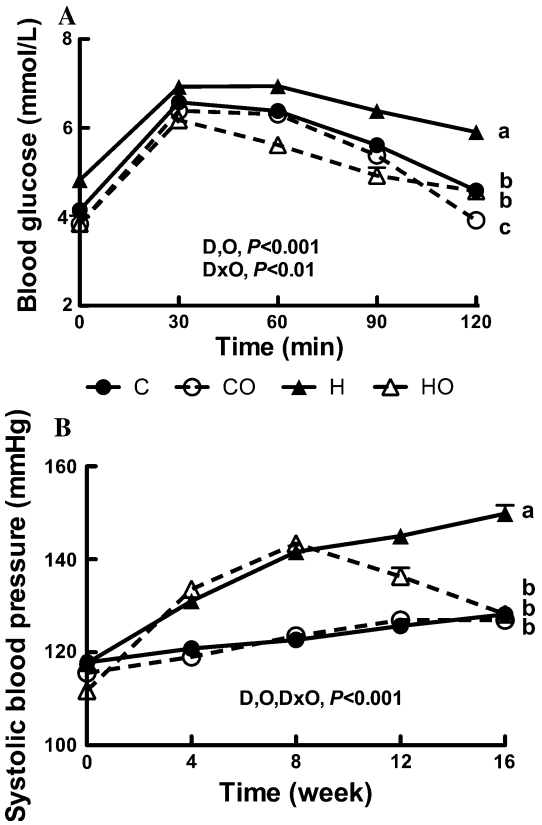

Fig. 1 Effects of OBE on oral glucose tolerance (**a**) and systolic blood pressure (**b**). Values are mean ± SEM and $n = 10$ for each group. End-point means without a common letter differ, $P < 0.05$. *D*, *O* and $D \times O$ represent effects of diet, OBE and interaction of diet and OBE. *C* cornstarch diet–fed rats, *CO* cornstarch diet + OBE–fed rats, *H* high-carbohydrate, high-fat diet–fed rats, *HO* high-carbohydrate, high-fat diet + OBE–fed rats

lower plasma activities of ALT, AST, ALP and LDH than H rats (Table 3). Plasma bilirubin concentrations were higher in H rats than in C rats, whereas HO rats had lower plasma bilirubin concentrations than H rats (Table 3). No changes were observed in plasma concentrations of albumin between the groups (Table 3).

Oxidative stress and inflammatory markers

H rats had higher plasma malondialdehyde concentrations and lower plasma glutathione peroxidase activities than C rats. HO rats showed lower plasma malondialdehyde concentrations and higher plasma glutathione peroxidase activities than H rats (Table 2). There was no difference between the plasma C-reactive concentrations of C and H rats. C-reactive protein concentrations in plasma were lower in HR and higher in CR rats when compared to H and C rats, respectively (Table 2). The heart and the liver from H rats showed up-regulation of NF-κB expression and down-regulation of Nrf2 expression. These changes in the expression of NF-κB and Nrf2 were normalised in HO rats (Fig. 5).

Effects of OBE in SHRs

Physiological and metabolic parameters

OBE extract did not affect body weight, water, food and energy intake and plasma lipid components in SO rats (Online Resource 1). Basal blood glucose concentrations, abdominal obesity and plasma malondialdehyde concentrations were lower in SO rats when compared to S rats (Online Resource 1). Oral glucose tolerance was improved in SO rats (Online Resource 2).

Cardiovascular structure and function

SO rats had lower systolic blood pressure after 6 weeks of treatment, and systolic blood pressure was lower in SO rats than in S rats until 12 weeks of treatment (Fig. 6a). LVIDd and systolic volumes were lower in SO rats than in S rats without affecting LVPWd (Table 4). Fractional shortening and ejection fraction were higher, whereas left ventricular masses were lower in SO rats than in S rats (Table 4). Left ventricular diastolic stiffness was lower in SO rats when compared to S rats as shown by lower diastolic stiffness constant (Table 4). Vascular responses to noradrenaline (Fig. 6b), acetylcholine (Fig. 6c) and sodium nitroprusside (Fig. 6d) were improved in SO rats when compared with S rats. Infiltration of inflammatory cells was not observed in SO rats, and fibrosis was lower in SO rats than in S rats (Online Resource 3).

Oxidative stress and inflammatory markers

SO rats had lower plasma malondialdehyde concentrations and higher plasma glutathione peroxidase activities than S rats (Online Resource 1). Plasma C-reactive protein concentrations were lower in SO rats when compared to S rats (Online Resource 1).

Discussion

Red wine contains many polyphenols with potential biological activities [26], especially resveratrol and catechins [8, 26, 27], but few other compounds from red wine have been evaluated. This study has shown that an ellagitannin mixture extracted from European oak bark used in red wine maturation produces both cardiac and liver protection as well as improved metabolic profile in high-carbohydrate, high-fat diet–fed Wistar rats. In addition, OBE improved cardiovascular structure and function in the SHR.

Limited studies with related ellagitannins from other sources, such as punicalagin from pomegranates, have shown cardiovascular improvement in rat models of

Table 3 Effects of OBE on cardiovascular and hepatic variables in H-induced metabolic syndrome in C, CO, H and HO rats

Variables	C	CO	H	HO	*P* value		
					Diet	OBE	Diet × OBE
Cardiovascular variables							
LVIDd (mm)	6.46 ± 0.16^{b}	6.40 ± 0.15^{b}	7.21 ± 0.20^{a}	6.56 ± 0.18^{b}	<0.05	<0.05	>0.05
LVPWd (mm)	1.58 ± 0.03^{b}	1.60 ± 0.04^{b}	1.75 ± 0.05^{a}	1.66 ± 0.05^{ab}	<0.05	>0.05	>0.05
Systolic volume (μL)	54.6 ± 5.1^{b}	50.2 ± 4.0^{b}	115.1 ± 4.8^{a}	58.9 ± 4.1^{b}	<0.001	<0.001	<0.001
Relative wall thickness	0.49 ± 0.01	0.48 ± 0.01	0.49 ± 0.01	0.49 ± 0.01	>0.05	>0.05	>0.05
Fractional shortening (%)	53.2 ± 1.3^{a}	52.2 ± 1.4^{a}	42.4 ± 1.0^{b}	51.0 ± 1.5^{a}	<0.001	<0.01	<0.001
Ejection fraction (%)	85.1 ± 1.2^{a}	82.6 ± 1.4^{a}	73.0 ± 1.1^{b}	82.1 ± 1.4^{a}	<0.001	<0.05	<0.001
Estimated left ventricular mass (g)	0.69 ± 0.02^{b}	0.68 ± 0.02^{b}	0.82 ± 0.03^{a}	0.71 ± 0.02^{b}	<0.01	<0.05	<0.05
Left ventricular + septum wet weight (mg/mm tibial length)	20.1 ± 0.8^{b}	19.4 ± 0.7^{b}	22.5 ± 0.8^{a}	19.9 ± 0.6^{b}	>0.05	<0.05	>0.05
Right ventricular weight (mg/mm tibial length)	4.1 ± 0.3	4.3 ± 0.3	4.5 ± 0.4	4.2 ± 0.3	>0.05	>0.05	>0.05
Left ventricular diastolic stiffness constant, κ	19.1 ± 0.8^{c}	18.4 ± 0.9^{c}	28.2 ± 1.1^{a}	22.8 ± 0.8^{b}	<0.001	<0.01	<0.05
Hepatic variables							
Liver wet weight (mg/mm tibial length)	265 ± 10^{b}	232 ± 11^{b}	297 ± 9^{a}	256 ± 10^{b}	<0.01	<0.001	>0.05
Plasma ALT (U/L)	35.6 ± 0.7^{b}	32.5 ± 0.6^{c}	48.2 ± 0.6^{a}	36.9 ± 0.9^{b}	<0.001	<0.001	<0.001
Plasma AST (U/L)	75.1 ± 4.8^{b}	77.8 ± 5.3^{b}	101.2 ± 6.6^{a}	82.3 ± 5.9^{b}	<0.05	>0.05	>0.05
Plasma ALP (U/L)	165 ± 15^{b}	172 ± 15^{b}	257 ± 20^{a}	192 ± 18^{b}	<0.01	>0.05	<0.05
Plasma LDH (U/L)	220 ± 20^{b}	206 ± 22^{b}	451 ± 28^{a}	262 ± 25^{b}	<0.001	<0.001	<0.001
Plasma albumin (g/L)	27.9 ± 0.3	28.1 ± 0.3	28.5 ± 0.3	28.0 ± 0.3	>0.05	>0.05	>0.05
Plasma total bilirubin (μmol/L)	2.0 ± 0.1^{b}	1.9 ± 0.1^{b}	2.5 ± 0.1^{a}	2.0 ± 0.1^{b}	<0.01	<0.01	>0.05

Values are mean ± SEM and $n = 8$–10 for each group. Means without a common letter differ, $P < 0.05$

C cornstarch diet–fed rats, *CO* cornstarch diet + OBE–fed rats, *H* high-carbohydrate, high-fat diet–fed rats, *HO* high-carbohydrate, high-fat diet + OBE–fed rats

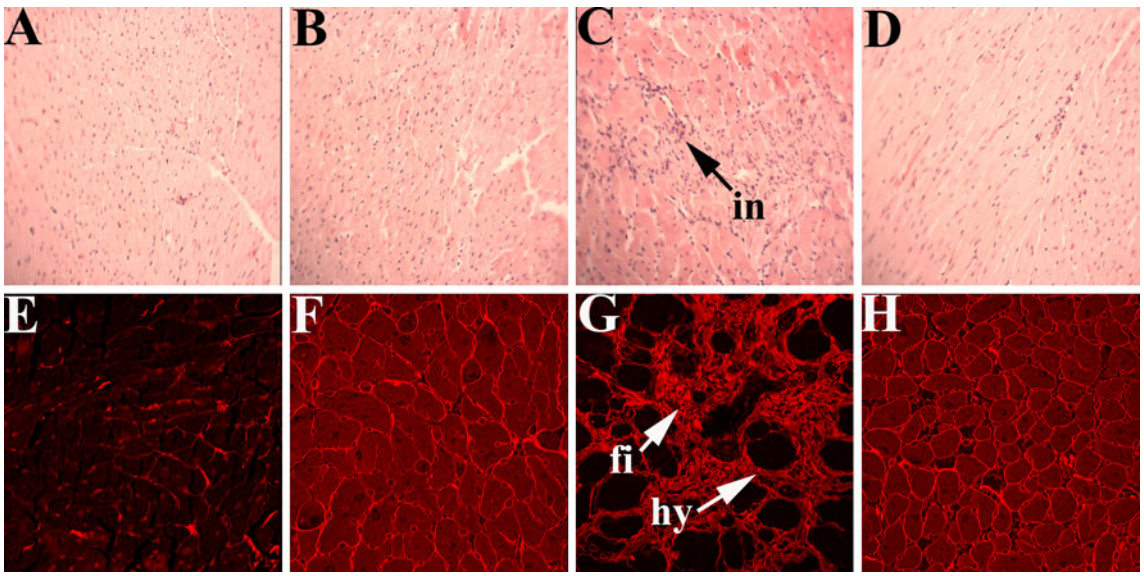

Fig. 2 Effects of OBE on inflammation and fibrosis in the heart induced by H feeding. Haematoxylin and eosin staining of left ventricle showing infiltration of inflammatory cells (**a**–**d**, inflammatory cells as dark spots surrounding the myocytes marked as *in*) (×40) from cornstarch diet–fed rats (**a**), cornstarch diet + OBE–fed rats (**b**), high-carbohydrate, high-fat diet–fed rats (**c**) and high-carbohydrate, high-fat diet + OBE–fed rats (**d**). Picrosirius red staining of left ventricle showing collagen deposition (**e**–**h**, fibrosis marked as *fi* and hypertrophied cardiomyocytes as *hy*) (×40) from cornstarch diet–fed rats (**e**), cornstarch diet + OBE–fed rats (**f**), high-carbohydrate, high-fat diet–fed rats (**g**) and high-carbohydrate, high-fat diet + OBE–fed rats (**h**)

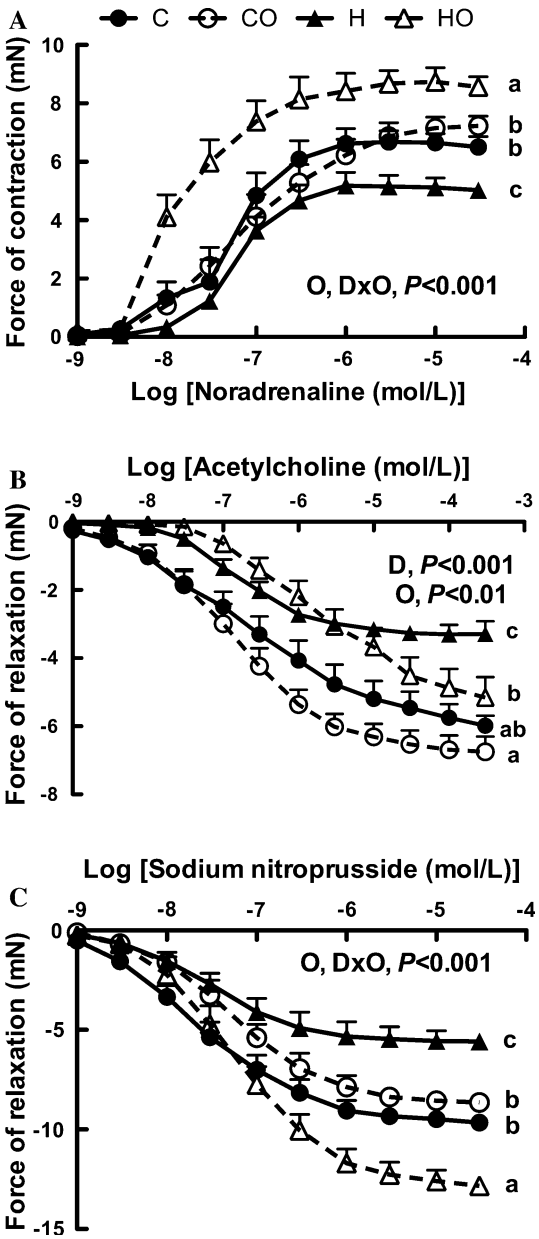

Fig. 3 Effects of OBE on vascular responses in rats. Noradrenaline-induced contraction (**a**), acetylcholine-induced relaxation (**b**) and sodium nitroprusside-induced relaxation (**c**) in thoracic aortic rings from C, CO, H and HO rats. Values are mean ± SEM and $n = 10$ for each group. End-point means without a common letter differ, $P < 0.05$. *D*, *O* and *DxO* represent effects of diet, OBE and interaction of diet and OBE. *C* cornstarch diet–fed rats, *CO* cornstarch diet + OBE–fed rats, *H* high-carbohydrate, high-fat diet–fed rats, *HO* high-carbohydrate, high-fat diet + OBE–fed rats

isoproterenol-induced cardiac necrosis and angiotensin-induced hypertension [12, 13] with no adverse effects even at high doses [28]. The pharmacokinetics of ellagitannins from pomegranate have been studied in humans as well as in rats [29, 30]. Ellagitannins are not absorbed directly due to their large molecular size [29, 30]. Earlier studies showed that urolithins, as metabolites of ellagitannins, were found in the urine of human subjects even 48 h after pomegranate juice consumption [9]. Ellagic acid, the hydrolysis product of ellagitannins, was not found in the blood within a few hours after pomegranate juice consumption [29]. Thus, the bioactive metabolites from ellagitannins are likely to be urolithins rather than ellagic acid.

H feeding in rats leads to the development of signs of metabolic syndrome and associated end-organ damage [20]. H rats developed abdominal obesity, hypertension, dyslipidaemia and impaired glucose tolerance. These changes were accompanied by cardiovascular remodelling and non-alcoholic steatohepatitis [20]. Thus, rats fed with H diet are suitable as a model to demonstrate the major changes found in human metabolic syndrome [20, 21]. We have previously shown that these diet-induced signs of the metabolic syndrome can be reversed by natural products with antioxidant and anti-inflammatory properties including rutin, olive leaf, purple carrots and chia seeds [23, 31–33]. Ellagitannins from oak bark showed similar biological activity to these other natural products.

The protective effects mediated by the oak-derived ellagitannins could relate to free radical scavenging and anti-inflammatory properties as with pomegranate-derived ellagitannins [34, 35]. The reduced plasma malondialdehyde concentrations and increased plasma glutathione peroxidase activity along with increased expression of Nrf2 in both the heart and the liver strongly support an antioxidant mechanism. The antioxidant activity of ellagitannins may lead to higher NO bioavailability by removal of superoxide, leading to reduction in blood pressure. Increased expression of eNOS, as shown with punicalagin in hypercholesterolaemic mice [36], would also increase NO bioavailability. Further, the improvement in vascular relaxation responses of OBE-treated rats is supportive of a reduction in blood pressure, especially the improvement in acetylcholine-induced relaxation, by a response dependent on endothelium-derived NO. In our study, the anti-inflammatory mechanism is supported by the lower expression of NF-κB, lower infiltration of inflammatory cells in the heart and lower collagen deposition in OBE-treated rats. These outcomes are associated with lower ventricular stiffness, possibly improving ventricular function. Similar anti-inflammatory activities of ellagitannins from pomegranate have been reported in a model of rheumatoid arthritis and in a colon inflammation model [35, 37]. The hepatoprotective responses with oak-derived ellagitannins are shown by reduced plasma activities of transaminases, attenuation of fat deposition and fibrosis, and inhibition of infiltration of inflammatory cells in the liver. The altered expression of NF-κB and Nrf2 in the liver confirms the antioxidative and anti-inflammatory effects of

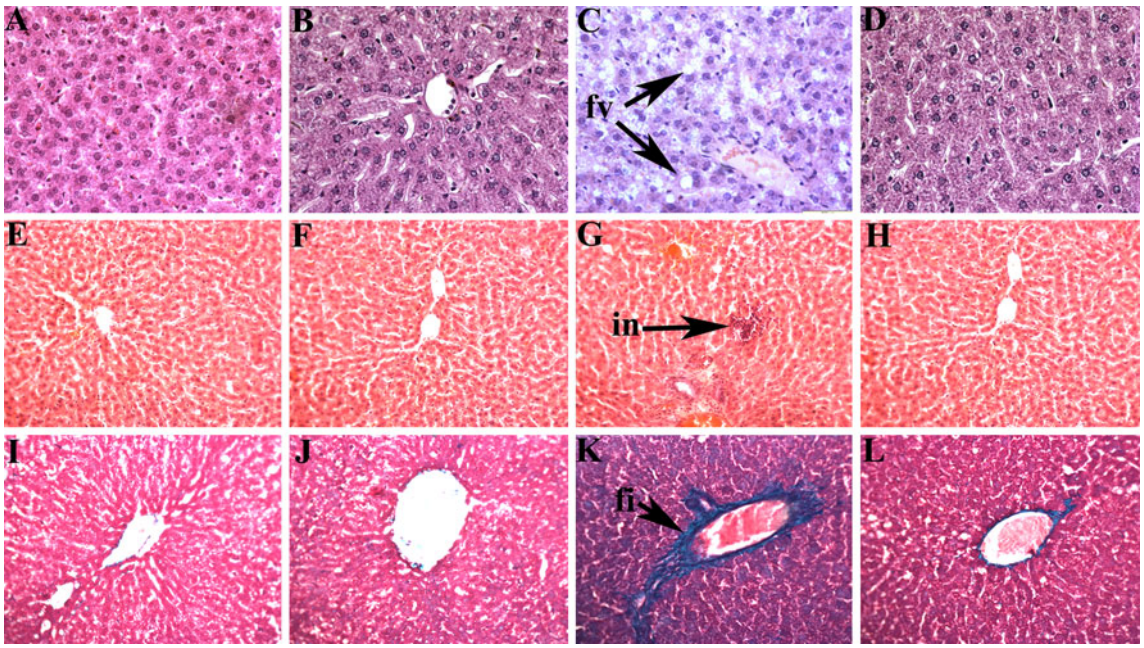

Fig. 4 Effects of OBE on fat deposition, inflammation and fibrosis in rat livers. Haematoxylin and eosin staining of liver showing enlarged fat vacuoles (**a**–**d**, marked as *fv*) (×40) and inflammatory cells (**e**–**h**, marked as *in*) (×20) from cornstarch diet–fed rats (**a**, **e**), cornstarch diet + OBE–fed rats (**b**, **f**), high-carbohydrate, high-fat diet–fed rats (**c**, **g**) and high-carbohydrate, high-fat diet + OBE–fed rats (**d**, **h**) rats. Milligan's Trichrome staining of hepatic portal regions showing fibrosis (**i**–**l**, marked as *fi*) (×20) from cornstarch diet–fed rats (**i**), cornstarch diet + OBE–fed rats (**j**), high-carbohydrate, high-fat diet–fed rats (**k**) and high-carbohydrate, high-fat diet + OBE–fed rats (**l**)

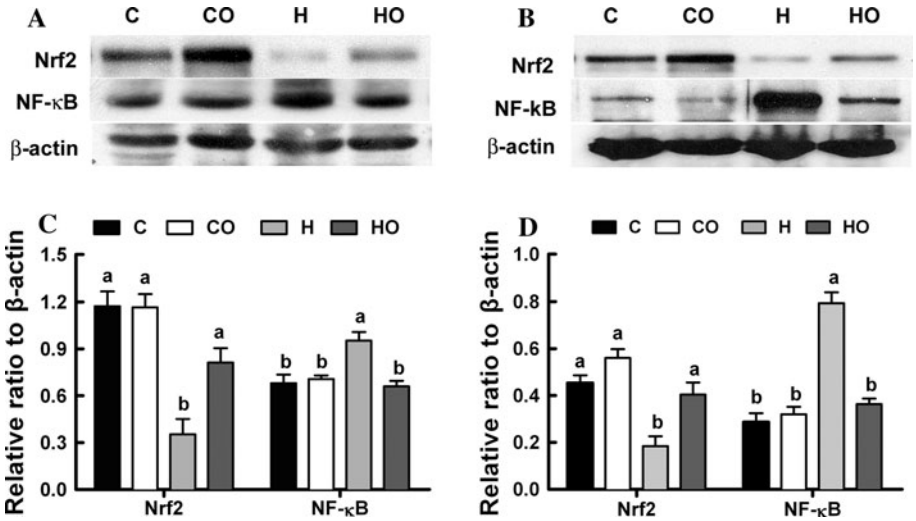

Fig. 5 Effects of OBE on expression of Nrf2 and NF-κB in the heart (**a**) and the liver (**b**). For quantitative analysis, the expression of these proteins was normalised against the expression of *β*-actin in the heart (**c**) and the liver (**d**). Values are mean ± SEM, $n = 3$. Means without a common letter differ, $P < 0.05$. *C* cornstarch diet–fed rats, *CO* cornstarch diet + OBE–fed rats, *H* high-carbohydrate, high-fat diet–fed rats, *HO* high-carbohydrate, high-fat diet + OBE–fed rats

ellagitannins from OBE. Thus, ellagitannins derived from oak bark ameliorated the changes associated with diet-induced cardiovascular remodelling and non-alcoholic fatty liver disease probably by both antioxidant and anti-inflammatory mechanisms.

OBE-treated rats presented improved metabolic parameters including lower abdominal fat deposition and improved glucose tolerance, as well as protection of the heart and the liver. Tannins from pomegranate leaf produced antiobesity effects by inhibiting energy intake in high–fat diet–fed mice [38], unlike the unchanged energy intake in our study. Decreased oxidative stress and inflammation may also be the mechanism responsible for improving metabolic parameters [39].

The improved cardiovascular parameters were also measured in adult SHR, the genetic model of choice to mimic human essential hypertension with extensive cardiovascular remodelling [19, 22]. OBE-treated rats had lower systolic blood pressure, ventricular collagen deposition and diastolic cardiac stiffness. Similar effects were previously reported with the ellagitannin-rich pomegranate juice in isoproterenol-induced cardiac necrosis model and

Table 4 Effects of OBE on cardiovascular structure and function in SHR

Variables	S	SO
LVIDd (mm)	8.01 ± 0.28	7.05 ± 0.22*
LVPWd (mm)	1.86 ± 0.08	1.72 ± 0.06
Systolic volume (μL)	138 ± 4	112 ± 4***
Relative wall thickness	0.54 ± 0.01	0.53 ± 0.01
Fractional shortening (%)	32.2 ± 1.2	38.5 ± 1.4**
Ejection fraction (%)	68.1 ± 1.0	74.6 ± 1.2***
Estimated left ventricular mass (g)	0.96 ± 0.04	0.82 ± 0.04*
Left ventricular + septum wet weight (mg/mm tibial length)	28.2 ± 0.8	25.3 ± 0.6**
Right ventricular weight (mg/mm tibial length)	4.5 ± 0.3	4.5 ± 0.2
Left ventricular diastolic stiffness constant, κ	32.4 ± 1.3	28.6 ± 1.1*

Values are mean ± SEM and $n = 8$–10 for each group. * versus S and $P < 0.05$, ** versus S and $P < 0.01$, *** versus S and $P < 0.001$. *S* SHR, *SO* SHR supplemented with OBE

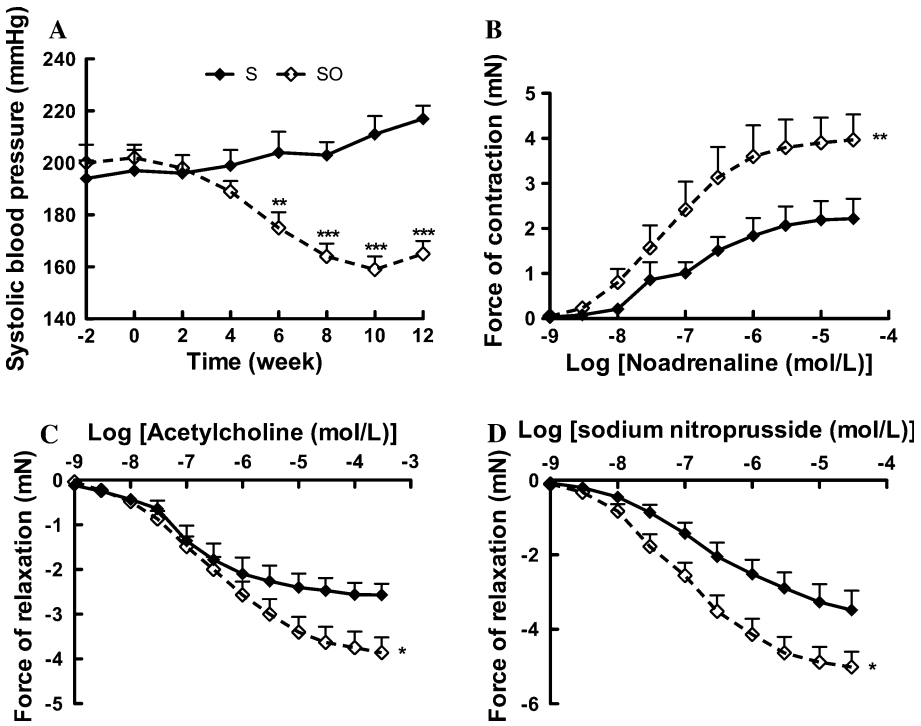

Fig. 6 Effects of OBE on systolic blood pressure (**a**), vascular contraction by noradrenaline (**b**) and vascular relaxation by acetylcholine (**c**) and sodium nitroprusside (**d**) in SHR (S) and SHR supplemented with OBE (SO). Values are mean ± SEM and $n = 10$ for each group. * versus S ($P < 0.05$), ** versus S ($P < 0.01$) and *** versus S ($P < 0.001$)

in angiotensin II-induced hypertension in streptozotocin-induced diabetic rats [12, 13]. Improvement in vascular responses in OBE-treated SHR also suggests higher bioavailability of NO [36]. Hence, these results with SHR support the cardioprotective roles of ellagitannins derived from oak bark.

In conclusion, the health effects of red wine have been attributed to polyphenols such as resveratrol, rather than to oak-derived ellagitannins. This study has defined the protective effects of an oak bark extract containing vescalagin, castalagin, grandinin and roburin E on the heart and the liver together with an improved metabolic profile in two rat models of human metabolic syndrome and human hypertension. These results imply that the ellagitannins from oak may be important mediators of the benefits of red wine in humans due to their antioxidant and anti-inflammatory responses. Clinical trials should be considered with these ellagitannin extracts.

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Conflict of interest No conflicts of interest.

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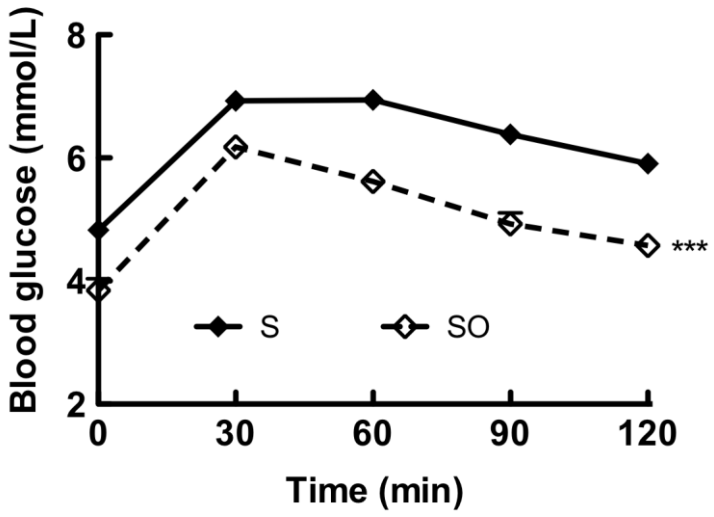
Online Resource

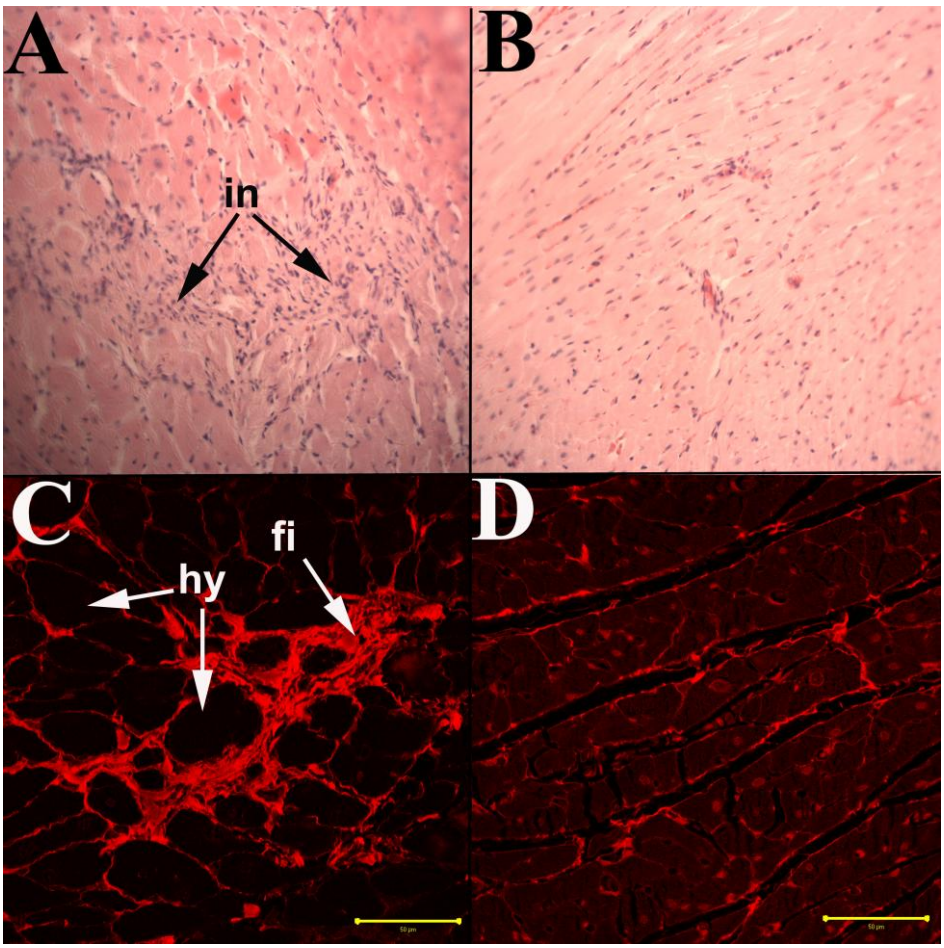
Online Resource 1. Effects of OBE on physiological, metabolic, oxidative stress and inflammatory parameters in SHR.

Variables	*S*	*SO*
Physiological variables		
Initial body weight (g)	426 ± 10	418 ± 6
Final body weight (g)	467 ± 6	466 ± 8
Water intake (mL/day)	34.5 ± 0.8	36.4 ± 1.1
Food intake (g/day)	30.3 ± 0.8	30.4 ± 0.6
Energy intake (kJ/day)	418 ± 11	450 ± 14
BMI (g/cm^2)	0.78 ± 0.01	0.76 ± 0.01
Feed efficiency (g/kJ)	0.10 ± 0.01	0.11 ± 0.01
Abdominal circumference (cm)	21.8 ± 0.4	22.3 ± 0.5
Abdominal fat pads (mg/mm tibial length)	226 ± 17	147 ± 20**
Metabolic variables		
Basal blood glucose (mmol/L)	4.8 ± 0.3	4.0 ± 0.2*
AUC (mmol/L·min)	745 ± 15	668 ± 13**
Plasma total cholesterol (mmol/L)	1.7 ± 0.1	1.5 ± 0.1
Plasma triglycerides (mmol/L)	0.6 ± 0.1	0.5 ± 0.1
Plasma non-esterified fatty acids (mmol/L)	1.8 ± 0.1	1.6 ± 0.1
Plasma urea (mmol/L)	4.0 ± 0.2	4.4 ± 0.3
Plasma uric acid (μmol/L)	44.2 ± 2.2	40.2 ± 1.8
Oxidative stress and inflammatory markers		
Plasma malondialdehyde (μmol/L)	32.5 ± 1.1	28.1 ± 1.2*
Plasma glutathione peroxidase activity (U/L)	926 ± 31	1015 ± 22*
Plasma C-reactive protein (μmol/L)	3.53 ± 0.15	2.12 ± 0.16***

Values are mean ± SEM and n = 8 - 10 for each group. * vs. S and $P < 0.05$, ** vs. S and $P < 0.01$, *** vs. S and $P < 0.001$. S, SHR; SO, SHR supplemented with OBE.



Online Resource 2. Effects of OBE on oral glucose tolerance in S and SO rats. Values are mean ± SEM and n = 10 for each group. *** vs. S ($P < 0.001$). S, SHR; SO, SHR supplemented with OBE.



Online Resource 3. Inflammation and fibrosis in the hearts from SHR and SHR supplemented with OBE. Haematoxylin and eosin staining of left ventricle showing infiltration of inflammatory cells (A & B, inflammatory cells as dark spots surrounding the myocytes marked as "in") (×40) from SHR (A) and SHR supplemented with OBE (B) rats. Picrosirius red staining of left ventricle showing collagen deposition (C & D, fibrosis marked as "fi" and hypertrophied cardiomyocytes as "hy") (×40) from SHR (C) and SHR supplemented with OBE (D) rats.

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ORIGINAL CONTRIBUTION

Chapter 5.2

Ellagic acid attenuates high-carbohydrate, high-fat diet-induced metabolic syndrome in rats

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Abstract
Background Fruits and nuts may prevent or reverse common human health conditions such as obesity, diabetes and hypertension; together, these conditions are referred to as metabolic syndrome, an increasing problem. This study has investigated the responses to ellagic acid, present in many fruits and nuts, in a diet-induced rat model of metabolic syndrome.
Methods Eight- to nine-week-old male Wistar rats were divided into four groups for 16-week feeding with cornstarch diet (*C*), cornstarch diet supplemented with ellagic acid (CE), high-carbohydrate, high-fat diet (*H*) and high-carbohydrate, high-fat diet supplemented with ellagic acid (HE). CE and HE rats were given 0.8 g/kg ellagic acid in food from week 8 to 16 only. At the end of 16 weeks, cardiovascular, hepatic and metabolic parameters along with protein levels of Nrf2, NF-κB and CPT1 in the heart and the liver were characterised.
Results High-carbohydrate, high-fat diet-fed rats developed cardiovascular remodelling, impaired ventricular function, impaired glucose tolerance, non-alcoholic fatty liver disease with increased protein levels of NF-κB and decreased protein levels of Nrf2 and CPT1 in the heart and the liver. Ellagic acid attenuated these diet-induced symptoms of metabolic syndrome with normalisation of protein levels of Nrf2, NF-κB and CPT1.
Conclusions Ellagic acid derived from nuts and fruits such as raspberries and pomegranates may provide a useful dietary supplement to decrease the characteristic changes in metabolism and in cardiac and hepatic structure and function induced by a high-carbohydrate, high-fat diet by suppressing oxidative stress and inflammation.

Keywords Cardiovascular remodelling · Ellagic acid · Metabolic syndrome · Non-alcoholic fatty liver disease · Obesity

Introduction

Metabolic syndrome is the presence of multiple risk factors for the development of cardiovascular disease and non-alcoholic fatty liver disease (NAFLD) including hypertension, insulin resistance, obesity and dyslipidaemia [1–4]. The prevalence of metabolic syndrome, cardiovascular disease and NAFLD is increasing throughout the world, in both developed and developing countries [5–7]. Thus, it is important to establish simple therapeutic concepts, such as dietary interventions, that decrease the incidence and symptoms of metabolic syndrome, as well as cardiovascular disease and NAFLD. One of the target strategies in the treatment of NAFLD is to increase the oxidation of fat in the liver, for example, by activation of the mitochondrial regulation of fatty acid oxidation by carnitine palmitoyltransferase 1 (CPT1) [8, 9]. Further targets include the regulators of oxidative stress and inflammation such as Nrf2 and NF-κB, respectively, that lead to cardiac and

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hepatic damage [10, 11] associated with cardiovascular disease and NAFLD [12–17]. Normalising the protein levels of these two transcription factors could prevent organ damage in these conditions.

A potential dietary intervention is ellagic acid, a polyphenol found in a wide variety of nuts as well as in fruits such as raspberries, pomegranates, grapes and blackcurrants [18, 19]. Ellagic acid is a dilactone formed from two moieties of gallic acid. Ellagic acid, a radical scavenger [20–22], induced cardioprotection against isoproterenol-induced myocardial infarction [23, 24] and also showed anti-proliferative effects in cancerous cell lines [25]. Further, ellagic acid reduced the damage in a rat model of Crohn's disease, alleviated the oxidative events and returned the levels of pro-inflammatory proteins to basal levels probably through MAPKs and NF-κB signalling pathways [26]. Increased Cu and Zn concentrations in the liver and serum of cholestatic rats were controlled by ellagic acid [27]. Hepatoprotective effects with ellagic acid were measured in vitro and in vivo in models of hepatic damage [28, 29]. We have previously reported that oak ellagitannins, complex esters of ellagic acid with glucose, improved the symptoms of metabolic syndrome in diet-induced obese rats [30].

This study has defined the responses following chronic treatment with ellagic acid on the components of metabolic syndrome and the associated complications including cardiovascular remodelling and fatty liver in a rodent model of diet-induced metabolic syndrome. Rats were fed with either cornstarch or high-carbohydrate, high-fat diets for 16 weeks. These diets were supplemented with ellagic acid for the last 8 weeks of the protocol. At the end of 16 weeks, metabolic parameters and structure and function of the heart and the liver were assessed. Protein levels of CPT1, NF-κB and Nrf2 in the heart and the liver were measured to study potential mechanisms for the action of ellagic acid.

Materials and methods

Rats and diets

All experimental protocols were approved by The University of Queensland Animal Experimentation Ethics Committee, under the guidelines of the National Health and Medical Research Council of Australia. Male Wistar rats (8–9 weeks old, 337 ± 2 g, $n = 47$) were obtained from The University of Queensland Biological Resources facility. Rats were randomly divided into four groups: cornstarch diet-fed rats (*C*; $n = 12$), cornstarch diet-fed rats supplemented with ellagic acid (CE; 0.8 g/kg food; $n = 11$; MP Biomedicals, Seven Hills, Australia), high-carbohydrate, high-fat diet-fed rats (*H*; $n = 12$) and high-carbohydrate, high-fat diet-fed rats supplemented with ellagic acid (HE; 0.8 g/kg food; $n = 12$). *C* and *H* rats were fed with cornstarch and high-carbohydrate, high-fat diets, respectively, for 16 weeks. CE and HE rats were also fed with cornstarch and high-carbohydrate, high-fat diets, respectively, for 16 weeks with the diets supplemented with ellagic acid (0.8 g/kg food) for the last 8 weeks of the protocol. The cornstarch and high-carbohydrate, high-fat diets have been previously described in detail [31–33]. Drinking water for *H* and HE rats was supplemented with 25 % fructose, whereas *C* and CE rats were given drinking water without any additive. All the rats were individually housed in temperature-controlled 12-h light/dark conditions and were given ad libitum access to food and water.

Physiological measurements

Body weight, food and water intakes were measured daily for all rats. Abdominal circumference and body length were measured at the end of the protocol using a standard measuring tape under light anaesthesia with Zoletil (tiletamine 10 mg/kg, zolazepam 10 mg/kg, i.p.; Virbac, Peakhurst, Australia) [31]. Energy intake, body mass index and feed efficiency were calculated as in previous study [31].

Systolic blood pressure measurements

Systolic blood pressure of rats was measured at the end of the protocol under light anaesthesia with Zoletil (tiletamine 10 mg/kg, zolazepam 10 mg/kg, i.p.), using an MLT1010 Piezo-Electric Pulse Transducer (ADInstruments, Sydney, Australia) and inflatable tail cuff connected to a MLT844 Piezo-Electric Pressure Transducer (ADInstruments, Sydney, Australia) and PowerLab data acquisition unit (ADInstruments, Sydney, Australia) [31].

Echocardiography

Echocardiographic examinations (Phillips iE33, 12-MHz transducer) were performed in all rats at the end of protocol as previously described [31]. Briefly, rats were anaesthetised using Zoletil (tiletamine 25 mg/kg and zolazepam 25 mg/kg, i.p.; Virbac, Peakhurst, Australia) and Ilium Xylazil (xylazine 15 mg/kg, i.p.; Troy Laboratories, Smithfield, Australia) and positioned in dorsal recumbency. Electrodes attached to the skin overlying the elbows and right stifle facilitated the simultaneous recording of a lead II electrocardiogram [31].

Body composition measurements

Dual-energy X-ray absorptiometric measurements were performed at the end of the protocol using a Norland XR36

DXA instrument (Norland Corp, Fort Atkinson, WI) under anaesthesia with Zoletil (tiletamine 25 mg/kg and zolazepam 25 mg/kg, i.p.) and Ilium Xylazil (xylazine 15 mg/kg, i.p.). Scans were analysed using the manufacturer's recommended software for use in laboratory animals (Small Subject Analysis Software, version 2.5.3/1.3.1; Norland Corp) as previously described [34]. The precision error of lean mass for replicate measurements, with repositioning, was 3.2 %.

Oral glucose tolerance test

At the end of the protocol, rats were deprived of food for 12 h for oral glucose tolerance testing. During this food deprivation period, fructose-supplemented drinking water in *H* and HE groups was replaced with normal drinking water. Oral glucose tolerance tests were performed after determining basal blood glucose concentrations in tail vein blood using Medisense Precision Q.I.D. glucose meters (Abbott Laboratories, Bedford, MA). 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 min after oral glucose administration [31]. Blood glucose concentrations over the period of 120 min were used to calculate area under the curve.

Terminal experiments

Rats were euthanised with Lethabarb (pentobarbitone sodium, 100 mg/kg, i.p.; Virbac, Peakhurst, Australia). After euthanasia, heparin (200 IU; Sigma-Aldrich Australia, Sydney, Australia) was injected through the right femoral vein. The abdomen was then opened and blood ($\sim$5 mL) was withdrawn from the abdominal aorta and collected into heparinised tubes. Blood was centrifuged at 5,000$\times g$ for 15 min to obtain plasma. Hearts were removed and were used as an isolated Langendorff heart preparation.

Isolated Langendorff heart preparation

The isolated Langendorff heart preparation assessed left ventricular function of the rats in all the groups as in previous studies [31–33]. Hearts isolated from euthanised 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 (ADInstruments, Sydney, Australia). All left ventricular end-diastolic pressure values were measured during pacing of the heart at 250 beats per min using an electrical stimulator. End-diastolic pressures were obtained from 0 to 30 mmHg for the calculation of diastolic stiffness constant (κ, dimensionless) as described in previous studies [31–33].

Vascular reactivity

Thoracic aortic rings ($\sim$4 mm in length; $n = 11$–12 from each group) 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 $\sim$10 mN. Cumulative concentration–response curves (contraction) were obtained for noradrenaline (Sigma-Aldrich Australia, Sydney, Australia) and cumulative concentration–response curves (relaxation) were obtained for sodium nitroprusside (Sigma-Aldrich Australia, Sydney, Australia) and acetylcholine (Sigma-Aldrich Australia, Sydney, Australia) following submaximal (70 %) contraction to noradrenaline [31].

Organ weights

After isolated heart perfusion studies, hearts ($n = 8$–9 from each group) were separated into left ventricles (with septum) and right ventricles and weighed. Livers ($n = 8$–9 from each group) were isolated and weighed. Retroperitoneal, epididymal and omental abdominal fat pads were removed separately and weighed. These organ weights were normalised against the tibial length (48.2 $\pm$ 0.1 mm, $n = 35$) at the time of organ removal and expressed as mg/mm of tibial length [31].

Histology

Histology of the heart

Hearts were removed from the rats ($n = 3$ from each group) soon after euthanasia and these hearts were fixed in 10 % neutral buffered formalin for 3 days. The samples were then dehydrated and embedded in paraffin wax. Thin sections (5 μm) of left ventricle were cut and stained with haematoxylin and eosin to study infiltration of inflammatory cells and picrosirius red to study collagen deposition [31].

Histology of the liver

Liver portions were isolated ($n = 3$) and fixed in 10 % neutral buffered formalin for three days. These tissue samples were dehydrated and then embedded in paraffin wax. Thin sections (5 μm) of these tissues were cut and stained with haematoxylin and eosin for the determination

of inflammatory cell infiltration (20×) and for determining the fat vacuoles (40×) in liver. Liver sections were also stained with Milligan's trichrome stain to determine portal fibrosis (20×) [31].

Plasma biochemistry

Plasma concentrations of total cholesterol and triglycerides were determined using kits and controls supplied by Olympus using an Olympus analyser (AU 400, Tokyo, Japan) [31]. Non-esterified fatty acids (NEFA) in plasma were determined using a commercial kit (Wako, Osaka, Japan) [31]. Plasma activity of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and the concentrations of albumin, total bilirubin, urea and uric acid were determined using kits and controls supplied by Olympus using an Olympus analyser (AU 400, Tokyo, Japan) [31]. Plasma C-reactive protein (BD Bioscience, Brisbane, Australia) concentrations were measured using commercial kits according to manufacturer-provided standards and protocols.

Regulatory protein levels in the heart and the liver

Heart and liver samples isolated from rats ($n = 3$ from each group) were stored at −80 °C. These samples were homogenised and sonicated after adding cell lysis buffer followed by ultracentrifugation at 100,000×g for 30 min at 4 °C. Supernatants were used to measure protein concentration in each sample by bicinconinic acid method (Thermo Scientific). Supernatants with equal protein amounts (40 µg) from each group were used in Western blot analysis to study the protein levels of CPT-1, Nrf2 (antibodies from Santa Cruz Biotechnology, Santa Cruz, CA), NF-κB (antibody from Cell Signaling Technology, Danvers, MA) and β-actin (antibody from Sigma-Aldrich Corp., St. Louis, MO) in the heart and the liver.

Statistical analysis

Values 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 function) prior to statistical analyses. All the groups were tested for effects of diet (D), treatment (E) and their interactions ($D \times E$) by two-way ANOVA. When interaction and/or the main effects were significant, means were compared using Newman–Keuls multiple comparison post-test. Mean ellagic acid intakes between CE and HE groups were compared using Student's t-test. $P < 0.05$ was considered significant. All statistical analyses were performed using GraphPad Prism version 5.00 for Windows.

Results

Physiological and metabolic parameters

Table 1 presents the effects of ellagic acid on physiological and metabolic parameters in rats. Mean ellagic acid intake was higher in CE rats (63.4 ± 1.8 mg/kg/day) compared with HE rats (35.6 ± 1.0 mg/kg/day) due to differences in food intake and body weights. Food intake and water intake were decreased in *H* rats compared with *C* rats; ellagic acid reduced food intake in HE rats without changing water intake, whereas it reduced water intake in CE rats without changing food intake. Energy intake was increased due to higher energy content of *H* diet and H rats showed higher feed efficiency than *C* rats. Energy intake was unaffected by ellagic acid supplementation in both CE and HE rats. Feed efficiency was lower in HE rats than in *H* rats. *H* rats gained more weight than *C* rats over the period of 16 weeks. The whole-body fat mass was almost doubled in *H* rats compared to *C* rats without any change in lean mass. The increase in abdominal fat deposition, as indicated by increases in abdominal circumference and body mass index, included the increases in the retroperitoneal, epididymal and omental fat deposits. Body weight and lean mass were lowered with ellagic acid supplementation in HE rats without any change in whole-body fat mass. Body weight and lean mass were unchanged in CE rats. Retroperitoneal and omental fat were normalised in HE rats, while epididymal fat was lowered. Retroperitoneal and omental fat were lowered in CE rats compared to *C* rats without any change in epididymal fat. Total abdominal fat deposition was lowered in both HE and CE rats compared with *H* and *C* rats, respectively. Basal blood glucose concentrations were increased in *H* rats compared with *C* rats. During oral glucose tolerance test, area under the curve was increased for *H* rats compared with *C* rats. During oral glucose tolerance test, ellagic acid supplementation lowered fasting blood glucose concentrations along with area under the curve in HE rats compared with *H* rats. Plasma concentrations of triglycerides, total cholesterol and NEFA were increased in *H* rats compared with *C* rats. Plasma concentrations of triglycerides, total cholesterol and NEFA were decreased in HE rats. Plasma concentrations of total cholesterol were decreased in CE rats without any changes in plasma concentrations of triglycerides and NEFA. Plasma uric acid concentrations were increased, whereas plasma urea concentrations were decreased in *H* rats. Plasma uric acid concentrations were decreased, whereas plasma urea concentrations were increased in HE rats; these parameters were unaffected in CE rats. Plasma C-reactive protein concentrations were higher in *H* rats than in *C* rats and were decreased in HE rats compared with *H* rats.

Table 1 Effects of ellagic acid on physiological and metabolic variables in *C*, CE, *H* and HE rats

Variables	*P* value						
	C	CE	*H*	HE	*D*	*E*	*D* × *E*
Physiological variables							
Initial body weight (g)	338 ± 2	339 ± 2	342 ± 2	336 ± 2	0.77	0.14	0.06
Final body weight (g)	421 ± 4^{c}	427 ± 8^{c}	515 ± 8^{a}	473 ± 11^{b}	<0.0001	0.031	0.005
Food intake (g/day)	30.9 ± 0.7^{a}	31.6 ± 0.5^{a}	23.2 ± 0.6^{b}	21.0 ± 0.4^{c}	<0.0001	0.19	0.014
Water intake (mL/day)	32.1 ± 0.7^{a}	30.1 ± 0.5^{b}	20.2 ± 0.7^{c}	19.0 ± 0.4^{c}	<0.0001	0.01	0.50
Energy intake (kJ/day)	359 ± 8^{b}	355 ± 5^{b}	466 ± 8^{a}	447 ± 6^{a}	<0.0001	0.11	0.29
Feed efficiency (g/kJ)	0.23 ± 0.01^{c}	0.25 ± 0.02^{c}	0.39 ± 0.01^{a}	0.31 ± 0.02^{b}	<0.0001	0.06	0.003
Body mass index (g/cm^{2})	0.63 ± 0.01^{b}	0.66 ± 0.01^{b}	0.72 ± 0.01^{a}	0.72 ± 0.02^{a}	<0.0001	0.27	0.27
Abdominal circumference (cm)	19.6 ± 0.4^{c}	19.6 ± 0.2^{c}	23.3 ± 0.4^{a}	21.3 ± 0.3^{b}	<0.0001	0.005	0.005
Retroperitoneal fat (mg/mm tibial length)	231 ± 13^{b}	146 ± 12^{c}	378 ± 31^{a}	247 ± 13^{b}	<0.0001	<0.0001	0.24
Epididymal fat (mg/mm tibial length)	120 ± 10^{c}	91 ± 7^{c}	240 ± 12^{a}	157 ± 11^{b}	<0.0001	<0.0001	0.012
Omental fat (mg/mm tibial length)	103 ± 9^{b}	67 ± 5^{c}	199 ± 10^{a}	116 ± 7^{b}	<0.0001	<0.0001	0.006
Total abdominal fat (mg/mm tibial length)	454 ± 20^{b}	303 ± 24^{c}	817 ± 32^{a}	520 ± 29^{b}	<0.0001	<0.0001	0.009
Whole-body fat mass (g)	81 ± 7^{b}	113 ± 12^{b}	151 ± 11^{a}	156 ± 16^{a}	<0.0001	0.13	0.27
Whole-body lean mass (g)	307 ± 8^{ab}	298 ± 8^{b}	326 ± 9^{a}	281 ± 4^{b}	0.89	0.001	0.023
Metabolic variables							
Basal blood glucose (mmol/L)	4.1 ± 0.1^{b}	4.3 ± 0.1^{b}	5.1 ± 0.1^{a}	3.9 ± 0.2^{b}	0.03	0.0005	<0.0001
Area under the curve (mmol/L·min)	685 ± 16^{b}	666 ± 14^{b}	776 ± 12^{a}	696 ± 11^{b}	<0.0001	0.0006	0.028
Plasma triglyceride (mmol/L)	0.5 ± 0.1^{b}	0.7 ± 0.1^{b}	1.2 ± 0.1^{a}	0.5 ± 0.1^{b}	0.017	0.017	<0.0001
Plasma total cholesterol (mmol/L)	1.5 ± 0.1^{a}	0.8 ± 0.1^{c}	2.1 ± 0.2^{a}	1.5 ± 0.1^{b}	<0.0001	<0.0001	0.71
Plasma NEFA (mmol/L)	1.3 ± 0.2^{b}	1.4 ± 0.1^{b}	2.9 ± 0.4^{a}	1.7 ± 0.2^{b}	0.0006	0.037	0.014
Plasma uric acid (μmol/L)	36.5 ± 3.3^{b}	40.5 ± 3.9^{b}	58.9 ± 6.2^{a}	44.3 ± 3.8^{b}	0.005	0.24	0.043
Plasma urea (mmol/L)	6.0 ± 0.3^{a}	6.9 ± 0.3^{a}	3.3 ± 0.2^{b}	6.2 ± 0.4^{a}	<0.0001	<0.0001	0.002
Plasma C-reactive protein (μmol/L)	2.22 ± 0.09^{b}	2.35 ± 0.07^{b}	2.69 ± 0.08^{a}	2.40 ± 0.07^{b}	0.002	0.31	0.011

Values are mean ± SEM and $n = 8$–12 for each group. Mean values within a row with unlike superscript letters are significantly different ($P < 0.05$)

C cornstarch diet-fed rats, *CE* cornstarch diet-fed rats supplemented with ellagic acid, *H* high-carbohydrate, high-fat diet-fed rats, *HE* high-carbohydrate, high-fat diet-fed rats supplemented with ellagic acid

Cardiovascular structure and function

H rats showed cardiovascular abnormalities as increases in systolic blood pressure, left ventricular internal diameter during diastole and systole (LVIDd and LVIDs, respectively), systolic volume and diastolic stiffness along with increased infiltration of inflammatory cells and increased collagen deposition (Table 2; Fig. 1c, g) compared to *C* rats (Fig. 1a, e). These changes were associated with decreases in left ventricular function including fractional shortening and ejection fraction (Table 2). Ellagic acid reduced systolic blood pressure in both CE and HE rats compared with *C* and *H* rats, respectively (Table 2), whereas it improved structural and functional parameters in HE rats without changing those parameters in CE rats. Left ventricular wet weights (with septum) and right ventricular wet weights were unchanged between the groups (Table 2). Infiltration of inflammatory cells and collagen deposition were reduced in left ventricle of HE rats (Fig. 1d, h).

H rats showed reduced aortic contractile responses to noradrenaline and relaxation responses to sodium nitroprusside and acetylcholine. Ellagic acid improved these responses in HE rats without changing responses in CE rats (Fig. 2a–c).

Hepatic structure and function

Livers from *H* rats showed infiltration of inflammatory cells, steatosis and portal fibrosis (Fig. 3c, g, k). These livers also showed higher wet weights along with increased plasma activities of ALT, AST, ALP and LDH indicating the liver damage caused by *H* diet (Table 2). Ellagic acid supplementation in the diet attenuated the high-carbohydrate, high-fat diet-induced changes in the liver and plasma activities of the enzymes from HE rats (Fig. 3d, h, l; Table 2). Plasma concentrations of albumin and total bilirubin were unchanged between the groups (Table 2).

Table 2 Effects of ellagic acid on cardiovascular and hepatic variables in *C*, CE, *H* and HE rats

Variables	*P* value						
	C	CE	*H*	HE	*D*	*E*	*D* × *E*
Cardiovascular variables							
Systolic blood pressure (mmHg)	129 ± 1^{c}	112 ± 1^{d}	145 ± 1^{a}	133 ± 2^{b}	<0.0001	<0.0001	0.07
LVIDd (mm)	6.62 ± 0.14^{b}	6.28 ± 0.14^{b}	7.39 ± 0.10^{a}	6.66 ± 0.12^{b}	<0.0001	0.0001	0.13
LVIDs (mm)	3.35 ± 0.20^{b}	3.46 ± 0.15^{b}	4.84 ± 0.14^{a}	3.70 ± 0.26^{b}	<0.0001	0.012	0.003
Systolic volume (μL)	42.5 ± 7.4^{b}	45.3 ± 5.5^{b}	109.2 ± 8.3^{a}	59.3 ± 6.3^{b}	<0.0001	0.002	0.0005
Fractional shortening (%)	51.9 ± 1.2^{a}	53.1 ± 2.4^{a}	38.6 ± 1.5^{b}	48.4 ± 2.3^{a}	<0.0001	0.006	0.029
Ejection fraction (%)	86.6 ± 1.8^{a}	82.5 ± 2.0^{a}	71.5 ± 1.3^{b}	81.1 ± 2.7^{a}	0.0002	0.18	0.002
Estimated left ventricular mass (g)	0.67 ± 0.02^{c}	0.66 ± 0.02^{c}	0.91 ± 0.03^{a}	0.79 ± 0.03^{b}	<0.0001	0.015	0.038
Left ventricular + septum wet weight (mg/mm tibial length)	20.5 ± 0.6	21.1 ± 0.7	22.1 ± 0.7	21.0 ± 0.4	0.23	0.68	0.17
Right ventricular wet weight (mg/mm tibial length)	4.63 ± 0.21	4.58 ± 0.16	4.48 ± 0.25	4.56 ± 0.19	0.68	0.94	0.76
Left ventricular diastolic stiffness constant, κ	20.5 ± 0.8^{b}	21.2 ± 1.1^{b}	27.3 ± 1.3^{a}	23.1 ± 0.9^{b}	0.0002	0.10	0.025
Hepatic variables							
Liver wet weight (mg/mm tibial length)	259 ± 11^{b}	270 ± 8^{b}	302 ± 10^{a}	272 ± 5^{b}	0.015	0.29	0.025
Plasma ALT activity (U/L)	35.3 ± 2.7^{b}	34.4 ± 2.1^{b}	57.6 ± 3.5^{a}	34.5 ± 2.9^{b}	0.0003	0.0001	0.0004
Plasma AST activity (U/L)	77 ± 6^{b}	87 ± 4^{b}	105 ± 8^{a}	86 ± 5^{b}	0.007	0.22	0.04
Plasma ALP activity (U/L)	177 ± 14^{b}	174 ± 16^{b}	279 ± 18^{a}	149 ± 18^{b}	0.026	0.0002	0.0004
Plasma LDH activity (U/L)	272 ± 33^{b}	283 ± 23^{b}	417 ± 31^{a}	287 ± 27^{b}	0.014	0.047	0.019
Plasma albumin concentration (g/L)	28.1 ± 0.5	28.6 ± 0.3	28.5 ± 0.5	28.5 ± 0.4	0.73	0.57	0.57
Plasma total bilirubin (μmol/L)	2.3 ± 0.1	2.6 ± 0.2	2.5 ± 0.2	2.5 ± 0.1	0.75	0.34	0.34

Values are mean ± SEM and $n = 8$–12 for each group. Mean values within a row with unlike superscript letters are significantly different ($P < 0.05$)

C cornstarch diet-fed rats, *CE* cornstarch diet-fed rats supplemented with ellagic acid, *H* high-carbohydrate, high-fat diet-fed rats, *HE* high-carbohydrate, high-fat diet-fed rats supplemented with ellagic acid

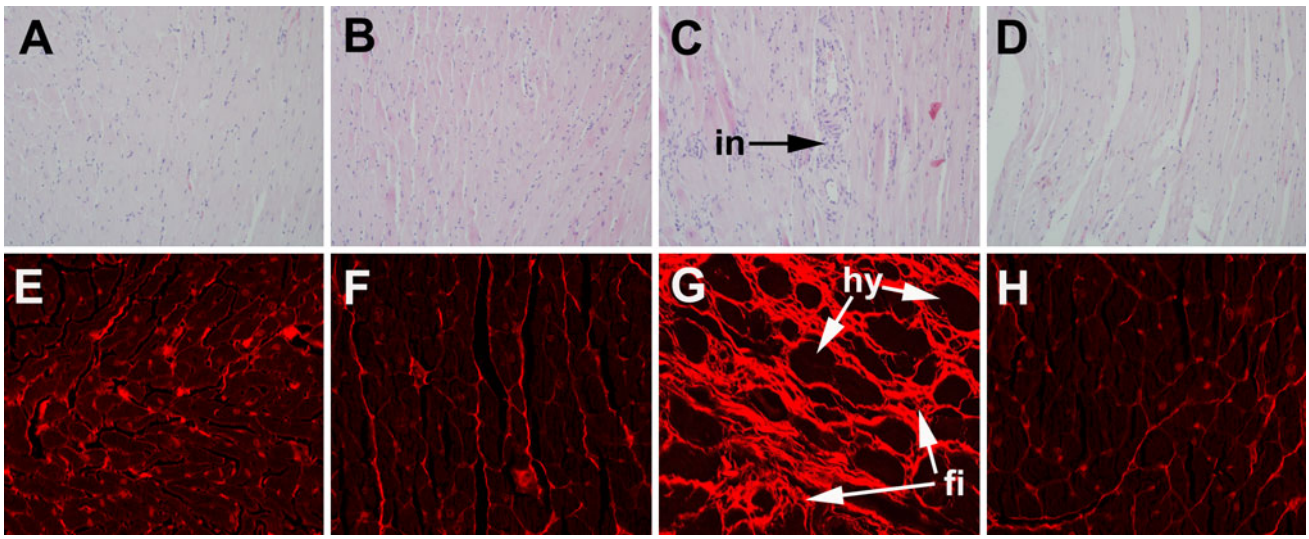
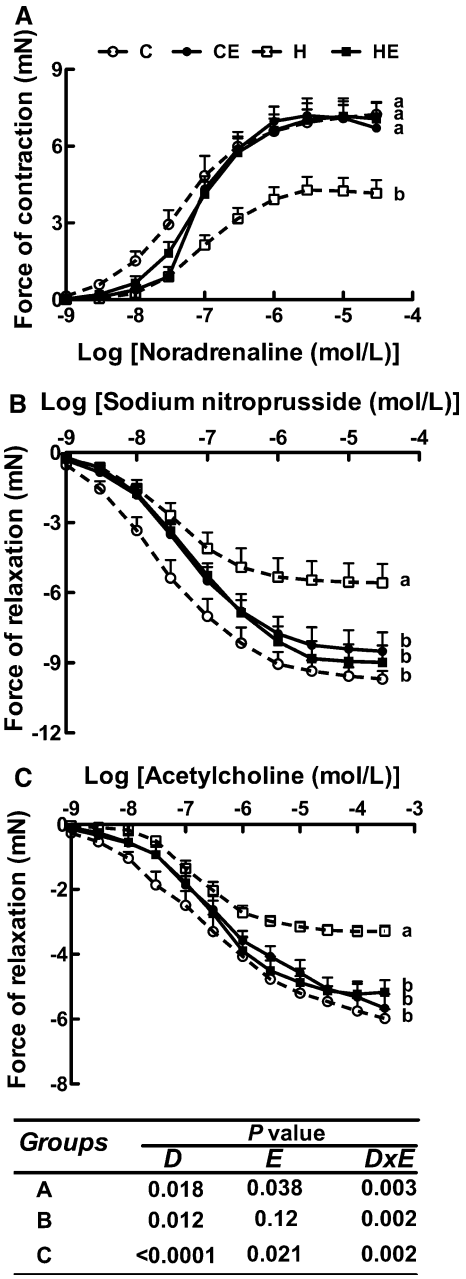


Fig. 1 Effects of ellagic acid supplementation on inflammation and fibrosis in the heart ($n = 3$ per group). Haematoxylin and eosin staining of left ventricle showing infiltration of inflammatory cells (**a**–**d**, inflammatory cells marked as 'in'; ×20) from cornstarch diet-fed rats (**a**), cornstarch diet-fed rats supplemented with ellagic acid (**b**), high-carbohydrate, high-fat diet-fed rats (**c**) and high-carbohydrate, high-fat diet-fed rats supplemented with ellagic acid (**d**). Picrosirius red staining of left ventricle showing collagen deposition and hypertrophy (**e**–**h**, fibrosis marked as 'fi' and hypertrophied cardiomyocytes as 'hy'; ×40) from cornstarch diet-fed rats (**e**), cornstarch diet-fed rats supplemented with ellagic acid (**f**), high-carbohydrate, high-fat diet-fed rats (**g**) and high-carbohydrate, high-fat diet-fed rats supplemented with ellagic acid (**h**)



Groups	P value		
	D	E	DxE
A	0.018	0.038	0.003
B	0.012	0.12	0.002
C	<0.0001	0.021	0.002

Fig. 2 Effects of ellagic acid supplementation on vascular responses. Noradrenaline-induced contraction (**a**), sodium nitroprusside-induced relaxation (**b**) and acetylcholine-induced relaxation (**c**) in thoracic aortic preparations from *C*, CE, *H* and HE rats. Values are mean ± SEM, $n = 10$–12. End-point means without a common letter differ, $P < 0.05$. *C*, cornstarch diet-fed rats; *CE*, cornstarch diet-fed rats supplemented with ellagic acid; *H*, high-carbohydrate, high-fat diet-fed rats; *HE*, high-carbohydrate, high-fat diet-fed rats supplemented with ellagic acid

Regulatory protein levels in the heart and the liver

In the hearts of *H* rats, protein levels of NF-κB were higher than in *C* rats, whereas the protein levels of CPT1 were lower than in *C* rats. These changes were normalised in HE rats. There was no difference in the cardiac protein levels of Nrf2 between the groups (Fig. 4a, c). In the liver from *H* rats, protein levels of Nrf2 and CPT1 were decreased, whereas NF-κB protein levels were enhanced compared with *C* rats. Protein levels of Nrf2 and CPT1 in the liver were increased in both CE and HE rats compared with *C* and *H* rats, respectively, whereas NF-κB protein levels were lowered in both CE and HE rats compared with *C* and *H* rats, respectively (Fig. 4b, d).

Discussion

Metabolic syndrome is the clustering of risk factors for cardiovascular disease, NAFLD and type 2 diabetes. These risk factors, such as hypertension and dyslipidaemia, are also responsible for the increased morbidity and mortality in humans. Thus, it is important to establish biological targets for the reduction of risk factors and treatment of this syndrome. Natural products that are rich in phytochemicals may be effective against this syndrome. Our previous studies with natural products including oak bark extract (rich in ellagitannins) [30], purple carrot juice (rich in anthocyanins) [35], olive leaf extract (rich in oleorupein) [32], chia seeds (rich in α-linolenic acid) [33] and studies with pure phytochemicals from natural products including rutin [36] and piperine [37] in diet-induced obese rats have shown promising results against metabolic syndrome. These studies have also defined the in vivo antioxidant and anti-inflammatory effects of these natural products. Ellagic acid is a phytochemical found in nuts and fruits and is part of the human diet. Thus, we investigated the responses to dietary ellagic acid supplementation on the targets in metabolic syndrome.

Rats fed with *H* diet for 16 weeks presented with the symptoms as well as the associated complications of metabolic syndrome, including central obesity, dyslipidaemia, hypertension and impaired glucose tolerance. The cardiovascular complications included inflammation, cardiac hypertrophy, fibrosis, increased diastolic stiffness, increased ventricular dimensions, decreased ventricular function and decreased vascular responses. The hepatic complications included steatosis, inflammation and portal fibrosis along with increased plasma activities of transaminases. These results have previously been characterised in detail [31]. These changes were accompanied by decreased protein levels of Nrf2 and CPT1 and increased protein levels of NF-κB in the heart and the liver together with increased plasma C-reactive protein concentrations.

Ellagic acid improved hepatic and cardiovascular structure and function, and normalised metabolic parameters such as glucose tolerance, blood lipid components, central obesity and physiological parameters such as body

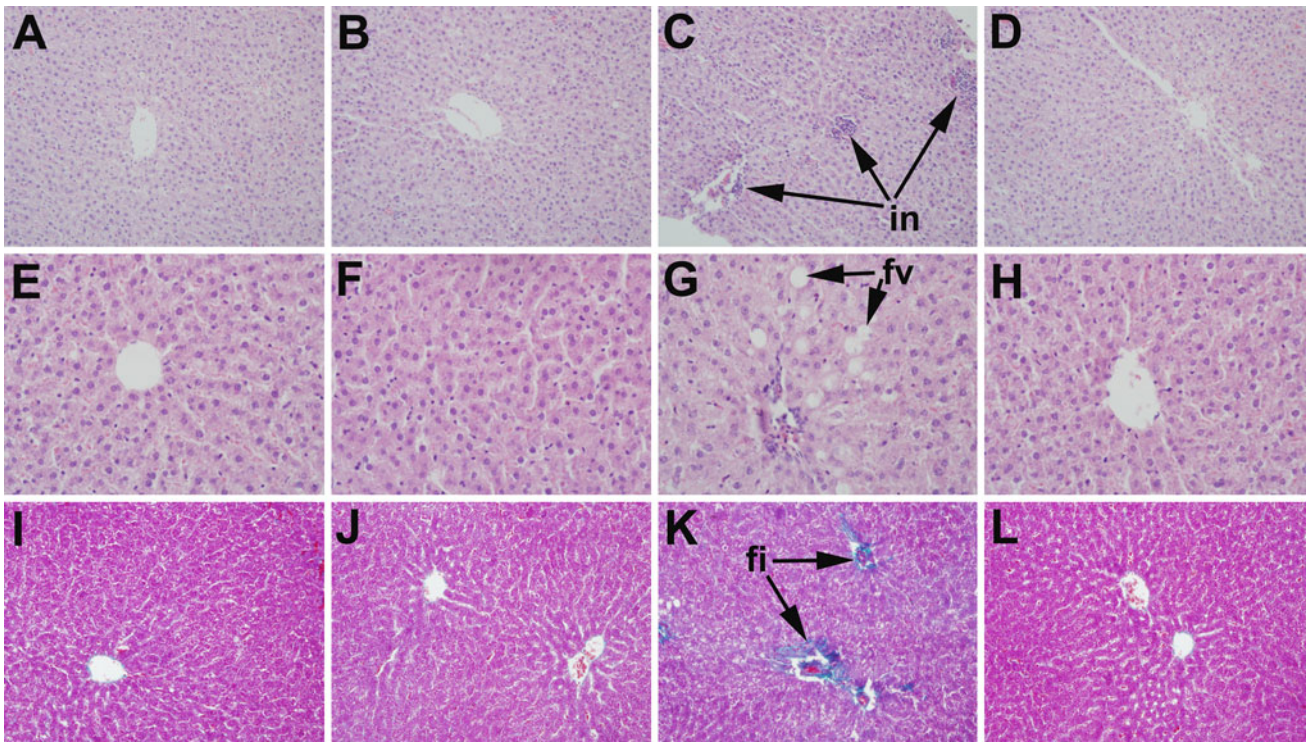


Fig. 3 Effects of ellagic acid supplementation on inflammation, fat deposition and fibrosis in the liver ($n = 3$ per group). Haematoxylin and eosin staining of the liver showing inflammatory cells (**a**–**d**, marked as 'in'; ×20) and enlarged fat vacuoles (**e**–**h**, marked as 'fv'; ×40) from cornstarch diet-fed rats (**a**, **e**), cornstarch diet-fed rats supplemented with ellagic acid (**b**, **f**), high-carbohydrate, high-fat diet-fed rats (**c**, **g**) and high-carbohydrate, high-fat diet-fed rats supplemented with ellagic acid (**d**, **h**) rats. Milligan's Trichrome staining of the liver showing fibrosis in the hepatic portal region (**i**–**l**, marked as 'fi'; ×20) from cornstarch diet-fed rats (**i**), cornstarch diet-fed rats supplemented with ellagic acid (**j**), high-carbohydrate, high-fat diet-fed rats (**k**) and high-carbohydrate, high-fat diet-fed rats supplemented with ellagic acid (**l**)

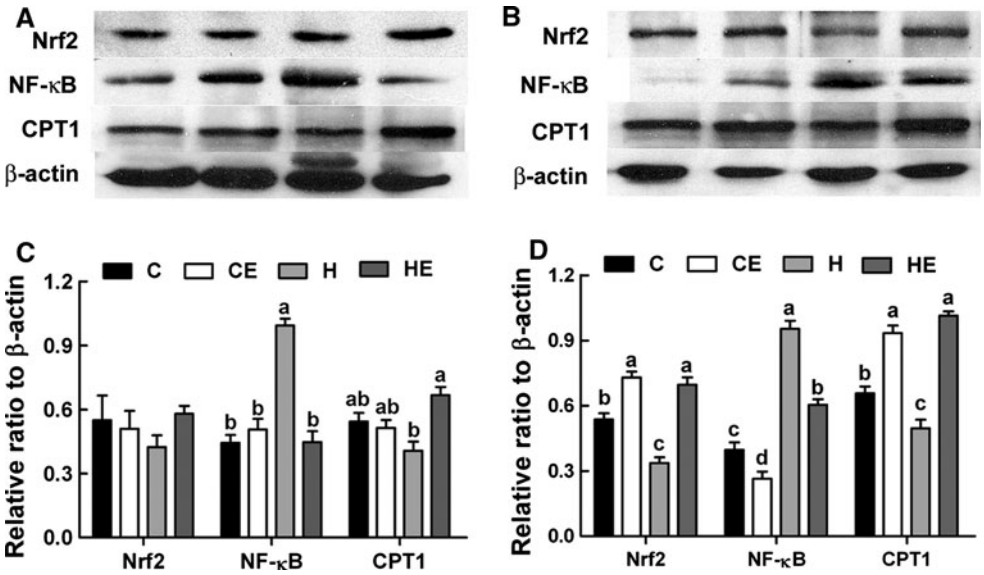


Fig. 4 Effects of ellagic acid supplementation on protein levels of Nrf2, NF-κB and CPT1 in the heart (**a**) and the liver (**b**). For quantitative analysis, the protein levels of these proteins were normalised against the protein levels of β-actin in the heart (**c**) and the liver (**d**). Values are mean ± SEM, $n = 3$. Means without a common letter differ, $P < 0.05$. *C*, cornstarch diet-fed rats; *CE*, cornstarch diet-fed rats supplemented with ellagic acid; *H*, high-carbohydrate, high-fat diet-fed rats; *HE*, high-carbohydrate, high-fat diet-fed rats supplemented with ellagic acid

weight. Reduced abdominal fat deposition without change in whole-body fat indicates lipid redistribution as seen with α-linolenic acid-rich chia seeds [33]. This redistribution of fat was accompanied by reduction in the blood lipid components and hepatic steatosis. The increased CPT1 protein levels in both the heart and the liver indicate that the redistribution of fat was accompanied by increased fatty acid oxidation.

Oxidative stress and inflammation, causing damage to the heart and the liver, were also targeted with ellagic acid

in this study. Nrf2 and NF-κB are important regulators of the oxidative stress and inflammation pathways, respectively [10, 38]. *H* diet decreased the protein levels of Nrf2 in the liver and increased NF-κB protein levels in the heart and the liver. Supplementation with ellagic acid reversed these changes and attenuated oxidative stress and inflammation in the heart and the liver.

The rats were given 0.8 g ellagic acid/kg of food for a dose of $\sim$50 mg/kg body weight/day. This dose corresponds to $\sim$1 g ellagic acid/day in a 70-kg human according to scaling equation [39] or $\sim$0.6 g/day according to body surface area comparisons between rats and humans [40]. Although the average daily human intake of ellagic acid is not known, the total intake of polyphenols is $\sim$1 g/day [41]. If the majority of polyphenols in the diet are taken from the fruits and nuts containing ellagic acid, then the above-mentioned dose is realistic in humans. One viable approach could be to provide this required amount through nutraceutical products containing partly purified ellagic acid from fruits and nuts as the major constituent.

In conclusion, high-carbohydrate, high-fat diet-induced symptoms of metabolic syndrome in rats were reversed by ellagic acid, accompanied by changes in protein levels of Nrf2, CPT1 and NF-κB. These results indicated that these proteins play important roles in the damage associated with metabolic syndrome and targeting these proteins with natural products can attenuate the complications in metabolic syndrome. Since the prevalence of metabolic syndrome, NAFLD and cardiovascular disease is still increasing in the population, the use of either purified ellagic acid as a complementary medicine or an increased dietary intake of fruits and nuts could be potentially effective intervention strategies.

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Conflict of interest No conflict of interest.

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Chapter 6.1

Coffee Extract Attenuates Changes in Cardiovascular and Hepatic Structure and Function without Decreasing Obesity in High-Carbohydrate, High-Fat Diet-Fed Male Rats[1–3]

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Abstract

Coffee, a rich source of natural products, including caffeine, chlorogenic acid, and diterpenoid alcohols, has been part of the human diet since the 15th century. In this study, we characterized the effects of Colombian coffee extract (CE), which contains high concentrations of caffeine and diterpenoids, on a rat model of human metabolic syndrome. The 8–9 wk old male Wistar rats were divided into four groups. Two groups of rats were fed a corn starch-rich diet whereas the other two groups were given a high-carbohydrate, high-fat diet with 25% fructose in drinking water for 16 wk. One group fed each diet was supplemented with 5% aqueous CE for the final 8 wk of this protocol. The corn starch diet contained ~68% carbohydrates mainly as polysaccharides, whereas the high-carbohydrate, high-fat diet contained ~68% carbohydrates mainly as fructose and sucrose together with 24% fat, mainly as saturated and monounsaturated fat from beef tallow. The high-carbohydrate, high-fat diet-fed rats showed the symptoms of metabolic syndrome leading to cardiovascular remodeling and nonalcoholic fatty liver disease. CE supplementation attenuated impairment in glucose tolerance, hypertension, cardiovascular remodeling, and nonalcoholic fatty liver disease without changing abdominal obesity and dyslipidemia. This study suggests that CE can attenuate diet-induced changes in the structure and function of the heart and the liver without changing the abdominal fat deposition. J. Nutr. 142: 690–697, 2012.

Introduction

Increased intake of cafeteria or Western diets with higher energy content correlates with the deposition of excess energy in the form of fat in adipose tissue, leading to obesity (1,2). The risk of cardiovascular disease and nonalcoholic fatty liver disease increases with obesity (3–5), increasing the burden on health costs (6). Coffee, a widely consumed beverage in Western diets (7), contains the alkaloid caffeine, the diterpenoid alcohols cafestol and kahweol, potassium, niacin, magnesium, and antioxidant substances such as chlorogenic acid and tocopherols (8–11). Epidemiological studies on the effects of coffee drinking have been inconclusive, e.g., on hypertension (12), due to the association of coffee drinking with smoking, higher food intake, less fruit intake, and sedentary lifestyle compared to tea drinkers (13). An inverse relationship was found between coffee consumption and serum uric acid concentration, a risk factor for cardiovascular disease (14).

Coffee consumption may reduce the risks of type 2 diabetes, cardiovascular disease, and some cancers (7,15–18). However, nonfiltered, boiled coffee, in contrast to filtered coffee, increased the serum LDL cholesterol concentrations in mildly to moderately hypercholesterolemic middle-aged men and women without affecting HDL cholesterol, VLDL cholesterol, and TG concentrations in serum (19). This suggests that the brewing process plays an important role in the effects of coffee (11,20), because boiled coffee contains higher concentrations of diterpenoid alcohols compared to filtered coffee (20). Boiled coffee consumption has been correlated with the increase in serum cholesterol concentrations and subsequent increased risk of cardiovascular disease (11). In atherogenic diet-fed hamsters, coffee supplementation inhibited weight gain without changing the lipid profile (20). The individual constituents of coffee may alter components of metabolic syndrome. In Spontaneously Hypertensive Rats, 0.5% chlorogenic acid supplementation in the diet for 8 wk inhibited the increase in blood pressure, reduced oxidative stress, and improved bioavailability of NO (21). Caffeine treatment (0.1% in drinking water) to obese,

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[3] Supplemental Figures 1–3 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
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diabetic ZSF1 rats for 30 wk improved glucose and insulin responses but induced adverse effects on kidney function and increased plasma cholesterol concentrations (22).

This study measured the effects of CE on metabolic variables and the structure and function of the cardiovascular system and liver in high-carbohydrate, high-fat diet-fed rats. High-carbohydrate, high-fat diet-fed rats showed symptoms of metabolic syndrome with metabolic abnormalities, cardiovascular remodeling, and nonalcoholic steatohepatitis (23). Following CE supplementation for 8 wk, the structure and function of the heart were characterized through echocardiography, isolated Langendorff heart preparation, and histopathology, and the structure and function of the liver were characterized through histopathology and plasma biochemical analyses. In addition, metabolic function was characterized through glucose tolerance testing and plasma insulin concentrations.

Materials and Methods

Rats, experimental diets, and treatment with CE. All experimental protocols were approved by The University of Queensland Animal Experimentation Ethics Committee under the guidelines of the National Health and Medical Research Council of Australia. Male Wistar rats (8–9 wk old, weighing 337 ± 1 g, $n = 40$) were supplied by The University of Queensland Biological Resources facility. Rats were randomly divided into 4 experimental groups: corn starch-rich diet-fed rats (C[7]; $n = 10$), corn starch-rich diet-fed rats treated with CE (CC; $n = 10$), high-carbohydrate, high-fat diet-fed rats (H; $n = 10$), and high-carbohydrate, high-fat diet-fed rats treated with CE (HC, $n = 10$). The micronutrient and macronutrient compositions of these diets was previously described (23–25). H and HC rats were also given 25% fructose in drinking water along with the diets, whereas normal drinking water without any supplementation was given to C and CC rats. C and H rats were fed with respective diets for 16 wk. CC and HC rats were fed with C and H diets, respectively, for the first 8 wk and then their diets were supplemented with 5% CE for an additional 8 wk. CE was prepared by mixing Colombian coffee (50 g) with hot water (100 mL) and then filtering after 5 min of mixing to obtain 50 mL of extract. This extract was then mixed in the food by replacing 50 mL water/kg food. Fresh extract was prepared during each food preparation. All the rats consumed food and water ad libitum and were individually housed in temperature-controlled, 12-h-light/-dark conditions.

Rats were monitored daily for body weight and food and water intakes. Daily coffee intake was calculated from the daily food intake. Oral glucose tolerance tests and SBP measurements were performed on all group of rats every 4th wk starting at wk 0 as in previous study (23). Abdominal circumference and body length of rats were measured using a standard measuring tape during the period of anesthesia for SBP measurements (23). BMI and energy efficiency were calculated as in previous study (23). The concentrations of caffeine, chlorogenic acid, kahweol, and cafestol in coffee were measured by HPLC (**Supplemental Figs. 1–3**).

Echocardiography. Echocardiographic examinations (Phillips iE33, 12 MHz transducer) were performed to assess the cardiovascular structure and function in all rats at the end of protocol as previously described (23). Briefly, rats were anesthetized using Zoletil (25 mg/kg tiletamine and 25 mg/kg zolazepam, i.p.; Virbac) and Ilium Xylazil (15 mg/kg xylazine, i.p.; Troy Laboratories) and positioned in dorsal recumbency. Electrodes attached to the skin overlying the elbows and right stifle facilitated the simultaneous recording of a lead II electrocardiogram. A short-axis view of the left ventricle at the level of the papillary muscles was obtained and used to direct acquisition of M mode images of the left ventricle for measurement of IVSd, LVPWd, LVIDs, and LVIDd. Measurements were taken in accordance with the guidelines of the American Society of Echocardiography using the leading-edge method. Details of this method were previously described (23). The ventricular contractility indexes were calculated as described in previous studies (26–28). These indexes included the ratios of SBP:LVIDs, SBP:systolic volume, and ESS:LVIDs.

Isolated Langendorff heart preparation. Rats were killed with Lethabarb (pentobarbitone sodium, 100 mg/kg, i.p.; Virbac). After killing, heparin (200 IU; Sigma-Aldrich Australia) was injected through the right femoral vein. The abdomen was then opened and blood (~5 mL) was withdrawn from the abdominal aorta, collected into heparinized tubes, and centrifuged at 5000 × *g* for 15 min to obtain plasma. Plasma was stored at −20°C before further biochemical analysis. Hearts were removed and used in isolated Langendorff heart preparations to assess left ventricular function of the rats, as in a previous study (23).

Hearts isolated from killed rats were perfused with modified Krebs-Henseleit bicarbonate buffer containing (in mmol/L): NaCl, 119.1; KCl, 4.75; $MgSO_4$, 1.19; KH_2PO_4, 1.19; $NaHCO_3$, 25.0; glucose, 11.0; and $CaCl_2$, 2.16. Buffer was bubbled with 95% O_2–5% CO_2 and maintained at 35°C. Isovolumetric ventricular function was measured by inserting a latex balloon catheter into the left ventricle connected to a Capto SP844 MLT844 physiological pressure transducer and Chart software on a Maclab system (ADInstruments). All left ventricular end-diastolic pressure values were measured during pacing of the heart at 250 beats/min using an electrical stimulator. End-diastolic pressures were obtained from 0 to 30 mm Hg for 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 and maintained at 35°C and allowed to stabilize at a resting tension of ~10 mN (23). Cumulative concentration-response curves (contraction) were obtained for noradrenaline (Sigma-Aldrich Australia) and cumulative concentration-response curves (relaxation) were obtained for acetylcholine (Sigma-Aldrich Australia) and sodium nitroprusside (Sigma-Aldrich Australia) following submaximal (70%) contraction to noradrenaline (23).

Organ weights and histology. After performing Langendorff heart perfusion, hearts were separated into right ventricle and left ventricle (with septum) ($n = 8$) for weighing. Livers and abdominal fat pads (retroperitoneal, epididymal, and omental) were isolated and weighed ($n = 8$). These organ weights were normalized to the tibial length at the time of removal and expressed as mg of tissue/mm of tibial length (23). The heart and liver of rats ($n = 2$ from each group) were exclusively used for histopathological analysis as in previous study (23). For staining, thin sections (5 μm) of tissues were cut and fixed on slides. Heart sections were stained with picrosirius red to study collagen deposition and were analyzed using laser confocal microscopy (Zeiss LSM 510 upright confocal microscope). Hematoxylin and eosin stain was used to visualize infiltration of inflammatory cells in both the heart and the liver, whereas Milligan's stain was used to study the perivascular fibrosis in the liver. After staining with hematoxylin and eosin or Milligan's stains, pictures were taken with a Zeiss Microscope. From each tissue sample, three slides were prepared and two random, nonoverlapping fields were selected from each slide. A representative picture was randomly selected from each group.

Biochemical analysis of plasma samples. Plasma activities of ALT, AST, ALP, and LDH, and plasma concentrations of total cholesterol, TG, albumin, total proteins, total bilirubin, urea, and uric acid were determined. All these variables were measured using kits and controls supplied by Olympus using an Olympus analyzer (AU 400) (23). NEFA in plasma were determined using a commercial kit (Wako) (23). Plasma concentrations of insulin (Laboratory Diagnostics) were measured using

[7] Abbreviations used: ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; C, corn starch-rich diet-fed rats; CC, corn starch-rich diet-fed rats treated with coffee extract; CE, coffee extract; ESS, end-systolic wall stress; H, high-carbohydrate, high-fat diet-fed rats; HC, high-carbohydrate, high-fat diet-fed rats treated with coffee extract; IVSd, interventricular septum thickness during diastole; LDH, lactate dehydrogenase; LVIDd, left ventriclar internal diameter during diastole; LVIDs, left ventriclar internal diameter during systole; LVPWd, left ventricular posterior wall thickness during diastole; NEFA, nonesterified fatty acids; SBP, systolic blood pressure.

commercial kits according to manufacturer-provided standards and protocols using a Titertek Multiskan MCC/340 spectrophotometer (Flow Laboratories) (23).

Statistical analysis. All data are presented as mean ± SEM. All the groups 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. All groups were tested for the effects of diets, treatment, and their interactions by 2-way ANOVA. When interaction and/or the main effects were significant, means were compared using Newman-Keuls multiple comparison post-test. Mean daily doses of caffeine, chlorogenic acid, cafestol, and kahweol between CC and HC rats were compared using Student's *t* test. $P < 0.05$ was considered significant. All statistical analyses were performed using GraphPad Prism version 5.00 for Windows.

Results

Coffee composition and dosage. Coffee contained 12.5 ± 0.4 mg/g caffeine (Supplemental Fig. 1), 1.5 ± 0.1 mg/g chlorogenic acid (Supplemental Fig. 2), 4.2 ± 0.1 mg/g cafestol, and 4.9 ± 0.1 mg/g kahweol (Supplemental Fig. 3). The daily doses (mg/kg) were higher in CC rats (caffeine, 68.3 ± 2.6; chlorogenic acid, 8.2 ± 0.3; cafestol, 23.0 ± 0.9; kahweol, 26.8 ± 1.0) than in HC rats (caffeine, 32.0 ± 1.3; chlorogenic acid, 3.8 ± 0.2; cafestol, 10.8 ± 0.4; kahweol, 12.5 ± 0.5) due to higher food consumption by CC rats ($P < 0.0001$) (**Table 1**).

Physiological and metabolic variables. The H rats had higher body weights compared to C rats. HC rats had even higher body weights compared to H rats, whereas the body weight of CC rats did not differ from C rats (**Fig. 1***A*). Although H rats consumed less food and water compared to C rats, H rats had higher energy intake compared to C rats. CC rats had higher water intake compared to C rats, whereas water intake did not differ between H and HC rats. CC and HC rats consumed more food and had higher energy intake compared to C and H rats, respectively (Table 1). Energy efficiency and BMI were higher in H rats compared with C rats. These variables did not differ in CC and HC rats compared with C and H rats, respectively (Table 1).

Basal blood glucose concentrations were higher in H rats compared to C rats and these were normalized in HC rats (Table 1). H rats had lower glucose tolerance with a higher AUC compared to C rats. CE attenuated this impairment of glucose tolerance with lower AUC in HC rats compared to H rats (Fig. 1*B*; Table 1). Plasma insulin was higher in H rats compared to C rats and it was lower in HC rats than H rats (Table 1). Plasma concentrations of total cholesterol, TG, and NEFA were higher in H rats compared to C rats. Plasma concentrations of total cholesterol were lower in HC rats than in H rats (Table 1). Plasma TG concentrations were higher in HC rats compared to H rats, whereas their plasma NEFA concentrations did not differ. Plasma total cholesterol and TG concentrations did not differ between the C and CC rats, whereas plasma NEFA concentrations were higher in CC rats compared to C rats (Table 1).

Abdominal circumference was higher in H rats compared to C rats. Abdominal circumference was higher in CC rats compared to C rats, whereas it did not differ between H and HC rats (Fig. 1C). Similarly, the total abdominal fat deposition was higher in H rats compared to C rats. Total abdominal fat deposition as well as retroperitoneal, epididymal, and omental fat did not differ in CC and HC rats compared to C rats and H rats, respectively (Table 1).

Cardiovascular structure and function. SBP was higher in H rats compared to C rats. HC rats had lower SBP than H rats, whereas SBP in CC rats did not differ from that in C rats (Fig. 1*D*). LVIDd and LVPWd were higher in H rats compared to C rats. LVIDd was lower whereas LVPWd was higher in HC rats compared to H rats. These variables did not differ between CC and C rats (**Table 2**). Systolic volume was higher in H rats

TABLE 1 Effects of CE on physiological and metabolic variables in C, CC, H, and HC rats[1]

					P value		
Variables	C	CC	H	HC	Diet	CE	Diet × CE
Physiological variables							
Water intake, *mL/d*	32.1 ± 1.3^{b}	36.7 ± 2.0^{a}	18.8 ± 1.0^{c}	18.2 ± 0.9^{c}	<0.0001	0.15	0.07
Food intake, *g/d*	31.9 ± 0.7^{b}	40.6 ± 1.1^{a}	20.7 ± 0.5^{d}	23.4 ± 0.8^{c}	<0.0001	<0.0001	0.0007
Energy intake, *kJ/d*	358 ± 8^{c}	433 ± 12^{b}	442 ± 10^{b}	484 ± 10^{a}	<0.0001	<0.0001	0.11
Energy efficiency, *kJ/g*	0.23 ± 0.02^{b}	0.21 ± 0.02^{b}	0.40 ± 0.02^{a}	0.45 ± 0.03^{a}	<0.0001	0.52	0.14
BMI, *g/cm*^{2}	0.67 ± 0.02^{b}	0.70 ± 0.02^{b}	0.78 ± 0.02^{a}	0.82 ± 0.02^{a}	<0.0001	0.09	0.80
Metabolic variables							
Basal blood glucose, *mmol/L*	4.0 ± 0.2^{b}	3.6 ± 0.1^{b}	5.0 ± 0.2^{a}	4.1 ± 0.1^{b}	<0.0001	0.0002	0.12
Blood glucose AUC[2], *mmol/L · min*	680 ± 12^{b}	706 ± 15^{b}	782 ± 16^{a}	710 ± 12^{b}	0.0005	0.11	0.001
Plasma insulin, *pmol/L*	0.22 ± 0.01^{b}	0.24 ± 0.01^{b}	0.39 ± 0.03^{a}	0.26 ± 0.03^{b}	0.0002	0.020	0.002
Plasma total cholesterol, *mmol/L*	1.4 ± 0.1^{b}	1.5 ± 0.1^{b}	2.0 ± 0.1^{a}	1.6 ± 0.1^{b}	0.001	0.14	0.017
Plasma TG, *mmol/L*	0.4 ± 0.1^{b}	0.7 ± 0.1^{b}	0.7 ± 0.1^{b}	1.2 ± 0.2^{a}	0.0003	0.0003	0.32
Plasma NEFA, *mmol/L*	1.2 ± 0.2^{b}	2.3 ± 0.2^{a}	2.6 ± 0.3^{a}	2.6 ± 0.3^{a}	0.002	0.038	0.038
Retroperitoneal fat, *mg/mm tibial length*	184 ± 15^{b}	179 ± 14^{b}	381 ± 22^{a}	347 ± 22^{a}	<0.0001	0.30	0.44
Epididymal fat, *mg/mm tibial length*	110 ± 8^{b}	116 ± 11^{b}	232 ± 16^{a}	268 ± 17^{a}	<0.0001	0.13	0.27
Omental fat, *mg/mm tibial length*	89 ± 10^{b}	76 ± 9^{b}	183 ± 15^{a}	173 ± 16^{a}	<0.0001	0.38	0.91
Total abdominal fat, *mg/mm tibial length*	383 ± 35^{b}	370 ± 28^{b}	796 ± 56^{a}	788 ± 73^{a}	<0.0001	0.84	0.96

[1] Values are mean ± SEM, $n = 8$–10. Means in a row with superscripts without a common letter differ, $P < 0.05$. C, corn starch-rich diet-fed rats; CC, corn starch-rich diet-fed rats treated with coffee extract; CE, coffee extract; H, high-carbohydrate, high-fat diet-fed rats; HC, high-carbohydrate, high-fat diet-fed rats treated with coffee extract; NEFA, nonesterified fatty acids.
[2] AUC was calculated with *x* axis as the baseline.

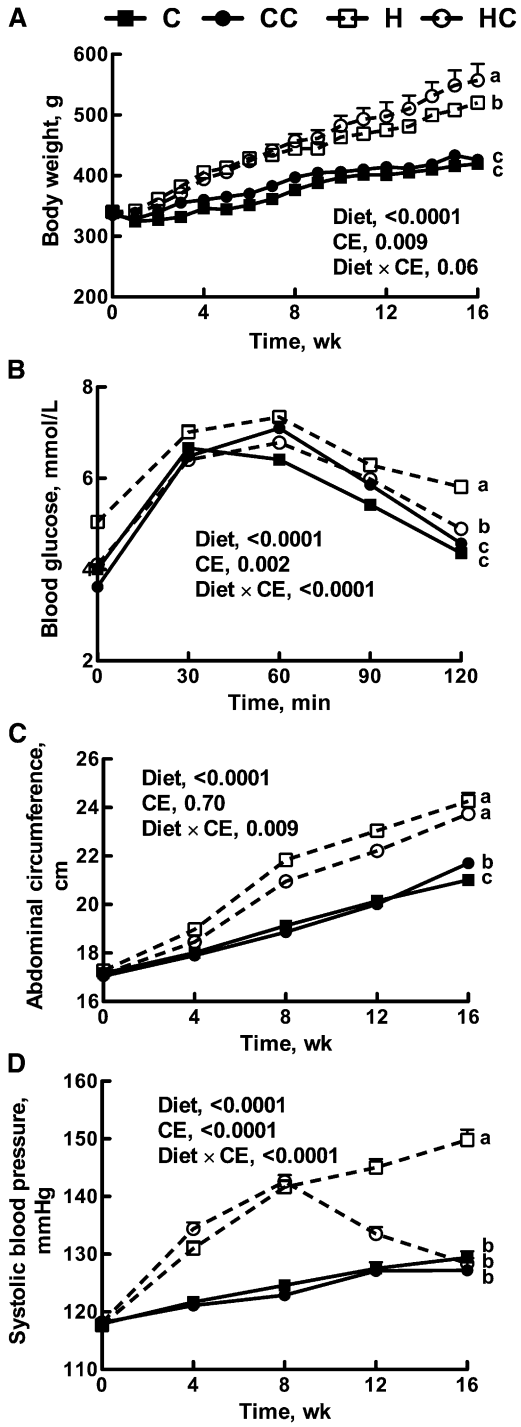

FIGURE 1 Effects of CE on body weight (*A*), oral glucose tolerance (*B*), abdominal circumference (*C*), and systolic blood pressure (*D*) in C, CC, H, and HC rats. Values are mean ± SEM, $n = 10$. End-point means without a common letter differ, $P < 0.05$. C, corn starch-rich diet-fed rats; CC, corn starch-rich diet-fed rats treated with coffee extract; CE, coffee extract; H, high-carbohydrate, high-fat diet-fed rats; HC, high-carbohydrate, high-fat diet-fed rats treated with coffee extract.

compared to C rats and it was lower in HC rats compared to H rats (Table 2). IVSd was higher in H rats than in C rats and it did not differ in CC and HC rats compared with C and H rats, respectively. LVIDs was higher in H rats than in C rats and this was normalized in HC rats (Table 2). The ratios of SBP:LVIDs and SBP:systolic volume were lower and ESS:LVIDs was higher in H rats compared to C rats. These indexes for ventricular contractility were normalized in HC rats. Although relative wall thickness did not differ between C and H rats, HC rats had a higher relative wall thickness (Table 2). Fractional shortening and ejection fractions were lower in H rats compared to C rats; these variables were higher in HC rats than in H rats and did not differ in CC rats compared to C rats (Table 2). Time from mitral valve closure to opening, ejection time, and deceleration time did not differ between the groups (Table 2). The ratio of early mitral inflow velocity to late mitral inflow velocity was lower in H rats compared to C rats, whereas it was higher in HC rats compared to H rats without being affected in CC rats compared to C rats (Table 2). Left ventricular mass estimated from echocardiographic examination was higher in H rats compared to C rats and it was lower in HC rats compared to H rats. Wet weight of left ventricle (with septum) was higher in H rats compared to C rats and was not affected by CE in both CC and HC rats (Table 2). Right ventricular wet weight did not differ between H and C rats, whereas right ventricular wet weights were higher in CC and HC rats compared to those in C and H rats, respectively (Table 2).

Left ventricle from H rats had a higher infiltration of inflammatory cells (**Fig. 2***C*) and collagen deposition (Fig. 2*G*) than C rats (Fig. 2*A*,*E*). CE prevented the infiltration of inflammatory cells (Fig. 2*D*) and collagen deposition (Fig. 2*H*) in left ventricle of HC rats. Diastolic stiffness measured through diastolic stiffness constant was higher in H rats compared to C rats and was lower in HC rats than in H rats (Table 2).

Noradrenaline-induced contraction (**Fig. 3***A*) and sodium nitroprusside- (Fig. 3*B*) and acetylcholine- (Fig. 3*C*) induced relaxations of isolated thoracic aortic rings were lower in H rats than in C rats; these responses were normalized in HC rats. In CC rats, acetylcholine-induced relaxation was higher than in C rats (Fig. 3*C*) and noradrenaline-induced contraction (Fig. 3*A*) and sodium nitroprusside-induced relaxation (Fig. 3*B*) did not differ from C rats.

Hepatic structure and function. Liver from H rats had a higher infiltration of inflammatory cells (**Fig. 4***C*) and presence of fat vacuoles (Fig. 4*G*) than did C rats (Fig. 4*A*,*E*) along with higher liver wet weights (**Table 3**). Portal fibrosis was higher in livers from H rats (Fig. 4*K*) compared to those from C rats (Fig. 4*I*). Although the fat deposition was attenuated in HC rats (Fig. 4*H*), the liver wet weights did not differ compared to H rats (Table 3). CE prevented the infiltration of inflammatory cell and portal fibrosis in HC rats (Fig. 4*D*,*L*).

Plasma activities of ALT, AST, ALP, and LDH were higher in H rats than in C rats (Table 3). HC rats had lower plasma activities of these enzymes compared to H rats (Table 3). CC rats had lower plasma activities of ALT and ALP than C rats where AST and LDH did not differ compared to C rats (Table 3). Plasma concentrations of albumin and total bilirubin did not differ between the groups (Table 3). Plasma urea concentrations were lower and plasma uric acid concentrations were higher in H rats than in C rats. These changes were attenuated in HC rats (Table 3).

Discussion

Coffee is a complex beverage with potential health benefits and problems, containing many bioactive compounds, including phenolic acids with antioxidant properties, diterpenoid alcohols, and caffeine (20). Thus, understanding the overall effect of the mixture of all the constituents in coffee is necessary. The brewing process determines the constituents of CE (11,20);

TABLE 2 Effects of CE on cardiovascular structure and function in C, CC, H, and HC rats[1]

Variables	C	CC	H	HC	*P* value		
					Diet	CE	Diet × CE
LVIDd, *mm*	6.61 ± 0.12^{b}	6.33 ± 0.14^{b}	7.46 ± 0.11^{a}	6.54 ± 0.27^{b}	0.004	0.001	0.07
LVPWd, *mm*	1.58 ± 0.03^{c}	1.62 ± 0.04^{c}	1.83 ± 0.05^{b}	2.06 ± 0.04^{a}	<0.0001	0.002	0.025
IVSd, *mm*	1.57 ± 0.02^{b}	1.62 ± 0.04^{b}	1.74 ± 0.04^{a}	1.78 ± 0.04^{a}	<0.0001	0.22	0.89
LVIDs, *mm*	3.31 ± 0.18^{b}	3.44 ± 0.17^{b}	4.86 ± 0.14^{a}	3.70 ± 0.23^{b}	<0.0001	0.008	0.001
Systolic volume, μL	41.1 ± 6.8^{b}	44.6 ± 6.2^{b}	120.2 ± 7.8^{a}	58.5 ± 7.0^{b}	<0.0001	0.0002	<0.0001
SBP:LVIDs	40.1 ± 2.1^{a}	38.0 ± 2.3^{a}	30.9 ± 1.4^{b}	36.8 ± 2.1^{a}	0.014	0.35	0.05
SBP:systolic volume	3930 ± 590^{a}	3400 ± 640^{a}	1250 ± 230^{b}	2950 ± 540^{a}	0.005	0.28	0.041
ESS:LVIDs	0.92 ± 0.08^{b}	0.98 ± 0.08^{b}	1.15 ± 0.07^{a}	0.75 ± 0.06^{b}	0.73	0.011	0.001
Relative wall thickness	0.48 ± 0.01^{b}	0.51 ± 0.02^{b}	0.48 ± 0.02^{b}	0.60 ± 0.02^{a}	0.017	0.0002	0.017
Fractional shortening, %	52.6 ± 1.6^{a}	52.9 ± 1.9^{a}	38.3 ± 1.4^{b}	47.6 ± 1.8^{a}	<0.0001	0.007	0.011
Ejection fraction, %	86.9 ± 1.6^{a}	83.3 ± 2.0^{ab}	72.1 ± 1.3^{c}	80.9 ± 1.4^{b}	<0.0001	0.11	0.0004
MCMO, *ms*	114 ± 4	114 ± 4	114 ± 4	104 ± 4	0.22	0.22	0.22
Ejection time, *ms*	91 ± 2	91 ± 3	88 ± 3	86 ± 2	0.13	0.70	0.70
E:A ratio	1.91 ± 0.12^{a}	1.89 ± 0.12^{a}	1.61 ± 0.11^{b}	1.78 ± 0.10^{a}	0.04	0.17	0.049
Deceleration time, *ms*	52.1 ± 2.0	55.0 ± 2.3	55.5 ± 4.0	61.2 ± 3.3	0.12	0.16	0.64
Estimated left ventricular mass, *g*	0.67 ± 0.02^{c}	0.66 ± 0.02^{c}	0.91 ± 0.03^{a}	0.81 ± 0.03^{b}	<0.0001	0.038	0.09
Left ventricle + septum wet weight, *mg/mm tibial length*	18.7 ± 0.8^{b}	19.9 ± 0.8^{ab}	22.1 ± 0.9^{a}	21.5 ± 1.0^{ab}	0.007	0.74	0.31
Right ventricular wet weight, *mg/mm tibial length*	4.21 ± 0.24^{b}	5.02 ± 0.27^{a}	4.49 ± 0.32^{b}	5.14 ± 0.29^{a}	0.48	0.014	0.78
Left ventricular diastolic stiffness constant (κ)	21.1 ± 1.3^{b}	20.2 ± 1.2^{b}	28.1 ± 1.4^{a}	24.1 ± 1.5^{b}	0.0003	0.07	0.28

[1] Values are mean ± SEM, n = 8–10. Means in a row with superscripts without a common letter differ, $P < 0.05$. C, corn starch-rich diet-fed rats; CC, corn starch-rich diet-fed rats treated with coffee extract; CE, coffee extract; E:A, ratio of early mitral inflow velocity to late mitral inflow velocity; ESS, end-systolic wall stress; H, high-carbohydrate, high-fat diet-fed rats; HC, high-carbohydrate, high-fat diet-fed rats treated with coffee extract; IVSd, interventricular septum thickness during diastole; LVIDd, left ventricular internal diameter during diastole; LVIDs, left ventricular internal diameter during systole; LVPWd, left ventricular posterior wall thickness during diastole; MCMO, time from mitral valve closure to opening; SBP, systolic blood pressure.

filtering was preferred to keep the phenolic acids intact and reduce the diterpenoid content. In most previous studies, coffee responses were characterized by focusing on one particular condition, e.g., hypercholesterolemia (11,14,19–21,29). To overcome this problem, we characterized the effects of CE in a model with most of the complications of metabolic syndrome, including obesity, hypertension, impaired glucose tolerance, cardiovascular remodeling, nonalcoholic fatty liver disease, and dyslipidemia (23).

High-carbohydrate, high-fat diet induced the symptoms of metabolic syndrome in rats as in our previous studies (23–25). We reported that these diet-induced symptoms can be either reversed or prevented from further progression by intervention with natural products such as purple carrots (30), olive leaf (24),

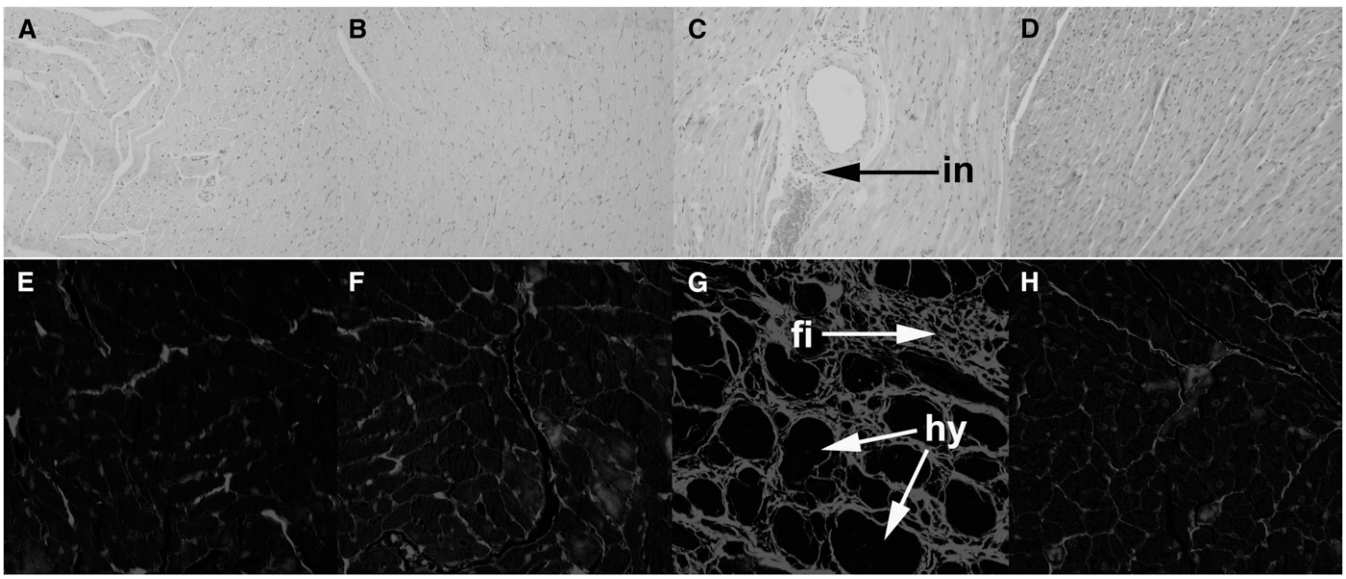

FIGURE 2 Effects of CE on inflammation and fibrosis in the heart. Hematoxylin and eosin staining of left ventricle showing infiltration of inflammatory cells (*A–D*, inflammatory cells marked as "in") from C (*A*), CC (*B*), H (*C*), and HC (*D*) rats. Picrosirius red staining of left ventricle showing collagen deposition and hypertrophy (*E–H*, fibrosis marked as "fi" and hypertrophied cardiomyocytes as "hy") from C (*E*), CC (*F*), H (*G*), and HC (*H*) rats. C, corn starch-rich diet-fed rats; CC, corn starch-rich diet-fed rats treated with coffee extract; CE, coffee extract; H, high-carbohydrate, high-fat diet-fed rats; HC, high-carbohydrate, high-fat diet-fed rats treated with coffee extract.

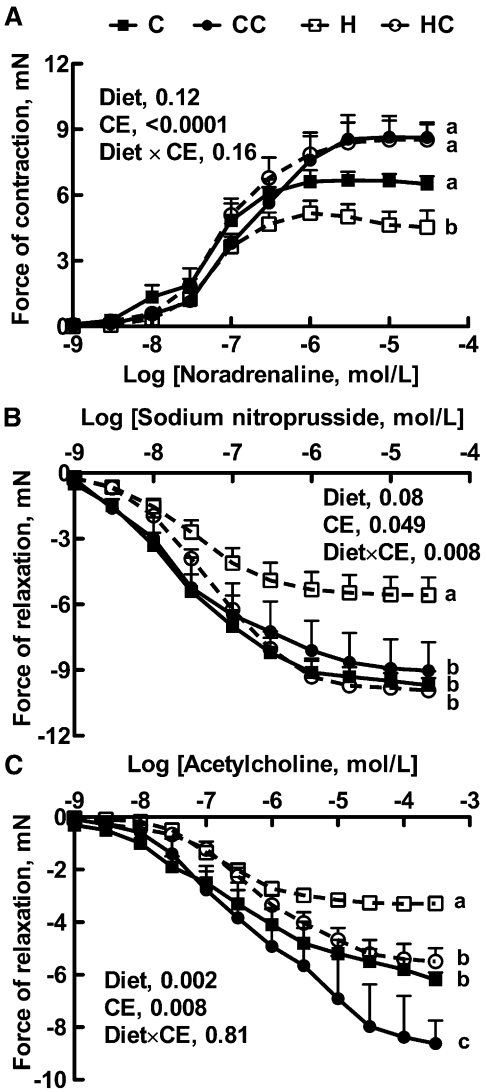

FIGURE 3 Effects of CE on noradrenaline-induced contraction (*A*), sodium nitroprusside-induced relaxation (*B*), and acetylcholine-induced relaxation (*C*) in thoracic aortic preparations from C, CC, H, and HC rats. Values are mean ± SEM, $n = 10$. End-point means without a common letter differ, $P < 0.05$. C, corn starch-rich diet-fed rats; CC, corn starch-rich diet-fed rats treated with coffee extract; CE, coffee extract; H, high-carbohydrate, high-fat diet-fed rats; HC, high-carbohydrate, high-fat diet-fed rats treated with coffee extract.

chia seeds (25), and rutin (31). The same protocol was used in this study to show that CE attenuated most of the changes in cardiovascular and liver structure and function and in glucose tolerance, whereas abdominal central obesity and dyslipidemia were unaffected. Although previous studies of coffee have shown higher serum cholesterol without changing TG concentrations (19), we found that cholesterol was lower, with higher concentrations of TG and NEFA. This inability to lower TG and NEFA may explain the lack of effect of CE on obesity.

In this study, CE supplementation in diet-induced metabolic syndrome in rats lowered blood pressure, although earlier studies characterizing the effects of coffee on hypertension in humans reported conflicting results (32–34). Earlier reports support a lowered risk for type 2 diabetes with coffee consumption (35). This study shows that coffee intake in rats is associated with lower basal blood glucose concentrations and attenuation of impairment in glucose tolerance despite the chronic consumption of a diet that impairs the glucose tolerance. Similarly, plasma insulin was lower in CE-supplemented rats.

The cardiovascular structure showed attenuation of the dilation and fibrosis in the left ventricle, whereas left ventricular wall thickness was higher, indicating the presence of hypertrophy. Apart from these structural changes, ventricular contractility was enhanced with CE supplementation. These conditions resemble the physiological hypertrophy seen in athletes where the heart becomes more efficient. Recent studies have also shown beneficial effects of coffee on liver disease, lower bright liver score indicating fatty liver and liver cancer, because an inverse relation was found between coffee consumption and the development of liver complications (36,37). In this study, we found protective effects of CE on the liver. In diet-induced metabolic syndrome, there is development of nonalcoholic fatty liver disease (23,31). Steatosis, inflammation, and fibrosis in the liver developed by the H diet were attenuated with CE supplementation along with improvements in liver function measured through plasma liver enzymes.

In nonobese healthy men and women, caffeinated coffee reduced flow-mediated dilation of brachial artery, indicating the induction of endothelial dysfunction. This response was induced by caffeine, because this detrimental effect was not seen with decaffeinated coffee (38,39). However, these studies measured acute effects. In healthy as well as diabetic women, long-term caffeinated and decaffeinated coffee consumption was inversely associated with the markers of inflammation and endothelial dysfunction (40). Our study has shown that coffee attenuated the vascular damage induced by the H diet. These results were shown as higher vascular contraction with noradrenaline and higher vascular relaxation with both sodium nitroprusside and acetylcholine after precontraction with noradrenaline.

Abdominal obesity has been associated with the occurrence of cardiovascular disease, nonalcoholic fatty liver disease, and impairment in metabolic function, including glucose tolerance (41,42). In this study, CE supplementation did not alter the amount of fat present in the abdominal areas and the plasma compared to H rats. However, the changes in structure and function of the heart, blood vessels, and the liver in H rats were attenuated. These results may classify the CE-supplemented rats as metabolically healthy. One of the possible reasons could be the change in types of fatty acid that were stored in the adipose tissues. A further study determining the types of fatty acid present in the abdominal area will help in understanding the mechanisms.

The diterpenoid alcohols cafestol and kahweol may cause deleterious effects of coffee (11,19,20,43,44), with the coffee polyphenols producing benefits (20) and caffeine showing contrasting results, including increases in cholesterol (22). Our results suggest that all these components were active and the effects observed were cumulative.

In this study, 50 g of Colombian coffee was used to make 50 mL of CE, which was then mixed in 1 kg of food. This provided a daily dose of ~3 g coffee/kg body weight in rats. This dose corresponds to 60 g coffee/d in a 70-kg human based on scaling equation (45) or 35 g/d based on body surface area comparisons between rats and humans (46). Generally, 7–8 g coffee is required to make a cup of coffee (19). Thus, the dose of coffee used in this study corresponds to ~4–5 cups/d of coffee according to body surface area comparisons and ~7–8 cups/d of coffee according to scaling equations. Similarly, the daily dose of caffeine from CE corresponds to 30–35 mg/kg in rats depending on their body weight. This dose corresponds to ~0.6 g/d for a 70-kg human (according to scaling equation) (45) and 0.35 g/d

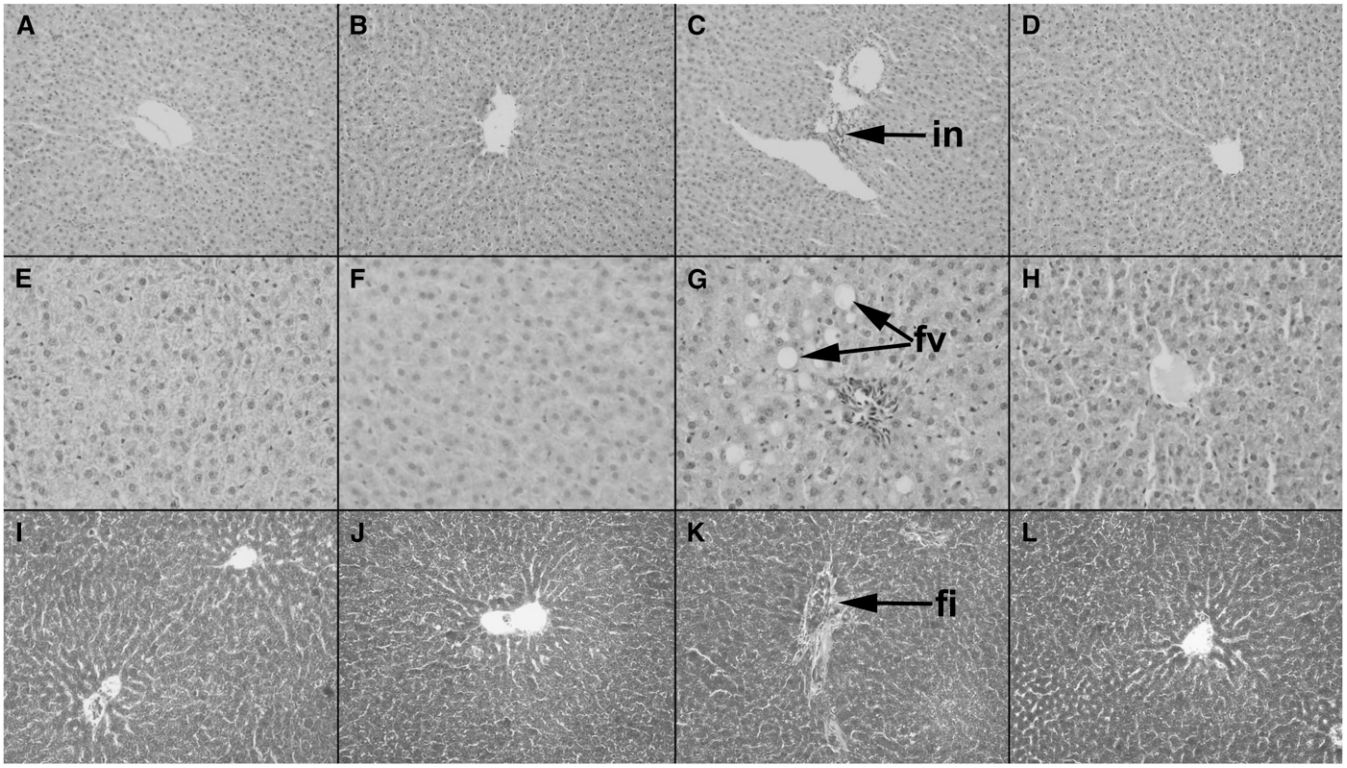

FIGURE 4 Effects of CE on inflammation, fat deposition, and fibrosis in the liver. Top and middle rows represent hematoxylin and eosin staining of the liver showing inflammatory cells (*A–D*, marked as "in") (×20) and enlarged fat vacuoles (*E–H*, marked as "fv") (×40) from C (*A,E*), CC (*B,F*), H (*C,G*), and HC (*D,H*) rats. Bottom row represents Milligan's Trichrome staining of the liver showing fibrosis in the hepatic portal region (*I–L*, marked as "fi") (×20) from C (*I*), CC (*J*), H (*K*), and HC (*L*) rats. C, corn starch-rich diet-fed rats; CC, corn starch-rich diet-fed rats treated with coffee extract; CE, coffee extract; H, high-carbohydrate, high-fat diet-fed rats; HC, high-carbohydrate, high-fat diet-fed rats treated with coffee extract.

(according to body surface area calculations) (46). An earlier report associated best performance with an intake of 0.4 g/d caffeine in humans (47).

In conclusion, CE attenuated hypertension and impairment in glucose homeostasis without affecting abdominal fat deposition and plasma lipid profile in diet-induced metabolic syndrome in rats. It also attenuated the changes in structure and function of the heart and the liver in these rats. A study in humans with >2 variables of metabolic syndrome could determine its beneficial effects. Because coffee is one of the major nonalcoholic beverages throughout the world, it is important to estimate the extent of its effects on human health.

Acknowledgments

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TABLE 3 Effects of CE on hepatic structure and function in C, CC, H, and HC rats[1]

Variables	C	CC	H	HC	*P* value		
					Diet	CE	Diet × CE
Liver wet weight, *mg/mm tibial length*	244 ± 11^{b}	237 ± 10^{b}	312 ± 15^{a}	336 ± 14^{a}	<0.0001	0.51	0.23
Plasma ALT activity, *U/L*	35.0 ± 1.3^{b}	28.6 ± 2.3^{c}	58.4 ± 2.0^{a}	32.7 ± 0.9^{bc}	<0.0001	<0.0001	<0.0001
Plasma AST activity, *U/L*	79.0 ± 4.8^{b}	79.5 ± 6.2^{b}	108.9 ± 7.0^{a}	84.2 ± 5.6^{b}	0.006	0.049	0.041
Plasma ALP activity, *U/L*	172 ± 11^{c}	125 ± 6^{d}	247 ± 12^{a}	206 ± 14^{b}	<0.0001	0.0004	0.79
Plasma LDH activity, *U/L*	252 ± 27^{b}	248 ± 21^{b}	438 ± 32^{a}	319 ± 35^{b}	<0.0001	0.043	0.06
Plasma albumin, *g/L*	28.0 ± 0.4	28.5 ± 0.4	28.7 ± 0.3	27.9 ± 0.3	0.89	0.67	0.07
Plasma total bilirubin, *μmol/L*	2.2 ± 0.1	2.3 ± 0.2	2.5 ± 0.1	2.0 ± 0.2	1.0	0.21	0.07
Plasma urea, *mmol/L*	5.8 ± 0.4^{a}	4.8 ± 0.5^{ab}	3.0 ± 0.3^{c}	4.3 ± 0.3^{b}	0.0001	0.69	0.005
Plasma uric acid, *μmol/L*	37.1 ± 2.6^{b}	41.2 ± 2.0^{b}	60.1 ± 3.8^{a}	38.8 ± 3.2^{b}	0.001	0.007	0.0001

[1] Values are mean ± SEM, n = 8–10. Means in a row with superscripts without a common letter differ, $P < 0.05$. ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; C, corn starch-rich diet-fed rats; CC, corn starch-rich diet-fed rats treated with coffee extract; CE, coffee extract; H, high-carbohydrate, high-fat diet-fed rats; HC, high-carbohydrate, high-fat diet-fed rats treated with coffee extract. LDH, lactate dehydrogenase.

data; J.W. measured the coffee constituents; S.K.P. and L.B. wrote the paper; and L.B. had primary responsibility for final content. All authors read and approved the final manuscript.

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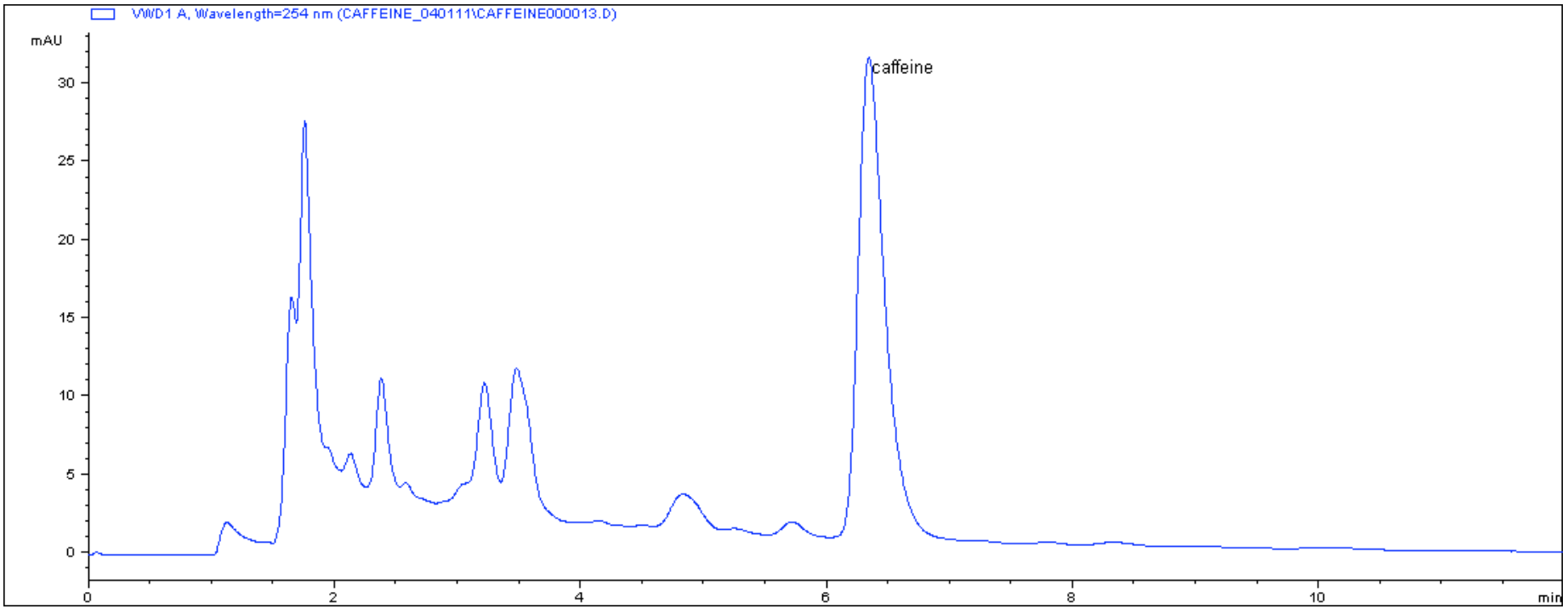
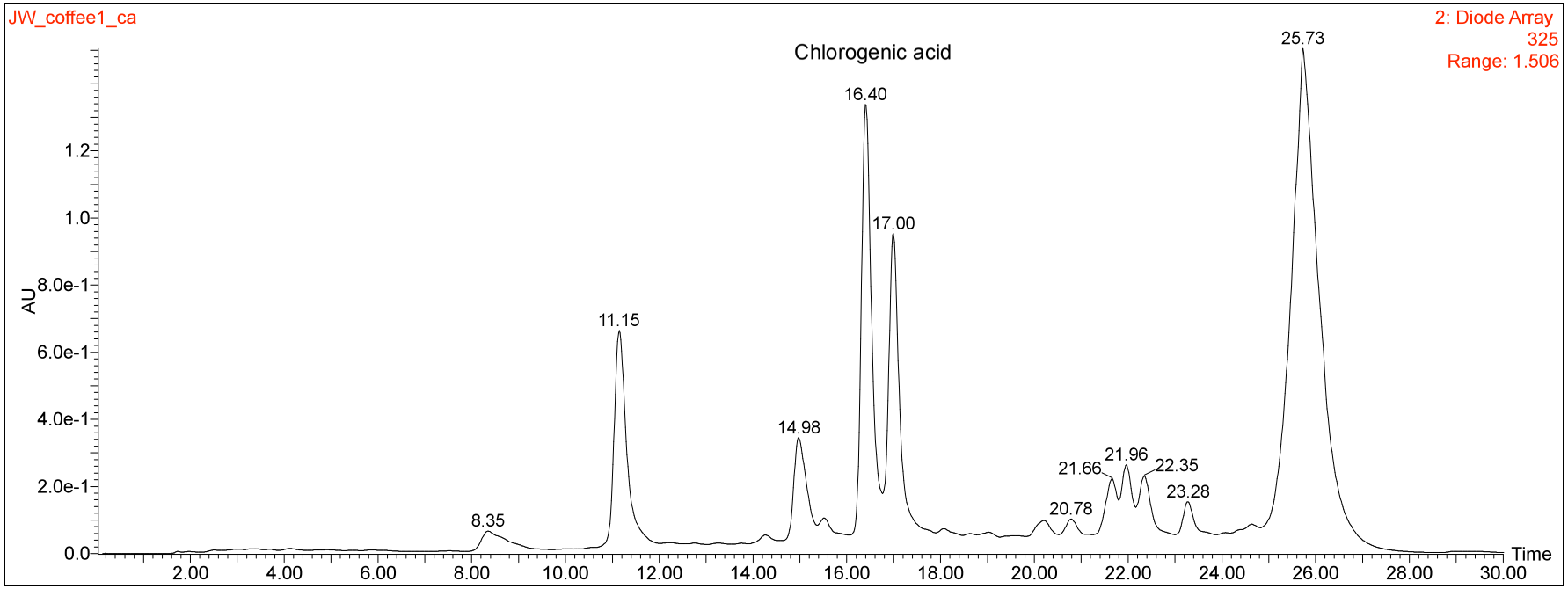


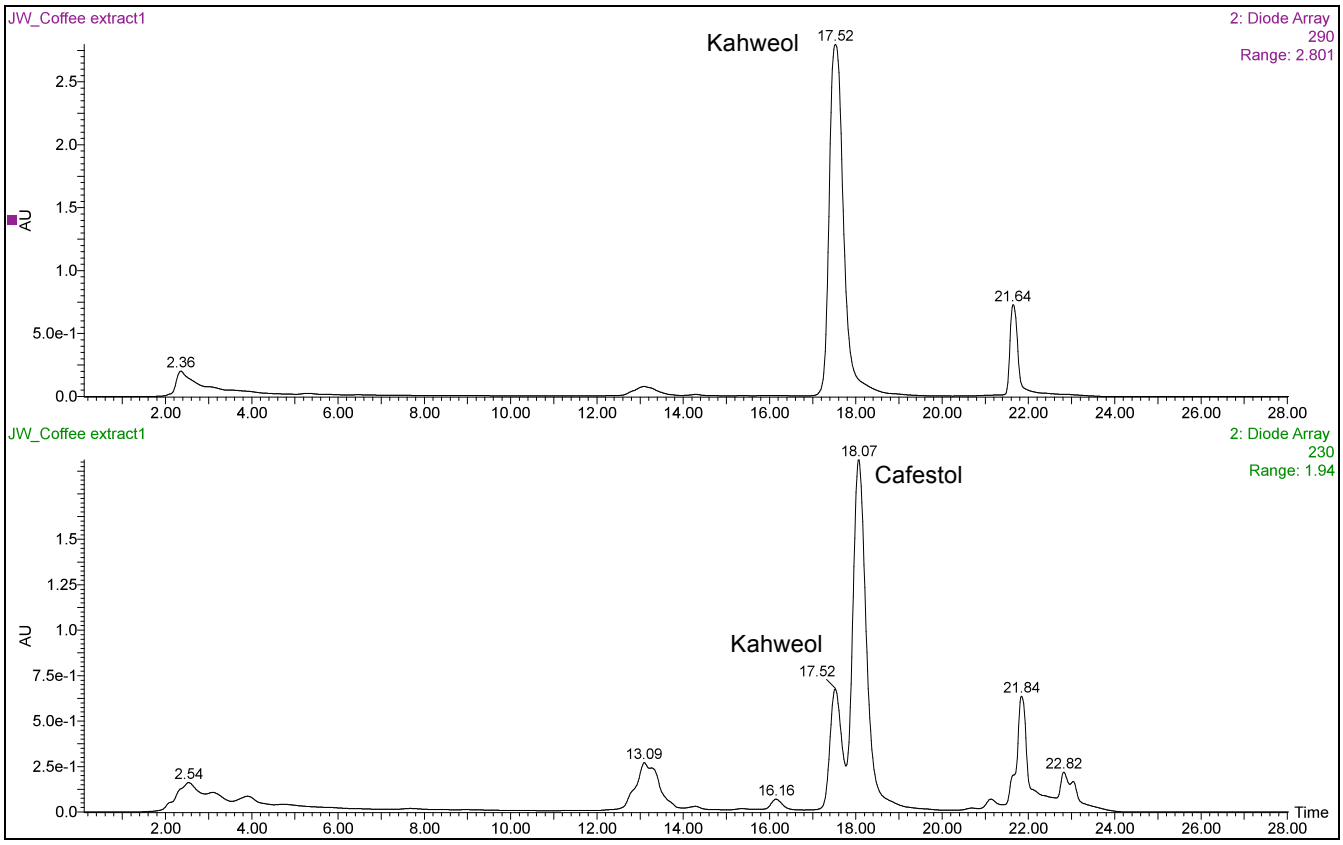
Supplemental Figure 1. HPLC analysis of caffeine in Colombian coffee.

Caffeine measurement: 0.2 g of coffee powder was accurately weighed. To this, 10 mL of water was added and the solution was heated at 80° C for one min. The liquid above the coffee solid was removed and placed in a 100 mL volumetric flask. The remaining coffee solid was extracted in this way a further four times and all the water extracts were removed and combined. The combined extracts were made up to 100 mL in water and the solution was filtered prior to injection into HPLC (Agilent Technologies, Colorado Springs, USA). HPLC analysis was performed on an Agilent 1100 series with Alltech Prevail C18 column (5 μm, 150 mm x 4.6 mm). A flow rate of 1 mL/min was used with 40% methanol and 0.5% phosphoric acid as solvent and injection volume of 15 μL. Compounds were detected at 254 nm. Caffeine (Sigma-Aldrich Australia, Sydney, Australia) was used as standard in this procedure and calibration for caffeine was carried out on concentrations between 5 and 100 mg/L.



Supplemental Figure 2. HPLC analysis of chlorogenic acid in Colombian coffee.

Chlorogenic acid measurement: 1 g of coffee powder was accurately weighed. To this, 10 mL of water was added and the solution was heated at 80° C for one min. 2 mL of the liquid above the coffee solids was filtered through a Millipore filter and placed into autosampler vial for analysis. HPLC analysis was performed on Waters 2690 with Waters XTerra C18 column (3.5 μm, 150 mm x 2.1 mm). A flow rate of 1 mL/min was used with 80% acetonitrile and 0.1% formic acid as solvents and injection volume of 10 μL; detection was at 325 nm. Chlorogenic acid (Sigma-Aldrich Australia) was used as standard in this procedure and calibration for chlorogenic acid was carried out on concentrations between 30 and 300 mg/L. This method measured the 3-caffeoylquinate form of chlorogenic acid.



Supplemental Figure 3. HPLC analysis of kahweol and cafestol in Colombian coffee.

Kahweol and cafestol measurements: 0.2 g of coffee powder was accurately weighed. To this, 2 mL of 2.5 mol/L KOH in ethanol was added and the solution was heated at 80° C for one hour to release the diterpenes from the fatty acids. 2 mL of water and 2 mL of methyl t-butyl ether was added. The sample was vortexed for 30 s and then centrifuged at 1800 × *g*. The upper ether layer was transferred to a separate tube and the sample was re-extracted with ether two more times. All ether extracts were combined and the ether was evaporated under nitrogen. Resulting solids were resuspended in 20 mL of 50% methanol in water and filtered through a Millipore filter and injected into the HPLC. HPLC analysis was performed on an Waters 2690 with Waters XTerra C18 column (3.5 μm, 150 mm x 2.1 mm). A flow rate of 1 mL/min was used with injection volume of 10 μL. For detection, 290 nm (kahweol) and 230 nm (cafestol) wavelengths were used. Cafestol (MP Biomedicals, Seven Hills, Australia) and kahweol (Santa Cruz Biotechnology, Santa Cruz, CA) were used as standards in this procedure and calibration for kahweol and cafestol were carried out on concentrations between 3 and 80 mg/L.

Chapter 6.2

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Basic nutritional investigation

Caffeine attenuates metabolic syndrome in diet-induced obese rats

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ABSTRACT

Objective: Caffeine is a constituent of many non-alcoholic beverages. The pharmacologic actions of caffeine include the antagonism of adenosine receptors and the inhibition of phosphodiesterase activity. The A1 adenosine receptors present on adipocytes are involved in the control of fatty acid uptake and lipolysis. In this study, the effects of caffeine were characterized in a diet-induced metabolic syndrome in rats.
Methods: Rats were given a high-carbohydrate, high-fat diet (mainly containing fructose and beef tallow) for 16 wk. The control rats were given a corn starch diet. These two treatment groups were given caffeine 0.5 g/kg of food for the last 8 wk of the 16-wk protocol. The structure and function of the heart and the liver were investigated in addition to the metabolic parameters including the plasma lipid components.
Results: The high-carbohydrate, high-fat diet induced symptoms of the metabolic syndrome, including obesity, dyslipidemia, impaired glucose tolerance, decreased insulin sensitivity, and increased systolic blood pressure, associated with the development of cardiovascular remodeling and non-alcoholic steatohepatitis. The treatment with caffeine in the rats fed the high-carbohydrate, high-fat diet decreased body fat and systolic blood pressure, improved glucose tolerance and insulin sensitivity, and attenuated cardiovascular and hepatic abnormalities, although the plasma lipid concentrations were further increased.
Conclusion: A decreased total body fat, concurrent with increased plasma lipid concentrations, reflects the lipolytic effects of caffeine in adipocytes, likely owing to the caffeine antagonism of A1 adenosine receptors on adipocytes.

Introduction

Caffeine is an alkaloid found in many food products including coffee and tea. It is probably the most widely used psychoactive drug [1]. Caffeine is a central nervous system stimulant, with additional effects including increases in resting energy expenditure, endurance, physical performance, and cognitive function and improvements in behavioral functions such as mood [2]. Caffeine from beverages is rapidly and completely absorbed from the gastrointestinal tract and reaches peak concentrations in the serum within 30 to 60 min [3]. Only 1% to 3% of caffeine is excreted unchanged in the urine. The rate of caffeine metabolism is variable, with a half-life of 4 to 6 h [3,4].

The most important mechanism of the action of caffeine appears to be the antagonism of adenosine receptors, although caffeine also inhibits phosphodiesterase [5]. Phosphodiesterase inhibition is minimal at the serum caffeine concentrations found after the consumption of beverages or food [5]. Adenosine receptors are found throughout the body, mainly in the brain, heart and blood vessels, respiratory tree, kidneys, adipose tissue, and gastrointestinal tract [6]. The cardiovascular responses to adenosine receptor activation have been studied for many years [7]. However, adenosine receptor densities are ~10-fold higher in adipose tissue than in cardiac tissue [8]. Mature adipocytes contain A1 adenosine receptors that inhibit lipolysis by decreasing adenylate cyclase activity, suggesting that adenosine receptors are a target for the control of obesity and diabetes [9].

Previous studies have shown the therapeutic effects of caffeine in hypertension, metabolic parameters, and hepatic fibrosis, which are components of the metabolic syndrome.

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Caffeine (0.1% in drinking water) decreased plasma glucose and insulin concentrations in rats fed high-sucrose and high-fat diets, respectively, with a decrease of mean arterial pressure in the two models [10]. Caffeine also decreased the increase in body weight and visceral adiposity observed in rats fed a high-fat diet [10]. A higher daily caffeine consumption in patients undergoing a liver biopsy for clinical indications was associated with less severe fibrosis at liver biopsy [11].

In our previous studies, rats fed a high-carbohydrate, high-fat diet showed symptoms of the metabolic syndrome, with metabolic abnormalities, cardiovascular remodeling, and non-alcoholic steatohepatitis [12]. In the present study, we have characterized the effects of caffeine in this diet-induced rat model of the metabolic syndrome. This study included the characterization of the structure and function of the cardiovascular system through echocardiography, systolic blood pressure measurements, an isolated Langendorff heart preparation, histopathology, and the vascular reactivity of the aorta. The structure and function of the liver were studied by histopathologic and plasma biochemical analyses. Similarly, body composition, abdominal fat deposition, and plasma lipid profiles were measured to assess the metabolic effects of caffeine.

Materials and methods

Rats and diets

All experimental protocols were approved by the University of Southern Queensland animal experimentation ethics committee under the guidelines of the National Health and Medical Research Council of Australia. Male Wistar rats (8–9 wk old, weight 337 ± 2 g, $n = 40$) were supplied by The University of Queensland biological resources facility. The rats were randomly assigned to one of four experimental dietary groups: a corn starch diet (C; $n = 10$), a corn starch diet plus caffeine (CC; caffeine 0.5 g/kg of food, $n = 10$), a high-carbohydrate, high-fat diet (H; $n = 10$), or a high-carbohydrate, high-fat diet plus caffeine (HC; caffeine 0.5 g/kg of food, $n = 10$). The composition of the corn starch and high-carbohydrate, high-fat diets has been described in detail in our previous studies [12,13]. The H and HC rats were given 25% fructose in drinking water in addition to their respective diets, whereas normal drinking water without any supplementation was given to the C and CC rats. The C and H rats were fed with the respective diets for 16 wk. The CC and HC rats were fed the on C and H diets, respectively, for the first 8 wk and then the diets were supplemented with caffeine (Sigma-Aldrich Australia, Sydney, Australia) for the last 8 wk of the protocol. All rats were given access ad libitum to food and water and were housed individually in temperature-controlled 12-h light/dark conditions.

The rats were monitored daily for body weight and food and water intakes. Systolic blood pressure measurements were performed on all rats at the end of protocol as described in our previous study [12]. The abdominal circumference and body length of the rats were measured using a standard measuring tape during the period of anesthesia for blood pressure measurements [12]. The body mass index, energy intake, and feed efficiency were calculated as described previously [12].

Oral glucose tolerance and insulin tolerance tests

The oral glucose tolerance and insulin tolerance tests were performed 2 d apart at 16 wk. For the oral glucose tolerance testing, the rats were food deprived for 12 h. The fructose-supplemented drinking water in the H and HC groups was replaced with normal water for the food-deprivation period. After the food-deprivation period, the basal blood glucose concentrations were measured in blood taken from the tail vein using a Medisense Precision Q.I.D. glucose meter (Abbott Laboratories, Bedford, USA). The rats were then given glucose 2 g/kg of body weight as a 40% solution by oral gavage. The tail vein blood samples were taken at 30, 60, 90, and 120 min after the glucose administration [12,13]. From these data, the area under the curve was calculated taking the x axis as the baseline.

For the insulin tolerance tests, the basal blood glucose concentrations were measured after 4 to 5 h of food deprivation as described earlier. The rats were injected with insulin-R 0.33 IU/kg (Eli Lilly Australia, West Ryde, Australia) intraperitoneally and the tail vein blood samples were taken at 30, 60, 90, and 120 min after an insulin injection. The rats were withdrawn from the test if the blood glucose concentration decreased below 1.1 mmol/L and the glucose solution 4 g/kg was immediately administered by oral gavage to prevent hypoglycemia [13].

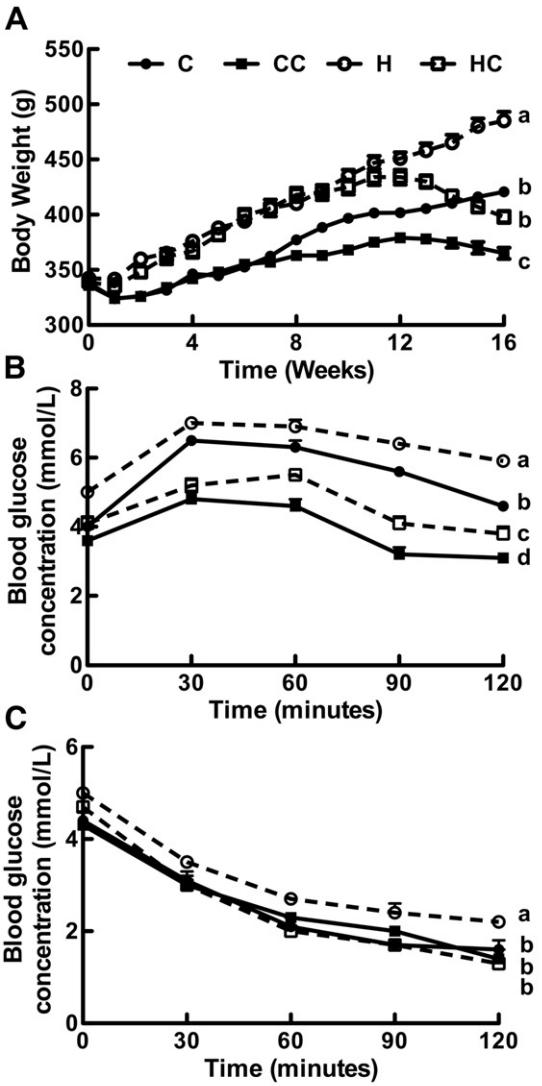

Figure	P value		
	Diet	Caff	DietxCaff
A	<0.0001	<0.0001	0.0007
B	<0.0001	<0.0001	0.029
C	0.11	0.001	0.027

Fig. 1. Effects of caffeine on body weight (A), oral glucose tolerance (B), and insulin sensitivity (C) in a diet-induced metabolic syndrome in rats. Values are presented as mean ± SEM ($n = 8$–10). Diet, Caff, and Diet × Caff represent effects of diet, caffeine, and the interaction between diet and caffeine, respectively. C, rats fed a corn starch diet; CC, rats fed a corn starch diet plus caffeine; H, rats fed a high-carbohydrate, high-fat diet; HC, rats fed a high-carbohydrate, high-fat diet plus caffeine.

Body composition measurements

The lean mass and fat masses were measured using dual-energy x-ray absorptiometry in all groups as previously described [12,13].

Isolated Langendorff heart preparation

The rats were euthanized with Lethabarb (pentobarbitone sodium; 100 mg/kg intraperitoneally; Virbac, Peakhurst, Australia). After euthanasia, heparin (200 IU; Sigma-Aldrich Australia) was injected through the right femoral vein. The abdomen was then opened and blood (~5 mL) was withdrawn from the abdominal aorta, collected into heparinized tubes, and centrifuged at 5000 × g for 15 min to obtain the plasma. The plasma was stored at −20°C before further

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Table 1
Effects of caffeine on physiologic and metabolic parameters

Variables	C	CC	H	HC	*P*		
					Diet	Caffeine	Diet × Caffeine
Initial body weight (g)	337 ± 2	336 ± 1	338 ± 2	339 ± 1	0.21	1.00	0.53
Final body weight (g)	395 ± 7^{b}	360 ± 5^{c}	480 ± 8^{a}	396 ± 6^{b}	<0.0001	<0.0001	0.0007
Water intake (mL/d)	31.3 ± 1.2^{a}	29.5 ± 1.9^{a}	18.8 ± 1.1^{b}	19.5 ± 1.4^{b}	<0.0001	0.70	0.39
Food intake (g/d)	30.8 ± 0.6^{b}	37.5 ± 0.5^{a}	23.2 ± 0.6^{c}	23.4 ± 0.4^{c}	<0.0001	<0.0001	<0.0001
Energy intake (kJ/d)	350 ± 14^{c}	421 ± 16^{b}	478 ± 11^{a}	492 ± 15^{a}	<0.0001	0.005	0.05
Feed efficiency (kJ/g)	0.16 ± 0.02^{b}	0.06 ± 0.01^{c}	0.32 ± 0.02^{a}	0.12 ± 0.01^{b}	<0.0001	<0.0001	0.003
BMI (g/cm^{2})	0.63 ± 0.01^{b}	0.59 ± 0.01^{c}	0.72 ± 0.01^{a}	0.62 ± 0.01^{b}	<0.0001	<0.0001	0.005
Caffeine intake (mg · kg^{-1} · d^{-1})	0	28.1 ± 0.5	0	47.9 ± 1.0*	—	—	—
Abdominal circumference (cm)	19.6 ± 0.4^{b}	17.9 ± 0.3^{c}	23.3 ± 0.4^{a}	19.2 ± 0.2^{b}	<0.0001	<0.0001	0.001
Whole-body lean mass (g)	317 ± 4^{b}	306 ± 5^{bc}	348 ± 4^{a}	295 ± 5^{c}	0.034	<0.0001	<0.0001
Whole-body fat mass (g)	71 ± 4^{b}	66 ± 6^{b}	152 ± 7^{a}	80 ± 6^{b}	<0.0001	<0.0001	<0.0001
Basal blood glucose concentration (mmol/L)	4.0 ± 0.1^{b}	3.6 ± 0.1^{c}	5.0 ± 0.1^{a}	4.1 ± 0.2^{b}	<0.0001	<0.0001	0.07
Area under curve (mmol · L^{-1} · min^{-1})	680 ± 13^{b}	480 ± 15^{d}	771 ± 10^{a}	562 ± 14^{c}	<0.0001	<0.0001	0.73
Plasma insulin (pmol/L)	0.41 ± 0.11	0.39 ± 0.07	0.55 ± 0.11	0.65 ± 0.06	0.05	0.66	0.51
Total cholesterol (mmol/L)	1.3 ± 0.1^{c}	2.0 ± 0.2^{b}	2.0 ± 0.1^{b}	2.6 ± 0.1^{a}	<0.0001	<0.0001	0.71
Triacylglycerols (mmol/L)	0.4 ± 0.1^{c}	0.5 ± 0.0^{c}	1.0 ± 0.1^{b}	1.5 ± 0.2^{a}	<0.0001	0.02	0.11
NEFA (mmol/L)	1.1 ± 0.2^{c}	2.2 ± 0.2^{b}	2.8 ± 0.3^{b}	5.1 ± 0.4^{a}	<0.0001	<0.0001	0.04
Retroperitoneal fat pads (mg/mm tibial length)	214 ± 9^{b}	132 ± 6^{c}	357 ± 21^{a}	198 ± 10^{b}	<0.0001	<0.0001	0.005
Epididymal fat pads (mg/mm tibial length)	129 ± 11^{b}	104 ± 8^{b}	225 ± 13^{a}	122 ± 7^{b}	<0.0001	<0.0001	0.0004
Omental fat pads (mg/mm tibial length)	93 ± 6^{b}	70 ± 5^{b}	194 ± 12^{a}	83 ± 6^{b}	<0.0001	<0.0001	<0.0001
Total abdominal fat pads (mg/mm tibial length)	435 ± 24^{b}	305 ± 18^{c}	775 ± 46^{a}	402 ± 21^{b}	<0.0001	<0.0001	0.0002

BMI, body mass index; C, rats fed a corn starch diet; CC, rats fed a corn starch diet plus caffeine; H, rats fed a high-carbohydrate, high-fat diet; HC, rats fed a high-carbohydrate, high-fat diet plus caffeine; NEFA, non-esterified fatty acid
Values are presented as mean ± SEM (n = 8–10). Means without a common superscript letter in a row differ ($P < 0.05$)
* $P < 0.0001$ (Student's t test) versus CC for caffeine intake.

biochemical analysis. The hearts were removed and used in the isolated Langendorff heart preparation to assess the left ventricular function of the rats in all groups, as in our previous study [12].

Vascular reactivity

The thoracic aortic rings (~4 mm in length) were suspended in an organ bath filled with Tyrode physiologic 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 [12]. The cumulative concentration–response curves (contraction) were obtained for noradrenaline (Sigma-Aldrich Australia) and the cumulative concentration–response curves (relaxation) were obtained for sodium nitroprusside (Sigma-Aldrich Australia) and acetylcholine (Sigma-Aldrich Australia) after a submaximal (~70%) contraction to noradrenaline [12].

Organ weights and histology

After performing the Langendorff heart perfusion studies, the hearts were separated into right and left ventricles (with the septum, n = 8). The two ventricles were weighed. The livers and abdominal fat pads (retroperitoneal, epididymal, and omental) were isolated and weighed (n = 8). These organ weights were normalized to the tibial length at the time of removal and expressed as milligrams of tissue per millimeter of tibial length [12]. The heart and liver from two rats in each group were used exclusively for the histopathologic analysis, as in our previous study [12].

Biochemical analysis of plasma samples

The plasma activities of alanine transaminase, aspartate transaminase, alkaline phosphatase, and lactate dehydrogenase and the plasma concentrations of total cholesterol, triacylglycerols, albumin, total proteins, total bilirubin, urea, and uric acid were determined using kits and controls supplied by Olympus using an Olympus analyzer (AU 400, Olympus, Tokyo, Japan) [12]. Non-esterified fatty acids (NEFAs) in the plasma were determined using a commercial kit (Wako, Osaka, Japan) [12]. The plasma concentrations of insulin (Laboratory Diagnostics, Kurnell, Australia) were measured with a commercial kit according to the manufacturer-provided standards and protocols using a Titertek Multiskan MCC/340 spectrophotometer (Flow Laboratories) [12].

Statistical analysis

All data are presented as mean ± standard error of the mean. All the groups were tested for variance using the Bartlett test and the variables that were not normally distributed were transformed (using the $\log_{10}$ function) before

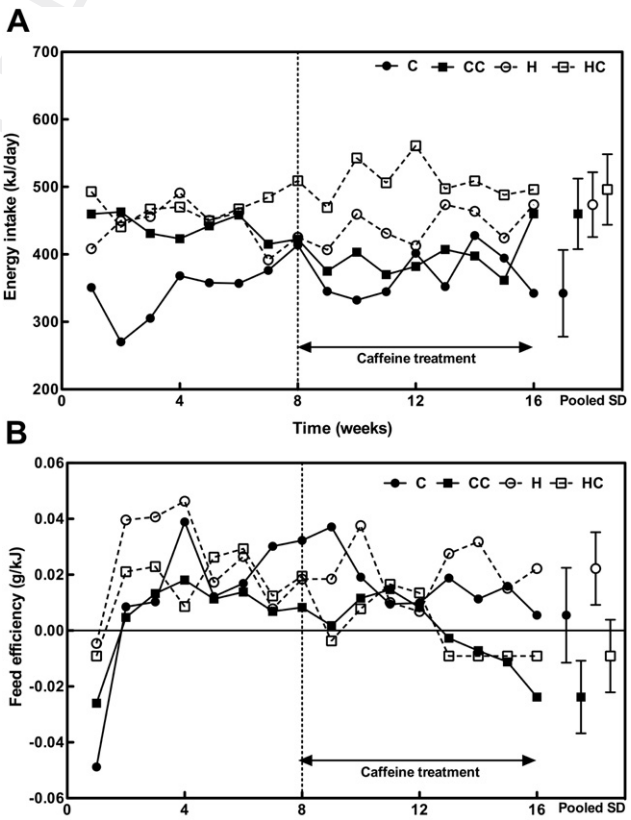

Fig. 2. Effects of caffeine on trends in weekly mean energy intake (A) and weekly feed efficiency (B) in a diet-induced metabolic syndrome in rats. Values are presented as mean and pooled SD at the end of the graph (n = 10). C, rats fed a corn starch diet; CC, rats fed a corn starch diet plus caffeine; H, rats fed a high-carbohydrate, high-fat diet; HC, rats fed a high-carbohydrate, high-fat diet plus caffeine.

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Table 2
Effects of caffeine on cardiovascular and hepatic functions

Variables	C	CC	H	HC	*P*		
					Diet	Caffeine	Diet × Caffeine
Systolic blood pressure (mmHg)	129 ± 1^{b}	118 ± 1^{c}	145 ± 1^{a}	118 ± 1^{c}	<0.0001	<0.0001	<0.0001
Left ventricular (with septum) wet weight (mg/mm tibial length)	20.3 ± 0.7^{ab}	19.0 ± 0.4^{b}	21.8 ± 1.1^{a}	18.2 ± 0.7^{b}	0.65	0.003	0.14
Right ventricular wet weight (mg/mm tibial length)	4.82 ± 0.23	4.57 ± 0.39	4.47 ± 0.32	3.81 ± 0.18	0.07	0.13	0.49
Left ventricular diastolic stiffness constant	21.1 ± 1.7^{b}	20.4 ± 1.4^{b}	28.2 ± 1.3^{a}	22.5 ± 1.6^{b}	0.005	0.043	0.11
Liver wet weight (mg/mm tibial length)	257 ± 13^{b}	245 ± 13^{b}	297 ± 11^{a}	331 ± 17^{a}	<0.0001	0.43	0.10
Plasma ALT activity (U/L)	38 ± 3^{b}	39 ± 4^{b}	55 ± 3^{a}	42 ± 3^{b}	0.004	0.08	0.04
Plasma AST activity (U/L)	75 ± 4^{b}	83 ± 6^{b}	102 ± 5^{a}	80 ± 5^{b}	0.02	0.17	0.005
Plasma ALP activity (U/L)	181 ± 13^{c}	148 ± 13^{c}	261 ± 18^{b}	363 ± 20^{a}	<0.0001	0.04	0.0002
Plasma LDH activity (U/L)	253 ± 29^{b}	218 ± 26^{b}	458 ± 31^{a}	233 ± 35^{b}	0.0009	0.0001	0.004
Plasma albumin (mg/ml)	28.1 ± 0.5	28.8 ± 0.4	28.6 ± 0.4	28.4 ± 0.6	0.92	0.61	0.36
Plasma total bilirubin (μmol/L)	2.2 ± 0.1^{ab}	1.9 ± 0.1^{bc}	2.4 ± 0.1^{a}	1.6 ± 0.2^{c}	0.71	0.0002	0.07
Plasma urea (mmol/L)	5.2 ± 0.2^{a}	5.0 ± 0.3^{a}	3.3 ± 0.3^{b}	4.6 ± 0.2^{a}	<0.0001	0.04	0.006
Plasma uric acid (μmol/L)	36 ± 3^{b}	33 ± 4^{b}	55 ± 4^{a}	30 ± 5^{b}	0.06	0.002	0.01

ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; C, rats fed a corn starch diet; CC, rats fed a corn starch diet plus caffeine; H, rats fed a high-carbohydrate, high-fat diet; HC, rats fed a high-carbohydrate, high-fat diet plus caffeine; LDH, lactate dehydrogenase
Values are presented as mean ± SEM ($n = 8$–10). Means without a common superscript letter in a row differ ($P < 0.05$)

statistical analyses. These groups were tested for the effects of diets, treatment, and/or their interactions by two-way analysis of variance. When the interaction and/or the main effects were significant, the group means were compared using the Newman–Keuls multiple-comparison post test. The weekly mean energy intake and weekly mean feed efficiency were tested with one-way repeated measures analysis of variance for trend analysis. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad Prism 5.00 for Windows (GraphPad, San Diego, CA, USA).

Results

Physiologic parameters and body composition

The H rats showed greater body weight, daily energy intake, body mass index, and abdominal circumference than the C rats, although they had lower daily water and food intakes compared with C rats (Fig. 1A, Table 1). The caffeine treatment decreased the body mass index and abdominal circumference in the CC and HC compared with the C and H rats, respectively (Table 1). The decrease in body weight started from the beginning of the caffeine treatment in the CC rats, whereas it started after 4 wk of treatment in the HC rats (Fig. 1A). The caffeine treatment in the CC and HC rats did not affect daily water intake compared with the C and H rats, respectively. The caffeine treatment increased the daily food intake in the CC rats compared with the C rats without the changing daily food intake of the HC rats compared with the H rats (Table 1). The caffeine did not affect the daily energy intake in the HC rats compared with the H rats, whereas it increased the daily energy intake in the CC rats by increasing daily food intake (Table 1). The whole-body lean mass and fat mass were greater in the H rats than in the C rats. The lean and fat masses were decreased in the HC rats compared with the H rats but not in the CC rats (Table 1).

Metabolic parameters

The basal blood glucose concentrations and the area under the curve for the oral glucose tolerance test were higher in the H

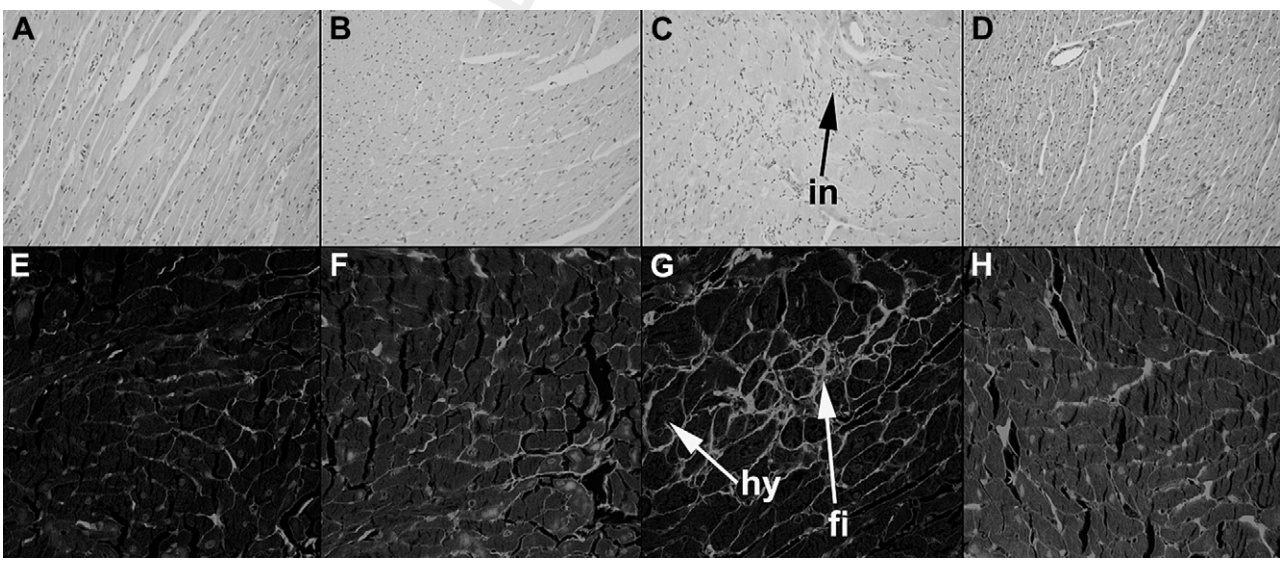

Fig. 3. Effects of caffeine on inflammation and fibrosis in the heart. (A–D) Hematoxylin and eosin staining of the left ventricle showing infiltration of inflammatory cells from rats fed a corn starch diet (A), a corn starch diet plus caffeine (B), a high-carbohydrate, high-fat diet (C), and a high-carbohydrate, high-fat diet plus caffeine (D). (E–H) Picrosirius red staining of the left ventricle showing collagen deposition and hypertrophy from rats fed a corn starch diet (E), a corn starch diet plus caffeine (F), a high-carbohydrate, high-fat diet (G), and a high-carbohydrate, high-fat diet plus caffeine (H). fi, fibrosis; hy, hypertrophied cardiomyocytes; in, inflammatory cells.

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rats than in the C rats, and these were lowered in the CC and HC rats compared with the C and H rats, respectively (Table 1, Fig. 1B). The decreased response to insulin in the H rats compared with the C rats was also improved in the HC rats (Fig. 1C). The plasma insulin concentrations were unchanged between the groups (Table 1). The plasma concentrations of total cholesterol, triacylglycerols, and NEFA were higher in the H rats than in the C rats. The plasma concentrations of total cholesterol and NEFA were higher in the CC and HC rats than in the C and H rats, respectively, whereas triacylglycerols were higher only in the HC rats than in H rats (Table 1). The retroperitoneal, epididymal, and omental fat pads were larger in the H rats than in the C rats. The HC rats showed a lowering of all three locations of abdominal fat, whereas the CC rats showed a decrease only in the retroperitoneal fat. The total abdominal fat pads were smaller in the CC and HC rats compared with the C and H rats, respectively (Table 1).

The weekly mean energy intakes and feed efficiencies are presented in Figure 2A and Figure 2B, respectively. For clarity, only the group mean values are presented, with animal variance indicated by the pooled standard deviations for each group. The feed efficiencies were negative during the first week of feeding the experimental diets in all groups but then recovered such that all were positive by week 2, with no significant differences between the treatment groups in weeks 5 to 8. The administration of caffeine from week 8 onward progressively decreased the feed efficiency in the CC and HC rats compared with the C and H rats, respectively, such that by weeks 13 through 16, the feed efficiencies were negative for the CC and HC rats but remained positive for the C and H rats ($P < 0.0001$). This decrease in feed conversion efficiency in the caffeine-fed animals paralleled the decreases in body weight seen in these animals (Fig. 1A).

Cardiovascular and hepatic parameters

The systolic blood pressure was higher in the H rats than the C rats, and this was normalized in the CC and HC rats (Table 2). The left ventricular wet weights were unchanged between the C and H rats, whereas this parameter was lowered in the HC rats compared with the H rats. The right ventricular wet weights were unchanged between the groups (Table 2). The heart tissue from the H rats had more infiltration of inflammatory cells (Fig. 3C) and collagen deposition (Fig. 3G) than from the C rats (Fig. 3A, E) and this was prevented in the HC rats (Fig. 3D, H). The left ventricular diastolic stiffness was greater in the H rats than in the C rats, and this was lowered in the HC rats compared with the H rats (Table 2). The H rats showed impaired vascular reactivity compared with the C rats, including deceased contractile responses to noradrenaline (Fig. 4A) and decreased relaxant responses to sodium nitroprusside (Fig. 4B) and acetylcholine (Fig. 4C). The CC and HC rats showed greater vascular responses to noradrenaline-induced contraction compared with the C and H rats, respectively (Fig. 4A). The HC rats showed normalized relaxant responses to sodium nitroprusside (Fig. 4B) and acetylcholine (Fig. 4C).

The livers from the H rats showed an increased infiltration of inflammatory cells (Fig. 5C) compared with the C rats (Fig. 5A). The fat vacuoles as evidence of steatosis were present in the livers from the H rats (Fig. 5G) but were absent in the livers from the C rats (Fig. 5E). The livers from the H rats but not the C rats showed mild portal fibrosis (Fig. 5K). Caffeine inhibited the infiltration of inflammatory cells (Fig. 5D), steatosis (Fig. 5H), and portal fibrosis (Fig. 5L) in the HC rats. The liver wet weights were greater for the H rats compared with the C rats, and this was unchanged by the caffeine administration in the CC and HC rats

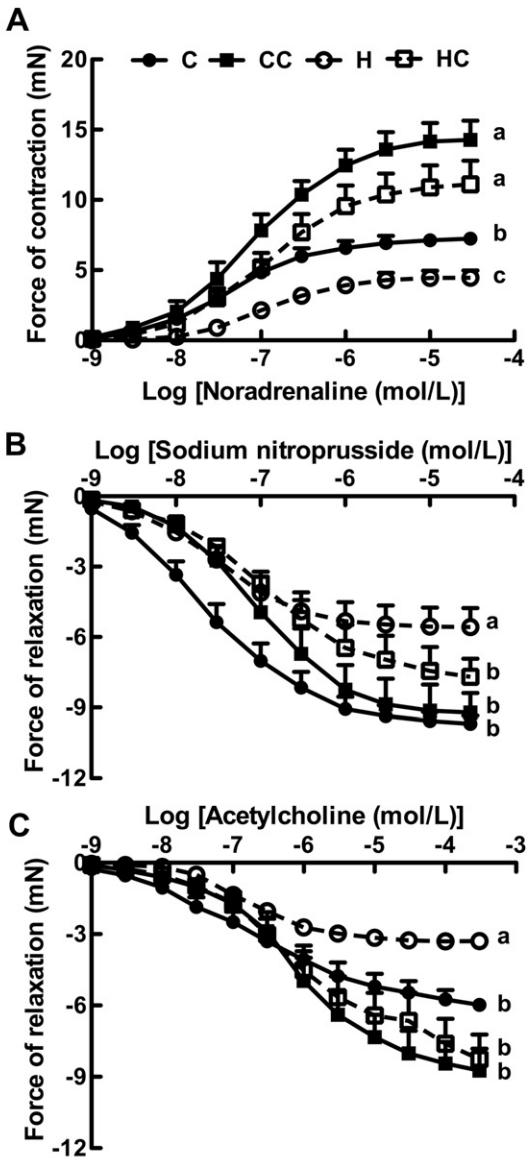

Figure	P value		
	Diet	Caff	DietxCaff
A	0.009	<0.0001	0.93
B	0.0004	0.27	0.08
C	0.046	<0.0001	0.16

Fig. 4. Effect of caffeine on concentration–response curves of the thoracic aortic rings to noradrenaline (A), sodium nitroprusside (B), and acetylcholine (C). Values are presented as mean ± SEM ($n = 8$–10). Diet, Caff, and Diet × Caff represent the effects of diet, caffeine, and the interaction between diet and caffeine, respectively. C, rats fed a corn starch diet; CC, rats fed a corn starch diet plus caffeine; H, rats fed a high-carbohydrate, high-fat diet; HC, rats fed a high-carbohydrate, high-fat diet plus caffeine.

(Table 2). The plasma activities of alanine transaminase, aspartate transaminase, alkaline phosphatase, and lactate dehydrogenase were greater in the H rats than in the C rats. Caffeine decreased the plasma activities of alanine transaminase, aspartate transaminase, and lactate dehydrogenase in the HC rats without affecting these activities in the CC rats, whereas caffeine

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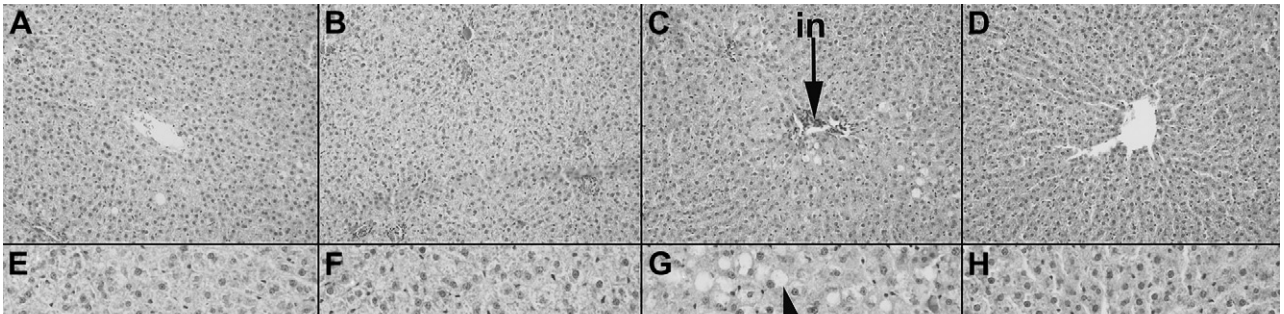

Fig. 5. Effects of caffeine on inflammation, fat deposition, and fibrosis in the liver. (A–H) Hematoxylin and eosin staining of the liver showing inflammatory cells (A–D, magnification 20×) and enlarged fat vacuoles (E–H, magnification 40×), respectively, from rats fed a corn starch diet (A, E), a corn starch diet plus caffeine (B, F), a high-carbohydrate, high-fat diet (C, G), and a high-carbohydrate, high-fat diet plus caffeine (D, H). (I–L) Milligan trichrome staining of the liver showing fibrosis in the hepatic portal region (magnification 20×) from rats fed a corn starch diet (I), a corn starch diet plus caffeine (J), a high-carbohydrate, high-fat diet (K), and a high-carbohydrate, high-fat diet plus caffeine (L). fi, fibrosis; fv, fat vacuoles; in, inflammatory cells.

increased the plasma activity of alkaline phosphatase in the HC rats without changing it in the CC rats (Table 2). Although total plasma bilirubin was unchanged between the C and H rats, the HC rats showed lower plasma concentrations of total bilirubin (Table 2). The plasma concentrations of urea decreased and uric acid increased in the H rats compared with the C rats. The plasma concentrations of urea and uric acid were normalized in the HC rats (Table 2).

Discussion

The metabolic syndrome is the clustering of various risk factors for cardiovascular disease and non-alcoholic fatty liver disease [14]. These risk factors include central obesity, hypertension, impaired glucose tolerance, dyslipidemia, and insulin resistance [14]. Because caffeine is readily available in the diet from widely consumed non-alcoholic beverages [15], it is important to understand its effects on the functioning of organs in chronic conditions such as the metabolic syndrome. Epidemiologic studies have suggested that the incidence of the metabolic syndrome is increasing in the worldwide population [16]. In this study, we have evaluated the responses to caffeine in rats fed a high-carbohydrate, high-fat diet as an appropriate model for the human metabolic syndrome.

The physiologic and pharmacologic effects of caffeine have been extensively studied and reviewed [1,2,17]. Although caffeine has beneficial effects, including central nervous system stimulant responses, the long-term consumption of caffeine has been associated with the development of tolerance to caffeine leading to lowered responses to caffeine after long-term administration [18]. Thus, it will be an important issue to adjust the dose of caffeine such that the development of tolerance can be delayed. The role of adenosine receptors in the development of tolerance to caffeine has been controversial [19,20]. There are also possible adverse effects of caffeine during pregnancy and fetal growth [21].

In this study, the rats fed the high-carbohydrate, high-fat diet developed most of the symptoms associated with the human metabolic syndrome, including central obesity, hypertension, dyslipidemia, impaired glucose tolerance, cardiovascular remodeling, and non-alcoholic fatty liver disease [12]. Caffeine supplementation in these rats attenuated the obesity, hypertension, cardiovascular remodeling, non-alcoholic fatty liver disease, and metabolic parameters, including glucose tolerance and insulin sensitivity. However, dyslipidemia was worsened.

Caffeine decreased the whole-body fat mass and the abdominal fat pads. This indicates that there was a removal of fat from the abdominal area, and this fat was not transported to the other fat-storing areas, including subcutaneous fat, unlike in chia seed-fed rats in which redistribution of abdominal fat occurs [13]. The increase in plasma lipid components, especially NEFAs, reflects the removal of fat from the abdomen after its conversion to NEFAs by the process of lipolysis. These results suggest that caffeine induced the lipolysis in adipocytes and, hence, decreased the abdominal fat pads. The overall decreases in the fat mass in the absence of a fat redistribution suggest that the excess plasma lipids are being metabolized rather than stored in the organs.

The rats, once adapted to the C or H diet, did not show any change in energy intake. With caffeine administration, the rats having a similar energy intake as their controls showed a decrease in feed efficiency and, hence, body weight. The decrease in feed efficiency may result from an increased energy expenditure, thermogenesis, maldigestion, or malabsorption. In

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previous studies, caffeine has shown thermogenic effects in lean and obese subjects [22]. In another study, 0.025% caffeine decreased the body fat in rats fed a high-fat diet by inducing lipolysis and, hence, increasing NEFAs in the blood [23].

Adenosine strongly inhibited hormone-induced lipolysis [9] and adenosine receptor agonists have been evaluated as lipid-lowering agents [6], lowering plasma NEFAs and triacylglycerols by suppressing lipolysis and triacylglycerol secretion [24]. In the cardiovascular system, the activation of A1 receptors decreased cardiac contractility and contributed to adverse ventricular remodeling [25]. In contrast, 8-cyclopentyl-1,3-dipropylxanthine, a selective A1 receptor antagonist, increased lipolysis in isolated rat adipocytes without affecting lipogenesis [26]. Caffeine acts as a non-selective antagonist on adenosine receptors [5]. The antagonism of A1 adenosine receptors with caffeine increased cyclic adenosine monophosphate production, thereby increasing lipolysis and, hence, the removal of fat from the adipocytes in the abdomen. This increased lipolysis was observed as a further increase in the plasma concentrations of triacylglycerols, total cholesterol, and NEFAs in the HC rats. The antiobesity and cardioprotective effects of caffeine in this study are consistent with the antagonism of adipocyte and cardiomyocyte A1 adenosine receptors. Further, the improvements in the liver structure and function could be due to liver A1 receptor antagonism because adenosine production and adenosine receptor activation in the liver play critical roles in the development of hepatic fibrosis [27,28]. Although serum concentrations of caffeine after food or beverage consumption have shown a minimal inhibition of phosphodiesterase activity [5], the inhibition of phosphodiesterases has shown promise against obesity and diabetes [29,30].

Although high NEFA concentrations increase the risk of cardiovascular dysfunction and hepatic steatosis [31], such complications did not present in this study despite much higher plasma concentrations of NEFAs in the caffeine-supplemented rats. These rats showed a decreased infiltration of inflammatory cells, decreased collagen deposition, and decreased diastolic stiffness in the left ventricle, an attenuation of non-alcoholic steatohepatitis, and high plasma NEFA concentrations.

In our previous study, we investigated the effects of the H diet on metabolic parameters [12]. Among these metabolic parameters, the basal blood glucose concentrations and glucose tolerance were improved with caffeine supplementation. Other changes included normalization of the plasma urea and uric acid concentrations. Previous studies have reported that caffeine increases serum urea concentrations [32], and the lowering of serum uric acid concentrations has been attributed to components of coffee other than caffeine [33].

The toxicity of caffeine is rarely associated with death, although psychologic disturbances have been reported with very high caffeine intakes in human studies [1,17,34]. The available data from extensive literature surveys of human studies with caffeine have indicated that the consumption of caffeine $\leq$0.4 g/d in adults is not associated with any adverse effects on health [2,17]. The dose used in this study corresponds to caffeine ~0.6 g/d according to a scaling equation [35] and ~0.35 g/d based on body surface area calculations [36]. These doses are achievable in humans and are in the range of safe doses for caffeine.

Conclusion

This study has defined the cardioprotective and hepatoprotective effects of caffeine in a diet-induced rat model of the human metabolic syndrome. These protective effects were accompanied by decreases in body weight and abdominal fat. The decrease in abdominal fat could be due to an antagonism by caffeine of A1 adenosine receptors present on adipocytes, leading to lipolysis. Although the protective effects of caffeine have been indicated with long-term feeding in the rats, the translation of these results to humans could be compromised by the development of caffeine tolerance after a long-term caffeine intake.

Acknowledgments

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American Journal of Physiology – Endocrinology and Metabolism

Chapter 7 *L-Carnitine attenuates symptoms of metabolic syndrome in high-carbohydrate, high-fat diet-fed rats*

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Running head: *L-Carnitine in diet-induced metabolic syndrome.*

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Abstract

Obesity and dyslipidemia are metabolic defects resulting from impaired lipid metabolism. In turn, these disorders are associated with the development of cardiovascular disease and non-alcoholic fatty liver disease. Measures to correct the defects in lipid metabolism may attenuate obesity, dyslipidemia, and associated complications. L-Carnitine supplementation was used in this study to enhance fatty acid oxidation to ameliorate these disturbances in lipid metabolism. Male Wistar rats (8-9 weeks old) were fed with either corn starch-rich or high-carbohydrate, high-fat diets for 16 week. Separate groups were selected for L-carnitine supplementation (1.2% in food) on either diet for the last 8 week of the protocol. High-carbohydrate, high-fat diet-fed rats showed central obesity, dyslipidemia, hypertension, impaired glucose tolerance, hyperinsulinemia, cardiovascular remodeling and nonalcoholic fatty liver disease. L-Carnitine supplementation attenuated these high-carbohydrate, high-fat diet-induced changes, together with modifications in lipid metabolism. This included inhibition of stearoyl-CoA desaturase-1 (SCD-1) activity in the tissues measured through SCD-1 activity index, reduced short-chain monounsaturated fatty acids storage in the tissues, and decreased polyunsaturated fatty acids contents in the tissues. Thus, L-carnitine supplementation attenuated obesity, dyslipidemia, and complications associated with metabolic syndrome through inhibition of SCD-1 activity, preferential oxidation of polyunsaturated fatty acids, and storage of saturated fatty acids and relatively inert oleic acid in the tissues.

Keywords: Metabolism, Fatty acids, L-carnitine, Obesity, Cardiovascular remodeling.

Introduction

Obesity is a chronic pathological condition characterized by excess fat deposition in the adipose tissue. A major reason for the increasing incidence of obesity around the world is the combination of a cafeteria-style diet with higher energy densities together with lower expenditure of this energy (21,24,34). Cafeteria/Western diet is rich in saturated fats and carbohydrates, mainly fructose and sucrose. Increasing intake of lipogenic carbohydrates such as fructose (18) and saturated fats can lead to disturbance in the metabolism of these macronutrients in the body (38). Decreased insulin sensitivity, hyperinsulinemia, and dyslipidemia are the other factors associated with obesity (15) that contribute towards the imbalance in metabolism of fat and carbohydrate (11,14). These metabolic disturbances can worsen the structure and function of tissues including the heart, liver, and skeletal muscle (2), including cardiovascular remodeling and nonalcoholic fatty liver disease (26).

L-Carnitine is a quaternary amine synthesized from lysine and methionine in the liver and kidneys. L-Carnitine is involved in fatty acid transport across mitochondrial membranes by carnitine palmitoyltransferase 1 (CPT1) for β-oxidation (4). In humans, L-carnitine is generally taken in the diet through meat products. If the requirement of L-carnitine is not fulfilled from the diet, as in vegetarians, it is synthesized in the body along with an increase in the absorption from the diet (9). Deficiency of L-carnitine in the body leads to pathophysiological complications including cardiomyopathy, encephalopathy, and skeletal muscle myopathy (8,37).

L-Carnitine enhanced oxidation of long chain fatty acids in the liver and the heart (10). Oral L-carnitine supplementation led to an increase in the total concentration of L-carnitine in humans (3). Further, L-carnitine supplementation decreased post-exercise venous concentrations of lactic acid (3). Athletes use L-carnitine for reducing body fat in order to lose weight by metabolism of long chain fatty acids (16).

Administration of L-carnitine could alleviate hypertension, obesity, dyslipidemia, insulin resistance, and fatty liver as the major signs of metabolic syndrome. L-Carnitine decreased blood pressure and improved the structure and function of the heart in DOCA-salt-induced cardiovascular remodeling in rats (23). In fructose-fed rats, L-carnitine reduced plasma concentrations of glucose, insulin, free fatty acids, and triglycerides, improved glucose tolerance, and showed protective effects on kidneys (32,33). A combination of either orlistat or sibutramine with L-carnitine improved lipid profile, insulin resistance, body weight, and inflammation in type 2 diabetic patients more than with either orlistat or sibutramine (5,6). L-Carnitine supplementation in type 2 diabetic patients did not change body weight, body mass index, fasting plasma glucose, and plasma total cholesterol, but reduced plasma triglycerides and total body fat (19). L-Carnitine reduced plasma activities of aspartate transaminase (AST), alanine transaminase (ALT), and γ-glutamyl transpeptidase (GGT) and plasma concentrations of total cholesterol, triglycerides, and inflammatory markers along with attenuation of nonalcoholic steatohepatitis in patients (20).

In this study, we increased dietary L-carnitine in high-carbohydrate, high-fat diet-fed male Wistar rats to increase the oxidation of stored fat. The metabolic effects of L-carnitine were characterized through measurements of body composition, glucose tolerance, and plasma lipid profile. Fatty acid composition of plasma, heart, liver, skeletal muscle, and retroperitoneal fat pads was measured to study the changes in metabolism and storage of fatty acids. Changes in cardiovascular structure and functions were evaluated through systolic blood pressure measurements, echocardiography, isolated Langendorff heart preparation, vascular responses including contraction and relaxation properties of thoracic aorta, and histopathology. Hepatic structure and function were determined through liver function tests and histopathology.

Experimental procedure

Rats, diets, and L-carnitine supplementation

All experimental protocols were approved by The University of Queensland and University of Southern Queensland Animal Experimentation Ethics Committees, under the guidelines of the National Health and Medical Research Council of Australia. Male Wistar rats (8-9 weeks old, weighing 339 ± 4 g, n = 48) were supplied by The University of Queensland Biological Resources facility. Rats were randomly divided into two experimental diet groups and were fed with either corn starch diet (C; n = 24) or high-carbohydrate, high-fat diet (H; n = 24) for 16 weeks. After 8 weeks of feeding with respective diets, 12 rats from each group were randomly separated and

treated with 1.2 % L-carnitine in food with same diet continued for further 8 weeks (CLC and HLC, respectively). The remaining 12 rats from both diet groups (C and H) were continued on the original diet without any treatment for further 8 weeks. The diet of C rats mainly consisted of corn starch (polysaccharides) while the diet of H rats contained beef tallow (saturated and monounsaturated fats), fructose, and condensed milk. Compositions of C and H diets used in this study have been described in detail in our previous studies (25,29,30). C and CLC rats were given drinking water without any additives whereas drinking water for H and HLC rats were supplemented with 25% fructose. All rats were provided *ad libitum* access to food and water and were individually housed in temperature-controlled 12 hour light-dark conditions.

Physiological and metabolic parameters

Rats were monitored daily for body weight, food and water intakes. Abdominal circumference, body mass index (BMI), energy intake, and feed efficiency were measured as described (25). At the end of the protocol, rats were food-deprived for 12 hours and oral glucose tolerance tests were performed as described (25). The blood glucose concentrations obtained from oral glucose tolerance tests were used to calculate the area under the curve by taking x-axis as the baseline. Body compositions of rats were measured at the end of the protocol (25,30). During terminal experiments, abdominal fat pads (separately as retroperitoneal, epididymal, and omental) were removed, weighed, and normalized to tibial length at the time of terminal experiments (25). Plasma concentrations of albumin, total bilirubin, urea, uric acid, total

cholesterol, and triglycerides were determined using kits and controls supplied by Olympus using an Olympus analyzer (AU 400, Tokyo, Japan) (25). Non-esterified fatty acids (NEFA) in plasma were determined using a commercial kit (Wako, Osaka, Japan) (25).

Cardiovascular structure and function

Systolic blood pressure measurements: Systolic blood pressures of rats were measured under light sedation with Zoletil (tiletamine 10 mg/kg, zolazepam 10 mg/kg intraperitoneally), using an MLT1010 Piezo-Electric Pulse Transducer (ADInstruments, Sydney, Australia) and inflatable tail-cuff connected to a MLT844 Physiological Pressure Transducer (ADInstruments, Sydney, Australia) and PowerLab data acquisition unit (ADInstruments, Sydney, Australia). These measurements were performed every fourth week (25).

Echocardiography: At the end of the protocol, echocardiographic examinations (Phillips iE33, 12MHz transducer) were performed to assess the cardiovascular structure and function in rats (25).

Isolated Langendorff heart preparation: During terminal experiments, rats were euthanized with Lethabarb (pentobarbitone sodium, 100 mg/kg intraperitoneally; Virbac, Peakhurst, NSW, Australia). After euthanasia, heparin (200 IU; Sigma-Aldrich Australia, Sydney, Australia) was injected through the right femoral vein. The abdomen was then opened and blood (~6-8 ml) was withdrawn from the abdominal aorta, collected into heparinized tubes, and

centrifuged at 5,000 × *g* for 15 minutes to obtain plasma. Plasma was stored at -20°C before further analysis. Hearts were removed and were used in isolated Langendorff heart preparation to assess left ventricular function of the rats (n = 9 from each group) (25). After performing Langendorff heart perfusion studies, hearts were separated into right ventricle and left ventricle (with septum). Both ventricles were weighed.

Vascular reactivity: Thoracic aortic rings from rats (~4 mm in length; n = 10-12 from each group) were suspended in an organ bath maintained at 35˚C and filled with Tyrode physiological salt solution bubbled with 95% O_2-5% CO_2 and allowed to stabilize at a resting tension of ~10 mN. Cumulative concentration-response curves (contraction) were obtained for norepinephrine (Sigma-Aldrich Australia, Sydney, Australia) and cumulative concentration-response curves (relaxation) were obtained for ACh (Sigma-Aldrich Australia) and sodium nitroprusside (Sigma-Aldrich Australia) following submaximal (70%) contraction to norepinephrine (25).

Histology of the heart: Three rats were exclusively used for histology from each group. Hearts were removed from these rats soon after euthanasia and were processed for histological assessments for inflammatory cells and collagen deposition (25).

Hepatic structure and function

Livers (n = 9 from each group) were isolated and weighed. Liver portions from histology rats were isolated (n = 3 from each group) and fixed in 10%

neutral buffered formalin for three days. These portions were used to determine fat deposition, infiltration of inflammatory cells, and portal fibrosis (25). Plasma activities of ALT, AST, alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) were measured to determine hepatic function (25).

Fatty acid analysis

Hearts (immediately after perfusion studies), liver portions (~6-8 g), retroperitoneal fat (~6-8 g), epididymal fat (~6-8 g), skeletal muscle (~6-8 g), and plasma (~2 ml) were isolated during terminal experiments (n = 6 from each group) and were stored at -20°C. These samples were then used for fatty acid analysis (30). The concentrations of the following fatty acids were determined in these samples: capric acid (C10:0), lauric acid (C12:0), dodecenoic acid (C12:1n-9), myristic acid (C14:0), myristoleic acid (C14:1n-5), palmitic acid (C16:0), palmitoleic acid (C16:1n-7), stearic acid (C18:0), vaccenic acid (C18:1*trans*-7), oleic acid (C18:1n-9), linoleic acid (C18:2n-6), α-linolenic acid (C18:3n-3), γ-linolenic acid (C18:3n-6), eicosanoic acid (C20:0), eicosenoic acid (C20:1n-9), eicosadienoic acid (C20:2n-6), dihomo-γ-linolenic acid (C20:3n-6), eicosatrienoic acid (C20:3n-3), arachidonic acid (C20:4n-6), eicosapentaenoic acid (C20:5n-3), behenic acid (C22:0), erucic acid (C22:1n-9), docosadienoic acid (C22:2n-6), docosatetraenoic acid (C22:4n-6), docosapentaenoic acid (C22:5n-3), docosahexaenoic acid (C22:6n-3), lignoceric acid (C24:0), and nervonic acid (C24:1n-9). Margaric acid (C17:0) was used as an internal standard in this procedure. All fatty acids were

expressed as % of total recovered fatty acids. The n-3:n-6 ratio and stearoyl-CoA desaturase-1 (SCD-1) activity index were calculated from fatty acid concentrations obtained (30).

Statistical analysis

All data are presented as mean ± SEM. Groups of rats 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. These groups were tested for effects of diet, L-carnitine, and their interactions by two-way ANOVA. When interaction and/or the main effects were significant, means were compared using Newman-Keuls multiple comparison post-test. $P < 0.05$ was considered significant. All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (San Diego, California, USA).

Results

Physiological, compositional, and metabolic parameters

H rats had higher body weight compared to C rats at 16 week and HLC rats showed lower body weight than H rats at 16 week. Food and water intakes were lower in H rats compared to C rats. Food and water intakes were unchanged in both HLC and CLC rats. Abdominal circumference along with retroperitoneal, epididymal, and omental fat pads were higher in H rats compared to C rats. These parameters were lowered in both CLC and HLC rats compared to C and H rats, respectively. HLC rats had lower fat mass compared

to H rats with no difference in lean mass between the groups. Basal blood glucose concentrations, AUC during oral glucose tolerance test, and plasma insulin concentrations were normalized in HLC rats. Plasma lipid components including total cholesterol, triglycerides, and non-esterified fatty acids were normalized in HLC rats (Table 1).

Plasma fatty acid composition: C16:0 and C16:1n-7 were increased and decreased, respectively, in plasma by L-carnitine in both CLC and HLC rats compared to C and H rats, respectively. C18:0 and C18:2n-6 concentrations were unchanged between the groups. C18:1n-9 was increased while C18:1*trans*-7 was decreased in HLC rats compared to H rats. C20:4n-6 concentrations were lower in H rats than in C rats and were unaffected by L-carnitine in plasma of both CLC and HLC rats. The remaining fatty acids were undetectable in plasma in all groups. Overall, saturated fatty acids (SFA) that were decreased in H rats compared to C rats were increased with L-carnitine supplementation in both CLC and HLC rats whereas monounsaturated fatty acids (MUFA) were decreased in CLC and HLC rats compared to C and H rats, respectively. Polyunsaturated fatty acids (PUFA) were unchanged between the groups. SCD-1 activity was decreased in both CLC and HLC rats compared to C and H rats, respectively (Table 1).

Cardiovascular structure and function

Hearts from H rats showed presence of inflammatory cells (Figure 1C) and collagen deposition (Figure 1G). The infiltration of inflammatory cells and

collagen deposition were inhibited in HLC rats (Figure 1D and 1H). Systolic blood pressure was higher in H rats and lowered in HLC rats. Hearts from H rats showed higher left ventricular internal diameter during diastole (LVIDd), indicative of ventricular dilation, increasing systolic volume. These changes affected the ventricular functional parameters including fractional shortening, ejection fraction, E/A ratio, and left ventricular diastolic stiffness (Table 2). HLC rats showed normalized LVIDd, systolic volume, ejection fraction, fractional shortening, E/A ratio, and left ventricular stiffness (Table 2). Thoracic aortic responses to norepinephrine (Figure 2A), sodium nitroprusside (Figure 2B), and ACh (Figure 2C) were diminished in H rats but improved in thoracic aorta from HLC rats (Figure 2A, 2B, and 2C).

Cardiac fatty acid composition: C16:0, C18:0, and C18:1n-9 were increased while C14:1n-5, C16:1n-7, C18:1*trans*-7, C18:3n-6, and C20:4n-6 were decreased in both CLC and HLC rats. C18:2n-6 was increased only in CLC. Other fatty acids were not detected in any of the groups. Total concentrations of SFA in cardiac tissue were increased in both CLC and HLC rats whereas MUFA and PUFA were decreased in both CLC and HLC rats. Within PUFA, n-3 fatty acids were lower than n-6 fatty acids in both CLC and HLC rats. This was indicated by the decreased n-3:n-6 ratio. SCD-1 activity was completely inhibited in both CLC and HLC rats (Table 2).

Hepatic structure and function

H rats showed presence of inflammatory cells (Figure 4C), fat vacuoles (Figure 4G), and mild portal fibrosis (Figure 4K) in the liver. HLC rats showed inhibition of infiltration of inflammatory cells (Figure 4D) and absence of fat vacuoles (Figure 4H) and portal fibrosis (Figure 4L). Livers from H rats showed higher wet weight and this was unaffected by L-carnitine supplementation in HLC rats. However, the markers of hepatic function in plasma including ALT, AST, ALP, and LDH, which were increased in H rats, were normalized in HLC rats. No differences were observed in the plasma concentrations of albumin and total bilirubin between the groups. Plasma uric acid concentrations were higher while plasma urea concentrations were higher in H rats than in C rats. These biochemical changes were attenuated in HLC rats (Table 3).

Hepatic fatty acid composition: C16:0, C18:0, and C18:1n-9 were increased whereas C14:1n-5, C16:1n-7, C18:1*trans*-7, and C18:2n-6 were decreased in both CLC and HLC rats. C14:0 was lower only in CLC rats whereas C20:3n-6 was unchanged between the groups. C20:4n-6 was lower in HLC rats. Other fatty acids were undetectable in liver. Overall, SFA were increased in both CLC and HLC rats while MUFA were increased only in HLC rats. PUFA were decreased in both CLC and HLC rats. n-3:n-6 fatty acids ratio was lower in both CLC and HLC rats compared to C and H rats, respectively (Table 3).

Skeletal muscle

Fatty acid composition: C16:0, C18:0, and C18:1n-9 were increased whereas C14:0, C14:1n-5, C16:1n-7, C18:2n-6, and C18:3n-6 were decreased in both CLC and HLC rats. C18:1*trans*-7 was increased only in CLC rats. C20:4n-6 was lower in H rats than in C rats and it was increased in HLC compared to H rats. Total SFA and MUFA were increased while total PUFA were decreased in both CLC and HLC rats. n-3:n-6 ratio was unchanged between the groups. SCD-1 activities were lower in both CLC and HLC rats (Table 4).

Fatty acid composition of retroperitoneal fat

C14:0, C16:0, C18:0, and C18:3n-3 were increased while C14:1n-5, C16:1n-7, C18:2n-6, and C18:3n-3 were decreased in retroperitoneal fat pads from both CLC and HLC. In CLC rats, mostly C18:1n-9 was stored whereas in HLC rats, C18:1*trans*-7 was stored in the retroperitoneal fat pads. C20:0 was lowered in CLC whereas it was increased in HLC rats. All other fatty acids were not detected in retroperitoneal fat pads. Total SFA and MUFA were increased in retroperitoneal fat pads whereas PUFA were decreased in both CLC and HLC rats. n-3:n-6 ratio was unchanged between the groups. SCD-1 activity was lowered in both CLC and HLC rats (Table 5).

Discussion

This study has characterized the metabolic and physiological effects of L-carnitine in a model of diet-induced metabolic syndrome in rats. This model mimics the human metabolic syndrome including the development of cardiovascular remodeling, nonalcoholic steatohepatitis, renal damage, pancreatic hypertrophy, and hyperinsulinemia (25). L-Carnitine supplementation in these rats attenuated the symptoms of metabolic syndrome including hypertension, impaired glucose tolerance, central obesity, dyslipidemia, and hyperinsulinemia. L-Carnitine also attenuated ventricular remodeling including dilation and fibrosis, as well as hepatic steatosis and fibrosis, and improved the function of the left ventricle and the liver.

Obesity is a chronic condition where excess fat is deposited in the body, especially the abdomen. Deposition of this excess fat in the abdomen is associated with the development of inflammatory organ damage including cardiovascular remodeling and nonalcoholic fatty liver disease (7,39). The presence of these complications is increasing throughout the world and the cost of health management is proportionately increasing (1).

Removal of fat from the abdomen, with reduced triglycerides and NEFA in plasma confirms increased β-oxidation in obese rats due to L-carnitine supplementation. In the heart, fatty acids are the major fuel source for energy supply (12). With addition of L-carnitine to the diet, increased availability of L-carnitine may shift the energy supply in the heart to fatty acid oxidation. In our

previous study, L-carnitine supplementation in an acute model of cardiovascular remodeling improved cardiac function (23). Reduced β-oxidation of fatty acids coupled with increased lipogenesis in the liver leads to steatosis followed by steatohepatitis (36) while increasing fatty acid oxidation by L-carnitine attenuated steatohepatitis (20,40).

L-Carnitine decreased the proportion of pro-inflammatory n-6 and *trans* fatty acids in the heart and the liver leading to the accumulation of C18:1n-9 in all tissues. However, C20:4n-6 and C18:1*trans*-7 were increased in the skeletal muscle suggesting a substrate bias in different tissues. Importantly, 18:2n-6 in the adipose tissue of L-carnitine-supplemented rats was substituted with equivalent amounts of C18:1n-9 and C18:1*trans*-7, respectively. This result strongly suggests that L-carnitine selectively facilitated the transport and oxidation of C18:2n-6 therefore limiting the production of C20:4n-6 to exert its pro-inflammatory effects. All the tissues showed extremely low indexes for SCD-1 activity indicating the inhibition of conversion of SFA to MUFA. Thus, SFA were increased in the plasma and the tissues. At the same time, only plasma and the heart showed reductions in total MUFA whereas the liver and the skeletal muscle preferentially stored MUFA in the form of C18:1n-9. Retroperitoneal fat from CLC rats stored C18:1n-9 whereas HLC rats stored C18:1*trans*-7 in retroperitoneal fat. Similar results were obtained in our previous study with chia seed in obese rats (30). Higher SCD-1 activity has been implicated in obesity and cardiovascular disease with inhibition of SCD-1 activity inducing an increase in fatty acid oxidation and decrease in body fat

(29). These results have been confirmed in this study with the decrease in the activity index of SCD-1 in all the tissues and attenuation of obesity and cardiovascular disease. Further study will be required to determine the mechanism of inhibition of SCD-1 by L-carnitine.

In fructose-fed rats, L-carnitine supplementation reduced plasma concentrations of glucose, insulin, triglycerides, and NEFA, and the liver and muscle content of triglycerides and NEFA (31). Also, glycogen content in the liver and the muscle were increased along with reduced activity of glycogen phosphorylase. Similarly, L-carnitine-treated rats showed reduced gluconeogenesis from different sources such as lactic acid, pyruvic acid, etc. (31). These observations clearly suggest that L-carnitine supplementation increased glycogenesis and reduced glycogenolysis and gluconeogenesis in fructose-fed rats. These effects of L-carnitine were observed in this study as lower blood glucose concentrations and plasma concentrations of insulin, triglyceride, and NEFA. These effects are supported by a recent study in piglets supplemented with L-carnitine where 563 genes were differentially expressed by L-carnitine supplementation, especially genes for the proteins and enzymes encoding for fatty acid uptake, fatty acid activation, and fatty acid oxidation (17). Similarly, genes for glycolysis were up-regulated and genes involved in gluconeogenesis were down-regulated (17).

This study along with earlier studies supports that L-carnitine supplementation attenuates the complications in carbohydrate and lipid metabolism in high carbohydrate, high fat-fed rats (4,22,27). Also, these

metabolic complications have been implicated in oxidative stress (35) including diminishing aortic relaxant responses (13). L-Carnitine supplementation attenuated the metabolic complications and hence improved aortic responses to sodium nitroprusside and acetylcholine were observed in this study.

In conclusion, high-carbohydrate, high fat diet-fed rats serve as a suitable animal model for the metabolic, cardiovascular and hepatic complications seen in human metabolic syndrome. L-Carnitine supplementation in these rats attenuated the symptoms of metabolic syndrome, cardiovascular remodeling and non-alcoholic fatty liver disease. Also, SCD-1 activity was inhibited by L-carnitine and hence reduced short chain MUFA in the tissues. At the same time, PUFA, specially C18:2n-6, were preferentially subjected to oxidation whereas SFA and C18:1n-9 were stored in the tissues.

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Author contribution

S.K.P. and L.B. designed the research protocol; S.K.P. conducted animal experiments; H.P. and J.W. conducted fatty acid analyses; L.W. performed body-composition measurements and advised on nutrition; K.K. assisted with plasma biochemical analyses, especially insulin measurements; S.K.P. and L.B. wrote the paper; and L.B. had primary responsibility for final content. All authors read and approved the final manuscript.

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Disclosures

None.

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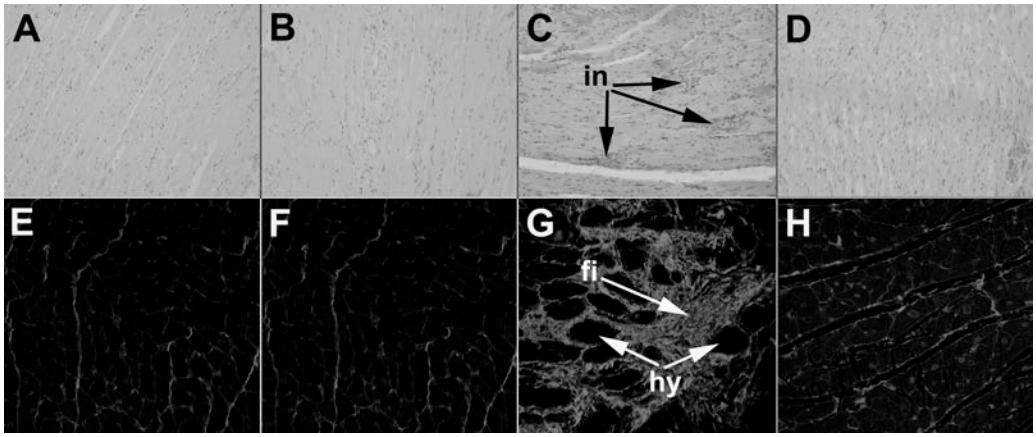

Figure 1. Effects of L-carnitine supplementation on inflammation and fibrosis in rat heart. Top row represents hematoxylin and eosin staining of left ventricle showing infiltration of inflammatory cells (A-D, inflammatory cells marked as "in"; ×20) from C (A), CLC (B), H (C), and HLC (D) rats. Bottom row represents picrosirius red staining of left ventricle showing collagen deposition and hypertrophy (E-H, fibrosis marked as "fi" and hypertrophied cardiomyocytes as "hy"; ×40) from C (E), CLC (F), H (G), and HLC (H) rats.

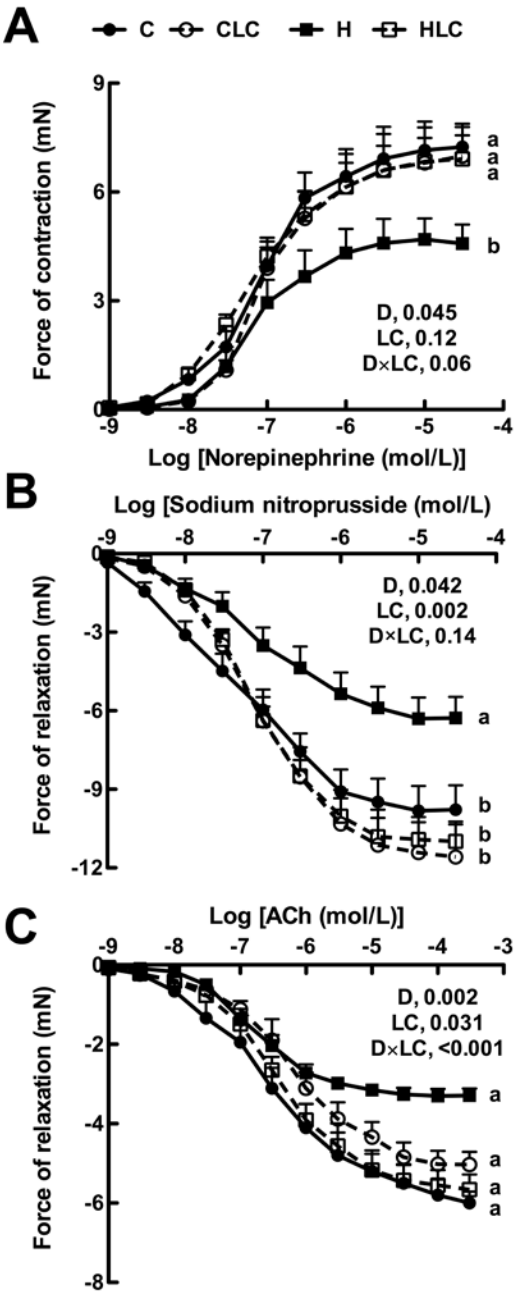

Figure 2. Effects of L-carnitine supplementation on va scular responses of thoracic aortic preparations. Norepinephrine-induced contraction (A), sodium nitroprusside-induced relaxation (B), and ACh-induced relaxation (C). Values are mean ± SEM, n = 10 - 12. End-point means without a common letter differ, $P < 0.05$. D, LC, and D×LC represent effects of diet, effects of L-carnitine, and interaction between effects of diet and L-carnitine, respectively.

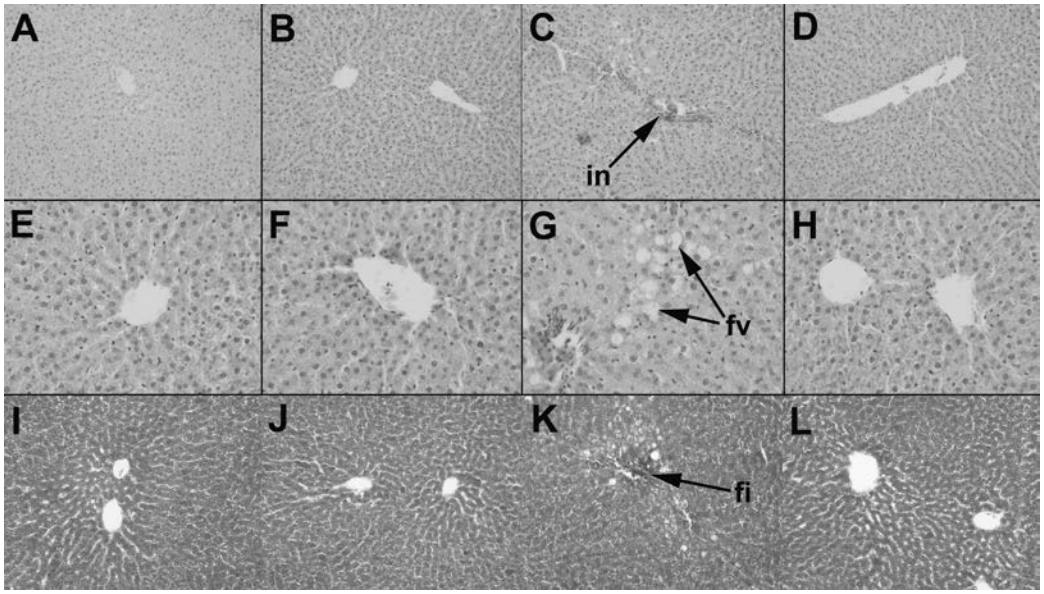

Figure 3. Effects of L-carnitine supplementation on inflammation, fat deposition, and fibrosis in the liver. Top and middle rows represent hematoxylin and eosin staining of the liver showing inflammatory cells (A-D, marked as "in"; ×20) and enlarged fat vacuoles (E-H, marked as "fv"; ×40), respectively, from C (A,E), CLC (B,F), H (C,G), and HLC (D,H) rats. Bottom row represents Milligan's trichrome staining of the liver showing fibrosis in the hepatic portal region (I-L, marked as "fi"; ×20) from C (I), CLC (J), H (K), and HLC (L) rats.

Table 1. Effects of L-carnitine on physiological, compositional, and metabolic parameters including plasma fatty acid composition

Variables					*P value*		
	C	*CLC*	*H*	*HLC*	*Diet*	*L-Carnitine*	*Diet × L-Carnitine*
Physiological, compositional, and metabolic parameters (n = 9-12)							
Body weight at 16 week, *g*	432 ± 6^{c}	418 ± 6^{c}	508 ± 9^{a}	465 ± 8^{b}	<0.0001	0.0004	0.055
Water intake, *ml/d*	33.1 ± 1.1^{a}	31.4 ± 1.0^{a}	21.2 ± 0.8^{b}	22.2 ± 0.7^{b}	<0.0001	0.70	0.15
Food intake, *g/d*	30.8 ± 1.0^{a}	31.0 ± 1.1^{a}	23.5 ± 0.8^{b}	22.7 ± 0.6^{b}	<0.0001	0.74	0.58
Energy intake, *kJ/d*	349 ± 13^{a}	353 ± 14^{a}	498 ± 19^{b}	485 ± 18^{b}	<0.0001	0.78	0.60
Abdominal circumference, *cm*	20.4 ± 0.3^{b}	19.6 ± 0.3^{b}	22.7 ± 0.5^{a}	20.7 ± 0.4^{b}	<0.0001	0.0007	0.13
Abdominal fat pads (total), *mg/mm tibial length*	410 ± 31^{b}	203 ± 24^{c}	750 ± 36^{a}	421 ± 37^{b}	<0.0001	<0.0001	0.07
Retroperitoneal fat, *mg/mm tibial length*	191 ± 13^{b}	92 ± 10^{c}	337 ± 20^{a}	185 ± 16^{b}	<0.0001	<0.0001	0.09
Epididyml fat, *mg/mm tibial length*	123 ± 10^{b}	69 ± 7^{c}	247 ± 14^{a}	143 ± 13^{b}	<0.0001	<0.0001	0.33
Omental fat, *mg/mm tibial length*	97 ± 9^{b}	42 ± 7^{c}	169 ± 10^{a}	93 ± 8^{b}	<0.0001	<0.0001	0.23
Total body fat mass, *g*	81 ± 7^{c}	60 ± 9^{c}	155 ± 13^{a}	121 ± 12^{b}	<0.0001	0.014	0.54
Total body lean mass, *g*	310 ± 6	309 ± 8	323 ± 8	301 ± 5	0.72	0.11	0.14
Bone mineral content, *g*	12.2 ± 0.3	12.4 ± 0.6	12.8 ± 0.4	13.2 ± 0.7	0.19	0.57	0.85
Bone mineral density, g/cm^2	0.15 ± 0.00	0.16 ± 0.00	0.16 ± 0.00	0.16 ± 0.01	0.32	0.32	0.32
Basal blood glucose concentration, *mmol/L*	3.97 ± 0.10^{c}	3.98 ± 0.12^{c}	4.80 ± 0.09^{a}	4.30 ± 0.06^{b}	<0.0001	0.013	0.010

AUC, *mmol/L·min*	646 ± 10[b]	657 ± 8[b]	718 ± 7[a]	666 ± 10[b]	<0.0001	0.025	0.0009
Plasma insulin, *μg/L*	1.73 ± 0.16[b]	1.48 ± 0.15[b]	3.95 ± 0.29[a]	2.38 ± 0.47[b]	<0.0001	0.005	0.035
Plasma total cholesterol, *mmol/L*	1.3 ± 0.1[b]	1.4 ± 0.1[b]	2.1 ± .0.1[a]	1.4 ± 0.1[b]	0.0002	0.004	0.0002
Plasma triglycerides, *mmol/L*	0.4 ± 0.1[b]	0.5 ± 0.1[b]	1.0 ± 0.1[a]	0.5 ± 0.1[b]	0.004	0.05	0.004
Plasma non-esterified fatty acids, *mmol/L*	1.1 ± 0.1[b]	1.0 ± 0.1[b]	2.7 ± 0.2[a]	1.4 ± 0.1[b]	<0.0001	<0.0001	<0.0001
Fatty acids, g/100g of total fatty acid content (n = 6)							
C14:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.00	1.00	1.00
C14:1n-5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.00	1.00	1.00
C16:0	26.60 ± 2.67[c]	47.77 ± 2.06[a]	23.06 ± 2.04[c]	37.13 ± 2.71[b]	0.008	<0.0001	0.15
C16:1n-7	30.97 ± 1.42[a]	6.94 ± 1.74[b]	27.66 ± 2.97[a]	8.67 ± 3.05[b]	0.75	<0.0001	0.31
C18:0	12.89 ± 1.51	13.26 ± 0.92	8.93 ± 1.74	12.51 ± 0.59	0.08	0.14	0.22
C18:1*trans*-7	0.00 ± 0.00[b]	0.38 ± 0.38[b]	14.23 ± 4.79[a]	0.55 ± 0.36[b]	0.007	0.012	0.009
C18:1n-9	7.59 ± 1.30[b]	13.15 ± 1.68[b]	10.54 ± 1.98[b]	21.36 ± 1.97[a]	0.005	0.0001	0.15
C18:2n-6	8.07 ± 3.13	8.36 ± 1.35	7.14 ± 1.62	9.31 ± 1.42	1.00	0.55	0.65
C18:3n-3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.00	1.00	1.00
C18:3n-6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.00	1.00	1.00
C20:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.00	1.00	1.00
C20:3n-6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.00	1.00	1.00
C20:4n-6	11.98 ± 1.21[a]	9.04 ± 1.15[ab]	6.41 ± 1.38[b]	5.21 ± 0.71[b]	0.0005	0.09	0.46
Total SFA	39.49 ± 3.11[c]	61.89 ± 1.76[a]	32.28 ± 2.35[d]	54.04 ± 2.16[b]	0.005	<0.0001	0.90

Total MUFA	40.49 ± 1.92[b]	20.71 ± 2.63[d]	54.17 ± 2.3[a]	31.44 ± 2.55[c]	<0.0001	<0.0001	0.54
Total PUFA	20.03 ± 2.37	17.40 ± 2.40	13.55 ± 0.59	14.52 ± 1.52	0.021	0.66	0.35
n3:n6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.00	1.00	1.00
SCD-1 index	1.23 ± 0.13[a]	0.14 ± 0.04[b]	1.21 ± 0.09[a]	0.24 ± 0.09[b]	0.67	<0.0001	0.53

Values are mean ± SEM. Mean values within a row with unlike superscript letters are significantly different, $P < 0.05$.

AUC, area under the curve; *C*, corn starch diet-fed rats; *CLC*, corn starch diet-fed rats supplemented with L-carnitine; *H*, high-carbohydrate, high-fat diet-fed rats; *HLC*, high-carbohydrate, high-fat diet-fed rats supplemented with L-carnitine; *MUFA*, monounsaturated fatty acids; *PUFA*, polyunsaturated fatty acids; *SCD-1*, steroyl Co-A desaturase-1; *SFA*, saturated fatty acids.

Table 2. Effects of L-carnitine on structure, function, and fatty acid composition of the heart

Variables	*C*	*CLC*	*H*	*HLC*	*P value*		
					Diet	*L-Carnitine*	*Diet × L-Carnitine*
Structural and functional parameters of the heart (n = 9-12)							
Systolic blood pressure, *mmHg*	129 ± 2^{bc}	127 ± 2^{c}	156 ± 3^{a}	135 ± 2^{b}	<0.0001	<0.0001	0.0002
Heart rate, *beats per min*	252 ± 6^{a}	228 ± 6^{b}	243 ± 6^{ab}	258 ± 6^{a}	0.09	0.46	0.002
LVIDd, *mm*	6.64 ± 0.10^{b}	6.02 ± 0.12^{c}	7.27 ± 0.09^{a}	6.39 ± 0.16^{b}	0.0002	<0.0001	0.29
LVPWd, *mm*	1.65 ± 0.04	1.81 ± 0.07	1.81 ± 0.06	1.87 ± 0.09	0.11	0.11	0.46
Ejection time, *msec*	92 ± 2	91 ± 2	88 ± 2	87 ± 2	0.05	0.62	1.00
E/A	1.88 ± 0.10^{a}	1.99 ± 0.12^{a}	1.53 ± 0.09^{b}	1.83 ± 0.09^{a}	0.015	0.048	0.35
Deceleration time, *msec*	53 ± 2	63 ± 3	55 ± 3	55 ± 2	0.44	0.12	0.12
Systolic volume, *μl*	43 ± 6^{b}	39 ± 4^{b}	101 ± 6^{a}	49 ± 7^{b}	<0.0001	<0.0001	0.0002
Relative wall thickness	0.48 ± 0.01^{b}	0.60 ± 0.03^{a}	0.48 ± 0.01^{b}	0.55 ± 0.02^{a}	0.20	<0.0001	0.20
Fractional shortening, *%*	53 ± 2^{a}	51 ± 2^{a}	39 ± 2^{b}	57 ± 2^{a}	0.05	0.0002	<0.0001
Ejection fraction, *%*	87 ± 2^{a}	88 ± 2^{a}	72 ± 2^{b}	89 ± 3^{a}	0.004	0.0003	0.001
MCMO, *msec*	114 ± 4^{a}	117 ± 2^{a}	114 ± 3^{a}	103 ± 2^{b}	0.019	0.17	0.019
Estimated left ventricular mass, *g*	0.67 ± 0.03^{b}	0.71 ± 0.05^{b}	0.91 ± 0.03^{a}	0.73 ± 0.05^{b}	0.003	0.10	0.011
Left ventricle + septum wet weight, *mg/mm*	19.5 ± 0.6	20.7 ± 0.5	21.0 ± 0.9	19.7 ± 0.8	0.73	0.95	0.09

tibial length							
Right ventricle wet weight, *mg/mm tibial length*	4.2 ± 0.2	4.5 ± 0.2	4.3 ± 0.2	4.5 ± 0.2	0.80	0.22	0.80
Left ventricular diastolic stiffness constant (κ)	20.2 ± 1.1[b]	21.0 ± 1.4[b]	28.3 ± 1.5[a]	22.6 ± 1.3[b]	0.0007	0.07	0.019
Fatty acids, g/100g of total fatty acid content (n = 6)							
C14:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.00	1.00	1.00
C14:1n-5	1.32 ± 0.28[a]	0.00 ± 0.00[b]	1.49 ± 0.31[a]	0.00 ± 0.00[b]	0.69	<0.0001	0.69
C16:0	2.12 ± 0.12[b]	22.39 ± 0.37[a]	5.70 ± 4.09[b]	22.00 ± 0.33[a]	0.45	<0.0001	0.35
C16:1n-7	25.95 ± 1.49[a]	1.56 ± 0.09[b]	21.85 ± 3.59[a]	0.37 ± 0.16[b]	0.19	<0.0001	0.46
C18:0	0.86 ± 0.06[c]	21.18 ± 0.28[a]	1.51 ± 0.10[b]	22.71 ± 0.61[a]	0.005	<0.0001	0.21
C18:1*trans*-7	22.50 ± 0.48[b]	4.63 ± 0.10[c]	24.61 ± 0.43[a]	2.64 ± 0.03[d]	0.86	<0.0001	<0.0001
C18:1n-9	0.92 ± 0.06[c]	10.90 ± 0.57[b]	0.65 ± 0.14[c]	13.99 ± 1.00[a]	0.025	<0.0001	0.009
C18:2n-6	11.77 ± 1.12[b]	14.89 ± 0.59[a]	10.12 ± 0.53[b]	10.56 ± 0.45[b]	0.0005	0.023	0.08
C18:3n-3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.00	1.00	1.00
C18:3n-6	12.97 ± 0.48[a]	0.00 ± 0.00[c]	8.91 ± 1.78[b]	0.00 ± 0.00[c]	0.040	<0.0001	0.040
C20:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.00	1.00	1.00
C20:3n-6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.00	1.00	1.00
C20:4n-6	19.64 ± 0.75[a]	17.03 ± 0.48[b]	19.50 ± 0.38[a]	16.99 ± 0.72[b]	0.88	0.0004	0.93
Total SFA	3.54 ± 0.32[b]	43.57 ± 0.36[a]	8.05 ± 4.35[b]	44.71 ± 0.45[a]	0.21	<0.0001	0.45

Total MUFA	50.70 ± 1.53[a]	24.51 ± 0.26[b]	48.59 ± 4.05[a]	27.74 ± 0.91[b]	0.80	<0.0001	0.24
Total PUFA	45.76 ± 1.41[a]	31.92 ± 0.39[b]	43.36 ± 0.61[a]	27.55 ± 0.54[c]	0.0006	<0.0001	0.25
n3:n6	0.03 ± 0.00[ab]	0.00 ± 0.00[b]	0.10 ± 0.05[a]	0.00 ± 0.00[b]	0.18	0.017	0.18
SCD-1 index	12.43 ± 0.94[a]	0.07 ± 0.00[b]	13.47 ± 2.94[a]	0.02 ± 0.01[b]	0.75	<0.0001	0.73

Values are mean ± SEM. Mean values within a row with unlike superscript letters are significantly different, $P < 0.05$.

C, corn starch diet-fed rats; *CLC*, corn starch diet-fed rats supplemented with L-carnitine; *E/A*, ratio of early mitral inflow velocity to late mitral inflow velocity; *H*, high-carbohydrate, high-fat diet-fed rats; *HLC*, high-carbohydrate, high-fat diet-fed rats supplemented with L-carnitine; *LVIDd*, left ventricular internal diameter during diastole; *LVPWd*, left ventricular posterior wall thickness during diastole; *MCMO*, time from mitral valve closure to opening; *MUFA*, monounsaturated fatty acids; *PUFA*, polyunsaturated fatty acids; *SCD-1*, steroyl Co-A desaturase-1; *SFA*, saturated fatty acids.

Table 3. Effects of L-carnitine on hepatic function and fatty acid composition

Variables	*C*	*CLC*	*H*	*HLC*	*P value*		
					Diet	*L-Carnitine*	*Diet × L-Carnitine*
Hepatic functions (n = 9-12)							
Liver wet weight, *mg/mm tibial length*	231 ± 11^{b}	221 ± 8^{b}	291 ± 10^{a}	302 ± 9^{a}	<0.0001	0.96	0.28
Plasma ALT, *U/L*	37 ± 4^{b}	33 ± 3^{b}	55 ± 3^{a}	39 ± 2^{b}	0.0003	0.002	0.06
Plasma AST, *U/L*	81 ± 4^{b}	80 ± 3^{b}	108 ± 4^{a}	88 ± 3^{b}	<0.0001	0.005	0.010
Plasma ALP, *U/L*	176 ± 11^{b}	157 ± 7^{b}	240 ± 12^{a}	171 ± 10^{b}	0.0004	<0.0001	0.018
Plasma LDH, *U/L*	240 ± 21^{b}	230 ± 17^{b}	416 ± 28^{a}	295 ± 18^{b}	<0.0001	0.004	0.013
Plasma albumin, *g/L*	28.0 ± 0.4	27.3 ± 0.5	28.0 ± 0.4	27.3 ± 0.3	1.00	0.09	1.00
Plasma total bilirubin, *μmol/L*	2.2 ± 0.1	2.4 ± 0.2	2.5 ± 0.1	2.2 ± 0.1	0.71	0.71	0.07
Plasma urea, *mmol/L*	5.4 ± 0.2^{a}	5.2 ± 0.2^{a}	3.5 ± 0.2^{c}	4.6 ± 0.1^{b}	<0.0001	0.016	0.0008
Plasma uric acid, *μmol/L*	34 ± 2^{c}	40 ± 3^{bc}	56 ± 4^{a}	47 ± 3^{b}	<0.0001	0.63	0.019
Fatty acids, g/100g of total fatty acid content (n = 6)							
C14:0	7.06 ± 1.19^{a}	0.00 ± 0.00^{c}	3.11 ± 0.59^{b}	1.18 ± 0.10^{bc}	0.05	<0.0001	0.001
C14:1n-5	0.74 ± 0.13^{a}	0.00 ± 0.00^{b}	0.79 ± 0.09^{a}	0.00 ± 0.00^{b}	0.76	<0.0001	0.76
C16:0	0.03 ± 0.03^{c}	24.37 ± 0.61^{a}	0.25 ± 0.05^{c}	23.20 ± 0.31^{b}	0.18	<0.0001	0.06
C16:1n-7	26.35 ± 0.99^{a}	4.56 ± 0.64^{c}	23.33 ± 0.75^{b}	1.87 ± 0.18^{d}	0.0006	<0.0001	0.82

C18:0	3.39 ± 3.39^{bc}	13.27 ± 1.13^{a}	0.32 ± 0.07^{c}	7.89 ± 0.51^{b}	0.030	0.0001	0.53
C18:1*trans*-7	15.06 ± 2.49^{a}	4.26 ± 0.28^{b}	10.87 ± 3.37^{a}	1.81 ± 0.09^{b}	0.13	0.0001	0.68
C18:1n-9	0.00 ± 0.00^{c}	25.96 ± 2.36^{b}	1.52 ± 1.52^{c}	51.71 ± 1.57^{a}	<0.0001	<0.0001	<0.0001
C18:2n-6	27.06 ± 4.47^{b}	8.21 ± 0.58^{c}	44.31 ± 4.94^{a}	4.28 ± 0.23^{c}	0.06	<0.0001	0.005
C18:3n-3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.00	1.00	1.00
C18:3n-6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.00	1.00	1.00
C20:0	0.40 ± 0.12^{a}	0.00 ± 0.00^{c}	0.19 ± 0.02^{b}	0.48 ± 0.03^{a}	0.044	0.39	<0.0001
C20:3n-6	0.67 ± 0.10	0.85 ± 0.16	0.73 ± 0.11	0.52 ± 0.7	0.72	0.97	0.60
C20:4n-6	10.91 ± 2.91^{a}	14.02 ± 1.19^{a}	7.96 ± 2.31^{ab}	3.84 ± 0.30^{b}	0.003	0.80	0.08
Total SFA	11.26 ± 4.14^{b}	37.64 ± 1.00^{a}	3.92 ± 0.61^{c}	32.76 ± 0.67^{a}	0.011	<0.0001	0.58
Total MUFA	42.15 ± 2.40^{b}	39.28 ± 2.48^{b}	36.69 ± 1.85^{b}	57.93 ± 1.24^{a}	0.004	0.0002	<0.0001
Total PUFA	46.59 ± 6.43^{b}	23.07 ± 1.68^{c}	59.39 ± 2.07^{a}	9.31 ± 0.61^{d}	0.89	<0.0001	0.001
n3:n6	0.20 ± 0.02^{a}	0.00 ± 0.00^{c}	0.10 ± 0.02^{b}	0.00 ± 0.00^{c}	0.002	<0.0001	0.002
SCD-1 index$^{\Delta}$	-	0.19 ± 0.02	78.84 ± 2.44	0.08 ± 0.01	-	-	-

Values are mean ± SEM. Mean values within a row with unlike superscript letters are significantly different, $P < 0.05$.

ALP, alkaline phosphatase; *ALT*, alanine transaminase; *AST*, aspartate transaminase*C*, corn starch diet-fed rats; *CLC*, corn starch diet-fed rats supplemented with L-carnitine; *H*, high-carbohydrate, high-fat diet-fed rats; *HLC*, high-carbohydrate, high-fat diet-fed rats supplemented with L-carnitine; *LDH*, lactate dehydrogenase; *MUFA*, monounsaturated fatty acids; *PUFA*, polyunsaturated fatty acids; *SCD-1*, steroyl Co-A desaturase-1; *SFA*, saturated fatty acids.

$^{\Delta}$ Negligible C16:0 fatty acid detected in C diet-fed group therefore showing a very high stearoyl-CoA 9-desatururation index in this group.

Table 4. Effects of L-carnitine on fatty acid composition of skeletal muscle

Variables					*P value*		
	C	*CLC*	*H*	*HLC*	*Diet*	*L-Carnitine*	*Diet × L-Carnitine*
Fatty acids, g/100g of total fatty acid content (n = 6)							
C14:0	14.35 ± 3.23[a]	1.59 ± 0.10[b]	6.26 ± 1.58[b]	2.35 ± 0.10[b]	0.06	0.0002	0.023
C14:1n-5	1.76 ± 0.10[a]	0.00 ± 0.00[b]	2.50 ± 0.23[a]	0.00 ± 0.00[b]	0.008	<0.0001	0.008
C16:0	0.59 ± 0.15[c]	28.82 ± 0.44[a]	0.43 ± 0.03[c]	26.26 ± 0.67[b]	0.003	<0.0001	0.008
C16:1n-7	28.41 ± 0.89[a]	7.92 ± 0.70[c]	20.94 ± 1.28[b]	2.37 ± 0.13[d]	<0.0001	<0.0001	0.28
C18:0	0.33 ± 0.07[c]	7.30 ± 0.82[b]	0.58 ± 0.03[c]	10.46 ± 0.54[a]	0.003	<0.0001	0.008
C18:1*trans*-7	0.67 ± 0.67[b]	4.06 ± 0.08[a]	1.07 ± 1.07[b]	2.15 ± 0.06[b]	0.25	0.002	0.08
C18:1n-9	4.24 ± 1.02[c]	36.24 ± 1.95[b]	5.27 ± 1.06[c]	44.91 ± 2.12[a]	0.007	<0.0001	0.028
C18:2n-6	36.64 ± 3.98[b]	7.57 ± 0.37[c]	50.53 ± 3.51[a]	6.44 ± 0.36[c]	0.027	<0.0001	0.011
C18:3n-3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.00	1.00	1.00
C18:3n-6	7.41 ± 1.52[a]	0.00 ± 0.00[b]	10.79 ± 2.18[a]	0.00 ± 0.00[b]	0.22	<0.0001	0.22
C20:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.00	1.00	1.00
C20:3n-6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.00	1.00	1.00
C20:4n-6	2.94 ± 0.73[a]	3.69 ± 0.74[a]	0.84 ± 0.18[b]	2.67 ± 0.47[a]	0.014	0.037	0.36

Total SFA	15.58 ± 3.45^{b}	37.71 ± 0.97^{a}	7.56 ± 1.55^{c}	39.06 ± 1.01^{a}	0.11	<0.0001	0.031
Total MUFA	35.35 ± 1.18^{b}	51.03 ± 1.97^{a}	30.08 ± 1.56^{c}	51.83 ± 1.75^{a}	0.19	<0.0001	0.08
Total PUFA	49.07 ± 3.42^{b}	11.26 ± 1.05^{c}	62.36 ± 2.44^{a}	9.11 ± 0.79^{c}	0.020	<0.0001	0.002
n3:n6	0.04 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.20	0.20	0.20
SCD-1 index	63.09 ± 13.71^{a}	0.28 ± 0.02^{b}	49.07 ± 1.18^{a}	0.09 ± 0.01^{b}	0.31	<0.0001	0.33

Values are mean ± SEM. Mean values within a row with unlike superscript letters are significantly different, $P < 0.05$.

C, corn starch diet-fed rats; *CLC*, corn starch diet-fed rats supplemented with L-carnitine; *H*, high-carbohydrate, high-fat diet-fed rats; *HLC*, high-carbohydrate, high-fat diet-fed rats supplemented with L-carnitine; *MUFA*, monounsaturated fatty acids; *PUFA*, polyunsaturated fatty acids; *SCD-1*, steroyl Co-A desaturase-1; *SFA*, saturated fatty acids.

Table 5. Effects of L-carnitine on fatty acid composition of retroperitoneal fat

Variables					*P value*		
	C	*CLC*	*H*	*HLC*	*Diet*	*L-Carnitine*	*Diet × L-Carnitine*
Fatty acids, g/100g of total fatty acid content (n = 6)							
C14:0	0.00 ± 0.00^{c}	1.71 ± 0.02^{b}	0.00 ± 0.00^{c}	2.55 ± 0.09^{a}	<0.0001	<0.0001	<0.0001
C14:1n-5	2.17 ± 0.04^{b}	0.00 ± 0.00^{c}	2.81 ± 0.10^{a}	0.00 ± 0.00^{c}	<0.0001	<0.0001	<0.0001
C16:0	0.22 ± 0.05^{c}	28.40 ± 0.27^{a}	0.42 ± 0.08^{c}	20.84 ± 0.12^{b}	<0.0001	<0.0001	<0.0001
C16:1n-7	31.51 ± 1.06^{a}	9.41 ± 0.59^{c}	22.40 ± 0.21^{b}	2.55 ± 0.08^{d}	<0.0001	<0.0001	0.08
C18:0	0.22 ± 0.03^{d}	3.66 ± 0.18^{b}	0.62 ± 0.02^{c}	6.92 ± 0.08^{a}	<0.0001	<0.0001	<0.0001
C18:1*trans*-7	3.46 ± 0.23^{c}	3.91 ± 0.08^{c}	6.97 ± 0.45^{b}	60.96 ± 0.58^{a}	<0.0001	<0.0001	<0.0001
C18:1n-9	0.00 ± 0.00^{c}	46.30 ± 0.43^{a}	0.00 ± 0.00^{c}	1.37 ± 0.32^{b}	<0.0001	<0.0001	<0.0001
C18:2n-6	51.23 ± 0.96^{b}	6.24 ± 0.19^{c}	60.76 ± 0.47^{a}	3.87 ± 0.12^{d}	<0.0001	<0.0001	<0.0001
C18:3n-3	0.00 ± 0.00^{b}	0.35 ± 0.01^{a}	0.07 ± 0.07^{b}	0.31 ± 0.02^{a}	0.69	<0.0001	0.15
C18:3n-6	10.12 ± 0.32^{a}	0.00 ± 0.00^{c}	5.18 ± 0.08^{b}	0.00 ± 0.00^{c}	<0.0001	<0.0001	<0.0001
C20:0	0.66 ± 0.13^{a}	0.00 ± 0.00^{c}	0.35 ± 0.02^{b}	0.63 ± 0.02^{a}	0.026	0.010	<0.0001
C20:3n-6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.00	1.00	1.00
C20:4n-6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.00	1.00	1.00
Total SFA	1.10 ± 0.14^{c}	33.78 ± 0.37^{a}	1.48 ± 0.13^{c}	30.94 ± 0.16^{b}	<0.0001	<0.0001	<0.0001

Total MUFA	37.24 ± 0.86[c]	59.63 ± 0.48[b]	32.40 ± 0.43[d]	64.88 ± 0.23[a]	0.71	<0.0001	<0.0001
Total PUFA	61.66 ± 0.92[b]	6.59 ± 0.19[c]	66.12 ± 0.48[a]	4.18 ± 0.13[d]	0.07	<0.0001	<0.0001
n3:n6	0.00 ± 0.0[c]	0.06 ± 0.00[b]	0.00 ±0.00[c]	0.08 ± 0.00[a]	<0.0001	<0.0001	<0.0001
SCD-1 index	129.55 ± 16.75[a]	0.32 ± 0.03[c]	44.38 ± 0.51[b]	0.13 ± 0.00[c]	<0.0001	<0.0001	<0.0001

Values are mean ± SEM. Mean values within a row with unlike superscript letters are significantly different, $P < 0.05$.

C, corn starch diet-fed rats; *CLC*, corn starch diet-fed rats supplemented with L-carnitine; *H*, high-carbohydrate, high-fat diet-fed rats; *HLC*, high-carbohydrate, high-fat diet-fed rats supplemented with L-carnitine; *MUFA*, monounsaturated fatty acids; *PUFA*, polyunsaturated fatty acids; *SCD-1*, steroyl Co-A desaturase-1; *SFA*, saturated fatty acids.

Chapter 8: Chronic ethanol consumption in rats fed a high-carbohydrate, high-fat diet

Introduction

Ethanol consumption is very common throughout the world as part of many beverages including beer, wine and whiskey [1]. Ethanol is a substantial source of energy (29.7 kJ/g), providing more energy than carbohydrates or proteins. Being a rich source of energy, it interferes with the handling of other nutrients and is responsible for malnutrition through impairment of gastrointestinal absorption and hepatic metabolism [2]. Chronic ethanol consumption leads to changes in the structure and function of the liver, including lipid accumulation (steatosis), steatohepatitis (steatosis, fibrosis and inflammation in the liver) and ultimately cirrhosis (advanced fibrosis and liver degeneration) [3]. Hepatic steatosis may originate from dietary lipids as chylomicrons, from adipose tissue as non-esterified fatty acids or from synthesis in the liver. Ethanol increases peripheral fat mobilisation, enhances hepatic triglyceride synthesis, decreases lipid oxidation in the liver and decreases hepatic lipoprotein release [4]. Fibrosis starts in the liver once these changes are initiated and the ethanol intake is chronic. Severe fibrosis leads to cirrhosis of the liver [4].

We have shown that a high-carbohydrate, high-fat diet induces non-alcoholic steatohepatitis with fat accumulation, infiltration of inflammatory cells, perivascular fibrosis and increased plasma markers of liver function as part of diet-induced metabolic syndrome [5-8]. Other researchers have reported similar outcomes with diets rich in simple carbohydrates (such as fructose and sucrose) and animal fat [9-13]. Since ethanol causes a similar range of liver changes, ethanol administration may accelerate the changes in high-carbohydrate, high-fat diet-fed rats, in the liver as well as in the heart and on metabolic function. The combination of ethanol with a high carbohydrate, high fat diet, analogous to modern dietary practice in countries such as Australia,

would then be predicted to worsen the myriad changes in metabolic syndrome.

In contradiction to these findings, moderate consumption of red wine as part of the Mediterranean diet has been associated with decreased cardiovascular disease [14]; moderate, regular ethanol intake could then be a key ingredient in the French paradox. Further, ethanol administration decreased infarct size in ischaemia-reperfusion injury in rat hearts [15,16]. Thus, the responses to chronic moderate ethanol intake are difficult to predict, but could attenuate or worsen the responses to a high-carbohydrate, high-fat diet.

This study measured the effects of chronic moderate ethanol feeding on metabolic parameters and structure and function of cardiovascular system and the liver in high-carbohydrate, high-fat diet-fed rats. The effects of ethanol feeding on the structure and function of the heart were characterised through echocardiography, isolated Langendorff heart preparation and histopathology while the structure and function of the liver were characterised through histopathology and plasma biochemical analyses. In addition, metabolic function was characterised through glucose tolerance testing and plasma lipid profile assessment.

Methods and materials

Rats and diets

All experimental protocols were approved by University of Southern Queensland Animal Experimentation Ethics Committee, under the guidelines of the National Health and Medical Research Council of Australia. Male Wistar rats (8-9 weeks old, weighing 337 ± 1 g, n = 48) were obtained from The University of Queensland Biological Resources facility. The rats were randomly divided into four experimental groups and were fed with one of the following diets; corn starch diet with normal drinking water (C[1]; n = 12), corn

[1] Abbreviations used: ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; CS, corn starch diet-fed rats; CE, corn starch diet-fed rats given 10% ethanol; DXA, Dual-energy X-ray absorptiometry; H, high-carbohydrate, high-fat diet-fed rats; HE,

starch diet with 10% (v/v) ethanol in drinking water (CE; n = 12), high-carbohydrate, high-fat diet with 25% (w/v) fructose-supplemented drinking water (H; n = 12) and high-carbohydrate, high-fat diet with 10% (v/v) ethanol in 25% (w/v) fructose-supplemented drinking water (HE; n = 12). All groups were fed with these diets for 16 weeks. Compositions of C and H diets have been previously described in detail [5,7,8]. All the rats were given *ad libitum* access to food and water and were individually housed in temperature-controlled 12-hour light/dark conditions.

Physiological parameters

Rats were monitored daily for body weight, food and water intakes. Oral glucose tolerance tests and systolic blood pressure measurements were performed on a ll groups of rats after 16 w eek as in previous study [5]. Abdominal circumference of rats was measured using a standard measuring tape while sedated for systolic blood pressure measurements [5].

Body composition measurements

Dual-energy X-ray absorptiometric (DXA) measurements were performed on the rats after 16 weeks of feeding (2 days before rats were euthanased for pathophysiological assessments) using a Norland XR36 DXA instrument (Norland Corp., Fort Atkinson, USA). DXA scans were analysed using the manufacturer's recommended software for use in laboratory animals (Small Subject Analysis Software, version 2.5.3/1.3.1, Norland Corp., Fort Atkinson, USA) as previously described [17]. The precision error of lean mass for replicate measurements, with repositioning, was 3.2%.

Echocardiography

high-carbohydrate, high-fat diet-fed rats given 10% ethanol; IVSd, interventricular septum thickness during diastole; LDH, lactate dehydrogenase; LVIDd, left ventriclar internal diameter during diastole; LVIDs, left ventriclar internal diameter during systole; LVPWd, left ventricular posterior wall thickness during diastole; MCMO, time from mitral valve closure to opening; NEFA, non-esterified fatty acids.

Echocardiographic examinations (Phillips iE33, 12MHz transducer) were performed to assess the cardiovascular structure and function in all rats at the end of protocol as previously described [5]. Briefly, rats were anaesthetised using Zoletil (tiletamine 25 mg/kg and zolazepam 25 mg/kg, i.p.; Virbac, Peakhurst, Australia) and Ilium Xylazil (xylazine 15 m g/kg, i.p.; Troy Laboratories, Smithfield, Australia) and positioned in dorsal recumbency. Electrodes attached to the skin overlying the elbows and right stifle facilitated the simultaneous recording of a lead II ECG. A short-axis view of the left ventricle at the level of the papillary muscles was obtained and used to direct acquisition of M mode images of the left ventricle for measurement of interventricular septum thickness during diastole (IVSd), left ventricular posterior wall thickness during diastole (LVPWd), left ventricular internal diameter during systole (LVIDs) and left ventricular internal diameter during diastole (LVIDd). Measurements were taken in accordance with the guidelines of the American Society of Echocardiography using the leading-edge method. Details of this method have been described previously [5,18].

Isolated Langendorff heart preparation

Rats were euthanised with Lethabarb (pentobarbitone sodium, 100 mg/kg, i.p.; Virbac, Peakhurst, Australia). After euthanasia, heparin (200 IU; Sigma-Aldrich Australia) was injected through the right femoral vein. The abdomen was then opened and blood (~5 mL) was withdrawn from the abdominal aorta, collected into heparinised tubes and centrifuged at 5000 × g for 15 minutes to obtain plasma. Plasma was stored at -20°C before further biochemical analysis. Hearts were removed and were used in isolated Langendorff heart preparations to assess left ventricular function of the rats as in previous study [5].

Hearts isolated from euthanased rats (n = 9) were perfused with modified Krebs–Henseleit bicarbonate buffer, containing (in millimolar): NaCl, 119.1; KCl, 4.75; MgSO4, 1.19; KH_2PO_4, 1.19; $NaHCO_3$, 25.0; glucose, 11.0; and $CaCl_2$, 2.16. Buffer was bubbled with 95% O_2-5% CO_2 and maintained at

35°C. Isovolumetric ventricular function was measured by inserting a latex balloon catheter into the left ventricle connected to a Capto SP844 MLT844 physiological pressure transducer and Chart software on a Maclab system (ADInstruments). 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 up to 30 mmHg for calculation of diastolic stiffness constant (κ, dimensionless) [5].

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 and maintained at 35˚C and allowed to stabilise at a resting tension of approximately 10 mN [5]. Cumulative concentration-response curves (contraction) were obtained for noradrenaline (Sigma-Aldrich Australia) and cumulative concentration-response curves (relaxation) were obtained for acetylcholine (Sigma-Aldrich Australia) and sodium nitroprusside (Sigma-Aldrich Australia) following submaximal (70%) contraction to noradrenaline [5].

Organ weights and histology

After performing Langendorff heart perfusion, hearts were separated into right ventricle and left ventricle (with septum) (n = 9) for weighing. Livers and abdominal fat pads (retroperitoneal, epididymal and omental) were isolated and weighed (n = 9). These organ weights were normalised to the tibial length at the time of removal and expressed as mg of tissue/mm of tibial length [5]. The heart and the liver of rats (n = 3 from each group) were exclusively used for histopathological analysis as in previous study [5]. Tissues were fixed in 10% neutral buffered formalin for 3 days. The tissue samples were then dehydrated and embedded in paraffin wax. For staining, thin sections (5 μm) of tissues were cut and fixed on slides. Heart sections were stained with picrosirius red to study collagen deposition and were analysed using laser confocal microscopy

(Zeiss LSM 510 upright Confocal Microscope). Haematoxylin and eosin stain was used to visualise infiltration of inflammatory cells in both the heart and the liver whereas Milligan's stain was used to study the perivascular fibrosis in the liver. Haematoxylin and eosin stain was also used to visualise the presence of fat vacuoles in the liver. After staining with haematoxylin and eosin or Milligan's stains, pictures were taken with a Zeiss Microscope. From each tissue sample, three slides were prepared and two random, non-overlapping fields were selected from each slide. A representative picture was randomly selected from each group.

Biochemical analysis of plasma samples

Plasma activities of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) and plasma concentrations of total cholesterol, triglycerides, albumin, total bilirubin, urea and uric acid were determined. These variables were measured using kits and controls supplied by Olympus using an Olympus analyser (AU 400, Tokyo, Japan) [5]. Plasma non-esterified fatty acids (NEFA) were determined using a commercial kit (Wako, Osaka, Japan) [5].

Statistical analysis

All data are presented as mean ± SEM. All the groups 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. All groups were tested for effects of diet, ethanol, and their interactions by two-way ANOVA. When interaction and/or the main effects were significant, means were compared using Newman-Keuls multiple comparison post-test. Mean daily ethanol intake of CE and HE rats were compared using Student's *t* test. $P < 0.05$ was considered significant. All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (San Diego, California, USA).

Results

Physiological and metabolic parameters

As a preliminary study, rats fed with standard chow diet were given 5%, 10% and 20% ethanol (n = 8-10 for each group) in drinking water for 16 weeks. No changes were observed in body parameters, cardiovascular and hepatic functions compared to chow-fed controls with 5% ethanol in drinking water (results not shown). With 20% ethanol in drinking water, rats did not gain body weight and showed less physical activity (results not shown). With 10% ethanol in drinking water, rats showed slight decreases in body weight. In terms of blood and plasma characteristics, there were minor differences between 10% and 20% ethanol feeding (results not shown). Thus 10% ethanol was selected for the study of the effects of a combination of ethanol and high-carbohydrate, high-fat diet.

High-carbohydrate, high-fat diet-fed rats (H) had higher body weight than corn starch diet-fed rats (C). H rats fed ethanol (HE) had lower body weight compared to H rats whereas the body weights of C and corn starch rats fed ethanol (CE) did not differ (Table 1). C rats consumed more water than H rats. Water consumption was higher in HE rats than in H rats whereas water consumption of C and CE rats did not differ (Table 1). C rats ate more food than H rats. Food consumption of CE and HE rats did not differ compared to C and H rats, respectively (Table 1). Abdominal circumference was higher in H rats compared to C rats. Abdominal circumference was greater in CE rats than in C rats whereas abdominal circumference did not differ between H and HE rats (Table 1). Daily ethanol intake of CE rats was 6.31 ± 0.28 g/kg body weight and 4.49 ± 0.18 g/kg body weight in HE rats.

Basal blood glucose concentrations were higher in H rats compared to C rats. CE and HE rats had lower blood glucose concentrations than C and H rats, respectively (Table 1). During oral glucose tolerance test, H rats showed impaired glucose tolerance indicated by higher blood glucose concentrations

compared to C rats after 120 minutes of glucose load. After 120 minutes of glucose loading, CE rats had lower blood glucose concentrations than C rats whereas blood glucose concentrations did not differ between H and HE rats (Figure 1A). Plasma total cholesterol, plasma triglyceride and plasma NEFA concentrations were higher in H rats compared to C rats, and lower in HE rats compared to H rats. Plasma total cholesterol and plasma triglycerides concentrations did not differ between C and CE rats whereas CE rats had higher plasma NEFA concentrations than C rats (Table 1). Retroperitoneal, epididymal and omental fat pads were increased in H rats compared to C rats, and lower in HE rats than in H rats. In CE rats, retroperitoneal fat was lower than in C rats whereas epididymal and omental fat content did not differ between C and CE rats (Table 1). Whole-body fat mass and lean mass were higher in H rats than in C rats. CE and HE rats did not differ in whole-body fat mass compared to C and H rats, respectively whereas the lean masses were lower in CE and HE rats compared to C and H rats, respectively (Table 1).

Cardiovascular structure and function

Systolic blood pressure was higher in H rats than in C rats after 16 weeks of the protocol. Systolic blood pressure did not differ in CE and HE rats compared to C and H rats, respectively (Figure 1B). LVIDd and LVIDs were higher in H rats compared to C rats, but did not differ in CE and HE rats compared to C and H rats, respectively (Table 2). Although LVPWd did not differ between C and H rats, CE and HE rats had lower LVPWd than C and H rats, respectively (Table 2). Relative wall thickness did not differ between C and H rats. CE rats had higher relative wall thickness than C rats whereas H and HE rats did not differ in relative wall thickness (Table 2). Fractional shortening and ejection fraction were lower in H rats compared to C rats. Ethanol feeding did not change fractional shortening in CE and HE rats compared to C and H rats, respectively. Ejection fraction was lower in CE rats than in C rats while it was higher in HE rats compared to H rats (Table 2). Ejection time did not differ

Table 1. Effects of combination of high-carbohydrate, high-fat diet and 10% ethanol on physiological and metabolic variables in rats

Variables					*P value*		
	C	***CE***	***H***	***HE***	***Diet***	***Ethanol***	***Diet × Ethanol***
Initial body weight, *g*	336 ± 2	337 ± 2	336 ± 2	337 ± 2	1.00	0.62	1.00
Final body weight, *g*	399 ± 7^{c}	394 ± 7^{c}	499 ± 7^{a}	457 ± 10^{b}	<0.0001	0.005	0.023
Water intake, *ml/day*	31.8 ± 1.7^{a}	29.6 ± 1.2^{a}	18.3 ± 0.3^{c}	22.2 ± 0.8^{b}	<0.0001	0.45	0.010
Food intake, *g/day*	31.9 ± 0.6^{a}	33.6 ± 0.8^{a}	20.9 ± 0.3^{b}	22.3 ± 0.6^{b}	<0.0001	0.014	0.80
Abdominal circumference, *cm*	19.6 ± 0.4^{c}	21.3 ± 0.4^{b}	23.8 ± 0.6^{a}	23.5 ± 0.4^{a}	<0.0001	0.13	0.035
Basal blood glucose, *mmol/L*	4.0 ± 0.1^{b}	3.4 ± 0.1^{c}	5.0 ± 0.1^{a}	4.2 ± 0.1^{b}	<0.0001	<0.0001	0.32
Plasma total cholesterol, *mmol/L*	1.43 ± 0.11^{b}	1.40 ± 0.11^{b}	1.99 ± 0.07^{a}	1.63 ± 0.09^{b}	0.0002	0.049	0.09
Plasma triglycerides, *mmol/L*	0.42 ± 0.06^{b}	0.63 ± 0.07^{ab}	0.79 ± 0.09^{a}	0.49 ± 0.07^{b}	0.12	0.54	0.001
Plasma NEFA, *mmol/L*	1.15 ± 0.14^{c}	2.09 ± 0.23^{b}	2.80 ± 0.21^{a}	1.89 ± 0.24^{b}	0.001	0.94	<0.0001
Retroperitoneal fat, *mg/mm tibial length*	213 ± 9^{c}	139 ± 13^{d}	357 ± 21^{a}	286 ± 30^{b}	<0.0001	0.0007	0.94
Epididymal fat, *mg/mm tibial length*	129 ± 11^{c}	111 ± 5^{c}	225 ± 14^{a}	179 ± 18^{b}	<0.0001	0.017	0.28
Omental fat, *mg/mm tibial length*	93 ± 6^{c}	73 ± 9^{c}	194 ± 12^{a}	148 ± 17^{b}	<0.0001	0.007	0.27
Total abdominal fat, *mg/mm tibial length*	435 ± 24^{c}	323 ± 25^{c}	775 ± 46^{a}	613 ± 63^{b}	<0.0001	0.003	0.56
Whole-body fat mass, *g*	74 ± 6^{b}	79 ± 5^{b}	155 ± 9^{a}	151 ± 18^{a}	<0.0001	0.96	0.68
Whole-body lean mass, *g*	312 ± 6^{b}	292 ± 3^{c}	339 ± 6^{a}	260 ± 10^{d}	0.71	<0.0001	0.0001

Values are mean ± SEM, n = 8-12. Means without a common superscript letter in a row differ, $P< 0.05$.

Abbreviations used: C, corn starch diet-fed rats; *CE*, corn starch diet-fed rats given 10% ethanol; *H*, high-carbohydrate, high-fat diet-fed rats; *HE*, high-carbohydrate, high-fat diet-fed rats given 10% ethanol; *NEFA*, non-esterified fatty acids.

between C and H rats while it was lower in CE rats than in C rats. Ejection time did not differ between H and HE rats. HCHF-fed rats had higher estimated left ventricular mass compared to CHOW- and CS-fed rats. Estimated left ventricular mass was higher in H rats than in C rats and this was unaffected by ethanol feeding (Table 2). Left and right ventricular wet weights did not differ between the groups (Table 2). Left ventricular diastolic stiffness was higher in H rats compared to C rats. Left ventricular diastolic stiffness did not change with ethanol feeding in both CE and HE rats (Table 2). Left ventricles from H rats showed infiltration of inflammatory cells (Figure 2C), fibrosis and hypertrophy (Figure 2G). These changes were absent in the left ventricles from C rats (Figure 2A & 2E). Left ventricles from HE rats showed infiltration of inflammatory cells (Figure 2D) whereas fibrosis was attenuated with ethanol feeding in HE rats (Figure 2H). Ethanol feeding did not affect infiltration of inflammatory cells, fibrosis and hypertrophy in CE rats (Figure 2B & 2F).

Thoracic aortic contraction with noradrenaline was lower in H rats compared to C rats. Thoracic aortic rings from HE rats had higher contractile response to noradrenaline than H rats while this contractile response did not differ between C and CE rats (Figure 3A). Sodium nitroprusside-induced relaxation was lower in H rats compared to C rats. HE rats showed higher relaxant response to sodium nitroprusside than H rats while this response did not differ between C and CE rats (Figure 3B). H rats had lower relaxant response to acetylcholine compared to C rats. CE and HE rats showed higher relaxant responses to acetylcholine compared to C and H rats, respectively (Figure 3C).

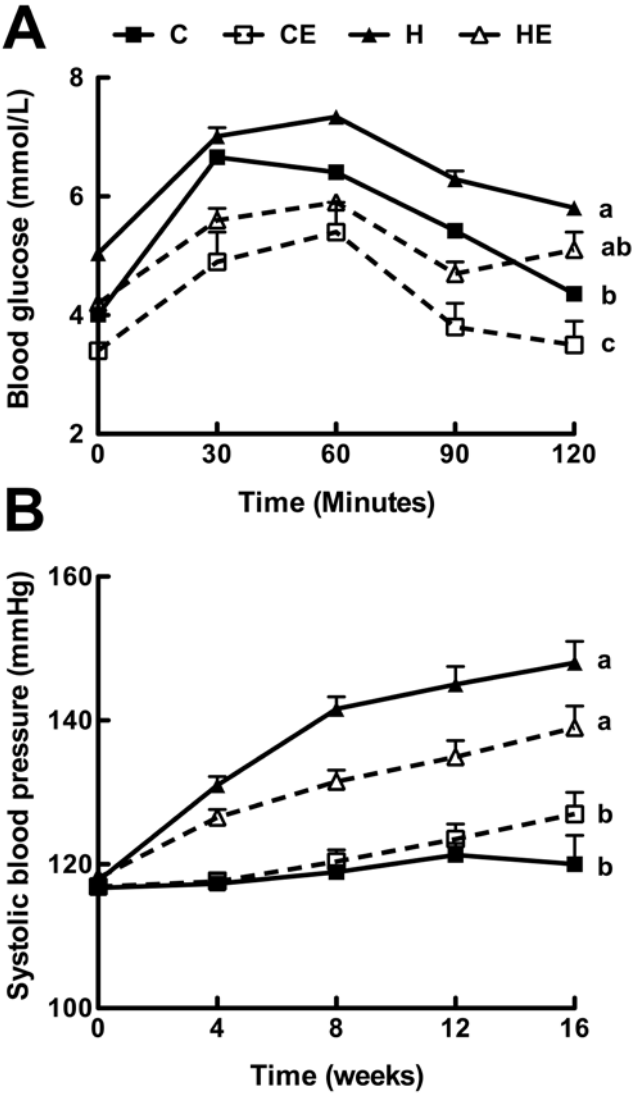

Figure	*P* value		
	Diet	*Ethanol*	*Diet* × *Ethanol*
A	<0.0001	0.005	0.78
B	<0.0001	0.76	0.019

Figure 1. Effects of combination of high-carbohydrate, high-fat diet and ethanol on oral glucose tolerance (A) and systolic blood pressure (B) in rats. Values are mean ± SEM, $n = 12$. E nd-point means without a common letter differ, $P < 0.05$.

C, corn starch diet-fed rats; *CE*, corn starch diet-fed rats given 10% ethanol; *H*, high-carbohydrate, high-fat diet-fed rats; *HE*, high-carbohydrate, high-fat diet-fed rats given 10% ethanol.

Table 2. Effects of combination of high-carbohydrate, high-fat diet and 10% ethanol on cardiovascular structure and function in rats

Variables	*C*	*CE*	*H*	*HE*	*P value*		
					Diet	*Ethanol*	*Diet × Ethanol*
LVIDd, *mm*	6.61 ± 0.12^{b}	6.46 ± 0.47^{b}	7.46 ± 0.11^{a}	7.89 ± 0.14^{a}	0.0001	0.59	0.27
LVIDs, *mm*	3.31 ± 0.18^{b}	3.56 ± 0.07^{b}	4.60 ± 0.09^{a}	4.56 ± 0.14^{a}	<0.0001	0.42	0.27
LVPWd, *mm*	2.69 ± 0.14^{a}	1.85 ± 0.07^{b}	2.85 ± 0.15^{a}	1.75 ± 0.04^{b}	0.79	<0.0001	0.25
Relative wall thickness	0.48 ± 0.01^{b}	0.59 ± 0.05^{a}	0.48 ± 0.02^{b}	0.45 ± 0.01^{b}	0.018	0.16	0.018
Fractional shortening, *%*	52.6 ± 1.3^{a}	46.7 ± 4.2^{ab}	38.3 ± 1.4^{c}	42.2 ± 1.2^{bc}	0.0005	0.68	0.049
Ejection fraction, *%*	86.9 ± 1.6^{a}	78.8 ± 1.8^{b}	72.1 ± 1.3^{c}	80.6 ± 1.2^{b}	0.0002	0.89	<0.0001
Ejection time, *msec*	91 ± 2^{a}	74 ± 5^{b}	88 ± 3^{a}	89 ± 4^{a}	0.11	0.038	0.021
Estimated left ventricular mass, *g*	0.67 ± 0.02^{b}	0.79 ± 0.07^{b}	0.97 ± 0.04^{a}	0.98 ± 0.03^{a}	<0.0001	0.15	0.22
Left ventricle + septum wet weight, *mg/mm tibial length*	19.3 ± 0.9	20.7 ± 0.8	21.8 ± 1.2	19.5 ± 0.9	0.50	0.64	0.07
Right ventricular wet weight, *mg/mm tibial length*	4.14 ± 0.27	4.92 ± 0.50	4.48 ± 0.37	4.55 ± 0.56	0.97	0.34	0.43
Left ventricular diastolic stiffness constant (κ)	21.2 ± 1.5^{b}	23.1 ± 1.2^{ab}	28.2 ± 1.8^{a}	27.6 ± 1.3^{a}	0.0004	0.66	0.40

Values are mean ± SEM, n = 8-12. Means without a common superscript letter in a row differ, $P<0.05$.

Abbreviations used: C, corn starch diet-fed rats; *CE*, corn starch diet-fed rats given 10% ethanol; *H*, high-carbohydrate, high-fat diet-fed rats; *HE*, high-carbohydrate, high-fat diet-fed rats given 10% ethanol; *LVIDd*, left ventricular internal diameter during diastole; *LVIDs*, left ventricular internal diameter during systole; *LVPWd*, left ventricular posterior wall thickness during diastole.

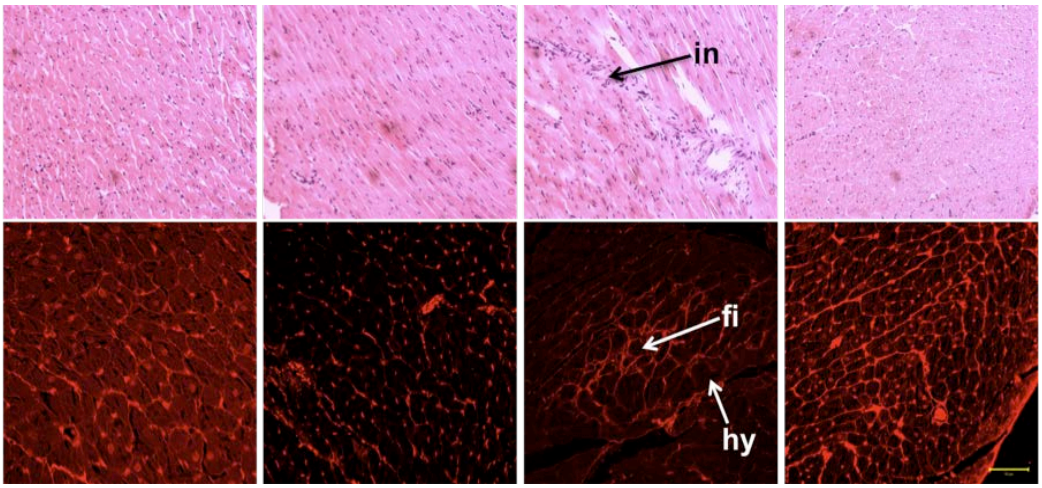

Figure 2. Effects of combination of high-carbohydrate, high-fat diet and ethanol on inflammation and fibrosis in the heart from rats fed with different diets. Haematoxylin and eosin staining of left ventricle showing infiltration of inflammatory cells (A-D, inflammatory cells marked as "in") from corn starch diet-fed rats (A), corn starch diet-fed rats given 10% ethanol (B), high-carbohydrate, high-fat diet-fed rats (C) and high-carbohydrate, high-fat diet-fed rats given 10% ethanol (D). Picrosirius red staining of left ventricle showing collagen deposition and hypertrophy (E-H, fibrosis marked as "fi" and hypertrophied cardiomyocytes as "hy") from corn starch diet-fed rats (E), corn starch diet-fed rats given 10% ethanol (F), high-carbohydrate, high-fat diet-fed rats (G) and high-carbohydrate, high-fat diet-fed rats given 10% ethanol (H).

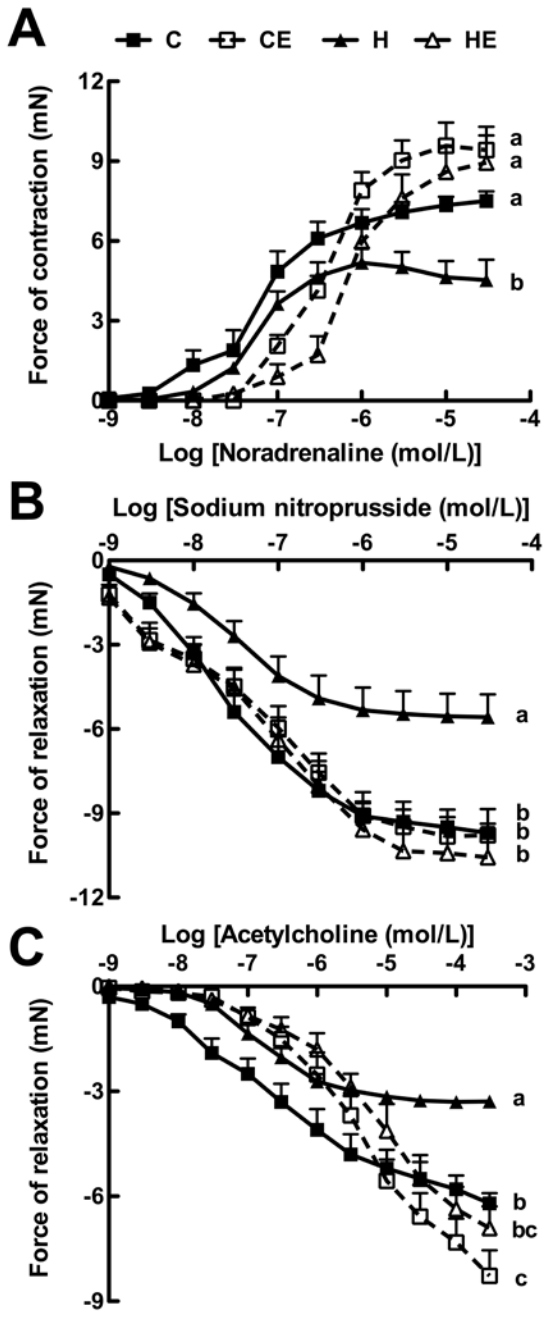

Figure	*P* value		
	Diet	*Ethanol*	*Diet* × *Ethanol*
A	0.037	0.0003	0.13
B	0.036	0.002	0.003
C	<0.0001	<0.0001	0.12

Figure 3. Effects of combination of high-carbohydrate, high-fat diet and ethanol on noradrenaline- induced contraction (A), sodium nitroprusside-induced relaxation (B) and acetylcholine-induced relaxation (C) in thoracic aortic preparations from rats. Values are mean ± SEM, $n = 12$. End-point means without a common letter differ, $P < 0.05$.

C, corn starch diet-fed rats; *CE*, corn starch diet-fed rats given 10% ethanol; *H*, high-carbohydrate, high-fat diet-fed rats; *HE*, high-carbohydrate, high-fat diet-fed rats given 10% ethanol.

Hepatic structure and function

Livers from H rats showed infiltration of inflammatory cells (Figure 4C), fat deposition (Figure 4G) and perivascular fibrosis (Figure K). These changes were not seen in the livers from C rats (Figure 4A, 4E & 4I). In HE rats, livers showed infiltration of inflammatory cells (Figure 4D), a further increase in fat deposition in the form of fat vacuoles (Figure 4H) as well as perivascular fibrosis (Figure 4L). In CE rats, infiltration of inflammatory cells (Figure 4B) and fibrosis (Figure 4F) were not observed whereas fat vacuoles were present (Figure 4J).

Liver wet weight was higher in H rats compared to C rats. CE rats showed higher liver wet weight than C rats whereas HE rats showed lower liver wet weights compared to H rats (Table 3). H rats had higher plasma activities of ALT, AST, ALP and LDH compared to C rats, while these activities were lower in HE rats than H rats. C and CE rats did not differ in plasma activities of ALT, AST and LDH whereas plasma activity of ALP was lower in CE rats than in C rats (Table 3). Plasma albumin concentrations did not differ between C and H rats. HE rats had lower plasma albumin concentrations than H rats whereas plasma albumin concentrations did not differ between C and CE rats (Table 3). Plasma total bilirubin concentrations did not differ between C and H rats. CE rats had lower plasma concentrations of total bilirubin than C rats while plasma total bilirubin concentrations did not differ between H and HE rats (Table 3). Plasma urea concentrations were lower in H rats than in C rats. Plasma urea concentrations were higher in HE rats compared to H rats, although this parameter was not normalised. Plasma urea concentrations did not differ between C and CE rats. Plasma uric acid concentrations were higher in H rats compared to C. Plasma uric acid concentrations did not differ in CE and HE rats compared to C and H rats, respectively (Table 3).

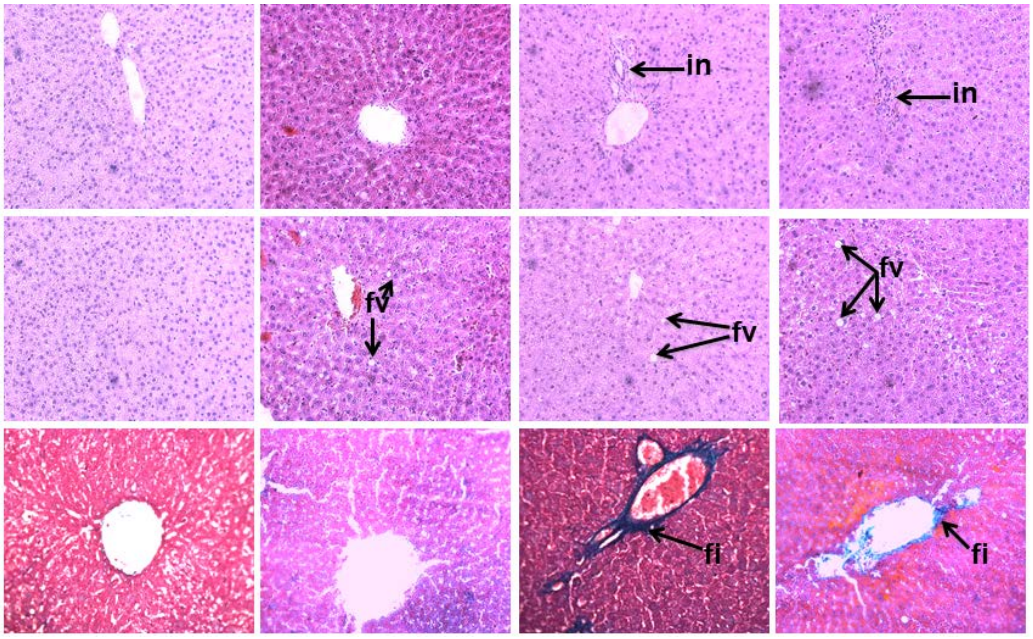

Figure 4. Effects of combination of high-carbohydrate, high-fat diet and ethanol on inflammation, fat deposition and fibrosis in the liver from rats fed with different diets. Top and middle rows represent hematoxylin and eosin staining of the liver showing inflammatory cells (A-D, marked as "in") (×20) and enlarged fat vacuoles (E-H, marked as "fv") (×40) from corn starch diet-fed rats (A,E), corn starch diet-fed rats given 10% ethanol (B,F), high-carbohydrate, high-fat diet-fed rats (C,G) and high-carbohydrate, high-fat diet-fed rats given 10% ethanol (D,H). Bottom row represents Milligan's trichrome staining of the liver showing fibrosis in the hepatic portal region (I-L, marked as "fi") (×20) from corn starch diet-fed rats (I), corn starch diet-fed rats given 10% ethanol (J), high-carbohydrate, high-fat diet-fed rats (K) and high-carbohydrate, high-fat diet-fed rats given 10% ethanol (L).

Table 3. Effects of combination of high-carbohydrate, high-fat diet and 10% ethanol on hepatic structure and function in rats

Variables					*P value*		
	C	*CE*	*H*	*HE*	*Diet*	*Ethanol*	*Diet × Ethanol*
Liver wet weight, *mg/mm tibial length*	234 ± 14^{b}	283 ± 7^{a}	287 ± 12^{a}	209 ± 6^{b}	0.31	0.17	<0.0001
Plasma ALT activity, *U/L*	37 ± 1^{b}	39 ± 5^{b}	60 ± 1^{a}	35 ± 1^{b}	0.0008	<0.0001	<0.0001
Plasma AST activity, *U/L*	73 ± 6^{b}	72 ± 3^{b}	105 ± 9^{a}	82 ± 3^{b}	0.0008	0.045	0.07
Plasma ALP activity, *U/L*	174 ± 19^{b}	104 ± 13^{c}	251 ± 21^{a}	96 ± 8^{c}	0.038	<0.0001	0.011
Plasma LDH activity, *U/L*	204 ± 23^{b}	172 ± 23^{b}	497 ± 14^{a}	182 ± 20^{b}	<0.0001	<0.0001	<0.0001
Plasma albumin, *g/L*	28.0 ± 0.6^{ab}	27.0 ± 0.5^{b}	28.7 ± 0.3^{a}	27.0 ± 0.2^{b}	0.42	0.003	0.42
Plasma total bilirubin, *μmol/L*	2.42 ± 0.09^{a}	2.08 ± 0.11^{b}	2.30 ± 0.06^{ab}	2.02 ± 0.09^{b}	0.32	0.001	0.74
Plasma urea, *mmol/L*	5.95 ± 0.16^{a}	5.81 ± 0.26^{a}	3.36 ± 0.12^{c}	4.02 ± 0.28^{b}	<0.0001	0.23	0.07
Plasma uric acid, *μmol/L*	32.1 ± 2.3^{b}	46.6 ± 4.8^{ab}	60.2 ± 3.1^{a}	50.0 ± 8.9^{ab}	0.006	0.69	0.027

Values are mean ± SEM, n = 9-12. Means without a common superscript letter in a row differ, $P<0.05$.

Abbreviations used: ALP, alkaline phosphatase; *ALT*, alanine transaminase; *AST*, aspartate transaminase; *C*, corn starch diet-fed rats; *CE*, corn starch diet-fed rats given 10% ethanol; *H*, high-carbohydrate, high-fat diet-fed rats; *HE*, high-carbohydrate, high-fat diet-fed rats given 10% ethanol; *LDH*, lactate dehydrogenase.

Discussion

Ethanol consumption is very common in most countries. In Australia, approximately 3,000 people die and 65,000 people are hospitalised every year as a result of excess ethanol consumption [19,20]. Ethanol-related deaths in Australia included a significant involvement of end-stage alcoholic liver cirrhosis as a cause of death [21]. A web-based survey has shown a high prevalence of ethanol consumption among Australian University students [22]. Similar results have been obtained from different countries [23-25]. National Health and Medical Research Council guidelines recommend no more than two standard drinks (20 g alcohol/day) on any day for healthy men and women to reduce the risk of harm from alcohol-related diseases [26]. It is proposed that cirrhosis occurs faster in the presence of conditions such as viral hepatitis, obesity or iron overload [21].

Ethanol is absorbed very rapidly and without metabolism from the gastrointestinal tract (about 20% in stomach and 80% in small intestine). After absorption, it is distributed throughout the body and then metabolised. The main organ responsible for the metabolism of ethanol is the liver [27]. Initially, ethanol is converted to acetaldehyde by alcohol dehydrogenase in the cytoplasm of the hepatocytes. Nicotinamide adenine dinucleotide (NAD) acts as an electron acceptor in this reaction. Liver microsomes can oxidise alcohol with the use of NADP instead of NAD. Acetaldehyde is then converted to acetic acid by aldehyde dehydrogenase, using NAD as the electron acceptor. The acetic acid thus produced is then converted to acetyl-CoA, which is an intermediate of Krebs cycle and *de novo* lipogenesis [27]. Many mechanisms have been validated for the development of fatty liver with chronic ethanol consumption [28].

Acetic acid produced from metabolism of ethanol decreases plasma non-esterified fatty acids [29]. A similar decrease occurs in plasma glycerol concentrations [30]. Also, being a major site for glycerol metabolism, liver had

reduced glycerol clearance in presence of ethanol [31]. These studies conclude that consumption of ethanol leads to decreased lipolysis in the peripheral tissues and hence lower concentrations of non-esterified fatty acids and glycerol in plasma. However, these effects are seen with a lower dose of ethanol. Non-esterified fatty acids have been involved increasing the risk of cardiovascular disease [32,33]. Thus, the reduction in plasma concentrations of non-esterified fatty acids with acute alcohol consumption may explain the cardioprotective effects of lower intake of alcohol. With higher dose of ethanol, the mobilisation of fatty acids from adipose tissue increased the plasma non-esterified fatty acid concentrations, although plasma triglycerides concentrations were lower [34].

The shift from NAD to NADH by ethanol inhibits the citric acid cycle and β-oxidation of fatty acids. At the same time, conversion of pyruvate to lactate is favoured. The increase in the ratios of NADH/NAD and lactate/pyruvate leads to inhibition of gluconeogenesis. These results were confirmed in both rat livers [35] as well as in humans [36]. When supply of glucose is limited as in the case of starvation, glucose is released from glycogen stores in the liver. In the case of chronic alcohol consumption, glycogenolysis is also impaired. Thus alcohol causes hypoglycaemia with impaired glycogenolysis and gluconeogenesis [36-38].

In our previous studies, we have shown that a high-carbohydrate, high-fat diet in rats induced symptoms of non-alcoholic fatty liver disease, including the presence of fat vacuoles in the liver, infiltration of inflammatory cells, perivascular fibrosis and increased plasma activities of liver enzymes including transaminases [5]. Although these changes were seen in high-carbohydrate, high-fat diet-fed rats, it was concluded that these symptoms were only mild, as expected with this diet for a relatively short period. Since ethanol is a dietary component that causes severe damage to the liver in humans when taken chronically [3], this study was designed to measure the effects of a combination of high-carbohydrate, high-fat diet and ethanol. It was hypothesised that this

combination would cause more damage to the liver than the high-carbohydrate, high-fat diet alone.

A combination of ethanol and high-carbohydrate, high-fat diet did not worsen the symptoms of metabolic syndrome in rats. Rather, some of the symptoms of metabolic syndrome including central obesity and dyslipidaemia were improved. Cardiovascular changes including left ventricular diameter, fractional shortening, ventricular diastolic stiffness and cardiac inflammation were similar to high-carbohydrate, high-fat diet alone whereas cardiac fibrosis and hypertrophy were attenuated with ethanol in high-carbohydrate, high-fat diet-fed rats. Vascular responses including smooth muscle contraction, endothelium-dependent and endothelium-independent vascular relaxations were improved in the ethanol and high-carbohydrate, high-fat diet-fed rats. Specifically, livers from high-carbohydrate, high-fat diet-fed rats given ethanol showed lower liver wet weight and plasma activities of liver enzymes compared to high-carbohydrate, high-fat diet-fed rats. However, the livers from ethanol-fed rats with both corn starch diet and high-carbohydrate, high-fat diet showed more fat vacuoles than high-carbohydrate, high-fat diet alone. These results in rats showed that ethanol feeding (5-6 g/kg body weight/day) in rats caused hepatic steatosis. This effect of ethanol was independent of high-carbohydrate, high-fat diet as it was also observed in corn starch diet-fed rats.

In conclusion, ethanol caused hepatic steatosis with both corn starch diet and high-carbohydrate, high-fat diet. However, hepatic inflammation and fibrosis were only observed with a combination of ethanol and high-carbohydrate, high-fat diet. Also, this combination either improved the cardiovascular complications or did not affect them, consistent with responses to ethanol expected as part of the Mediterranean diet. It can be concluded that a combination of high-carbohydrate, high-fat diet and 10% ethanol can be used to mimic alcoholic steatohepatitis, but this model does not mimic the cardiovascular complications observed with high-carbohydrate, high-fat diet alone due to the cardioprotective effect of ethanol.

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Chapter 9: Discussion and conclusion

This thesis has firstly characterised an appropriate rat model to mimic diet-induced metabolic syndrome in humans and secondly evaluated the potential therapeutic benefits of interventions with natural products found in foods. *Chapter 1* describes metabolic syndrome, cardiovascular remodelling and non-alcoholic fatty liver disease. Metabolic syndrome is prevalent throughout the world [1]. This combination of obesity, hypertension, dyslipidaemia and insulin resistance markedly increases the risk for development of cardiovascular disease, non-alcoholic fatty liver disease and diabetes, increasing both morbidity and mortality. The major players in the development of cardiovascular disease and non-alcoholic fatty liver disease are oxidative stress and inflammation.

Chapter 2 describes the potential therapeutic benefits of polyphenols from natural products and some of the Indian spices on the symptoms of metabolic syndrome. Polyphenols are plant secondary metabolites derived exclusively from the shikimate-derived phenylpropanoid and/or the polyketide pathway(s), featuring more than one phenolic ring and being devoid of any nitrogen-based functional group in their most basic structural expressions [2]. In this study, we focused on polyphenols or polyphenol-rich natural products as potential treatment strategies for metabolic syndrome.

Research in metabolic syndrome, as in other human diseases, relies on appropriate animal models to test possible interventions in humans. Many rodent models are available, including genetic/spontaneous models, genetically-engineered models, chemically-induced models and diet-induced models [1]. Since the human syndrome is mainly diet-induced rather than genetic, *Chapter 3.1* has argued that a diet-induced model in rats is the most appropriate model to mimic the human metabolic syndrome. Many different diets are available in the literature to induce obesity in rats and mice, usually with increased content of either simple carbohydrates such as fructose or sucrose, or increased

saturated fats. However, the high carbohydrate diet does not induce obesity and the high saturated fat diet does not mimic the human diet. The most appropriate model therefore incorporates a diet with increased simple sugars as well as saturated fats, including *trans* fats.

Characterisation of the chronic changes induced by this diet on metabolic, cardiovascular and liver parameters is given as *Chapter 3.2* of this thesis. Young adult male Wistar rats were given a diet of 20% beef tallow, 17.5% fructose and 39.5% condensed milk along with 25% fructose in drinking water for 16 weeks [3-5]. A corn starch rich diet was used as a control for this diet, aiming to provide the same proportion of carbohydrates in the form of polysaccharides. Corn starch is a slowly digestible carbohydrate whereas fructose and sucrose are simple lipogenic carbohydrates which are absorbed easily in the gastrointestinal tract. Also, corn starch did not increase blood glucose concentrations, systolic blood pressure, abdominal fat deposition, plasma insulin or plasma lipid concentrations [3]. Thus, corn starch diet served as an appropriate control diet for comparison with the high-carbohydrate, high-fat diet [3-5]. High-carbohydrate, high-fat diet-fed rats developed progressive increases in body weight, energy intake, central obesity, systolic blood pressure, dyslipidaemia and hyperinsulinaemia [3]. These changes were associated with development of cardiovascular remodelling including ventricular dilatation, ventricular stiffness and fibrosis and reduced ventricular function and non-alcoholic steatohepatitis including fat deposition, inflammation and fibrosis in the liver with increases in the plasma activities of marker enzymes for liver dysfunction [3]. This model was used to investigate the potential therapeutic effects of natural products since pathophysiological responses mimicking human metabolic syndrome were shown. The different responses to natural products used in this study have been summarised in *Table 1*.

Table 1. Effects of natural products on components of metabolic syndrome

Components of metabolic syndrome	***Central obesity***	***Glucose tolerance***	***Hypertension***	***Dyslipidaemia***	***Cardiovascular remodelling***	***Non-alcoholic steatohepatitis***	***Body weight***	***Energy intake***
Treatment								
Rutin (100 mg/kg body weight/day)	√	√	√	√	√	√	√	×
Quercetin (50 mg/kg body weight/day)	√	√	√	×	√	√	×	×
Oak bark extract (0.5 ml/kg food)	√	√	√	√	√	√	√	×
Ellagic acid (50 mg/kg body weight/day)	√	√	√	√	√	√	√	×
Coffee extract (5% in food)	×	√	√	×	√	√	×	×
Caffeine (0.5 g/kg food)	√	√	√	×	√	√	√	×
L-Carnitine (1.2 % in food)	√	√	√	√	√	√	√	×

High-carbohydrate, high-fat diet-fed rats showed symptoms of metabolic syndrome along with cardiovascular and hepatic complications. This table represents the effects of the treatments investigated in this thesis. No treatments decreased the energy intake but all improved some or most of the components of metabolic syndrome. √ indicates that the complication was affected by the treatment whereas × indicates no effect of treatment on the complication.

Initially, two flavonoids, rutin and its aglycone, quercetin, were studied for their effects in diet-induced metabolic syndrome in rats. Both rutin and quercetin inhibited infiltration of inflammatory cells in the heart and the liver, reduced collagen deposition in the left ventricle and perivascular fibrosis in the liver, improved ventricular and liver functions, reduced diastolic stiffness, improved vascular contraction and relaxation, prevented hepatic steatosis and improved glucose tolerance, although they had different effects on central obesity and dyslipidaemia. Rutin (100 mg/kg body weight/day) reduced abdominal fat, body weight and plasma lipid contents suggesting that the excess fat was either oxidised or excreted from the body (*Chapter 4.1*). In contrast, quercetin (50 mg/kg body weight/day) reduced abdominal fat without changing body weight and plasma lipid components. This suggests that the fat was moved from the abdominal area and either stored in subcutaneous areas or converted into muscle mass, hence producing no change in body weight (*Chapter 4.2*). Although the only chemical difference between rutin and quercetin is the presence of glucose and rhamnose moieties in rutin, there were differences in the responses to these compounds in diet-induced metabolic syndrome. These differences might arise from differences in the absorption and metabolism of these phytochemicals [6]. Molecular weights of rutin and quercetin are 610 and 302, respectively. Since rutin is almost double the molecular weight of quercetin and one molecule of rutin contains only one quercetin, the dose of rutin given was double the dose of quercetin. This was done in order to ensure that the effects seen from both rutin and quercetin were equivalent in terms of active constituents as the glycosidic part of rutin is cleaved before it is absorbed in the gastrointestinal tract.

Similar results were shown with ellagic acid and ellagitannins. Ellagitannins are complex esters of ellagic acid with glucose while ellagic acid is a dilactone formed from 2 molecules of gallic acid. Ellagitannins induced reduction in body weight as well as abdominal fat, which indicates that the abdominal fat was either oxidised or excreted from the body, an effect also observed with rutin (*Chapter 5.1*). Unlike ellagitannins, ellagic acid-

supplemented rats did not show reductions in total body fat whereas the abdominal fat was reduced (*Chapter 5.2*). This clearly indicates that the fat was removed from the abdominal area and stored in the subcutaneous areas without being converted into muscle mass. Thus, there were differences between the effects of ellagic acid and ellagitannins on body fat and body weight. However, both ellagitannins and ellagic acid prevented infiltration of inflammatory cells in the heart and the liver, reduced collagen deposition in the left ventricle and perivascular fibrosis in the liver, improved left ventricular and liver functions, reduced ventricular diastolic stiffness, improved vascular contraction and relaxation, prevented hepatic steatosis and improved glucose tolerance.

Colombian coffee extract (*Chapter 6.1*) containing caffeine, chlorogenic acid and diterpenoids such as cafestol and kahweol was the only treatment in this thesis that did not reduce abdominal fat content. High-carbohydrate, high-fat diet-fed rats given 5% coffee extract showed attenuation of cardiovascular remodelling and dysfunction, non-alcoholic steatohepatitis, impaired glucose tolerance and hypertension whereas central obesity and dyslipidaemia were unaffected. Other studies have shown correlations between presence of excess abdominal fat and dyslipidaemia with the occurrence of cardiovascular disease and non-alcoholic fatty liver disease [7-10]. Although abdominal fat and plasma lipids were not reduced by coffee extract, there were improvements in glucose tolerance, left ventricular function and liver function, prevention of cardiac and hepatic infiltration of inflammatory cells, prevention of hepatic steatosis and lowering of ventricular dimensions and ventricular diastolic stiffness. Among the major constituents of coffee, chlorogenic acid has been studied for its beneficial effects in metabolic syndrome [11-13]. Results with caffeine have been inconclusive [14-17] and there have been no studies with pure diterpenoids to show any effects in metabolic syndrome. Thus, we tested caffeine at approximately the same dose given through coffee extract (~30 mg/kg body weight/day). When caffeine was given to the high-carbohydrate, high-fat diet-fed rats, they showed normalisation of body fat, glucose tolerance and insulin sensitivity along with lowering of systolic blood pressure, body

weight and abdominal fat. These effects were also accompanied by improvements in glucose tolerance, liver function, prevention of cardiac and hepatic infiltration of inflammatory cells, prevention of hepatic steatosis and lowering of ventricular diastolic stiffness. However, plasma lipid components were increased. These effects of caffeine were attributed to the antagonism of A_1 adenosine receptors by caffeine (*Chapter 6.2*). Inhibition of A_1 adenosine receptors activates lipolysis in the adipocytes hence reducing the abdominal fat and increasing plasma non-esterified fatty acids. The comparison between the two studies indicates that the effects of caffeine in reducing abdominal fat in coffee-fed rats were overpowered by responses to other constituents. Previously, diterpenoids from coffee have been reported to induce hyperlipidaemia [18-22]. Thus, the lack of change in abdominal fat with coffee extract is probably due to the opposing responses to caffeine and the diterpenoids.

High fructose and fat in the diet are responsible for the increased incidence and prevalence of metabolic syndrome and associated complications. In obesity, *de novo* lipogenesis is increased and fat oxidation is decreased. L-Carnitine, the fatty acid transporter across the mitochondrial membrane, was given to the rats to increase the availability of L-carnitine for increased fatty acid oxidation. L-Carnitine treatment for the last 8 weeks of the protocol after induction of metabolic syndrome in high-carbohydrate, high-fat diet fed-rats reduced central obesity and dyslipidaemia, improved glucose tolerance and insulin sensitivity, and improved cardiovascular and hepatic structure and function. These improvements were accompanied by reduced stearoyl CoA desaturase-1 activity in the plasma, heart, liver, skeletal muscle and abdominal fat pads. Also, polyunsaturated fatty acids, mainly linoleic acid (C18:2n-6), were preferred for oxidation and saturated fatty acids and oleic acid were stored in the body (*Chapter 7*). Thus, L-carnitine supplementation in obese rats attenuated the symptoms of metabolic syndrome, cardiovascular remodelling and non-alcoholic fatty liver disease through inhibition of stearoyl CoA desaturase-1 activity leading to reduced short chain monounsaturated fatty acids

in the tissues. Further, L-carnitine directed C18:2n-6 preferentially for oxidation hence reducing n-6 fatty acid pathway to reduce inflammatory responses.

The Western diet includes cafeteria diet combined with the consumption of ethanol, commonly as beer, wine or spirits. Both cafeteria diet and ethanol ingestion can induce steatohepatitis yet ethanol has been claimed to be cardioprotective. In combination, ethanol (10% in drinking water) and high-carbohydrate, high-fat diet worsened hepatic steatosis without worsening inflammation or fibrosis. However, the combination diet improved left ventricular fibrosis and function but high-carbohydrate, high-fat induced changes in cardiac dimensions and diastolic stiffness were unchanged in ethanol-fed rats. These results support the French paradox where ingestion of alcoholic beverages such as wine with high-fat diet reduces the risk of cardiovascular disease (*Chapter 8*).

Overall, this thesis has examined the effects of individual polyphenolic compounds or extracts produced from a single natural product. Some of the possible mechanisms of actions of these natural products have been described in *Table 2*. Literature studies have examined the effects of a combination of various natural products. A recent study investigated the effects of a combination of curcumin with piperine and quercetin in high-fat diet and low-dose streptozotocin-induced diabetic rats [23]. In this study, *Curcuma longa*, *Piper nigrum and Allium cepa* were used to provide curcumin, piperine and quercetin, respectively. This combination of compounds improved plasma lipid profile and also reduced plasma glucose concentrations in diabetic rats [23]. In another study, a combination of quercetin (10 mg/kg/day) and α-tocopherol (10 mg/kg/day) was effective in attenuating the isoproterenol-induced cardiac changes [24]. An *in vitro* study with MOLT-4 cells suggested that combination of quercetin and ellagic acid increased the activation of p53 and $p21^{cip1/waf1}$ and the MAP kinases, JNK1/2 and p38, in a more than additive manner. This study suggested that quercetin and ellagic acid synergistically induced apoptosis in cancer cells [25]. The synergistic effects of fish oil and green tea extract with

querectin have recently been reviewed indicating the positive effects of the addition of these components with quercetin in the diet [26]. In contrast, another study with a combination of quercetin and coenzyme Q_{10} did not support the synergistic effects of these two molecules. This study indicated that the combination of quercetin and coenzyme Q_{10} was as effective as the individual compounds in diabetic rats [27].

Table 2. Possible mechanisms of action for treatments used in this thesis

Treatment	***Possible mechanisms of action***
Rutin (1.6 g/kg in food)	Increased energy expenditure, anti-oxidative and anti-inflammatory effects, increased apoptosis in liver
Quercetin (0.8 g/kg in food)	Fat distribution away from abdomen, anti-oxidative and anti-inflammatory effects, increased oxidation of fat, increased apoptosis in liver
Oak bark extract (0.5 ml/kg food)	Anti-oxidative and anti-inflammatory effects
Ellagic acid (0.8 g/kg in food)	Fat distribution away from abdomen, anti-oxidative and anti-inflammatory effects, increased oxidation of fat
Coffee extract (5% in food)	Anti-oxidative and anti-inflammatory effects
Caffeine (0.5 g/kg food)	Inhibition of A_1 adenosine receptors in adipocytes, cardiomyocyte and hepatocytes
L-Carnitine (1.2 % in food)	Increased fat oxidation, stearoyl-CoA desaturase-1 inhibition, preferential oxidation of *n*-6 fatty acids

The results from studies described in this thesis were used to deduce the possible mechanisms of actions.

In conclusion, natural products, that are part of food, can be used as medicines to reduce the symptoms of metabolic syndrome, cardiovascular disease and fatty liver disease. Onions, apples, red wine, grapes, coffee and berries have been part of the human diet since antiquity. This study has certainly shown that these constituents of diet or the individual components of these constituents can improve many or even all of the symptoms of the metabolic syndrome and associated complications. However, every natural product has a different type of action and these actions can be seen to a different extent with each natural product. Overall, food can be medicine if taken in the proper amount and in the proper way.

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Appendix A

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Comparison of purple carrot juice and β-carotene in a high-carbohydrate, high-fat diet-fed rat model of the metabolic syndrome

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Anthocyanins, phenolic acids and carotenoids are the predominant phytochemicals present in purple carrots. These phytochemicals could be useful in treatment of the metabolic syndrome since anthocyanins improve dyslipidaemia, glucose tolerance, hypertension and insulin resistance; the phenolic acids may also protect against CVD and β-carotene may protect against oxidative processes. In the present study, we have compared the ability of purple carrot juice and β-carotene to reverse the structural and functional changes in rats fed a high-carbohydrate, high-fat diet as a model of the metabolic syndrome induced by diet. Cardiac structure and function were defined by histology, echocardiography and in isolated hearts and blood vessels; liver structure and function, oxidative stress and inflammation were defined by histology and plasma markers. High-carbohydrate, high-fat diet-fed rats developed hypertension, cardiac fibrosis, increased cardiac stiffness, endothelial dysfunction, impaired glucose tolerance, increased abdominal fat deposition, altered plasma lipid profile, liver fibrosis and increased plasma liver enzymes together with increased plasma markers of oxidative stress and inflammation as well as increased inflammatory cell infiltration. Purple carrot juice attenuated or reversed all changes while β-carotene did not reduce oxidative stress, cardiac stiffness or hepatic fat deposition. As the juice itself contained low concentrations of carotenoids, it is likely that the anthocyanins are responsible for the antioxidant and anti-inflammatory properties of purple carrot juice to improve glucose tolerance as well as cardiovascular and hepatic structure and function.

Purple carrots: Anthocyanins: β-Carotene: Metabolic syndrome: High-carbohydrate, high-fat diet-fed rats

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Diet plays an important role in the aetiology and prevention of the risk factors of the metabolic syndrome[1]. Many epidemiological studies have shown a strong inverse association between an increased consumption of fruits and vegetables and a decreased incidence of CVD[2–5], especially stroke[3], IHD[2,3], CHD[4] and blood pressure[5]. Although the risk of type 2 diabetes is not related to the consumption of fruit or vegetables, the intake of antioxidant phytochemicals has been associated with reduction in the risk of type 2 diabetes[6,7]. Close relationships between obesity, the metabolic syndrome and the development of non-alcoholic fatty liver disease (NAFLD) have been described, with most NAFLD patients displaying multiple components of the metabolic syndrome[8]. However, the effects of increased intakes of fruit and vegetables on NAFLD patients have not been studied.

Glucosinolates, flavonoids, tannins, carotenoids, phytates and phyto-oestrogens represent the major classes of phytochemicals present in fruits and vegetables[9]. These nutrients may improve health through many mechanisms, such as reducing oxidative stress and inflammation, improving lipid profiles, lowering blood pressure and enhancing glucose metabolism[9]. Anthocyanins, a subclass of flavonoids, are pigments of red fruits such as cherries, plums, strawberries, raspberries, blackberries, grapes, red and black currants and, together with the phenolic acids, are the major phytochemicals in the human diet[10]. Although research on the therapeutic uses of the phytochemicals in carrots started with the demonstration of pro-vitamin A activity by Moore in 1929[11], few data exist on possible therapeutic benefits from carrot varieties other than orange carrots where β-carotene is the predominant phytochemical component.

Carrot varieties differ in colour from white to orange, yellow, red and purple and contain different combinations and quantities of macronutrients, fibre, vitamins, minerals and phytochemicals including carotenoids, phenolic acids, anthocyanins, isocoumarins, terpenes and sesquiterpenes[12,13]. The colour of orange carrots is due to higher concentrations of α- and β-carotene; red carrots contain increased lycopene and yellow carrots contain increased lutein concentrations while white carrots have lower concentrations of all carotenoids[13]. The anthocyanidins and chlorogenic acid are the major antioxidants in purple carrots[13,14]. The colour of purple carrots (*Daucus carota* L. ssp. *sativus* var. *atrorubens* Alef.) comes from the presence of

Abbreviations: CRP, C-reactive protein; H (8 weeks or 16 weeks), high-carbohydrate, high-fat; HC, high-carbohydrate, high-fat + β-carotene; HP, high-carbohydrate, high-fat + purple carrot juice; NAFLD, non-alcoholic fatty liver disease.

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anthocyanins such as cyanidin-3-(2″-xylose-6″-sinapoyl-glucose-galactoside)[13,15]. Additionally, at least forty phenolic acids are present in purple carrots including chlorogenic and caffeic acids as the predominant compounds[13]. Purple carrots also contain polyacetylene compounds such as falcarindiol, falcarindiol 3-acetate and falcarinol[16]. Purple carrots remain a sparsely studied potential dietary component that are rich in anthocyanins, phenolic acids and carotenoids[13].

β-Carotene, the best known of the large carotenoid family of phytochemicals, is the major dietary source of vitamin A (retinol). Epidemiological studies have inversely associated circulating β-carotene concentrations with the risk of hypertension[17,18] but not with type 2 diabetes[19,20]. The antioxidant as well as pro-oxidant actions of β-carotene are widely known[21,22]. Carotenoids, particularly β-carotene and lycopene, protect animals and humans against oxidative processes, especially in lipophilic environments such as cell membranes by acting as free radical scavengers[23]. Carotenoids have been proposed as a dietary strategy to prevent cancers, possibly by antioxidant actions and the stimulation of intercellular communication via gap junctions[23]. *In vivo*, β-carotene prevented ethanol-induced liver damage and improved hepatic antioxidant enzyme status in rats[24]. In streptozotocin-induced diabetic rats, β-carotene treatment increased hepatic and cardiac antioxidant enzymes[25], improved circulatory insulin and glucose concentrations[26] but increased lipid peroxidation[25].

Epidemiological studies have shown that plant-based diets protect against CVD and cancer; flavonoids, including the anthocyanins, occur widely in plants and may improve human health[27,28]. In particular, the anthocyanins may improve the symptoms of the metabolic syndrome such as dyslipidaemia, impaired glucose tolerance and hypertension[27,28]. Anthocyanin-rich plant extracts lowered plasma lipid concentrations in rat models of hyperlipidaemia[29,30] and an extract from black rice (*Oryza sativa* L. *indica*) lowered plasma lipid concentrations and improved insulin resistance in fructose-fed rats[31]. Spontaneously hypertensive rats fed on anthocyanin-rich purple maize, purple sweet potato and red radish showed reduced blood pressure and heart rate[32]. Other constituents of carrots such as the polyacetylenes may suppress inflammation in the metabolic syndrome by decreasing lipopolysaccharide-induced expression of inflammatory proteins in macrophages and endothelial cells[12].

Although the phytochemical profile of purple carrots has been reported, sparse information exists on the pharmacological activity of purple carrot juice. The aims of the present study were to determine whether purple carrot juice attenuates or reverses the cardiovascular, liver and metabolic changes produced in a high-carbohydrate, high-fat diet-fed rat model of the metabolic syndrome and whether these responses are comparable with that of β-carotene.

Diets with increased carbohydrates, mainly as fructose[33], increased fats[34] or a combination of both[35] in rats have been studied to mimic the human metabolic syndrome. Maize starch is a slowly digestible carbohydrate[36] and served as a control for the high-carbohydrate, high-fat diet in the present study where the primary carbohydrate is fructose. Unlike fructose, maize starch does not increase blood glucose, plasma insulin or NEFA concentrations[35,36].

The present study characterised structural changes in the rat heart by histology and echocardiography, while heart function was assessed *in vivo* using echocardiography and *ex vivo* in isolated perfused hearts. Systolic blood pressure was measured and isolated thoracic rings were used to measure vascular reactivity. Biochemical parameters (enzyme activities of alanine transaminase, aspartate transaminase and alkaline phosphatase as well as total plasma bilirubin concentrations) were assessed, and along with histology, were used to define structural and functional changes in the liver. Circulatory markers of impaired metabolism (lactate dehydrogenase, urea and oral glucose tolerance test), oxidative stress (malondialdehyde and uric acid) and inflammation (C-reactive protein; CRP) were measured.

Materials and methods

Purple carrot juice

Purple carrot juice was prepared by SDS Beverages Pty Ltd (Irymple, VIC, Australia). Purple carrots were crushed in a hammer mill with water acidified with citric acid. Following separation of solids, pectinases were added to the juice and mixed at 50–55°C for clarification. The juice was then subjected to pasteurisation, chilling and filtering through a 200 μm mesh before evaporation was carried out to concentrate the juice to 60 brix. This 60 brix purple carrot juice was processed through another 200 μm mesh and a metal analyser and stored at 4°C. This product was added as a 5 % supplement to either the maize starch or high-carbohydrate, high-fat diet described below (Table 1).

Total monomeric anthocyanins were determined using the pH differential method as previously described[37]. Absorbance was measured at 520 and 700 nm. Total monomeric anthocyanins were expressed as cyanidin-3-glucoside (molar extinction coefficient of 26 900 litres/cm per mol and molecular weight of 449·2 g/mol).

Carotenoid concentrations in purple carrot juice were analysed on an Agilent 1100 series HPLC system equipped with a photodiode array UV-visible detector (Agilent Technologies, Waldbronn, Germany). Separations were achieved on a 150 × 3·0 mm internal diameter, 3 μm particle size, analytical scale YMC C30 reversed-phase column (YMC, Wilmington, MA, USA). Elution was performed at a solvent flow rate of 1·0 ml/min and detection at 450 nm. The mobile phases consisted of acetone and Milli-Q water. For routine analysis, a 10–95 % linear gradient elution of acetonitrile and Milli-Q water was pumped through the column at 1·0 ml/min over 30 min. All solvents were HPLC grade and contained 0·005 % trifluoroacetic acid (Sigma-Aldrich Australia, Sydney, NSW, Australia) to enhance peak shape. Purple carrot juice was dissolved in 100 % analytical grade methanol and filtered through a Whatman polytetrafluoroethylene (PTFE) membrane filter (0·45 μm; Whatman PLC, Maidstone, Kent, UK) before loading the sample (10 μl injection volume). The UV spectra of the different carotenoid (all-*trans*-β-carotene, zeaxanthin and lutein) compounds were recorded with a diode array detector. Carotenoids were quantified using calibration curves of the corresponding standard compounds at the specific absorption maximum.

Table 1. Diet composition and energy contents of diets

Ingredient	M	MC	MP	H	HC	HP
Maize starch (g/kg)	570·00	570·00	570·00	–	–	–
Powdered rat feed (g/kg)*	155·00	155·00	155·00	155·00	155·00	155·00
HMW salt mixture (g/kg)†	25·00	25·00	25·00	25·00	25·00	25·00
Fructose (g/kg)	–	–	–	175·00	175·00	175·00
Beef tallow (g/kg)	–	–	–	200·00	200·00	200·00
Condensed milk (g/kg)	–	–	–	395·00	395·00	395·00
Water (ml/kg)	250·00	250·00	200·00	50·00	50·00	–
Purple carrot juice (ml/kg)	–	–	50·00	–	–	50·00
β-Carotene (mg/kg)	–	400	–	–	400	–
Energy (kJ/g)	11·23	11·23	11·67	17·93	17·93	18·37
Macronutrient composition‡						
Total carbohydrates (g/kg)	600·25	600·25	600·25	515·67	515·67	515·67
Total fat (g/kg)	8·07	8·07	8·07	239·04	239·04	239·04
Total proteins (g/kg)§	31·78	31·78	31·78	58·12	58·12	58·12
Total fibres (g/kg)	7·44	7·44	7·44	7·44	7·44	7·44
Total vitamins (g/kg)	0·32	0·32	0·32	0·32	0·32	0·32
Retinol (mg/kg)	4·0	4·0	4·0	4·0	4·0	4·0
Total minerals (g/kg)§	0·13	0·13	0·13	0·44	0·44	0·44
Ash (g/kg)	0·63	0·63	0·63	0·00	0·00	0·00
Total moisture (g/kg)	326·4	326·4	276·4	154·0	154·0	104·0

M, maize starch diet; MC, maize starch + β-carotene diet; MP, maize starch + purple carrot juice diet; H, high-carbohydrate, high-fat diet; HC, high-carbohydrate, high-fat + β-carotene diet; HP, high-carbohydrate, high-fat + purple carrot juice diet.

* Meat-free rat and mouse feed (Specialty Feeds) contained (g/kg feed): carbohydrate, 707·07; protein, 194·00; fat, 48·00; fibre, 48·00; total vitamins, 2·08; total minerals, 0·85.

† Hubble, Mendel and Wakeman salt mixture[54] (MP Biochemicals, Seven Hills, NSW, Australia).

‡ Derived from powdered rat feed.

§ H, HC and HP diets additionally derive these micronutrients from condensed milk.

Rats

The experimental groups consisted of eighty-four male Wistar rats (aged 8–9 weeks; weight 337 ± 5 g) supplied by The University of Queensland Biological Resources unit and individually housed at the School of Biomedical Sciences Animal House Facility. All experimental procedures were approved by the Animal Experimentation Ethics Committee of The University of Queensland under the guidelines of the National Health and Medical Research Council of Australia. All experimental groups were housed in a temperature-controlled, 12 h light–dark cycle environment with *ad libitum* access to water and the group-specific rat diet. Daily body weight, feed and water measurements were taken to monitor the day-to-day health of the rats. The rats were randomly divided into six separate groups based on their diet: maize starch (*n* 12); maize starch + β-carotene (*n* 12); maize starch + purple carrot juice (*n* 12); high-carbohydrate, high-fat (H; *n* 24); high-carbohydrate, high-fat + β-carotene (HC; *n* 12); high-carbohydrate, high-fat + purple carrot juice (HP; *n* 12). The full description of the diets is given in Table 1. In addition, the drinking water of all high-carbohydrate, high-fat diet-fed rats contained 25 % fructose. Both purple carrot juice and β-carotene (Sigma-Aldrich Australia) were administered for 8 weeks starting 8 weeks after the initiation of the maize starch or high-carbohydrate, high-fat diets. Twelve rats were killed from the high-carbohydrate, high-fat diet group at 8 weeks to assess the pathophysiological state before purple carrot or β-carotene intervention (H 8 weeks). Two rats per group were taken for histological analysis. Two slides were prepared per tissue specimen and two random, non-overlapping fields pictured for analysis. Organs were also collected from rats used for perfusion studies.

Systolic blood pressure was measured under light sedation with intraperitoneal injection of Zoletil (tiletamine 15 mg/kg, zolazepam 15 mg/kg; Virbac, Peakhurst, NSW, Australia). Measurements were taken using an MLT1010 Piezo-Electric Pulse Transducer (ADInstruments, Sydney, NSW, Australia) and inflatable tail-cuff connected to a MLT844 Physiological Pressure Transducer (ADInstruments) and PowerLab data acquisition unit (ADInstruments).

For the oral glucose tolerance test, basal blood glucose concentrations were measured in tail-vein blood using a Medisense Precision Q.I.D glucose meter (Abbott Laboratories, Bedford, MA, USA) after an overnight (10–12 h) feed deprivation. Fructose-supplemented drinking water in H, HC and HP groups was replaced with normal water for the overnight feed-deprivation period for the measurement of basal blood glucose concentrations. The rats were given glucose at 2 g/kg body weight as a 40 % solution via oral administration. Tail-vein blood samples were taken at 0, 30, 60, 90 and 120 min following glucose administration.

Echocardiography

Echocardiographic examination (Philips iE33 system, 12 MHz transducer; Philips Medical System, Andover, MA, USA) was performed at 8 and 16 weeks as previously described[35,38]. Briefly, rats were anaesthetised using intraperitoneal Zoletil (toletamine 15 mg/kg and zolazepam 15 mg/kg intraperitoneally; Virbac, Peakhurst, NSW, Australia) combined with xylazine (10 mg/kg) (Ilium Xylazil; Troy Laboratories, Sydney, NSW, Australia) and positioned in dorsal recumbency. Electrodes attached to the skin overlying the elbows and right stifle facilitated the simultaneous recording of a lead II electrocardiogram.

British Journal of Nutrition

A short axis view of the left ventricle at the level of the papillary muscles was obtained and used for direct acquisition of M-mode images of the left ventricle for measurement of diastolic posterior wall thickness, left ventricular internal systolic dimension and left ventricular end-diastolic diameter. For these parameters, diastole was defined by the beginning of the QRS complex on the simultaneously recorded electrocardiogram and systole identified as the nadir of systolic anterior wall motion independent of the electrocardiogram complex.

Pulsed-wave Doppler velocity profiles of mitral inflow was obtained from the left apical four-chamber view for measurement of early (E_M) and late (A_M) mitral inflow velocity, mitral inflow E-wave deceleration time, and time from mitral valve closure to opening (MCMO). A pulsed-wave Doppler velocity profile of the ascending aorta was obtained from a suprasternal view for measurement of aortic (Ao) velocity, ejection time (ET) and the diameter of the ascending aorta at the point of transition to the transverse aorta.

Derived indices of left ventricular systolic function (fractional shortening (FS%), ejection fraction (EF%)) were calculated using well-established formulae[38]. Left ventricular mass was estimated using the standard cube equation as previously described[35,38].

Isolated heart preparation

The left ventricular function of the rats in all treatment groups was assessed using the Langendorff heart preparation. Terminal anaesthesia was induced via intraperitoneal injection of pentobarbitone sodium (Lethabarb®, 100 mg/kg). After heparin (Sigma-Aldrich Australia) administration (100 IU) through the right femoral vein, blood (about 5 ml) was taken from the abdominal aorta. 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 (ADInstruments Australia and Pacific Islands, Bella Vista, NSW, Australia). All left ventricular end-diastolic pressure values were measured while pacing the heart at 250 beats per min using an electrical stimulator. End-diastolic pressures were obtained starting from 0 mmHg up to 30 mmHg. The diastolic stiffness constant (κ, dimensionless) was calculated as in previous studies[35,39].

Organ bath studies

Thoracic aortic rings (4 mm in length) were suspended in an organ bath chamber with a resting tension of 10 mN. Cumulative concentration–response (contraction) curves were measured for noradrenaline (Sigma-Aldrich Australia); concentration–response (relaxation) curves were measured for acetylcholine (Sigma-Aldrich Australia) and sodium nitroprusside (Sigma-Aldrich Australia) in the presence of a submaximal (70 %) contraction to noradrenaline[35].

Organ weights

The right and left ventricles were separated after perfusion experiments and weighed. Liver and abdominal fat were removed following heart removal and blotted dry for weighing. Perirenal, epididymal and omental fat were together weighed as abdominal fat. Organ weights were normalised relative to the tibial length at the time of their removal (in mg/mm).

Histology

Immediately after removal, heart and liver tissues were fixed in 10 % buffered formalin for 3 d with change of formalin every day to remove traces of blood from the tissue. The samples were then dehydrated and embedded in paraffin wax. Thin sections (10 μm) of left ventricle and the liver were cut and stained with haematoxylin and eosin stain for determination of inflammatory cell infiltration. Liver sections were also stained with Milligan's Trichrome stain to determine fibrosis. Collagen distribution was measured in the left ventricle with picrosirius red stain. Laser confocal microscopy (Zeiss LSM 510 upright Confocal Microscope)[35] with colour intensity quantitatively analysed using NIH-imageJ software (National Institutes of Health, Bethesda, MD, USA) was used to determine the extent of collagen deposition in selected tissue sections.

Plasma analysis

Briefly, blood was centrifuged at 5000 ***g*** for 15 min within 30 min after collection into heparinised tubes. Plasma samples were separated and transferred to Eppendorf tubes for analysis. Plasma enzymic activities and analyte concentrations were determined using kits and controls supplied by Olympus using an Olympus analyser (AU 400; Olympus, Tokyo, Japan): cholesterol, Olympus OSR6516 enzymic colour test; TAG, Olympus OSR 6133 enzymic colour test; lactate dehydrogenase, Olympus OSR6127 kinetic UV test; urea, Olympus OSR 6134 kinetic UV test; uric acid, Olympus OSR 6098 enzymic colour test; alanine transaminase, Olympus OSR 6107 kinetic UV test; aspartate transaminase, Olympus OSR 6109 kinetic UV test; alkaline phosphatase, Olympus OSR 6004 knetic UV test; albumin, Olympus OSR 6101 photometric colour test; total bilirubin, Olympus OSR 6111 photometric colour test. Globulin was calculated as total protein-albumin. Plasma malondialdehyde concentrations were measured by HPLC (Shimadzu®) as previously described[40]. Plasma CRP was estimated using a commercial kit (Kamiya Biomedical, CA, USA) according to manufacturer-provided standards and protocols using a Cobas-Mira automated analyzer (Roche Diagnostics, Basel, Switzerland).

Statistical analysis

All data are presented as mean values with their standard errors. Results were tested for variance using Bartlett's test and data that were not normally distributed were transformed (using log 10 function) before statistical analyses. Differences between the groups were determined by one-way ANOVA with the Newman–Keuls multiple comparison post test. For non-parametric data (total plasma cholesterol, plasma NEFA, lactate dehydrogenase, malondialdehyde and plasma urea concentrations and feed intake), Kruskal–Wallis tests were performed. All statistical analysis was performed using GraphPad Prism version 5.00 for Windows (San Diego, CA, USA). $P<0{\cdot}05$ was considered as statistically significant.

Results

Diet and supplement intake, body weight and metabolic parameters

Purple carrot juice contained anthocyanins (total 5·53 mg/ml) and traces of β-carotene (0·75 μg/ml), lutein (0·54 μg/ml) and zeaxanthin (4·4 μg/ml). The mean daily intakes of total anthocyanins and carotenoids were higher in the maize starch + purple carrot juice and maize starch + β-carotene groups than in the HP and HC groups, respectively, as the feed intake was higher in all maize starch diet-fed rats (Table 2). However, the carotenoid intakes in the maize starch + purple carrot juice and HP rats were extremely low due to the low carotenoid content of the juice (Table 2).

The high-carbohydrate, high-fat diet induced an increased body weight, especially with increased abdominal fat pads and abdominal circumference, compared with maize starch diet-fed rats (Table 2). Although high-carbohydrate, high-fat diet feeding reduced feed and water intake, rats on this diet had higher daily energy intake compared with maize starch diet-fed rats (Table 2). Neither purple carrot juice nor β-carotene supplementation altered daily feed, water or energy intake. However, purple carrot juice supplementation reduced percentage gain in body weight, abdominal fat pads and abdominal circumference (Table 2). The high-carbohydrate, high-fat diet produced impaired glucose tolerance, increased plasma concentrations of total cholesterol, TAG, NEFA, malondialdehyde, CRP and uric acid, increased lactate dehydrogenase activity and decreased plasma urea concentration (Table 2). Purple carrot juice supplementation improved oral glucose tolerance (Table 2) and reduced plasma total cholesterol, TAG, NEFA and CRP concentrations (Table 2). Although β-carotene improved oral glucose tolerance and reduced plasma CRP concentrations (Table 2), it further increased plasma TAG and NEFA concentrations (Table 2). Further, purple carrot juice supplementation attenuated the changes in lactate dehydrogenase activity, plasma uric acid, malondialdehyde and urea concentrations in high-carbohydrate, high-fat diet-fed rats; these parameters were unaltered by β-carotene supplementation (Table 2).

Cardiovascular structure and function

High-carbohydrate, high-fat diet-fed rats showed increased left ventricular diastolic internal diameter and relative wall thickness with increased estimated left ventricular mass and wet weight in comparison with the maize starch diet-fed rats (Table 3). Moreover, the high-carbohydrate, high-fat diet reduced fractional shortening and ejection fraction and increased ejection time (Table 3). Both β-carotene and purple carrot juice supplementation reduced estimated left ventricular mass and wet weight. In addition, β-carotene reduced left ventricular diastolic internal diameter, increased fractional shortening and ejection fraction and decreased ejection time (Table 3). High-carbohydrate, high-fat diet-fed rats showed sustained elevation in systolic blood pressure after 4 weeks of diet administration as compared with maize starch diet-fed rats (Table 3). Purple carrot juice as well as β-carotene supplementation normalised blood pressure as early as 4 weeks post-intervention (Table 3). Following high-carbohydrate, high-fat diet feeding for 16 weeks, diminished vascular responses were observed in isolated thoracic aortic rings to noradrenaline, sodium nitroprusside and acetylcholine when compared with maize starch diet-fed rats (Fig. 1). However, this reduced thoracic aortic reactivity was not observed after 8 weeks of high-carbohydrate, high-fat diet feeding. Purple carrot juice and β-carotene supplementation prevented any further decrease in sodium nitroprusside- and acetylcholine-dependent vasorelaxation (Fig. 1(B) and (C)) as compared with high-carbohydrate, high-fat diet-fed rats at 8 weeks but failed to attenuate the reduced contractility with noradrenaline (Fig. 1(A)).

Ex vivo cardiac function as measured in the Langendorff isolated heart showed an increased diastolic stiffness (Table 3) in the high-carbohydrate, high-fat diet-fed rats as compared with the maize starch diet-fed group (Table 3). Reduction in diastolic stiffness was only observed in purple carrot juice-supplemented rats. Histological analysis revealed that high-carbohydrate, high-fat diet-fed rats showed increased infiltration by inflammatory cells into the left ventricle (Fig. 2(E)) and increased interstitial collagen deposition and hypertrophy (Fig. 2(I); Table 3) when compared with the maize starch diet-fed rats (Fig. 2(A) and (H); Table 3). Both purple carrot juice- and β-carotene-supplemented high-carbohydrate, high-fat diet-fed rats showed normalised infiltration of inflammatory cells (Fig. 2(G); Fig. 2(F)) and reduced collagen deposition with normalised hypertrophy (Fig. 2(N); Fig. 2(M); Table 3). No major changes were observed in purple carrot juice- and β-carotene-supplemented maize starch diet-fed rats (Fig. 2(B), (C), (I) and (J); Table 3).

Hepatic structure and function

In comparison with maize starch diet-fed rats, high-carbohydrate, high-fat diet-fed rats had elevated plasma alanine transaminase, aspartate transaminase and alkaline phosphatase activities although the albumin:globulin ratio and plasma bilirubin concentrations remained unaffected (Table 4). Purple carrot juice supplementation reversed liver function abnormalities indicated by normalised activity of these enzymes (Table 4). Although β-carotene supplementation normalised transaminases activities in high-carbohydrate, high-fat diet-fed rats, alkaline phosphatase activity remained elevated (Table 4).

By histological examination, high-carbohydrate, high-fat diet feeding to rats caused macrovesicular steatosis (Fig. 3(E)) resulting from accumulation of lipids in large and small droplets within hepatocytes with markedly high portal inflammation (Fig. 3(M)) and portal fibrosis (Fig. 3(T); Table 4) as compared with the maize starch diet-fed rats (Fig. 3(A), (H) and (P); Table 4). The purple carrot juice-supplemented high-carbohydrate, high-fat diet-fed rats displayed minimal signs of macrovesicular steatosis (Fig. 3(G)), portal inflammation (Fig. 3(O)) or fibrosis (Fig. 3(V); Table 4). The β-carotene-supplemented high-carbohydrate, high-fat diet-fed rats displayed normalised portal inflammation (Fig. 3(N)) and fibrosis (Fig. 3(P); Table 4) but showed obvious hepatocyte ballooning and increased fat vacuole size and number (Fig. 3(F)). No major hepatic changes were observed in purple carrot juice- and β-carotene-supplemented maize starch diet-fed rats (Fig. 3(C), (K) and (R) and 3(B), (J) and (Q); Table 4).

Table 2. Changes in dietary intakes, body anthropometrics, blood and plasma biochemistry

(Mean values with their standard errors for ten animals per group)

	M		MC		MP		H (8 weeks)		H (16 weeks)		HC		HP	
Parameter	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Dietary intakes														
Feed (g/d)	$33{\cdot}0^{j,k}$	2·9	$34{\cdot}4^{l}$	1·5	$29{\cdot}0^{g,h,i,j}$	2·6	$21{\cdot}0^{c,d,e}$	2·0	$22{\cdot}4^{b,d,f,g}$	2·0	$23{\cdot}5^{a,e,f}$	0·6	$19{\cdot}6^{a,b,c,h}$	2·1
Water (ml/d)	$25{\cdot}5^{k,l}$	2·3	$30{\cdot}7^{l}$	0·8	$22{\cdot}9^{d,e,i,j,k}$	2·6	$18{\cdot}0^{c,e,f,g}$	2·0	$18{\cdot}4^{b,f,h,i}$	2·0	$17{\cdot}3^{a,b,c,d}$	0·4	$19{\cdot}1^{a,g,h,j}$	1·9
Total energy (kJ/d)	$375{\cdot}0^{a,b}$	15	$396{\cdot}1^{b,c}$	17·4	$343{\cdot}0^{a}$	16·0	$459{\cdot}0^{d}$	10·0	$454{\cdot}0^{d}$	6·0	$494{\cdot}8^{d}$	12·4	$431{\cdot}0^{c,d}$	15·0
Total anthocyanins (mg/kg)	$0{\cdot}0^{a}$	0·0	$0{\cdot}0^{a}$	0·0	24·4	1·0	$0{\cdot}0^{a}$	0·0	$0{\cdot}0^{a}$	0·0	$0{\cdot}0^{a}$	0·0	15·2	0·4
Total β-carotene (mg/kg)	$0{\cdot}0^{a}$	0·0	30·8	1·1	$0{\cdot}0027^{a}$	0·0001	$0{\cdot}0^{a}$	0·0	$0{\cdot}0^{a}$	0·0	22·1	0·6	$0{\cdot}0017^{a}$	0·00 002
Total lutein (mg/kg)	$0{\cdot}0^{a}$	0·0	$0{\cdot}0^{a}$	0·0	0·002	0·0001	$0{\cdot}0^{a}$	0·0	$0{\cdot}0^{a}$	0·0	$0{\cdot}0^{a}$	0·0	0·0012	0·00 002
Total zeaxanthin (mg/kg)	$0{\cdot}0^{a}$	0·0	$0{\cdot}0^{a}$	0·0	0·0164	0·0001	$0{\cdot}0^{a}$	0·0	$0{\cdot}0^{a}$	0·0	$0{\cdot}0^{a}$	0·0	0·0104	0·0002
Body anthropometrics														
Body-weight gain (%)*	$10{\cdot}8^{b,e,f}$	0·6	$10{\cdot}7^{c,d,e}$	1	5·4	0·9	30·0	0·8	$14{\cdot}3^{g}$	0·9	$12{\cdot}8^{a,d,f,g}$	0·7	$10{\cdot}6^{a,b,c}$	0·6
Abdominal circumference (cm)	$18{\cdot}4^{b,c,d}$	0·3	$17{\cdot}9^{a,b}$	0·2	$19{\cdot}3^{c,e}$	0·3	$18{\cdot}7^{a,d,e}$	0·4	22·9	0·3	21·1	0·2	20·2	0·4
Abdominal fat pads (mg/mm)	$401^{b,c,d}$	56	$211{\cdot}7^{a}$	19·4	264^{a}	18	$421^{d,e,f}$	2·6	781·0	48·0	$521{\cdot}6^{b,e,g}$	29·4	$452^{c,f,g}$	31
Oral glucose tolerance (AUC) (mmol/l × min)														
Week 0	$769{\cdot}0^{a}$	17·4	$763{\cdot}8^{a}$	15·4	$771{\cdot}5^{a}$	13·5	–	–	$738{\cdot}7^{a}$	25·5	$755{\cdot}4^{a}$	9·4	$761{\cdot}9^{a}$	7·0
Week 8	$682{\cdot}4^{a}$	18·7	$685{\cdot}9^{a}$	18·9	$683{\cdot}6^{a}$	20·0	–	–	$831{\cdot}4^{b}$	17·1	$826{\cdot}6^{b}$	24·0	$835{\cdot}6^{b}$	22·7
Week 16	$688{\cdot}7^{c,f,g}$	24·9	$651{\cdot}7^{a,e,f}$	20·1	$588{\cdot}1^{a,b}$	12·2	–	–	875·1	19·9	$625{\cdot}1^{b,c,d,f}$	24·5	$679{\cdot}6^{d,g}$	20·7
Plasma lipids (mmol/l)														
Total cholesterol	$1{\cdot}6^{b}$	0·07	$1{\cdot}6^{a,b,c}$	0·1	$1{\cdot}5^{a,b,c}$	0·05	$2{\cdot}0^{a,b,c}$	0·2	$2{\cdot}1^{c}$	0·2	$1{\cdot}9^{a,b,c}$	0·1	$1{\cdot}6^{a,b}$	0·06
TAG	$0{\cdot}5^{d,e,f,g}$	0·06	$0{\cdot}5^{c,g,h,i,j}$	0·05	$0{\cdot}3^{a,b,c,d}$	0·02	$0{\cdot}5^{b,f,j,k}$	0·2	$1{\cdot}2^{h}$	0·1	1·9	0·4	$0{\cdot}5^{a,e,i,k}$	0·1
NEFA	$0{\cdot}9^{b,c,d}$	0·1	$2{\cdot}4^{f}$	0·3	$0{\cdot}9^{a,d,e}$	0·1	$0{\cdot}7^{a,b}$	0·01	$2{\cdot}1^{f}$	0·06	4·8	0·7	$1{\cdot}0^{c,e}$	0·1
Plasma oxidative stress markers (μmol/l)														
Malondialdehyde	$27{\cdot}0^{a,b,c}$	0·6	$28{\cdot}2^{c,d,e,f}$	0·4	$28{\cdot}5^{b,f,g,h}$	0·7	$30{\cdot}7^{d,g,i,j,k}$	0·7	$32{\cdot}2^{j,l}$	1·2	$32{\cdot}1^{k,l}$	1·1	$28{\cdot}5^{a,e,h,i}$	1·1
Uric acid	$27{\cdot}2^{a}$	1·6	$53{\cdot}2^{d,h,i,j}$	6·4	$58{\cdot}9^{b,f,i,k}$	5·3	$42{\cdot}5^{b,c,d,e}$	3·1	$58{\cdot}1^{c,g,j,k}$	5·7	$46{\cdot}4^{e,f,g,h}$	2·0	$22{\cdot}7^{a}$	1·5
CRP (μmol/l)	$59{\cdot}3^{c,g,j,l}$	7·4	$49{\cdot}3^{a,d,g,h,i}$	10·6	$46^{b,c,d,e,f}$	7·3	$58{\cdot}1^{e,h,k,l}$	7·4	102·3	4·6	$57{\cdot}5^{f,i,j,k}$	6·2	$23{\cdot}5^{a,b}$	9·2
Lactate dehydrogenase (U/l)	$201{\cdot}0^{a,b,c,d}$	28·1	$277{\cdot}8^{b,f,j,k,l,m}$	35·1	$208{\cdot}9^{d,e,f,g}$	18·5	$279{\cdot}8^{a,e,i,m,n,o}$	23·2	$361{\cdot}8^{k,n,p}$	37·9	$329{\cdot}6^{h,l,o,p}$	39·1	$221{\cdot}6^{c,g,h,i,j}$	19·1
Urea (mmol/l)	$7{\cdot}3^{d,f}$	0·5	$4{\cdot}8^{a,b}$	0·4	$6{\cdot}6^{e,f}$	0·6	$5{\cdot}7^{b,c}$	0·2	$3{\cdot}8^{a}$	0·4	2·4	0·4	$6{\cdot}4^{c,d,e}$	0·1

M, maize starch diet; MC, maize starch + β-carotene diet; MP, maize starch + purple carrot juice diet; H, high-carbohydrate, high-fat diet; HC, high-carbohydrate, high-fat + β-carotene diet; HP, high-carbohydrate, high-fat + purple carrot juice diet; AUC, area under the curve; CRP, C-reactive protein.

[a–q] Mean values within a row with unlike superscript letters were significantly different ($P<0{\cdot}05$).

*Body-weight gain calculated as percentage of body weight increase from 8 weeks to 16 weeks for all groups and week 1 to week 8 for the high-carbohydrate, high-fat diet-fed rats (8 weeks).

Table 3. Changes in cardiovascular structure and function

(Mean values with their standard errors for ten animals per group)

	M		MC		MP		H (8 weeks)		H (16 weeks)		HC		HP	
Parameter	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
LVIDd (mm)	6·1^{a,b,c,j}	0·2	6·4^{c,d,e,f,g}	0·4	7·0^{f,i,j,k,l,m}	0·2	7·9^{k,n,p}	0·3	7·4^{d,l,o,p}	0·2	6·5^{b,g,h,i}	0·3	7·1^{a,e,h,m,n,o}	0·3
LVIDs (mm)	0·4^{b,c,d}	0·01	0·3^{a}	0·01	0·3^{a}	0·01	0·5	0·01	0·4^{d,e}	0·01	0·4^{c,e}	0·01	0·4^{b,e}	0·01
LVPWd (mm)	1·8^{a}	0·1	1·7^{a}	0·08	1·5^{a}	0·0	1·6^{a}	0·0	1·8^{a}	0·1	1·9^{a}	0·1	1·6^{a}	0·1
Relative wall thickness	0·4^{a,b,c,d}	0·02	0·5^{k,l}	0·06	0·4^{d,e,f,g}	0·01	0·4^{c,g,h,i}	0·01	0·6^{l}	0·02	0·5^{a,e,h,j,k}	0·02	0·4^{b,f,i,j}	0·02
Fractional shortening (%)	51·1^{a,b,c}	2·6	54·7^{b}	4·0	50·7^{a,b,c}	3·4	43·8^{a,b,c}	2·2	40·2^{c}	1·1	52·7^{b}	2·6	43·6^{a,b,c}	3·4
Ejection fraction (%)	88·0^{e,h,l,m}	2·0	90·5^{k,m,n}	2·2	85·3^{a,f,i,j,k,l}	1·8	81·6^{c,g,h,i}	2·2	79·1^{a,c,d}	1·6	90·7^{i,n}	1·6	80·4^{d,e,f,g}	3·4
Ejection time (ms)	84·7^{a}	1·7	95·5^{a}	3·0	99·5^{a}	4·0	97·7^{a}	5·4	93·0^{a}	1·7	86·2^{a}	2·7	92·0^{a}	4·4
Deceleration time (ms)	56·0^{a,b,c}	4·7	62^{b}	3·7	72·2^{a,b,c}	4·9	47·0^{c}	4·5	59·7^{a,b,c}	3·8	56·0^{a,b,c}	4·5	57·0^{a,b,c}	6·1
E_M (m/s)	0·7^{b,c,d,e,f}	0·04	0·7^{a,f,g,h,i}	0·05	0·9^{j,m,n}	0·05	0·6^{a,b}	0·03	0·8^{e,g,j,k,l}	0·01	0·8^{d,h,k,m,o}	0·02	0·8^{c,i,l,n,o}	0·05
A_M (m/s)	0·4^{a}	0·02	0·4^{a}	0·04	0·5^{a}	0·04	0·4^{a}	0·02	0·5^{a}	0·03	0·5^{a}	0·04	0·5^{a}	0·01
MCMO (ms)	131·3^{b}	7·3	117·6^{a,b,c}	7·4	121·0^{a,b,c}	7·0	119·0^{a,b,c}	6·9	105·4^{c}	4·1	105·2^{c}	3·0	118·8^{a,b,c}	3·5
Estimated left ventricle mass (g)	0·7^{a,d,e,f,g}	0·03	0·6^{a,b,c}	0·01	0·7^{b,g,h,i,j}	0·02	0·8^{d,h,k,m,n}	0·03	0·9^{m,o}	0·06	0·8^{e,i,l,n,o}	0·05	0·7^{c,f,j,k,l}	0·03
Left ventricle + septum wet weight (mg/mm)	19·4^{a,b,c}	0·8	20·8^{a,b,c}	0·5	19·3^{a,b,c}	0·6	22·5^{b}	1·5	22·3^{b}	1·3	20·7^{a,b,c}	0·8	17·2^{c}	0·7
Right ventricle wet weight (mg/mm)	3·7^{a}	0·5	4·5^{a}	0·1	3·7^{a}	0·2	3·4^{a}	0·1	3·9^{a}	0·3	3·6^{a}	0·2	3·6^{a}	0·4
Left ventricle fibrosis (% surface area)*	3·3^{a}	0·7	3·8^{a}	0·6	4·1^{a}	0·9	5·6^{a}	0·4	7·8	0·6	4·6^{a}	0·5	4·2^{a}	0·7
Diastolic stiffness (κ)	20·5^{a,b,c}	0·4	24·6^{a,d,f,h,i}	0·8	20·9^{b,d,e}	1·2	26·3^{g,h,j}	2·2	28·7^{i,j,k}	0·8	30·6^{k}	1·1	22·4^{c,e,f,g}	1·3
Systolic blood pressure (mmHg)	125·1^{a,e,f,g}	4·3	123·9^{a,b,c,d}	1·0	126·8^{b,e,h,i}	1·3	137·0^{k}	1·2	145·0	4·5	129·9^{c,f,h,j,k}	0·6	127·3^{d,g,i,j}	1·1

M, maize starch diet; MC, maize starch + β-carotene diet; MP, maize starch + purple carrot juice diet; H, high-carbohydrate, high-fat diet; HC, high-carbohydrate, high-fat + β-carotene diet; HP, high-carbohydrate, high-fat + purple carrot juice diet; LVIDd, left ventricular internal diameter in diastole; LVIDs, left ventricular internal diameter in systole; LVPWd, left ventricular posterior wall thickness in diastole; E_M, early mitral inflow velocity; A_M, late mitral inflow velocity; MCMO, mitral closing-mitral opening time.

[a–p] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

*Four animals per group.

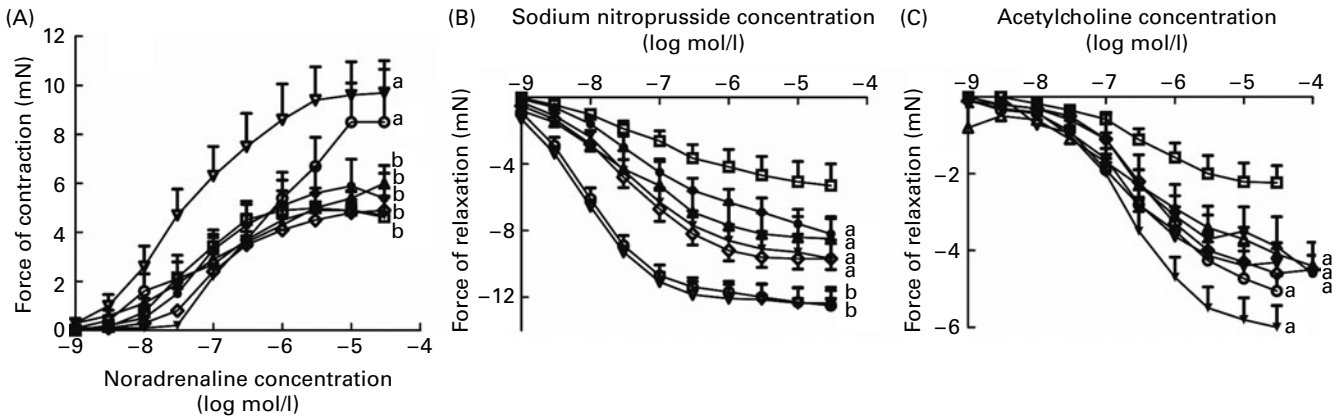

Fig. 1. Cumulative concentration–response curves for noradrenaline (A), sodium nitroprusside (B) and acetylcholine (C) in thoracic aortic rings from maize starch diet (○), maize starch diet + β-carotene (△), maize starch diet + purple carrot juice (◇), high-carbohydrate, high-fat diet for 8 weeks (▽), high-carbohydrate, high-fat diet for 16 weeks (□), high-carbohydrate, high-fat diet + β-carotene (●) and high-carbohydrate, high-fat diet + purple carrot juice (▼)-fed rats. Values are means for eight to ten rats per group, with standard errors represented by vertical bars. [a,b] Mean values with an unlike letter were significantly different ($P<0·05$).

Discussion

A combination of high carbohydrate and high fat in the diet was used in the present study to mimic human diets that have been associated with the development of the metabolic syndrome. This diet produced changes in metabolic, cardiovascular and hepatic structure and functions such as excessive abdominal fat deposition, impaired glucose tolerance, elevated blood pressure and increased plasma lipid concentrations together with increased plasma oxidative stress markers and liver enzymes compared with maize starch diet-fed rats. Further, histological evaluation of heart and liver showed increased infiltration of inflammatory cells and increased collagen deposition in addition to increased fat vacuoles in the liver. This was accompanied by increased left ventricular stiffness and decreased aortic reactivity.

The present study compares therapeutic responses from anthocyanin-rich purple carrot juice with responses to β-carotene in high-carbohydrate, high-fat diet-fed rats. Since carotenoids are sparsely soluble in water, unlike anthocyanins, the purple carrot juice was very low in carotenoids but high in total anthocyanins. The present results suggest that many responses to purple carrot juice differ from those following β-carotene supplementation. β-Carotene and purple carrot juice produced different responses on blood lipid profiles, lipid deposition in the liver, plasma alkaline phosphatase and lactate dehydrogenase activities, left ventricular stiffness and plasma markers of oxidative stress such as uric acid and malondialdehyde. The responses to β-carotene may be produced by vitamin A and its major metabolites, retinoic acid and retinaldehyde. Rats fed with vitamin A and retinoic acid showed a marked increase in hepatic and plasma concentrations of glycogen, cholesterol and acylglycerols with elevated plasma NEFA and increased glucose incorporation into liver lipids[41,42]. Vitamin A stimulated fatty acid mobilisation from adipose tissue and enhanced formation of glycerophosphate through glycolysis, with consequent increases in the acylglycerol synthesis in the liver[41]. Additionally, both retinoic acid and retinaldehyde inhibited adipogenesis[43]. Here we show that β-carotene prevented further increases in abdominal fat weight after treatment. It is therefore likely that β-carotene and its major metabolites suppress adipogenesis, thereby allowing excess accumulation of lipids in the liver and the plasma.

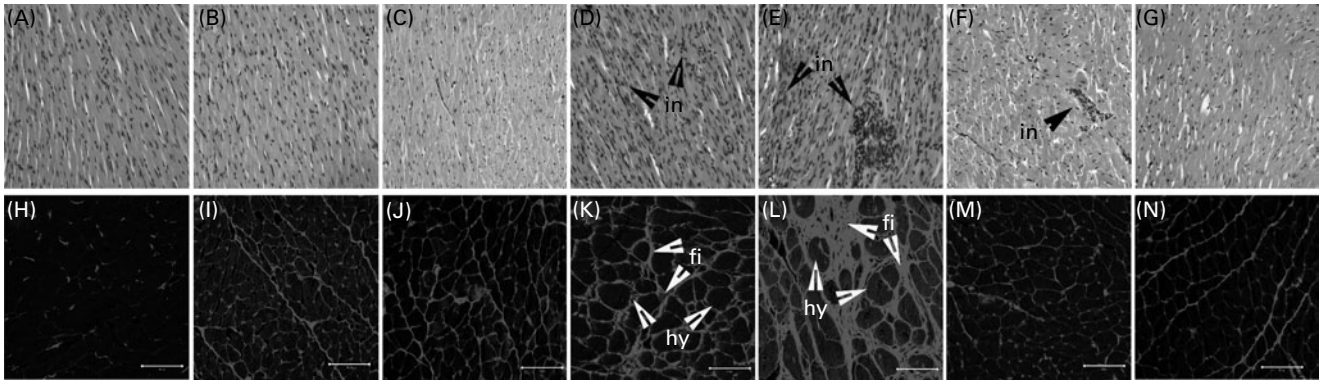

Fig. 2. Haematoxylin and eosin staining of left ventricle (× 20) showing inflammatory cells (marked as 'in') as dark spots outside the myocytes in maize starch diet (A), maize starch diet + β-carotene (B), maize starch diet + purple carrot juice (C), high-carbohydrate, high-fat diet for 8 weeks (D), high-carbohydrate, high-fat diet for 16 weeks (E), high-carbohydrate, high-fat diet + β-carotene (F) and high-carbohydrate, high-fat diet + purple carrot juice (G)-fed rats. Picrosirius red staining of left ventricular interstitial collagen deposition (40 ×) in maize starch diet (H), maize starch diet + β-carotene (I), maize starch diet + purple carrot juice (J), high-carbohydrate, high-fat diet for 8 weeks (K), high-carbohydrate, high-fat diet for 16 weeks (L), high-carbohydrate, high-fat diet + β-carotene (M) and high-carbohydrate, high-fat diet + purple carrot juice (N)-fed rats; fibrosis is marked as 'fi' and hypertrophied cardiomyocytes are marked as 'hy'.

British Journal of Nutrition

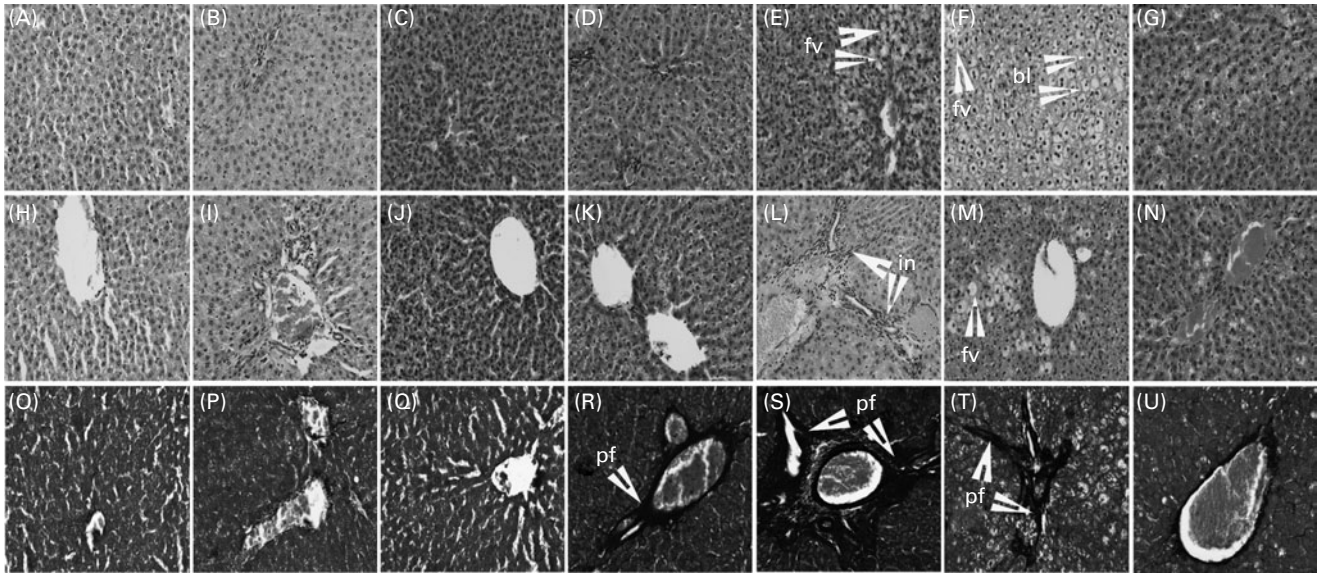

Fig. 3. Haematoxylin and eosin staining of hepatocytes (× 40) showing hepatocytes with enlarged fat vacuoles (marked as 'fv') and inflammatory cells (marked as 'in') (20 ×) from maize starch diet (A, H), maize starch diet + β-carotene (B, I), maize starch diet + purple carrot juice (C, J), high-carbohydrate, high-fat diet for 8 weeks (D, K), high-carbohydrate, high-fat diet for 16 weeks (E, L), high-carbohydrate, high-fat diet + β-carotene (F, M) and high-carbohydrate, high-fat diet + purple carrot juice (G, N)-fed rats. Milligan's trichrome staining of the hepatic portal regions showing collagen (marked as 'pf') (20 ×) in maize starch diet (O), maize starch diet + β-carotene (P), maize starch diet + purple carrot juice (Q), high-carbohydrate, high-fat diet for 8 weeks (R), high-carbohydrate, high-fat diet for 16 weeks (S), high-carbohydrate, high-fat diet + β-carotene (T) and high-carbohydrate, high-fat diet + purple carrot juice (U)-fed rats. bl, Hepatocyte ballooning.

In the heart, retinoic acid inhibited G protein-coupled receptor-mediated hypertrophy as well as angiotensin II-induced increases in intracellular Ca^{2+} in neonatal cardiomyocytes[44]. *In vivo*, retinoic acid suppressed myocardial cell hypertrophy[44] and prevented ventricular fibrosis and remodelling in spontaneously hypertensive rats[45]. β-Carotene and carotenoid breakdown products induced apoptosis in Jurkat E6.1 malignant T-lymphoblast cells[46] and human neutrophils[47] but impaired mitochondrial respiration and elevated accumulation of malondialdehyde[48]. Thus, β-carotene as a precursor of vitamin A and retinoic acid may increase lipid peroxidation, decrease cardiac hypertrophy, and prevent cardiac fibrosis and inflammation.

In contrast, purple carrot juice supplementation normalised plasma TAG, total cholesterol and NEFA concentrations, consistent with the hypolipidaemic responses to other anthocyanin-rich plant extracts such as blueberry leaf[29]. NEFA is involved both in insulin resistance and hypertension by inhibiting aortic endothelial NO synthase activity through an oxidative mechanism[49,50]. The present results demonstrated enhanced vascular relaxant responses, consistent with improved NO production and lowered blood pressure.

Table 4. Changes in hepatic structure and function

(Mean values with their standard errors for ten animals per group)

	M		MC		MP		H (8 weeks)		H (16 weeks)		HC		HP	
Parameter	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Liver wet weight (mg/mm)	$303{\cdot}3^{b,g,i}$	9·8	$222{\cdot}1^{a}$	7·0	$231{\cdot}2^{a}$	13·0	$302{\cdot}8^{c,f,h,i}$	12·9	$328{\cdot}5^{e,h}$	10·0	$297{\cdot}0^{d,e,f,g}$	8·9	$273{\cdot}7^{b,c,d}$	13·0
Liver fibrosis (% surface area)*	$0{\cdot}9^{a,b,c,d}$	0·02	$1{\cdot}1^{a,e,h,i}$	0·1	$1{\cdot}0^{b,e,f,g}$	0·1	$3{\cdot}6^{k}$	0·8	5·7	0·5	$2{\cdot}4^{c,f,h,j,k}$	0·3	$1{\cdot}8^{d,g,i,j}$	0·7
ALT (U/l)	$32{\cdot}9^{d,g,h,i}$	2·0	$59{\cdot}8^{k}$	7·0	$31{\cdot}0^{a,b,c,d}$	1·9	$36{\cdot}1^{c,f,h,j}$	2·2	$55{\cdot}3^{k}$	3·7	$31{\cdot}0^{a,e,f,g}$	3·6	$41{\cdot}2^{b,e,i,j}$	3·4
AST (U/l)	$71{\cdot}0^{a,h,g,i}$	4·0	$118{\cdot}1^{k}$	14·2	$70{\cdot}8^{a,b,c,d}$	5·1	$77^{b,f,i,j,k}$	2·5	101·7	5·4	$65{\cdot}2^{c,e,f,h}$	3·0	$75{\cdot}3^{d,e,g,j}$	3·8
ALP (U/l)	$154{\cdot}7^{a,d,e}$	2·0	$167{\cdot}6^{c,e}$	6·5	$133{\cdot}6^{a,b}$	7·1	$192{\cdot}4^{f}$	5·2	$208{\cdot}4^{f,g}$	0·6	$224{\cdot}8^{g}$	14·3	$153{\cdot}5^{b,c,d}$	6·2
Albumin (g/l)	$28{\cdot}5^{a,b}$	0·6	$27{\cdot}7^{a}$	0·4	$29{\cdot}1^{a,b}$	0·6	$28{\cdot}0^{a}$	0·6	$28{\cdot}0^{a}$	0·2	$27{\cdot}4^{a}$	0·2	$29{\cdot}9^{b}$	0·5
Globulin (g/l)	$29{\cdot}6^{a,b}$	1·0	$26{\cdot}2^{b}$	0·8	$29{\cdot}4^{a,b}$	0·8	$28{\cdot}2^{a,b}$	0·8	$28{\cdot}7^{a,b}$	0·7	$27{\cdot}6^{a,b}$	0·7	$29{\cdot}9^{a}$	1·0
Total bilirubin (μmol/l)	$2{\cdot}2^{a,b}$	0·1	$3{\cdot}0^{a}$	0·6	$1{\cdot}5^{b}$	0·07	$2{\cdot}2^{a,b}$	0·07	$2{\cdot}3^{a,b}$	0·05	$2{\cdot}1^{a,b}$	0·2	$2{\cdot}1^{a,b}$	0·1

M, maize starch diet; MC, maize starch + β-carotene diet; MP, maize starch + purple carrot juice diet; H, high-carbohydrate, high-fat diet; HC, high-carbohydrate, high-fat + β-carotene diet; HP, high-carbohydrate, high-fat + purple carrot juice diet; ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase.

$^{a-k}$ Mean values within a row with unlike superscript letters were significantly different ($P<0{\cdot}05$).

* Four animals per group.

British Journal of Nutrition

Left ventricular dysfunction has been correlated with metabolic disturbances, oxidative stress and the appearance of inflammatory cells preceding left ventricular fibrosis and left ventricular stiffness[51]. Purple carrot juice supplementation reduced left ventricular stiffness, the number of inflammatory cells and left ventricular collagen deposition. Echocardiographic assessment showed improved systolic function and decreased left ventricular dimensions following purple carrot treatment. Along with other results, reduction in lactate dehydrogenase activity and improved N homeostasis with purple carrot juice supplementation could be indicative of normalised metabolism leading to cardioprotective effects. Our data also showed that uric acid concentrations decreased in the purple carrot juice-treated rats. Uric acid, the endproduct of purine metabolism and a major endogenous water-soluble antioxidant[52], is increased in fructose-fed rats following increased production of reactive oxygen species[33]. Additionally, the reduction in plasma malondialdehyde concentrations, a major product of PUFA peroxidation, suggests *in vivo* antioxidant activity of purple carrot juice.

In contrast to β-carotene, purple carrot juice supplementation reduced liver wet weight, portal inflammation, fat deposition and portal fibrosis in the liver and showed total restoration of liver function. We have shown that purple carrot juice reversed the pathological features of NAFLD in the high-carbohydrate, high-fat diet-fed rats. Increased activities of the serum enzymes including aspartate transaminase, alanine transaminase and alkaline phosphatase reflect active liver damage. Inflammatory hepatocellular disorders result in extremely elevated transaminase levels[53]. Thus, purple carrot juice treatment has a significant role in the decrease or reversal of liver dysfunction since the activity of these plasma enzymes was lower than in high-carbohydrate-high-fat diet-fed rats and similar to maize starch diet-fed rats. Both β-carotene and purple carrot juice reduced plasma CRP concentrations, suggesting their possible *in vivo* anti-inflammatory effects.

The present study demonstrates for the first time that treatment of the metabolic syndrome induced by diet in rats with purple carrot juice attenuates or reverses the changes in cardiovascular and liver structure and functions as well as in metabolic parameters, especially abdominal fat deposition and plasma lipid profiles. As the juice itself contained low concentrations of carotenoids, it is likely that the anthocyanins are responsible for the antioxidant and anti-inflammatory properties of purple carrot juice. Furthermore, β-carotene alone produces limited and sometimes contradictory responses compared with purple carrot juice in this rat model of the metabolic syndrome.

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H. P. and L. B. developed the original study aims; H. P. and S. P. conducted the experiments and carried out the data analyses; data were interpreted by all authors. The preparation of the manuscript drafts has involved all authors and L. B. has been the corresponding author throughout the writing process.

There are no conflicts of interest.

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Appendix B

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Lipid redistribution by α-linolenic acid-rich chia seed inhibits stearoyl-CoA desaturase-1 and induces cardiac and hepatic protection in diet-induced obese rats

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Abstract

Chia seeds contain the essential fatty acid, α-linolenic acid (ALA). This study has assessed whether chia seeds attenuated the metabolic, cardiovascular and hepatic signs of a high-carbohydrate, high-fat (H) diet [carbohydrates, 52% (wt/wt); fat, 24% (wt/wt) with 25% (wt/vol) fructose in drinking water] in rats. Diets of the treatment groups were supplemented with 5% chia seeds after 8 weeks on H diet for a further 8 weeks. Compared with the H rats, chia seed-supplemented rats had improved insulin sensitivity and glucose tolerance, reduced visceral adiposity, decreased hepatic steatosis and reduced cardiac and hepatic inflammation and fibrosis without changes in plasma lipids or blood pressure. Chia seeds induced lipid redistribution with lipid trafficking away from the visceral fat and liver with an increased accumulation in the heart. The stearoyl-CoA desaturase-1 products were depleted in the heart, liver and the adipose tissue of chia seed-supplemented rats together with an increase in the substrate concentrations. The C18:1*trans*-7 was preferentially stored in the adipose tissue; the relatively inert C18:1n-9 was stored in sensitive organs such as liver and heart and C18:2n-6, the parent fatty acid of the n-6 pathway, was preferentially metabolized. Thus, chia seeds as a source of ALA induce lipid redistribution associated with cardioprotection and hepatoprotection.

Keywords: Chia seed; α-Linolenic acid; Metabolic syndrome; High-carbohydrate, high-fat diet

1. Introduction

All three nutritionally important n-3 fatty acids, α-linolenic acid (ALA; C18:3n-3), eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3), reduce one or more risk factors of cardiovascular disease as shown by epidemiological, human, animal and cell culture studies [1–5]. In human diets, ALA, the essential n-3 fatty acid, is usually derived from plant sources such as flax seed, while EPA and DHA are ingested from fish, fish oil supplements and other sea foods [1]. The richest botanical source of ALA is the oil from chia seeds containing about 60% (wt/vol) [6–10]. Chia seed (*Salvia hispanica* L.) was a major dietary component in the Mayan and Aztec populations [6]. In sucrose-fed rats, dietary chia seed prevented the onset of dyslipidemia and insulin resistance and reduced visceral adiposity without affecting glucose homeostasis [10]. Rats fed a 16% chia seed diet showed increased plasma ALA, EPA and DHA concentrations, decreased serum triglyceride content and increased serum high-density lipoprotein content [9]. In humans receiving conventional therapy for diabetes, chia seed reduced systolic blood pressure and C-reactive protein (CRP) concentrations and increased serum ALA and EPA concentrations without affecting body weight [11]. In another human intervention study, dietary chia seed increased plasma ALA concentrations, but EPA and DHA concentrations, body composition, biomarkers of inflammation and oxidative stress were unchanged [12].

Despite ALA being the primary fatty acid of the pathway, it has generated less scientific interest than EPA or DHA among the n-3 fatty acids possibly due to the inefficient conversion of ALA to EPA and DHA by humans [13]. Epidemiological and clinical evidence suggests

Abbreviations: ALA, α-linolenic acid; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; C, corn starch diet; CC, corn starch+chia seed diet; CK, creatine kinase; CPT-1, carnitine palmitoyltransferase I; CRP, C-reactive protein; DHA, docosahexaenoic acid; DXA, dual-energy X-ray absorptiometry; EPA, eicosapentaenoic acid; H, high–carbohydrate, high-fat diet; HC, high-carbohydrate, high-fat diet+chia seed; LDH, lactate dehydrogenase; LV, left ventricle; LVIDd, left ventricle internal diameter in diastole; LVIDs, left ventricle internal diameter in systole; LVPWd, left ventricle posterior wall thickness in diastole; NEFA, nonesterified fatty acids; SCD, stearoyl-CoA 9-desaturase; SREBP-1, sterol regulatory element binding protein-1.

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that higher consumption of ALA is associated with reduced risk of cardiovascular diseases [14–20]. A meta-analysis of five prospective cohort studies showed that increased ALA consumption reduced heart disease mortality [14]. The Lyon Heart Study concluded that diets rich in ALA were more efficient than presently used diets in the secondary prevention of cardiovascular diseases [15]. The cardiovascular health study [16]; The Nurses' Health study [17,18]; the National Heart, Lung, and Blood Institute Family Heart Study [19] and the Health Professionals Study [20] concluded that higher ALA intake was associated with a reduced risk of coronary heart disease and related mortalities.

Metabolic syndrome is a clustering of interrelated risk factors for cardiovascular disease and diabetes. These factors include hyperglycemia, hypertension, dyslipidemia, central adiposity and nonalcoholic fatty liver disease [21]. We have previously shown that rats fed a high-carbohydrate, high-fat diet mimic many signs of human metabolic syndrome [22,23]. Therefore, to determine the responses of chia seed as a rich source of ALA on the signs of metabolic syndrome, we investigated metabolic changes, cardiovascular and hepatic structure and function, lipid content and fatty acid profiles in the liver, retroperitoneal adipose tissue and the heart of rats fed either corn starch diet, high-carbohydrate, high-fat diet or either diet containing 5% whole chia seed supplement. Liver and adipose tissue were selected for their major role in fatty acid metabolism, while the heart was selected because fatty acids modulate its functions. Corn starch is a slowly digestible carbohydrate [24] and served as a control for the high-carbohydrate, high-fat diet in this study where the primary carbohydrates are fructose and sucrose. Unlike fructose, corn starch does not increase blood glucose, plasma insulin or nonesterified fatty acid (NEFA) concentrations [22,24].

2. Materials and methods

2.1. Animals and diets

The experimental groups consisted of 48 male Wistar rats (8–9 weeks old, 338±4 g) supplied by The University of Queensland Biological Resources unit and individually housed at the University of Southern Queensland Animal House. All experimentation was approved by the Animal Experimentation Ethics Committee of the University of Southern Queensland under the guidelines of the National Health and Medical Research Council of Australia. The rats were randomly divided into four separate groups (n=12 each) fed corn starch (C), corn starch+chia seed (CC), high-carbohydrate, high-fat (H) and high-carbohydrate, high-fat+chia seed (HC). All experimental groups were housed in a temperature-controlled, 12-h light/dark cycle environment with *ad libitum* access to water and the group-specific rat diet. Measurements of body weight and food and water intakes were taken daily to monitor the day-to-day health of the rats. Feed conversion efficiency (%) was calculated as [mean body weight gain [g]/daily energy intake (kJ)]×100 [25].

All group-specific diets were custom prepared in our laboratory. Corn starch diet was prepared by thorough mixing of corn starch, powdered rat feed (meat-free rat and mouse feed; Speciality Feeds, Glen Forrest, WA, Australia), Hubble, Mendel and Wakeman salt mixture (MP Biochemicals, Seven Hills, NSW, Australia) and water, while the corn starch and part of water were replaced with condensed milk, fructose and beef tallow in the high-carbohydrate, high-fat diet (Table 1). The drinking water in all high-carbohydrate, high fat-fed rats was augmented with 25% fructose. Chia seed-supplemented diets were prepared by replacing an equivalent amount of water in the diet with the whole seed (Table 1). The fatty acid composition of all group-specific diets and chia seed is described in Table 1. Five percent of chia seed supplementation was administered for 8 weeks starting 8 weeks after the initiation of the corn starch or high-carbohydrate, high-fat diet.

Table 1
Fatty acid profile and energy density of chia seed, C, CC, H and HC diets

Constituent	Chia seed	Diets			
		C	CC	H	HC
Corn starch, g/kg	–	570.00	570.00	–	–
Powdered rat feed[a], g/kg	–	155.00	155.00	155.00	155.00
HMW salt mixture, g/kg	–	25.00	25.00	25.00	25.00
Fructose, g/kg	–	–	–	175.00	175.00
Beef tallow, g/kg	–	–	–	200.00	200.00
Condensed milk, g/kg	–	–	–	395.00	395.00
Water, ml/kg	–	250.00	200.00	50.00	–
Chia seed, g/kg	–	–	50.00	–	50.00
Macronutrient composition					
Total carbohydrate, g/kg	–	600.25	600.25	515.67	515.67
Total fat, g/kg	–	8.07	8.07	239.04	239.04
Total protein, g/kg	–	31.78	31.78	58.12	58.12
Total fiber, g/kg	–	7.44	7.44	7.44	7.44
Total vitamins, g/kg	–	0.32	0.32	0.32	0.32
Total minerals, g/kg	–	0.13	0.13	0.44	0.44
Ash, g/kg	–	0.63	0.63	0.00	0.00
Total moisture, g/kg	–	326.38	296.38	153.98	123.98
Total calculated energy density, kJ/g	18.6	11.2	12.2	17.9	18.7
Total extractable lipids, g/kg (n=3)	272.6±0.1	6.2±0.1	11.6±0.1	187.0±2.3	193.9±0.4
Fatty acid, g/100g of total fatty acid content (n=3)					
C14:0	2.8±0.4	10.0±0.9	15.6±0.2	3.7±0.1	4.7±0.1
C16:0	7.6±0.2	17.5±0.4	14.5±0.1	24.6±0.5	26.9±0.5
C18	3.4±0.6	0.6±0.0	4.9±0.1	24.2±0.2	21.9±0.4
C18:1n-9	8.7±0.4	34.5±2.3	24.7±0.4	0.9±0.5	3.4±0.6
C18:1*trans*-7	0.0±0.0	0.0±0.0	0.0±0.0	40.8±0.8	34.1±0.2
C18:2n-6	20.5±0.2	30.5±0.8	25.5±0.0	2.7±0.1	3.0±0.1
C18:3n-3	56.1±0.5	4.7±0.1	12.6±0.0	0.1±0.1	2.1±0.4
C20:0	0.3±0.1	0.0±0.0	0.4±0.0	0.2±0.1	0.3±0.1
Total SFA	14.1±0.5	28.3±1.4	35.5±0.3	53.1±0.7	54.9±1.0
Total MUFA	9.3±0.6	35.7±2.3	25.5±0.4	44.1±0.6	40.0±0.7
Total PUFA	76.7±0.8	36.0±0.9	39.1±0.1	2.8±0.0	5.1±0.3

For the dietary fatty acid compositions, each value is a mean±S.E.M. Number of repetitive experiments are indicated within parenthesis. HMW, Hubble, Mendel and Wakeman; SFA, saturated fatty acid; PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid.

[a] Meat-free rat and mouse feed (Speciality Feeds) contains the following (in g/kg of feed): carbohydrates, 707.07; proteins, 194.00; fat, 48.00; fiber, 48.00; total vitamins, 2.08 and total minerals, 0.85.

2.2. Systolic blood pressure

Systolic blood pressure was measured as previously described [22] under light sedation following intraperitoneal (ip) injection of Zoletil (tiletamine 15 mg/kg, zolazepam 15 mg/kg; Virbac, Peakhurst, NSW, Australia). Measurements were taken using an MLT1010 Piezo-Electric Pulse Transducer (ADInstruments, Sydney, Australia) and inflatable tail-cuff connected to an MLT844 Physiological Pressure Transducer (ADInstruments) and PowerLab data acquisition unit (ADInstruments).

2.3. Oral glucose tolerance and insulin tolerance tests

The oral glucose tolerance and the insulin tolerance tests were performed 2 days apart from each other at 16 weeks. For oral glucose tolerance after 10–12 h of overnight food deprivation, basal blood glucose concentrations were measured in blood taken from the tail vein using Medisense Precision Q.I.D glucose meter (Abbott Laboratories, Bedford, MA, USA). Fructose-supplemented drinking water in the H and HC groups was replaced with normal water for the overnight food deprivation period. The rats were given 2 g/kg body weight of glucose as a 40% solution via oral gavage. Tail vein blood samples were taken at 30, 60, 90 and 120 min following glucose administration.

For insulin tolerance, basal blood glucose concentrations were measured after 4–5 h of food deprivation as above. The rats were injected ip 0.75 IU/kg insulin-R (Eli Lilly Australia, West Ryde, NSW, Australia), and tail vein blood samples were taken at 0, 30, 60, 90 and 120 min. Rats were withdrawn from the test if the blood glucose dropped below 1.1 mmol/L, and 4 g/kg glucose was immediately administered by oral gavage to prevent hypoglycemia.

2.4. Echocardiography

Echocardiographic examination (Phillips iE33, 12MHz transducer) was performed at 16 weeks as previously described [22,26]. Measurements were taken in accordance with the guidelines of the American Society of Echocardiography using the leading-edge method [27]. Briefly, rats were anesthetized using intraperitoneal Zoletil (tiletamine 15 mg/kg and zolazepam 15 mg/kg ip; Virbac) and Ilium Xylazil (xylazine 15 mg/kg ip; Troy Laboratories, Smithfield, NSW, Australia) and positioned in dorsal recumbency. Electrodes attached to the skin overlying the elbows and right stifle facilitated the simultaneous recording of a lead II electrocardiogram. A short-axis view of the left ventricle (LV) at the level of the papillary muscles was obtained and used for direct acquisition of M-mode images of the LV for measurement of diastolic posterior wall thickness (LVPWd), LV internal systolic dimension and LV end-diastolic dimension (LVIDd).

2.5. Body composition measurements

Dual-energy X-ray absorptiometric (DXA) measurements were performed on the rats after 8 and 16 weeks of feeding, 2 days before rats were killed for pathophysiological assessments, using a Norland XR36 DXA instrument (Norland Corp., Fort Atkinson, WI, USA). DXA scans were analyzed using the manufacturer's recommended software for use in laboratory animals (Small Subject Analysis Software, version 2.5.3/1.3.1; Norland Corp) as previously described [28]. The precision error of lean mass for replicate measurements, with repositioning, was 3.2%. Visceral adiposity index (%) was calculated as: ([retroperitoneal fat (g)+omental fat (g)+epididymal fat (g)]/[body weight (g)])×100 and expressed as adiposity percent [29].

2.6. Isolated heart preparation

The left ventricular function of the rats in all treatment groups was assessed using the Langendorff heart preparation. Terminal anesthesia was induced via ip injection of pentobarbitone sodium (Lethabarb, 100 mg/kgip). After heparin (Sigma-Aldrich Australia, Sydney, Australia) administration (200 IU) through the right femoral vein, blood (~5 ml) was taken from the abdominal aorta. Isovolumetric ventricular function was measured by inserting a latex balloon catheter into the LV of the isolated heart connected to a Capto SP844 MLT844 physiological pressure transducer and Chart software on a Maclab system (ADInstruments Australia and Pacific Islands, Bella Vista, NSW, Australia). All left ventricular end-diastolic pressure values were measured while pacing the heart at 250 beats/min using an electrical stimulator. End-diastolic pressures were obtained starting from 0 up to 30 mmHg. Diastolic stiffness constant (κ, dimensionless) was calculated as in previous studies [22,30]. $+dP/dt$ and $-dP/dt$ were calculated as the mean rate of contraction and relaxation, respectively, of at least 50 beats with the heart paced at 250 beats/min, and the end-diastolic pressure was maintained at approximately 10 mmHg.

2.7. Aortic contractility

Thoracic aortic rings (4 mm in length) were suspended in an organ bath chamber with a resting tension of approximately 10 mN. Cumulative concentration–response (contraction) curves were measured for noradrenaline (Sigma-Aldrich Australia); concentration–response (relaxation) curves were measured for acetylcholine (Sigma-Aldrich Australia) and sodium nitroprusside (Sigma-Aldrich Australia) in the presence of a submaximal (70%) contraction to noradrenaline [22].

2.8. Organ weights

The right and left ventricles were separated after perfusion experiments and weighed. Liver, retroperitoneal, epididymal and omental fat were removed following heart removal and blotted dry for weighing. Organ weights were normalized relative to the tibial length at the time of their removal (in mg/mm). Immediately after weighing, the LV, liver and retroperitoneal fat were stored at −20°C in a 50-ml polypropylene centrifuge tube for fatty acid analysis.

2.9. Fatty acid analysis

The extraction of tissue and dietary lipids was undertaken by manual solvent extraction using a 2:1 chloroform/methanol mixture with 0.1% butylated hydroxytoluene as an antioxidant. Approximately 1–5 g of retroperitoneal fat, heart and liver and 2 ml plasma were macerated into a 50-ml polypropylene centrifuge tube and were mixed on a rotating device for 40 min with 20 ml of chloroform/methanol solvent and then centrifuged at 2500 rpm for 5 min. The extraction procedure was repeated twice, combining all of the extracting solvent and subsequently washing with ddH_2O to remove all polar material. Chloroform was evaporated under a stream of nitrogen on a hot plate set at 60°C until the beakers reached constant weight. The beaker weights were recorded for the calculation of gravimetric extractable lipid content [31].

Approximately 15–20 mg of extracted lipid samples with 1 mg of heptadecanoic acid (C17) added as an internal standard was methylated in a clean 10-ml test tube. Saponified lipids were extracted with 2 ml heptane and then transferred into an autosampler vial for gas chromatography. Fatty acid methyl esters were analyzed on an Agilent J&W DB-23 column (60 m×0.25 mm×0.25 μm) (Agilent Technologies, Santa Clara, CA, USA) by a Shimadzu GC-17A equipped with a flame ionization detector. The injection and the detector temperatures were set at 250°C and 285°C, respectively. The column temperature was set at 100°C for 2 min, raised at 10°C/min to 180°C, held for 5 min, raised at 5°C/min to 240°C and held for 25 min. Carrier gas (helium) was passed at 110 kPa, with constant linear velocity of 15cm/s. A sample of 1 μL was injected with a split ratio of 25. A multi-acid standard mixture was used for checking the performance of the gas chromatography and as a recovery test for the sample preparation procedure. Quantitation of the fatty acids in all samples was based on a linear calibration equation obtained from the C17 standards. For identification purposes, a 28-fatty-acid methyl ester mixture standard (Nu-Check Prep. Inc, Elysian, MN, USA) was used for retention time (RT) calibration. A plot of carbon number versus log RT for the saturated series, 1° of unsaturation and 2° of unsaturation allowed a relationship to be developed for identification purposes.

All fatty acids were expressed as grams per 100 g of total recovered fatty acids. The n-3:n-6 ratio was derived using the following formula:

$$\text{n3:n6} = [\text{C18:3n-3} + \text{C20:3n-3} + \text{C20:5n-3} + \text{C22:5n-3} + \text{C22:6n-3}] / [\text{C18:2n-6} + \text{C18:3n-6} + \text{C20:2n-6} + \text{C20:3n-6} + \text{C20:4n-6} + \text{C22:2n-6} + \text{C22:4}]\text{n-6}$$

As the diets were rich in C18:1n-9, the stearoyl-CoA 9-desaturase (SCD) index was calculated as the ratio between C16:1n-7 and C16:0, and the ratio between C18:1n-9 and C18:0 was not considered.

2.10. Histology

Two rats per group were taken exclusively for histological analysis. Two slides were prepared per tissue specimen and two random, nonoverlapping fields per slide were taken to avoid biased analysis. Organs were also collected from rats used for perfusion studies. Immediately after removal, heart and liver tissues were fixed in 10% buffered formalin for 3 days and then dehydrated and embedded in paraffin wax as previously described [22]. Thin sections (7 μm) of LV and the liver were cut and stained with hematoxylin and eosin stain for determination of inflammatory cell infiltration. Liver sections were also stained with Milligan's trichrome stain to determine fibrosis. Collagen distribution was measured in the LV with picrosirius red stain. Laser confocal microscopy (Zeiss LSM 510 upright Confocal Microscope) with color intensity quantitatively analyzed using NIH-imageJ software (National Institute of Health, USA) was used to determine the extent of collagen deposition in selected tissue sections [22].

2.11. Plasma biochemistry

Briefly, blood was centrifuged at 5000×*g* for 15 min within 30 min of collection into heparinized tubes. Plasma was separated and transferred to Eppendorf tubes for storage at −20°C before analysis. Activities of plasma enzymes and analyte concentrations were determined using kits and controls supplied by Olympus using an Olympus analyzer (AU 400, Tokyo, Japan) as previously described [22]. Plasma CRP was estimated using a commercial kit (Kamiya Biomedical, Thousand Oaks, CA, USA) according to the manufacturer-provided standards and protocols using a Cobas-Mira automated analyser.

2.12. Statistical analysis

All data are presented as mean±S.E.M. 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. Data from C, CC, H and HC groups were tested by two-way analysis of variance. When interaction and/or the main effects were significant, means were compared using Newman-Keuls multiple-comparison post hoc test. Where transformations did not result in normality or constant variance, a Kruskal-Wallis nonparametric test was performed. A P value of <.05 was considered as statistically significant. All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (San Diego, CA, USA).

3. Results

3.1. Dietary intake, body parameters and plasma biochemistry

Food intake was decreased in H and HC rats compared with C and CC groups (Table 2). Chia seed supplementation did not change water intake in either group, but food consumption was increased in CC rats (Table 2). Chia seed supplementation increased energy intake compared with C and H diet-fed rats (Table 2). This increased energy intake in the chia seed-supplemented rats corresponded with increased body weight gain and feed conversion efficiency in both groups without any change in abdominal circumference or total body fat mass. However, chia seed supplementation reduced the visceral adiposity index and increased bone mineral content in both groups without altering the body lean mass (Table 2). The reduction in visceral adiposity index was due to decreased retroperitoneal and omental but not epididymal fat deposition in HC rats. In CC rats, selective lowering of omental fat was observed (Table 2). Chia seed supplementation decreased the total lipid content in the retroperitoneal fat and liver but increased the content in the heart (Table 2). This lipid redistribution induced by chia seed supplementation was associated with increased plasma triglycerides and NEFA, although plasma total cholesterol concentrations were not affected (Table 2). Chia seed supplementation also reduced heart wet weights.

Furthermore, chia seed supplementation normalized plasma urea concentrations suppressed by H diet feeding and reduced lactate

Table 2
Dietary intakes, body composition and anthropometrics, organ wet weights, tissue fatty acid composition and plasma biochemistry in C, CC, H and HC diet-fed rats

Variable	C	CC	H	HC	*P*		
					Diet	Treatment	Interaction
Food intake, g/d (n=10)	33.9±0.9^{b}	42.1±2.1^{a}	22.9±0.3^{c}	22.8±0.4^{c}	<.0001	.0014	.0011
Water intake, ml/d (n=10)	31.4±0.4^{a}	31.7±0.8^{a}	19.7±0.3^{b}	19.1±0.4^{b}	<.0001	.7714	.3856
Chia seed intake, g/d (n=10)	0.0±0.0^{c}	2.1±0.1^{a}	0.0±0.0^{c}	1.1±0.0^{b}	<.0001	<.0001	<.0001
Energy intake, kJ/d (n=10)	400.8±9.3^{b}	503.1±18.1^{a}	452.5±12.4^{b}	507.7±13.6^{a}	.0475	<.0001	.0946
Body weight gain (8–16 weeks), % (n=10)	11.0±1.3^{b}	22.6±1.5^{a}	15.2±1.9^{b}	23.8±1.7^{a}	.1033	<.0001	.3593
Feed conversion efficiency, % (n=10)	2.7± 0.1^{c}	4.5± 0.3^{a}	3.3± 0.2^{b}	4.6± 0.2^{a}	.0801	<.0001	.1913
Bone mineral content, g (n=10)	12.4±0.2^{c}	13.3±0.3^{bc}	13.9±0.1^{b}	15.4±0.6^{a}	<.0001	.0017	.4018
Total body fat mass, g (n=10)	75.5±4.4^{b}	82.8±8.7^{b}	153.9±6.6^{a}	153.1±12.3^{a}	<.0001	.7049	.6371
Total body lean mass, g (n=10)	325.9±4.5^{b}	343.5±9.2^{ab}	345.9±8.9^{ab}	368.1±10.0^{a}	.012	.0237	.7865
Abdominal circumference, cm (n=10)	20.0±0.3^{b}	20.4±0.3^{b}	22.2±0.4^{a}	22.0±0.2^{a}	<.0001	.7475	.3369
Visceral adiposity index, % (n=10)	4.4±0.3^{c}	2.1±0.2^{d}	7.9±0.5^{a}	5.9±0.3^{b}	<.0001	<.0001	.4479
Tissue wet weights[e], mg/mm (n=10)							
Retroperitoneal fat	169.4±12.6^{c}	126.0±14.5^{c}	387.6±26.0^{a}	296.6±19.1^{b}	<.0001	.001	.2141
Epididymal fat	105.3±7.0^{b}	85.8±10.5^{b}	192.8±13.5^{a}	169.6±13.4^{a}	<.0001	.0697	.8742
Omental fat	102.8±11.4^{b}	69.5±6.5^{c}	175.0±10.3^{a}	147.4±5.7^{b}	<.0001	.0014	.7469
Liver	204.2±22.2^{b}	223.0±6.6^{b}	318.5±12.0^{a}	288.6±5.8^{a}	<.0001	.6741	.0723
Heart	22.3±0.3^{b}	22.7±0.8^{b}	25.6±1.0^{a}	23.4±0.4^{b}	.006	.2077	.0555
Tissue lipid content, mg/g (n=6)							
Retroperitoneal adipose tissue	396.2±25.3^{c}	453.9±12.9^{c}	632.3±22.4^{a}	527.1±19.4^{b}	<.0001	.2676	.0008
Liver	25.9±2.4^{b}	26.0±3.2^{b}	60.2±2.9^{a}	29.8±3.2^{b}	<.0001	<.0001	<.0001
Heart	29.8±0.7^{b}	38.4±2.7^{b}	36.7±2.2^{b}	47.5±4.4^{a}	.0106	.0029	.7012
Plasma	11.5±0.8	13.9±1.7	13.5±2.8	14.2±1.1	.5385	.3834	.6233
Plasma urea, mmol/L (n=10)	5.6±0.6^{a}	5.1±0.4^{a}	3.7±0.3^{b}	2.6±0.2^{b}	<.0001	.0549	.4616
Plasma LDH, U/L (n=10)	191.1±18.2^{c}	122.0±12.2^{c}	386.6±34.8^{a}	302.7±38.0^{b}	<.0001	.0097	.793
Plasma uric acid, μmol/L (n=10)	25.9±1.4^{b}	24.0±1.9^{b}	54.7±5.2^{a}	32.9±3.7^{b}	<.0001	.0013	.0059
Plasma CRP, μmol/L (n=10)	59.3±7.4^{b}	58.2±5.9^{b}	102.3±4.6^{a}	54.6±2.6^{b}	.0009	<.0001	.0001
Plasma total cholesterol, mmol/L (n=10)	1.3±0.1^{b}	1.4±0.1^{b}	2.0±0.1^{a}	1.8±0.1^{a}	<.0001	.6201	.1423
Plasma triglyceride, mmol/L (n=10)	0.4±0.0^{c}	0.6±0.1^{c}	1.0±0.1^{b}	1.6±0.2^{a}	<.0001	.0024	.1112
Plasma NEFA, mmol/L (n=10)	1.2±0.1^{c}	1.7±0.3^{bc}	2.2±0.1^{b}	4.7±0.3^{a}	<.0001	<.0001	<.0001
Plasma fatty acid, g/100g of total fatty acid content (n=6)							
C14:0	75.56±2.32	73.24±3.27	75.20±1.37	71.79±3.44	.7436	.3063	.8437
C14:1n-5	1.01±0.18^{a}	0.00±0.00^{b}	0.80±0.07^{a}	0.29±0.11^{b}	.7227	<.0001	.0359
C16:0	0.02±0.02^{b}	2.75±0.2^{a}	0.14±0.14^{b}	2.34±0.31^{a}	.5064	<.0001	.2306
C16:1n-7	14.99±1.95^{a}	9.24±1.21^{b}	13.85±0.71^{a}	7.59±0.89^{b}	.2891	.0001	.8442
C18:1n-9	0.00±0.00^{b}	2.25±0.34^{a}	0.00±0.00^{b}	2.25±0.28^{a}	1	<.0001	1
C18:1*trans*-7	3.20±0.13^{b}	2.71±0.34^{b}	3.73±0.19^{b}	8.75±1.07^{a}	<.0001	.0008	.0001
C18:2n-6	1.71±0.18^{a}	0.00±0.00^{b}	2.02±0.22^{a}	0.00±0.00^{b}	.2884	<.0001	.2884
C18:3n-6	0.00±0.00	1.32±0.50	0.00±0.00	1.12±0.48	.7759	.0022	.7759
C18:3n-3	1.45±0.16^{a}	2.98±0.56^{b}	1.62±0.13^{a}	2.22±0.93^{b}	.3456	.0023	.1435
C20:3n-3	0.00±0.00^{b}	1.79±0.43^{a}	0.00±0.00^{b}	1.89±0.17^{a}	.831	<.0001	.831
C20:5n-3	2.07±0.07^{b}	0.86±0.09^{c}	2.65±0.34^{a}	0.57±0.06^{c}	.4345	<.0001	.0267
C24:0	0.00±0.00^{b}	0.33±0.02^{a}	0.00±0.00^{b}	0.28±0.05^{a}	.3642	<.0001	.3642
C22:6n-3	0.00±0.00^{c}	0.64±0.08^{b}	0.00±0.00^{c}	0.91±0.09^{a}	.0364	<.0001	.0364
Total SFA	75.58±2.31	78.22±1.90	75.34±1.35	74.41±3.17	.3853	.7118	.4431
Total MUFA	19.19±2.22	14.19±1.42	18.38±0.89	18.88±1.96	.2676	.2009	.1217
Total PUFA	5.23±0.30	7.59±0.73	6.28±0.65	6.71±1.26	.9176	.101	.2482
n3:n6 ratio	2.15±0.21^{b}	9.10±2.41^{a}	2.15±0.15^{b}	10.99±4.26^{a}	.5748	.0001	.5748

Each value is a mean±S.E.M. Means with superscript lettersa,b,c,d without a common letter differ (P <.05). Number of repetitive experiments are indicated within parenthesis. SFA, saturated fatty acid; PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid.

[e] Normalized against tibial length.

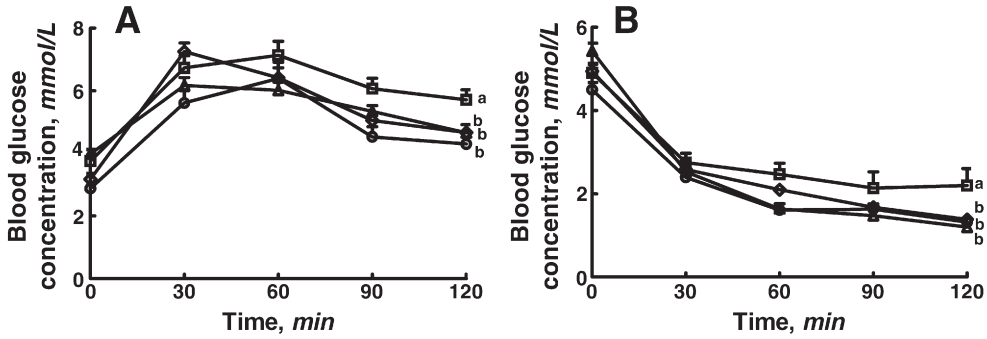

Figure	*P-value*		
	Diet	Chia	Diet x Chia
A	0.0055	0.1578	0.0058
B	0.0134	0.0286	0.0988

Fig. 1. Glucose (2 g/kg) (A) and insulin (0.75 IU/kg) (B) tolerance in C, CC, H and HC diet-fed rats. Data are shown as means±S.E.M. Means of values at 120 min without a common alphabet significantly differ. n=10/group.

dehydrogenase (LDH) activity as a marker of decreased metabolic activity, uric acid concentrations as a marker of fructose-induced oxidative stress and plasma CRP concentrations as a marker of inflammation (Table 1). In addition, chia seed-supplemented groups showed improved glucose and insulin tolerance (Fig. 1).

The plasma fatty acid profile suggests that the chia seed-supplemented rats resisted desaturation of C14:0 and C16:0 fatty acids by SCD (Table 2). The SCD index could not be calculated in C and H rats due to low concentrations of the saturated substrate. Both chia seed-supplemented groups had increased plasma concentrations of

Table 3
Changes in cardiovascular structure, function and fatty acid composition in C, CC, H and HC diet-fed rats

Variable	C	CC	H	HC	*P*		
					Diet	Treatment	Interaction
LVIDd, mm (n=10)	6.4±0.2^{b}	6.7±0.2^{b}	7.7±0.2^{a}	6.1±0.3^{b}	.1354	.0074	.0002
LVPWd, mm (n=10)	1.7±0.0^{b}	1.8±0.1^{b}	1.9±0.0^{ab}	2.1±0.1^{a}	.0011	.0408	.4841
Relative wall thickness (n=10)	0.4±0.01^{c}	0.5±0.01^{b}	0.6±0.02^{a}	0.4±0.01^{c}	.0006	.0006	<.0001
Fractional shortening, % (n=10)	60.8±1.4^{a}	64.0±1.9^{a}	50.5±2.1^{b}	59.2±2.2^{a}	.0004	.0038	.1617
Ejection fraction, % (n=10)	84.8±1.6	89.0±1.1	83.8±1.3	84.4±1.6	.0557	.0987	.2118
Ejection time, ms (n=10)	85.1±4.0	85.8±3.3	86.9±2.6	89.6±6.6	.528	.7011	.8212
Deceleration time, ms (n=10)	61.3±1.6^{c}	70.8±1.4^{b}	56.2±1.6^{c}	64.2±1.5^{a}	.0005	<.0001	.6264
LV developed pressure, mmHg (n=10)	64.7±6.1^{a}	51.3±4.5^{ab}	41.1±3.7^{b}	57.4±3.8^{a}	.0674	.7596	.003
+dP/dt, mmHg×s^{-1} (n=10)	1078.9±104.3^{a}	1009.1±62.8^{a}	753.6±37.3^{b}	1052.5±81.2^{a}	.0701	.1378	.0197
−dP/dt, mmHg×s^{-1} (n=10)	−613.5±66.2^{a}	−616.3±57.2^{a}	−373.9±21.9^{b}	−601.3±43.8^{a}	.0156	.0277	.0314
Diastolic stiffness (k), (n=10)	22.8±0.7^{c}	22.9±0.6^{c}	29.4±0.6^{a}	26.5±0.6^{b}	<.0001	.8741	<.0001
Estimated LV mass, g (n=10)	0.7±0.0^{b}	0.8±0.0^{b}	1.1±0.1^{a}	0.8±0.1^{b}	.0076	.1659	.0076
LV+septum wet weight[e], mg/mm (n=10)	19.0±0.4^{b}	19.4±0.7^{b}	21.4±1.0^{a}	19.2±0.3^{b}	.0847	.1503	.0466
Right ventricle wet weight[e], mg/mm (n=10)	3.2±0.2^{b}	3.3±0.2^{b}	4.2±0.2^{a}	4.2±0.2^{a}	<.0001	.8625	.7107
LV fibrosis, % surface area (n=4)	3.6±0.9^{b}	3.5±0.7^{b}	12.6±1.2^{a}	4.4±0.2^{a}	<.0001	.0003	.0004
Systolic blood pressure, mmHg (n=10)	123.7±1.8^{c}	138.3±3.3^{b}	150.7±3.6^{a}	153.2±5.6^{a}	<.0001	.0316	.1222
Fatty acids, g/100g of total fatty acid content (n=6)							
C14:0	42.49±3.73^{b}	53.44±3.72^{ab}	54.07±5.35^{ab}	58.05±1.18^{a}	.0458	.0635	.37
C14:1n-5	0.72±0.15^{a}	0.22±0.14^{b}	0.71±0.17^{a}	0.00±0.00^{b}	.3983	.0002	.4399
C16:0	1.22±0.13^{b}	8.45±0.70^{a}	2.26±1.49^{b}	6.75±0.34^{a}	.6996	<.0001	.1198
C16:1n-7	14.83±1.08^{a}	1.01±0.29^{c}	10.23±2.00^{b}	0.45±0.02^{c}	.0357	<.0001	.0932
C18:0	0.49±0.04^{a}	2.48±0.06^{b}	0.69±0.08^{c}	1.18±0.08^{d}	<.0001	<.0001	<.0001
C18:1n-9	0.53±0.05^{b}	11.28±0.65^{a}	0.31±0.07^{b}	11.90±0.65^{a}	.6695	<.0001	.3738
C18:1*trans*-7	12.96±1.00^{a}	2.05±0.09^{b}	11.41±1.57^{a}	1.58±0.04^{b}	.2914	<.0001	.5688
C18:2n-6	6.79±0.79	6.67±1.17	4.75±0.82	5.23±0.31	.0492	.8307	.7218
C18:3n-6	7.47±0.63^{a}	0.00±0.00^{b}	4.26±1.02^{a}	0.00±0.00^{b}	.0145	<.0001	.0145
C18:3n-3	0.00±0.00^{b}	0.60±0.15^{a}	0.0±0.0^{b}	0.41±0.07^{a}	.2646	<.0001	.2646
C20:4n-6	0.04±0.04^{a}	0.23±0.07^{b}	0.49±0.05^{c}	0.24±0.02^{b}	.0001	.543	.0002
C20:5n-3	11.31±0.95^{a}	6.19±0.34^{c}	8.92±0.95^{b}	6.44±0.05^{c}	.1385	<.0001	.0714
C22:5n-3	0.05±0.05^{b}	1.29±0.41^{a}	0.00±0.00^{b}	0.07±0.01^{b}	.006	.0048	.0103
C24:0	0.29±0.19^{c}	3.19±0.93^{a}	0.21±0.12^{c}	1.82±0.01^{b}	.1453	.0001	.1927
C22:6n-3	0.77±0.16^{b}	2.67±0.91^{c}	0.96±0.23^{b}	5.60±0.23^{a}	.0047	<.0001	.0111
Total SFA	44.53±3.60^{d}	67.73±1.43^{b}	57.33±5.98^{c}	67.95±0.87^{a}	.0847	.0001	.095
Total MUFA	29.04±1.79^{a}	14.56±0.89^{b}	22.66±3.55^{c}	13.93±0.63^{b}	.1046	<.0001	.1784
Total PUFA	26.43±2.18^{a}	17.71±1.07^{b}	20.01±2.55^{b}	18.12±0.3^{b}	.1053	.0072	.0682
n3:n6 ratio	0.86±0.06^{b}	2.05±0.05^{a}	1.28±0.28^{b}	2.27±0.15^{a}	.0645	<.0001	.5478
Stearoyl-CoA 9-desaturase index	12.43±0.94^{a}	0.11±0.02^{b}	13.47±2.94^{a}	0.07±0.00^{b}	.7493	<.0001	.7301

Each value is a mean±S.E.M. Means with superscript letters^{a,b,c,d} without a common letter differ (P <.05). Number of repetitive experiments are indicated within parenthesis. SFA, saturated fatty acid; PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid.

[e] Normalized against tibial length.

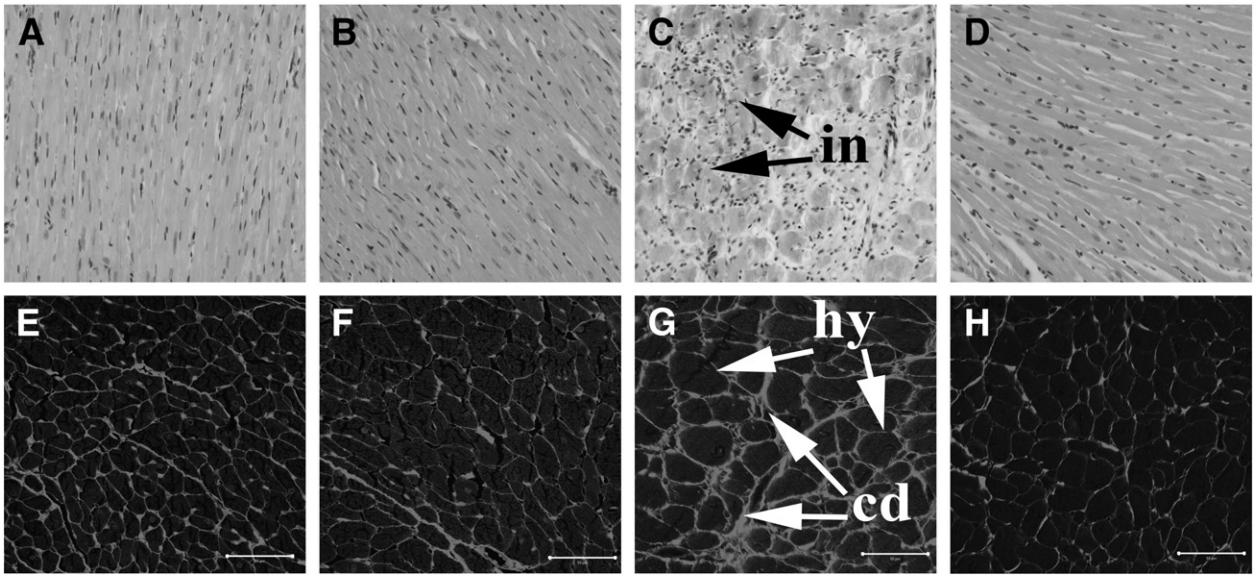

Fig. 2. Hematoxylin and eosin staining of LV (×20) showing inflammatory cells (marked as "in") as dark spots outside the myocytes; C (A), CC (B), H (C) and HC (D) diet-fed rats. Picrosirius red staining of left ventricular interstitial collagen deposition (×40) in C (E), CC (F), H (G) and HC (H) diet-fed rats. Collagen deposition is marked as "cd" and hypertrophied cardiomyocytes are marked as "hy."

C18:1n-9, but increased concentrations of C18:1*trans*-7 were observed only in HC group (Table 2). Both chia seed-supplemented groups had depleted plasma C18:2n-6 and C20:5n-3 but increased C18:3n-6, C18:3n-3, C20:4n-3 and C22:6 n-3 concentrations (Table 2). Although the total saturated and unsaturated fatty acids remained unchanged, the n3:n6 ratio was markedly enhanced in the plasma of the chia seed-supplemented groups (Table 2).

3.2. Cardiovascular changes

Compared with H rats, HC groups had smaller LV internal diameter with increased fractional shortening and deceleration time (Table 3). In addition, chia seed reduced LV wet weight and reduced estimated LV mass (Table 3). However, chia seed supplementation did not reduce blood pressure in either group.

After 16 weeks, H rats showed greater infiltration by inflammatory cells into the LV (Fig. 2C) as well as increased interstitial collagen deposition (Fig. 2G; Table 3) compared with C rats (Fig. 2A and E; Table 3). HC rats showed normalized inflammatory state (Fig. 2D) and markedly reduced collagen deposition (Fig. 2H; Table 3), but no changes in inflammatory cell infiltration or collagen deposition were seen in CC rats (Fig. 2B and F; Table 3). The reduction in LV fibrosis is consistent with reduced diastolic stiffness constant in HC diet-fed rats (Fig. 2D and H; Table 3).

Furthermore, H feeding diminished vascular responses in isolated thoracic aortic rings to noradrenaline, sodium nitroprusside and acetylcholine when compared with C rats (Fig. 3A-C). In isolated thoracic

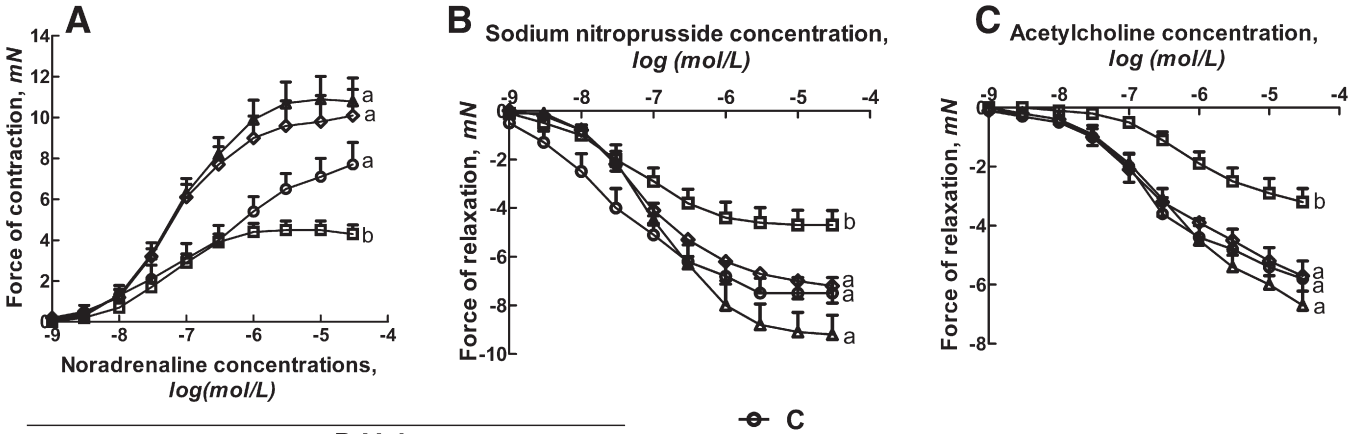

Figure	*P*-Value		
	Diet	Treatment	Interaction
A	0.0594	0.0002	0.2075
B	0.0016	0.005	0.5716
C	0.0074	0.011	0.2147

Fig. 3. Cumulative concentration–response curves for noradrenaline (A), sodium nitroprusside (B) and acetylcholine (C) in thoracic aortic rings from C, CC, H and HC diet-fed rats. Data are shown as means±S.E.M. Endpoint means without a common alphabet in each data set significantly differ. n=8–10/group.

aortic rings from HC rats, increased vasorelaxation was induced by sodium nitroprusside and acetylcholine (Fig. 3B and C) in addition to increased contractility to noradrenaline (Fig. 3A). Vascular responses in isolated thoracic aortic rings from CC rats remained unchanged compared with C rats (Fig. 3A-C).

Similar to the plasma fatty acid profile, chia seed-supplemented rats resisted SCD action on C14:0 and C16:0 fatty acids in the heart (Table 3). Both chia seed-supplemented groups had increased concentrations of C18:0, C18:1n-9 but decreased concentrations of C18:1*trans*-7 in the heart together with depleted C18:3n-6 and C20:5n-3 but increased C18:3n-3, 22:5n-3, C22:6n-3 and C24:0 concentrations (Table 3). As a result of decreased desaturation of C14:0 and C16:0, the total saturated fatty acid increased, and total unsaturated fatty acid content decreased (Table 3). However, the cardiac n-3:n-6 ratio was increased in the chia seed-supplemented groups (Table 3).

3.3. Hepatic structure and function

H diet feeding elevated plasma markers of liver function in comparison with C-fed rats (Table 4). HC diet feeding did not alter the elevated alanine transaminase (ALT) activity but decreased the aspartate transaminase (AST) activity (Table 4). ALP activity was increased in the HC group (Table 4). Creatine kinase (CK) activity decreased in both chia seed-supplemented groups, but none of the other enzymes were affected in CC group (Table 4).

C (Fig. 4A, E and I) and CC (Fig. 4B, F and J) groups showed negligible lipid accumulation, inflammatory cell infiltration or fibrosis in the liver. H feeding for 16 weeks increased the size of the fat vacuoles within the hepatocytes (Fig. 4C) with increased portal inflammatory cell infiltration (Fig. 4G) and portal fibrosis (Fig. 4K; Table 4). The HC diet-fed rats displayed normalized macrovesicular steatosis (Fig. 4D), portal inflammation (Fig. 4H) and fibrosis (Fig. 4I; Table 4).

Similar to cardiac and plasma fatty acid profile, chia seed-supplemented rats resisted stearoyl-CoA 9-desaturation of C14:0 and C16:0 fatty acids in the liver (Table 4). Both chia seed-supplemented groups had increased concentrations of C14:0, C16:0, C18:0 and C18:1n-9 but decreased concentrations of C18:1*trans*-7 and C18:2n-6 (Table 4). Although C20:4n-6 content in the liver remained unchanged across all groups, both chia seed-supplemented groups had depleted C18:3n-3 and C20:5n-3 but increased C22:5n-3, C22:6n-3 and C24:0 concentrations (Table 4). As a result of decreased desaturation of C14:0 and C16:0, the total saturated fatty acid increased and total unsaturated fatty acid content decreased (Table 4). However, the liver n-3:n-6 ratio was increased in the chia seed-supplemented groups (Table 4).

3.4. Retroperitoneal adipose tissue fatty acid composition

Similar to other tissues, chia seed-supplemented rats resisted stearoyl-CoA 9-desaturation of C14:0 and C16:0 fatty acids (Table 5) in the retroperitoneal adipose tissue. Both chia seed-supplemented groups had increased concentrations of C14:0, C16:0 and C18:1*trans*-7 but decreased concentrations of C18:2n-6 and C18:3n-6 (Table 5). C18:3n-3 was increased in HC group (Table 5) despite the extremely small amounts of fatty acids with chain length of more than 20 carbon atoms detected in the adipose tissue of all groups, making accurate quantification difficult. Consequently, the total saturated fatty acid increased and total unsaturated fatty acid content decreased, but the n-3:n-6 ratio in retroperitoneal fat was increased in the chia seed-supplemented groups (Table 5).

4. Discussion

The aim of this study was to assess the metabolic, cardiac and liver changes following 5% chia seed supplementation in high-carbohydrate, high-fat (H) diet-fed rats with low n-3 fatty acids.

Table 4
Changes in hepatic structure, function and fatty acid composition in C, CC, H and HC diet-fed rats

Variable	C	CC	H	HC	*P*		
					Diet	Treatment	Interaction
ALT, U/L (n=10)	31.0±1.8^{b}	31.6±3.0^{b}	46.2±3.8^{a}	45.4±4.9^{a}	.0002	.9777	.8452
AST, U/L (n=10)	76.0±3.5^{b}	75.0±3.2^{b}	104.8±5.9^{a}	76.4±8.1^{b}	.0099	.0118	.0183
ALP, U/L (n=10)	154.9±11.9^{b}	188.4±30.5^{b}	181.4±11.6^{b}	251.1±12.5^{a}	.0209	.0083	.3333
CK, U/L (n=10)	165.3±13.2^{b}	70.4±11.0^{c}	243.4±28.2^{a}	122.3±17.3^{bc}	.0013	<.0001	.4867
Liver fibrosis, % surface area (n=4)	1.6±0.2^{c}	1.8±0.1^{c}	7.4±0.5^{a}	3.6±0.2^{b}	<.0001	<.0001	<.0001
Fatty acid, g/100g of total fatty acid content (n=6)							
C14:0	6.69±0.98^{b}	14.16±1.30^{a}	3.10±0.59^{c}	9.92±1.56^{b}	.0031	<.0001	.7832
C14:1n-5	1.72±0.14^{a}	0.72±0.06^{b}	0.79±0.09^{a}	1.05±0.09^{c}	.0575	<.0001	.2051
C16	0.03±0.03^{b}	19.96±0.41^{a}	0.25±0.05^{b}	21.28±0.80^{a}	.1028	<.0001	.2363
C16:1n-7	25.40±1.10^{a}	3.74±0.75^{b}	23.30±0.75^{a}	1.31±0.32^{c}	.0088	<.0001	.8347
C18:0	0.00±0.00^{d}	1.04±0.14^{a}	0.32±0.07^{c}	0.64±0.05^{b}	.6316	<.0001	.0003
C18:1n-9	0.00±0.00^{c}	29.25±1.35^{b}	1.52±1.52^{c}	40.16±1.34^{a}	<.0001	<.0001	.001
C18:1*trans*-7	14.23±1.93^{a}	0.00±0.00^{b}	10.86±3.37^{a}	0.00±0.00^{b}	.3958	<.0001	.3958
C18:2n-6	26.58±4.73^{b}	8.60±0.96^{b}	44.26±4.93^{a}	7.18±0.29^{c}	.0289	<.0001	.0119
C18:3n-3	7.42±0.47^{a}	3.42±0.30^{c}	4.93±0.15^{b}	3.23±0.18^{c}	.0003	<.0001	.0011
C20:0	0.40±0.02^{c}	0.17±0.02^{b}	0.19±0.02^{b}	0.47±0.04^{a}	.1045	.356	<.0001
C20:4n-6	0.64±0.07	0.67±0.34	0.73±0.11	0.83±0.28	.5923	.7801	.8804
C20:5n-3	10.85±2.89^{a}	8.33±0.53^{b}	7.96±2.31^{b}	6.34±0.46^{c}	<.0001	.0001	.3082
C22:5n-3	0.16±0.07^{c}	2.81±0.19^{b}	0.05±0.03^{c}	1.56±0.08^{a}	<.0001	<.0001	<.0001
C24:0	0.39±0.13^{b}	4.11±0.68^{a}	0.06±0.06^{b}	3.64±0.77^{a}	.4495	<.0001	.894
C22:6n-3	0.24±0.15^{c}	2.16±0.16^{a}	0.03±0.03^{c}	1.38±0.13^{b}	.001	<.0001	.0381
Total SFA	10.26±3.20^{b}	39.46±1.40^{a}	3.91±0.61^{c}	35.96±1.85^{b}	.023	<.0001	.4843
Total MUFA	40.35±1.07^{ab}	33.75±1.83^{c}	36.65±1.86^{bc}	42.70±1.26^{a}	.1047	.8605	.0006
Total PUFA	49.38±3.93^{b}	26.79±0.82^{c}	59.43±2.08^{a}	21.34±1.16^{c}	.3362	<.0001	.0034
n3:n6 ratio	0.99±0.23^{b}	1.78±0.30^{a}	0.35±0.12^{c}	1.44±0.16^{ab}	.0329	.0003	.4911
Stearoyl-CoA 9-desaturase index^{d}	–	0.19±0.04	78.84±2.44	0.06±0.02	–	–	–

Each value is a mean±S.E.M. Means with superscript letters^{a,b,c,d} without a common letter differ (P <.05). Number of repetitive experiments is indicated within parenthesis. SFA, saturated fatty acid; PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid.

[d] Negligible C16:0 fatty acid was detected in C diet-fed group, therefore showing a very high stearoyl-CoA 9-desatururation index in this group.

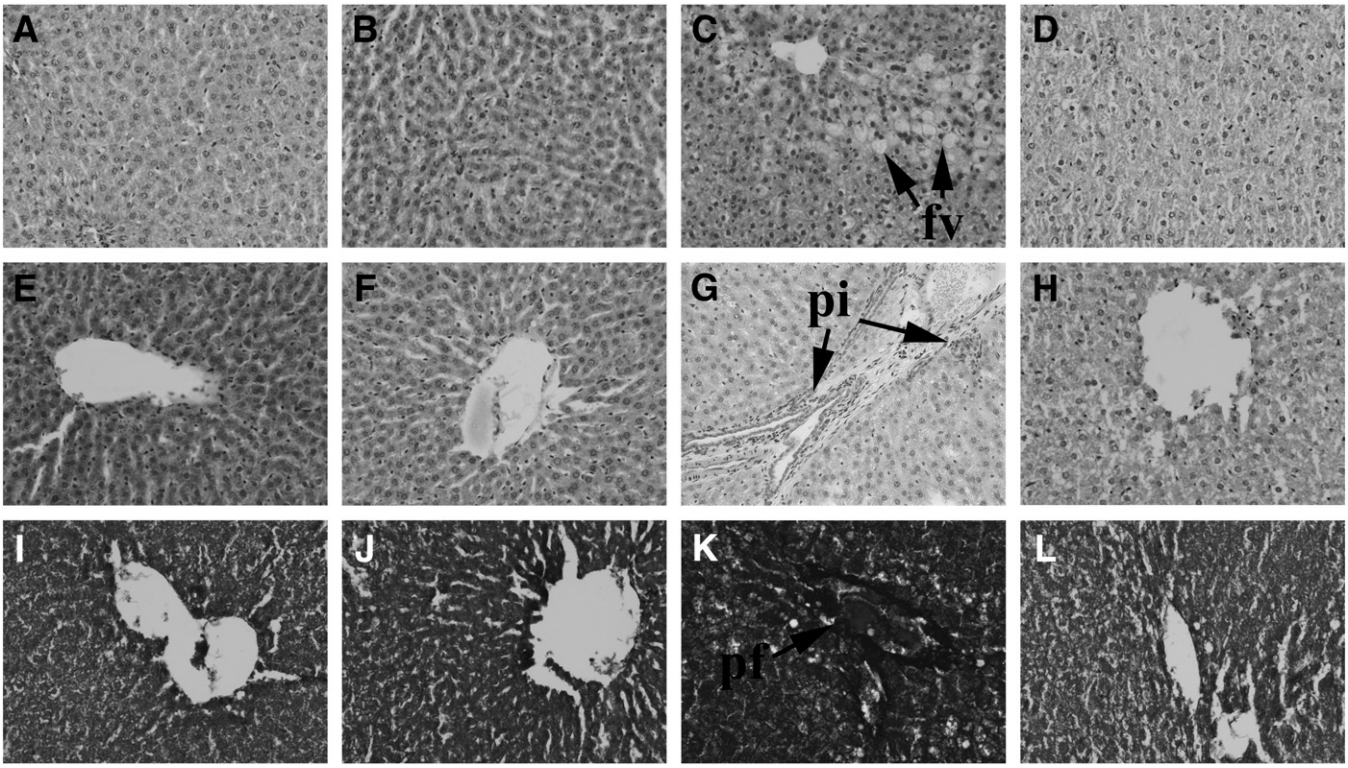

Fig. 4. Hematoxylin and eosin staining of hepatocytes (×20) showing hepatocytes with enlarged fat vacuole (marked as "fv") and inflammatory cells around the portal region (marked as "pi") (×20) from C (A, E), CC (B, F), H (C, G) and HC (D, H) diet-fed rats. Milligan's trichrome staining of the hepatic portal regions showing collagen (marked as "pf") (×20) in C (I), CC (J), H (K) and HC (L) diet-fed rats.

Rats fed H diet developed hypertension, impaired glucose and insulin tolerance, dyslipidemia, hepatic steatosis, cardiac fibrosis and functional deterioration, inflammation and abdominal obesity. With the exception of elevated blood pressure and some plasma markers of liver function, dietary chia seed supplementation attenuated structural and functional changes caused by H feeding. Chia seed supplementation caused lipid redistribution away from the abdominal cavity, suppressed stearoyl-CoA desaturase index and increased n-3:n-6 ratio in various tissues.

Chia seed-supplemented rats improved insulin and glucose tolerance, reduced visceral adiposity, decreased hepatic steatosis, reduced cardiac and hepatic fibrosis and inflammation without changes in plasma lipids or blood pressure. However, the most notable result from our study is the chia seed-induced lipid redistribution with lipid trafficking away from the visceral fat and liver with increased accumulation in the heart. Furthermore, the selectivity for different fatty acids for the unsaturated and the desaturated products of the 18 carbon length fatty acids was altered in different tissues by chia seed supplementation.

The relative abundance of the C18:1n-9 was increased in the heart and the liver of the chia seed-supplemented groups. The C18:1*trans*-7 was more selectively stored in the adipose tissue than the heart and the liver, and the C18:2n-6 or its elongated Δ^5 and Δ^6 desaturase products were depleted in all tissues suggesting that C18:2n-6 is

Table 5
Fatty acid composition of retroperitoneal fat and desaturase index from C, CC, H and HC diet-fed rats

Fatty acid, g/100g of total fatty acid content (n=6)	C	CC	H	HC	*P*		
					Diet	Treatment	Interaction
C14:0	0.00±0.00^{c}	1.75±0.08^{b}	0.00±0.00^{c}	2.55±0.11^{a}	<.0001	<.0001	<.0001
C14:1n-5	2.17±0.04^{b}	0.12±0.01^{c}	2.81±0.10^{a}	0.22±0.02^{c}	<.0001	<.0001	<.0001
C16:0	0.22±0.05^{b}	31.15±1.60^{a}	0.42±0.08^{b}	23.62±0.68^{c}	.0004	<.0001	.0003
C16:1n-7	31.48±1.06^{a}	2.44±0.27^{b}	22.40±0.21^{c}	1.47±0.46^{b}	<.0001	<.0001	<.0001
C18:0	0.22±0.03^{c}	0.47±0.09^{c}	0.62±0.02^{c}	1.10±0.10^{a}	<.0001	<.0001	.1143
C18:1*trans*-7	3.46±0.23^{c}	40.99±1.52^{b}	6.97±0.45^{d}	59.91±1.29^{a}	<.0001	<.0001	<.0001
C18:2n-6	51.18±0.96^{b}	10.78±0.80^{c}	60.76±0.47^{a}	6.51±0.49^{d}	.0013	<.0001	<.0001
C18:3n-6	0.08±0.04^{b}	0.33±0.21^{b}	5.18±0.08^{a}	0.09±0.01^{b}	<.0001	<.0001	<.0001
C18:3n-3	10.11±0.32^{a}	10.45±0.80^{a}	0.07±0.07^{c}	3.12±0.42^{b}	<.0001	.0021	.0106
C20:0	0.66±0.13^{a}	0.14±0.05^{c}	0.35±0.02^{bc}	0.54±0.04^{ab}	.5453	.0354	<.0001
Total SFA	1.10±0.14^{c}	33.78±1.44^{a}	1.48±0.13^{c}	28.09±0.50^{b}	.0025	<.0001	.0008
Total MUFA	37.21±0.86^{c}	43.98±1.37^{b}	32.40±0.43^{d}	62.03±1.23^{a}	<.0001	<.0001	<.0001
Total PUFA	61.70±0.92^{b}	22.18±0.78^{c}	66.12±0.48^{a}	9.85±0.93^{d}	<.0001	<.0001	<.0001
n3:n6 ratio	0.20±0.01^{c}	1.02±0.18^{a}	0.00±0.00^{c}	0.48±0.03^{b}	.0006	<.0001	.0776
Stearoyl-CoA 9-desaturase index	129.55±16.75^{a}	0.08±0.01^{c}	44.38±0.51^{b}	0.06±0.02^{c}	<.0001	<.0001	<.0001

Each value is a mean±S.E.M. Means with superscript letters^{a,b,c,d} without a common letter differ (P <.05). Number of repetitive experiments are indicated within parenthesis. SFA, saturated fatty acid; PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid.

preferentially oxidized rather than stored. Trans-fatty acids only undergo partial β-oxidation and are not the preferred substrate [32]. However, C18:1*trans*-7 can be efficiently converted to conjugated linoleic acid by the action of stearoyl-CoA desaturase 1 (SCD-*1*) [33], the activity of which is inhibited by C18:3n-3-enriched chia seed. The fatty acid homeostasis in tissues of the chia seed-supplemented rats was maintained so that the C18:1*trans*-7 was preferentially stored in the adipose tissue; the relatively inert C18:1n-9 was stored in sensitive organs such as liver and heart and the C18:2, n-6, the parent fatty acid of the n-6 pathway, was preferentially oxidized. It is therefore plausible that the mechanism of down-regulation of the n-6 pathway by n-3 fatty acids involves increasing the transport and oxidation of C18:2n-6 into the mitochondria.

Our results are consistent with the inhibition of SCD-1 by ALA-rich chia seed. SCD-1 is a Δ^9 fatty acid desaturase that catalyzes the rate-limiting step in the production of monounsaturated from saturated fatty acids [34]. In addition to reduced MUFA synthesis, SCD-1 deficiency or treatment with SCD-1-targeted antisense oligonucleotides induced protection from obesity, cellular lipid accumulation and insulin resistance in mice [35–37]. Fructose, being more lipogenic than glucose, is a potent inducer of hepatic *Scd-1* [38–40], and this enzyme plays a pivotal role in fructose-induced lipogenesis [41]. In addition to metabolic effects elicited by dietary carbohydrates, fatty acids also modulate the transcriptional activation of *Scd-1* and other lipogenic genes [42, 43]. The binding of sterol regulatory element binding protein-1 (SREBP-1) to the SREBP response element of the *Scd-1* promoter is decreased by dietary *n-3* polyunsaturated fatty acid from fish oil [44]. In addition, increased plasma palmitoleate, a product of SCD-1, in humans has been independently associated with both hypertriglyceridemia and abdominal adiposity [45]. Saturated fat-enriched diet induced lipogenic genes in wild-type mice, with the induction of the *Scd-1* and *Cpt-1* gene [46]. On the contrary, in *Scd-1*-deficient mice, the high saturated fat diet does not induce lipogenesis; instead, mitochondrial fatty acid oxidation is increased [46]. However, inhibition of SCD-1 in a low-fat diet could potentially increase the diet intake to supplement the loss of unsaturated fatty acid due to inhibition for normal metabolic function. This effect is clearly seen in CC diet-fed rats where the dietary intake increases following chia seed supplementation.

In *Scd-1*-deficient mice, a lack of functional SCD-1 decreases the accumulation of hepatic triglycerides and cholesterol esters, down-regulates *de novo* fatty acid synthesis in the liver and reduces adiposity [37]. Furthermore, *Scd-1*$^{-/-}$ mice are resistant to high-carbohydrate and high-fat diet-induced liver steatosis [47]. *Scd-1*$^{-/-}$ mice have increased fatty acid β-oxidation in the liver [48], skeletal muscle [49] and brown adipose tissue [50] and up-regulated AMP-activated protein kinase pathway [48,49]. In addition to the regulation of lipid metabolism, SCD-1 is involved in the regulation of carbohydrate metabolism. *Scd-1*$^{-/-}$ mice have increased whole-body glucose tolerance and elevated insulin sensitivity in skeletal muscle and brown adipose tissue [51,52]. However, in the heart, the lack of *Scd-1* decreased mitochondrial fatty acid uptake and oxidation while increasing glucose transport and oxidation [53]. In leptin-deficient *ob*/*ob* mice, disruption of *Scd-1* gene improved cardiac function by correcting systolic and diastolic dysfunction without altering plasma triglyceride and NEFA concentrations [54].

To the best of our knowledge, this is the first report of lipid redistribution with a rich dietary source of any n-3 fatty acid associated with cardioprotection and hepatoprotection. In addition, we report an intricate pattern of fatty acid distribution in various tissues from rats fed a chia seed-supplemented diet that would probably lead to an improved lipid homeostatic condition. The results warrant further research on the use of chia seed as a complimentary therapy for treating some signs of metabolic syndrome.

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Appendix C

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Review

Omega-3 fatty acids and metabolic syndrome: Effects and emerging mechanisms of action

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ABSTRACT

Epidemiological, human, animal, and cell culture studies show that *n*−3 fatty acids, especially α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), reduce the risk factors of cardiovascular diseases. EPA and DHA, rather than ALA, have been the focus of research on the *n*−3 fatty acids, probably due to the relatively inefficient conversion of ALA to EPA and DHA in rodents and humans. This review will assess our current understanding of the effects and potential mechanisms of actions of individual *n*−3 fatty acids on multiple risk factors of metabolic syndrome. Evidence for pharmacological responses and the mechanism of action of each of the *n*−3 fatty acid trio will be discussed for the major risk factors of metabolic syndrome, especially adiposity, dyslipidemia, insulin resistance and diabetes, hypertension, oxidative stress, and inflammation. Metabolism of *n*−3 and *n*−6 fatty acids as well as the interactions of *n*−3 fatty acids with nutrients, gene expression, and disease states will be addressed to provide a rationale for the use of *n*−3 fatty acids to reduce the risk factors of metabolic syndrome.

Contents

1. Introduction . . . 373
2. Metabolism of *n*−3 and *n*−6 series fatty acids. . . . 374
3. *n*−3 Fatty acids on risk factors of metabolic syndrome. . . . 374
   3.1. Adiposity and dyslipidemia . . . 374
   3.2. Glucose homeostasis and insulin resistance . . . 376
   3.3. Hypertension . . . 377
   3.4. Oxidative stress . . . 379
   3.5. Inflammation . . . 380
4. Safety and nutrient-gene/nutrient-drug interactions of *n*−3 PUFAs . . . 381
5. Discussion and conclusions . . . 381
   Conflict of interest . . . 382
   Acknowledgment . . . 382
   References . . . 382

Abbreviations: AA, arachidonic acid; ACE, angiotensin converting enzyme; ALA, α-linolenic acid; Apo-E, apolipoprotein E; COX, cyclooxygenase; CPT, carnitine palmitoyl transferase; CRP, C-reactive protein; Cx43, connexin 43; DGLA, dihomogamma linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; eNOS, endothelial nitric oxide synthase; GLA, γ-linolenic acid; GLUT, glucose transporter; HDL, high-density lipoprotein; HO-1, heme oxygenase-1; HSP, heat shock protein; IL, interleukin; IRS, insulin receptor substrate; JNK, c-Jun N-terminal kinase; LA, linoleic acid; LDL, low-density lipoprotein; LepR, leptin receptor; LOX, lipoxygenase; LT, leukotriene; MCP, monocyte chemotactic protein; MMP, matrix metalloproteinase; MUFA, monounsaturated fatty acid; NADPH, nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor kappa light chain enhancer of activated B cells; NO, nitric oxide; PAI, plasminogen activator inhibitor; PG, prostaglandins; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PPAR, peroxisome proliferator-activated receptors; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; SHR, spontaneously hypertensive rats; SREBP, sterol regulatory element binding protein; TAG, triacylglycerol; TIMP, tissue inhibitors of metalloproteinase; TNF, tumor necrosis factor; TX, thromboxane; VLDL, very low-density lipoprotein.

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1. Introduction

Metabolic syndrome has usually been defined as the clustering of interrelated risk factors for cardiovascular disease and type 2 diabetes, including hyperglycemia, insulin resistance, hypertension, hypertriglyceridemia, decreased HDL-cholesterol concentration, and obesity [1]. While the prevalence of metabolic syndrome is reaching pandemic proportions worldwide [2], studies have shown that dietary modifications including low carbohydrate diets, low fat diets, diets rich in fibers, Mediterranean diets and diets rich in phytochemicals such as flavonoids and phenolic acids reduce one or more risk factors of metabolic syndrome [3–5]. Another intervention to reduce risk in patients with metabolic syndrome may be an increase in the relative abundance of omega-3 ($n-3$) polyunsaturated fatty acids (PUFA) in the diet [5].

The cardioprotective effects of $n-3$ fatty acids, especially α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), have been defined by epidemiological, human, animal, and cell culture studies [5–9]. Although ALA, EPA, and DHA are grouped together as the $n-3$ PUFA, there is substantial evidence suggesting that the individual fatty acids may have selective and potentially independent effects on cardiovascular health [5–9]. In human diets, ALA is usually derived from botanical sources with chia seed and flax seed being the richest sources. Fish,

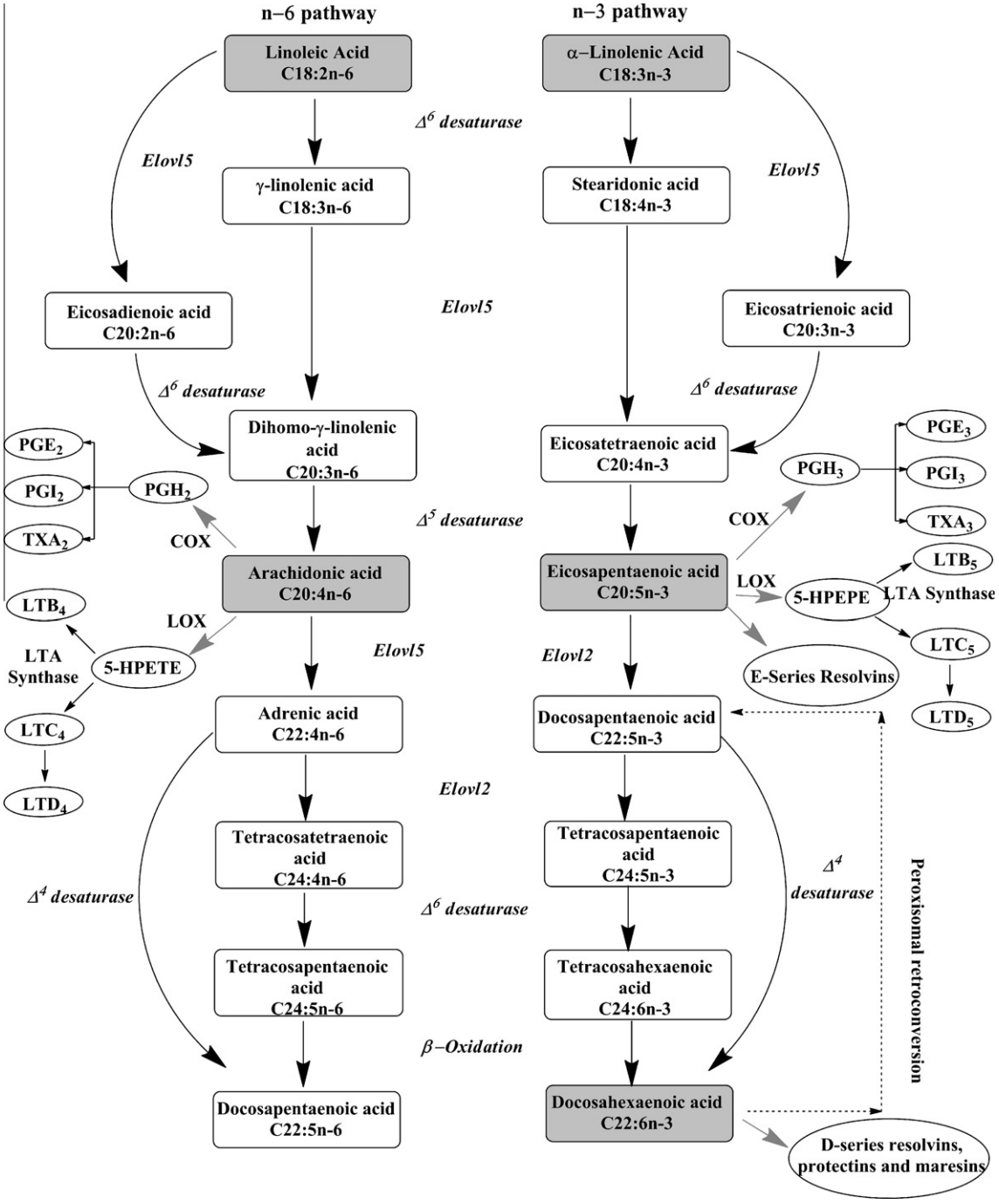

Fig. 1. The $n-3$ and $n-6$ PUFA metabolic pathways. PGE, prostaglandins; PGI, prostacyclins; LT, leukotriene; TX, thromboxane; 5-HPETE, 5-hydroperoxyeicosatetraenoic acid; 5-HPEPE, 5-hydroperoxyeicosapentaenoic acid.

fish oil supplements, and other sea foods primarily account for the EPA and DHA in human diets [6]. Fish do not synthesize these long chain *n*−3 fatty acids but accumulate EPA and DHA by consuming plankton and algae as a part of the marine food chain [10].

Although humans and animals can synthesize saturated (SFA) and monounsaturated (MUFA) fatty acids *de novo*, they lack the enzymes necessary to insert a *cis* double bond at the *n*−3 or the *n*−6 position of a fatty acid to synthesize ALA or linoleic acid (LA), respectively [11]. The two essential fatty acids, ALA (*n*−3) and LA (*n*−6), share a common metabolic pathway (Fig. 1) [12]. Consequently, these fatty acids compete for the Δ^6-desaturase in metabolism [13]. ALA as an essential fatty acid can be converted into EPA and DHA. LA is a direct precursor of the pro-inflammatory arachidonic acid (AA) [14]. The ratio of *n*−6 to *n*−3 fatty acids in early human diets was probably almost equal. However, due to changes in dietary habits, especially with the increased use of vegetable oils such as soybean, corn, sunflower, safflower oil, and cotton seed oils rich in LA [15], this ratio in the typical Western diet is now at least 10:1 [16].

Reducing the ratio of *n*−6:*n*−3 PUFA in the diet may reduce the risk factors of metabolic syndrome, although an optimal ratio is yet to be recommended [10,13,17]. However, whether *n*−6 fatty acids mitigate or worsen cardiovascular diseases is controversial [15,18–20]. Some studies attribute the cardioprotective properties of *n*−6 fatty acids to LDL-cholesterol lowering properties [19], while others contradict these conclusions [20] mostly due to the potent pro-inflammatory effects of specific eicosanoids derived from AA [15]. Irrespective of the amount of *n*−6 fatty acids in the diet, there is a growing consensus that incorporation of high levels of ALA and the metabolically more active EPA and DHA in the diet is important for reducing the risks of cardiovascular diseases [13,17].

This review will assess our current understanding of the effects and potential mechanisms of actions of *n*−3 fatty acids on multiple risk factors of metabolic syndrome leading to cardiovascular disease, especially hypertension, dyslipidemia, insulin resistance, oxidative stress, and inflammation.

2. Metabolism of *n*−3 and *n*−6 series fatty acids

The *n*−3 and *n*−6 PUFAs contain the first *cis* double bond between the third and fourth carbon atoms (*n*−3) or the sixth and seventh carbon atoms (*n*−6) from the methyl end of the fatty acids [11]. LA (18:2*n*−6) and ALA (18:3*n*−3) are the parent fatty acids of the *n*−6 and *n*−3 series [11]. Two series of PUFA are derived from either LA or ALA by an alternating series of desaturation and elongation reactions using the same enzyme complexes for both pathways [12] (Fig. 1). The first metabolite of the *n*−6 pathway in mammals is γ-linolenic acid (GLA; 18:3*n*–6) produced by the enzyme Δ^6 desaturase [11,12]. This enzyme acts on ALA to produce stearidonic acid (STA; 18:4*n*−3), the first metabolite of the *n*−3 pathway [11]. The major end-products of *n*−6 and *n*−3 pathways are AA (20:4*n*−6) and DHA (22:6*n*−3), respectively, produced by the action of Δ^5 desaturases [11,12] with EPA (20:5*n*−3) as an important intermediate of the *n*−3 pathway.

The lipoxygenase (LOX) and cyclooxygenase (COX) mediated metabolism of AA and EPA has been the target in the management of most inflammatory diseases. The action of LOX and COX on AA and EPA generates a family of derivatives collectively called eicosanoids which includes prostaglandins (PG), thromboxanes (TX), leukotrienes (LT), epoxides, and oxylipins [21]. The pro-inflammatory series-4 leukotrienes are derived from AA by LOX and series-2 prostaglandins by the action of COX [22]. LOX/COX action on EPA generates the anti-inflammatory series-5 leukotrienes and series-3 prostaglandins and thromboxanes [22] (Fig. 1). Dihomogamma linolenic acid (DGLA), the direct precursor of AA in the *n*−6 fatty acid pathway, competes with both AA and EPA for LOX and COX [23,24]. LOX/COX action on DGLA yields series-1 prostaglandins and thromboxanes that have potent anti-inflammatory, vasodilatory, and anti-aggregatory effects [23,24]. Apart from eicosanoid production, metabolism of EPA and DHA also generates resolution phase interaction products such as E series and D series resolvins, respectively [25–27]. Additionally, DHA metabolism yields protectins such as docosatrienes and maresins, a novel class of macrophage mediators [27,28]. All EPA and DHA-derived resolvins, protectins, and maresins induce pro-resolving actions during an inflammatory response [25–28]. AA and DHA are the major acyl components of structural lipids found in most tissue types [29]. Although the same set of enzymes synthesize AA and DHA from their parent fatty acids, it is now well established that increased dietary ALA inhibits the synthesis of AA from LA [30]. In rats fed equivalent amounts of LA and ALA, there was a 20–40% reduction in LA-derived PUFA incorporation into liver phosphoglycerides, and a higher dietary ALA:LA ratio reduced the LA-derived PUFA incorporation even further [30,31]. The increased dietary intake of ALA decreased the metabolites of the *n*−6 pathway, especially AA, the precursor of the 2-series prostaglandins. The metabolites of *n*−3 series increased, attributed to effective competition of ALA over LA for the Δ^6 desaturase [32]. Furthermore, rats receiving more dietary ALA showed reduced PG synthesis from AA than those receiving LA alone [33,34].

However, chronic dietary intervention as well as stable-isotope studies using ALA show that humans as well as rodents amongst other mammals do not efficiently convert medium chain ALA to long chain EPA or DHA [35–37]. In neonatal rats as well as guinea pigs, DHA concentrations in the brain, retina, liver, and heart remained comparable in animals fed either an ALA-rich diet or a high LA diet [36,37]. In guinea pigs fed an ALA-rich diet, increases in ALA and EPA but not DHA were observed in heart, liver, and adipose tissue [38]. In humans, ALA is converted to EPA in low amounts (∼8%) and in even lower amounts to DHA (<0.1%) [35,39,40]. While most radioactive tracer studies supporting this conclusion have used 5 min infusion and reported little or no newly synthesized EPA and/or DHA [41–43], long chain metabolites of ALA may only appear in the circulation after 60 min of infusion [44]. Also, studies with women and female rats show that females have higher conversion rates than males, an effect mostly attributed to estrogen-mediated up-regulation of Δ^5 and Δ^6 desaturases [45]. Nevertheless, in obese humans, ALA from flaxseed helped maintain plasma EPA and DHA concentrations without dietary fish or sea food and was associated with preventing increases in weight gain, body mass index, and hip circumference [46]. Therefore, it is plausible that the physiological responses with ALA may be independent of its conversion to the longer chain *n*−3 PUFA, particularly DHA. This would imply that the mechanism of action of ALA would also differ from the longer chain compounds. In subsequent sections, the differences in responses as well as the likely mechanisms of action of ALA (18C, medium chain) and the long chain *n*−3 PUFAs (>18C) will be evaluated.

3. *n*−3 Fatty acids on risk factors of metabolic syndrome

3.1. Adiposity and dyslipidemia

The *n*−3 fatty acids reduce the risk for cardiovascular disease partly by improving the blood lipid profile. However, both responses and mechanism of actions of the medium chain and long chain *n*−3 fatty acids appear to be independent. ALA exerts most of its effects by modulating lipoproteins, while EPA and DHA may reduce triacylglycerol (TAG) synthesis and adiposity.

Daily supplementation with 20–50 g ALA-rich flaxseed reduced total cholesterol and LDL-cholesterol concentrations in normolipi-

demic [47–49] as well as hypercholesterolemic patients [50,51]. Ground flaxseed (38–40 g/day) also reduced lipoprotein-A and apolipoproteins A-1 and B in postmenopausal women [52–54]. However, in overweight adults, ingestion of ALA-rich chia seed (50 g/day) for 12 weeks did not affect measures of body composition, lipoproteins, or plasma concentrations of EPA and DHA but increased plasma concentrations of ALA [55]. Medium flaxseed doses (12.5 g/day) prevented the increase in plasma TAG concentrations in hypercholesterolemic rabbits while low doses (0.04–0.2 g/day) prevented the increase in total cholesterol concentrations in LDL receptor-deficient mice [56,57]. In 1% cholesterol-fed rats, dietary supplementation with ALA-rich flax and pumpkin seeds (33% wt/wt) decreased total cholesterol, TAG in plasma and liver, plasma LDL-cholesterol, atherogenic index, and LDL/HDL ratio [58]. In addition, the ALA-rich diet increased PUFA (ALA and LA) and MUFA (oleic and eicosaenoic acids) but decreased SFA (palmitic and stearic acids) in total plasma and hepatic fatty acid content with reduced lipid storage in hepatocytes [58]. However, *n*−3 fatty acid-deprived rats, when given access to 5% flaxseed oil-enriched diet, displayed increased body weight and adipose tissue mass [59].

In patients with type 2 diabetes and mixed hyperlipidemia who were already receiving fluvastatin (80 mg/day), *n*−3 fatty acids (EPA + DHA 4 g/day) reduced TAG, VLDL, and TAG/HDL-cholesterol ratio and increased HDL-cholesterol in blood [60]. In healthy males, fish oil and DHA-oil supplementation (4 g/day) for 15 weeks lowered fasting plasma TAG concentrations and postprandial total chylomicron concentration while increasing the HDL2/HDL3-cholesterol ratio [61]. Increased HDL3 was associated with increased visceral fat deposition and impaired VLDL function [62]. The Look AHEAD (Action for Health in Diabetes) study examined the effects of marine *n*−3 fatty acid intake in individuals with diabetes, its association with adiposity, lipid profile and glucose homeostasis, and its changes with behavioral lifestyle intervention for weight loss in 2397 individuals [63]. Marine *n*−3 fatty acid intake was inversely associated with blood TAG concentrations and weakly associated with HDL-cholesterol concentrations but there was no association with non-HDL-cholesterol or adiposity [63]. In women with polycystic ovary syndrome, consumption of *n*−3 fatty acids (EPA + DHA 4 g/day) over 8 weeks reduced hepatic fat content quantified using proton magnetic resonance spectroscopy [64]. In addition, reductions in blood TAG concentrations and systolic and diastolic blood pressure were observed [64]. In obese individuals, body mass index, waist circumference, and hip circumference were inversely correlated with EPA and DHA intakes [65]. In patients with dyslipidemia, consumption of EPA + DHA (3 g/day) increased HDL-cholesterol and decreased TAG in blood after 3 and 6 months [66]. In another intervention study in patients with metabolic syndrome, purified fish oil *n*−3 fatty acids supplementation for 6 months reduced body weight and serum concentrations of LDL-cholesterol, total cholesterol and TAG at a relatively low dose (180 mg EPA + 120 mg DHA capsule/day) [67]. It has been suggested that the TAG-lowering effects of DHA and EPA may be caused by decreased hepatic TAG secretion and enhanced clearance of TAG from the plasma [61,68].

Ethyl esters of DHA from microalgae (0.6–5.0 g/kg/day) caused dose-dependent decreases in TAG and total cholesterol concentrations in rats fed a high-fructose diet [69]. In Sprague–Dawley rats, feeding of fish oil at 10% of total dietary fat for 3 weeks suppressed postprandial hypertriglyceridemia, increased lipoprotein lipase activity in heart and liver, and reduced hepatic triacylglycerol lipase activity [68]. Although dietary fish oil did not alter the rate of lymphatic absorption of TAG or influence the half-lives of chylomicron or its remnants, secretion of TAG from the liver of rats injected with Triton WR-1339 (an inhibitor of lipoprotein lipase) was lower in the rats fed with DHA [68]. In rats fed either 3% DHA or EPA-supplemented diets, total cholesterol concentrations were lower in the rats fed with DHA-supplemented diet, whereas serum TAG concentrations were lower only in the rats fed with EPA-supplemented diet [70]. In psychologically-stressed female BALB/c mice, serum TAG concentrations were reduced by dietary fish oil (17.6 g of DHA and 7.7 g of EPA per 100 g of fatty acid) [71]. In Zucker rats, both dietary fish and krill oil (0.5% EPA + DHA in the diet) reduced hepatic TAG concentration, but heart TAG concentrations were lower only in krill oil-fed rats [72].

In peroxisome proliferator-activated receptors-α (PPAR-α) null mice, fish oil diet (8% of the total dietary fat) increased hepatic diacylglycerol, TAG, and acyl-CoA [73]. However, in apolipoprotein E (Apo-E)-knockout mice supplemented with 1% dietary fish oil, atherosclerotic plaque formation was not prevented even though concentrations of DHA and EPA were increased in plasma in different forms (free fatty acids, TAG, phospholipids, and cholesteryl ester fraction) [74]. Apo-E is required for clearance of TAG-rich particles and it was therefore suggested that TAG-lowering effects of long chain *n*−3 fatty acids may be due to their Apo-E modulatory properties [74]. Apo-E-knockout mice fed on a fish oil diet showed 40% reduction in thrombin generation in addition to up-regulation of genes contributing to lipid catabolism and down-regulation of genes for γ-glutamyl carboxylase and transcription factors implicated in lipid synthesis [75].

Fish oil, when used at a dose providing 15% of total energy, lowered subcutaneous (inguinal) and visceral (retroperitoneal and epididymal) adipose tissue weights in high fat (33% lard)-fed Wistar rats [76]. Similarly, fish oil supplementation (providing 40% of energy) reduced epididymal [77,78] and retroperitoneal [78] fat in high fat-fed rats. Reduction in fat accumulation with *n*−3 rich diet in rats was associated with reduction in adipocyte hypertrophy [78,79]. In rats fed a high fat cafeteria style diet (30% palmitic and 40% oleic acid) or 20% safflower oil, EPA (1 g/kg/day) [80] and EPA + DHA mixture (5% dietary supplementation) [81] increased leptin gene expression and plasma leptin concentrations with reduced retroperitoneal fat and food intake. However, in high fat-fed C57BL/6J mice (20% dietary flax or corn oil), dietary EPA/DHA (1–12% wt/wt of dietary lipids) reduced mostly epididymal fat by limiting both hypertrophy and hyperplasia of adipocytes with lower EPA/DHA ratio favouring this effect [82].

The prevention of hypertrophy or hyperplasia of adipocytes would ideally have to be followed by increased metabolism of the dietary fat to prevent the accumulation of lipids in the liver or circulation as there is decreased storage of lipids in the adipose tissue. This is usually achieved by increased fatty acid oxidation in liver, intestine, cardiac muscle, and skeletal muscle. Mitochondrial carnitine palmitoyl transferase 1 (CPT1) facilitates fatty acid transport in mitochondria for oxidation in these tissues. The mitochondrial expression of CPT1 is in turn regulated by PPARs [83]. In C57BL/6J mice, feeding of ALA-rich semisynthetic high fat diet (20% flax seed oil) with EPA/DHA (6% EPA, 51% DHA) concentrate was associated with a three-fold stimulation of the expression of genes encoding regulatory factors for mitochondrial biogenesis and oxidative metabolism, mainly PPAR-γ coactivator-1α and nuclear respiratory factor-1 [84]. These effects were associated with decreased lipogenesis in epididymal but not subcutaneous fat [84]. Furthermore, rats fed with a non-purified high fat diet supplemented with 15%, 20% or 44% fish oil had increased heart, skeletal muscle, and adipocyte CPT1 specific activity and tissue capacity, and a lower sensitivity of CPT1 to malonyl-CoA inhibition [84,85]. Malonyl-CoA-mediated inhibition of CPT1 is reduced by malonyl-CoA decarboxylase, which is regulated by AMP kinase [85]. In primary cultured rat adipocytes, the stimulatory effect of EPA on mRNA expression and secretion of visfatin, an adipokine highly expressed in visceral adipose tissue, was dependent on activation of AMP kinase by EPA [86].

An alternative pathway for fat oxidation is mediated by peroxisomal acyl-CoA oxidase, a relatively inefficient pathway for fat oxidation, yielding over 30% more heat and therefore less ATP than mitochondrial β-oxidation [87,88]. Dietary fish oil (40% energy) in Fisher 344 rats induced peroxisomal acyl-CoA oxidase gene expression 2–3 fold in liver, skeletal muscle, and heart [77].

To reduce body fat storage, diet-derived or stored TAGs need to be hydrolyzed into fatty acids for both CPT1 and peroxisomal acyl-CoA oxidase to utilize. Lipoprotein lipase and adipose triacylglycerol lipase play central roles in catalysing the hydrolysis of TAG into fatty acids and monoacylglycerol in lipoproteins, skeletal muscle and adipose tissue, respectively [89,90]. The evidence presented in this review strongly suggests that *n*−3 fatty acids upregulate both these enzymes to exert their hypotriacylglycerolemic and lipolytic effects in the adipocytes. The expression of both lipoprotein lipase and adipose triacylglycerol lipase seems to be regulated by PPAR-α and PPAR-γ [89,91,92]. There is enough evidence to suggest that *n*−3 fatty acids and their metabolites are highly potent ligands for PPAR-α and PPAR-γ [73,93–97]. Even more intriguing is the existence of functional PPAR-α response elements in genes encoding peroxisomal acyl-CoA oxidase [98], CPT [99], HMG-CoA synthase [100], and mitochondrial uncoupling proteins [101]. Furthermore, *n*−3 fatty acids suppressed the hepatic expression of sterol regulatory element-binding protein-1 (SREBP-1), a transcription factor responsible for activating genes involved in fatty acid synthesis, in a PPAR-α dependent process [94]. Therefore, PPARs could be one of the early targets of *n*−3 fatty acids to maintain lipid homeostasis in the body by mechanisms outlined in Fig. 2.

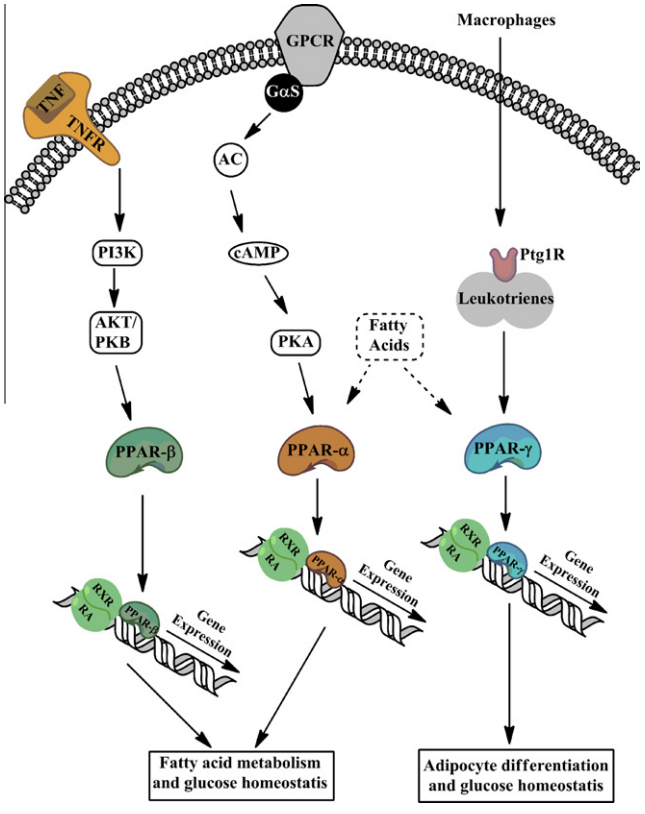

Fig. 2. Control of lipid and glucose metabolism at an expressional level by the *n*−3 fatty acids. TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; PI3K, phosphoinositide 3-kinase; AKT/PKB, thymoma viral proto-oncogene designated as protein kinase B; PPAR, peroxisome proliferator-activated receptors; RXR, retinoid X receptor; RA, retinoic acid; GPCR, G-protein coupled receptor; AC, adenylyl cyclases; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A.

Clearly, enough data has been accumulated over the last decade to understand the mechanisms of action of the long chain *n*−3 fatty acids in reducing adiposity and dyslipidemia. However, the mechanism of action of ALA is not completely understood. Due to the differences in pharmacological response to ALA and EPA/DHA, it is plausible that ALA may exert its effects through alternate mechanisms and this warrants further investigation.

3.2. Glucose homeostasis and insulin resistance

Data with *n*−3 fatty acids for glucose homeostasis and insulin resistance from humans and animals studied are inconsistent. Human studies suggest that none of the nutritionally important *n*−3 fatty acids improve insulin sensitivity. This is probably due to the inverse association of insulin resistance and Δ^5 desaturase activity that could in turn reduce bioavailability of *n*−3 PUFA [102].

In obese, hypertensive patients, dietary fish or fish oil supplementation had no independent effects on insulin or glucose [103,104]. In mildly obese subjects, 15 mL/day of fish oil did not alter the metabolic clearance rates of glucose at plasma insulin concentrations of approximately 100 and 1400 μU/mL during a hyperinsulinemic, isoglycemic clamp [105]. Also, no changes were reported in insulin binding to erythrocytes [105]. In healthy elderly patients, maintaining dietary SFA and MUFA with *n*−6 and *n*−3 PUFA ratio adjusted by replacing *n*−6 PUFA with ALA, EPA, DHA, or a mixture of EPA and DHA produced no differences in fasting blood glucose, fasting insulin, or HOMA-IR [106]. In healthy individuals consuming fish oil (*n*−3 fatty acids; 3.6 g/day), minimal effects on insulin sensitivity, first phase insulin response, and glucose tolerance were reported [107]. In non-diabetic elderly subjects, fish oil capsule supplementation for 6 months (1 g/day, 180 mg EPA and 120 mg DHA) had no effect on insulin sensitivity [108]. In type 2 diabetic adults, neither milled flaxseed nor flaxseed oil (13 g/day for 12 weeks) altered glycemic control [109].

Although the Inuit Health in Transition Study reported an inverse association between erythrocyte membrane EPA and DHA concentrations and the *n*−3/*n*−6 ratio with insulin resistance, the study showed a positive association between ALA concentrations and insulin resistance [110]. However, in young iron-deficient women, an oily fish diet decreased plasma insulin concentrations with improved insulin sensitivity as compared to a red meat diet [111]. In patients with myocardial infarction or unstable ischemic attacks, multiple bioactive lipid components including ceramides, lysophosphatidylcholines and diacylglycerols, which are potential mediators of lipid-induced insulin resistance and inflammation, were decreased in groups fed with fatty fish diet, whereas in the groups fed with lean fish, cholesterol esters and specific long-chain TAG were increased [112].

In contrast, data from animal studies show that the *n*−3 fatty acids strongly reduce insulin resistance and improve glucose tolerance, especially in murine models of metabolic syndrome and type-2 diabetes. ALA deprivation in rats increased adipose tissue mass, plasma glucose and insulin concentrations, and insulin resistance index [59]. Rats deprived of *n*−3 fatty acids given access to flaxseed oil-enriched diet displayed increased body weight and adipose tissue mass, whilst the plasma glucose concentrations and insulin resistance index decreased [59]. In rats fed high fat diets (59% safflower oil), replacement of only 6% of LA from safflower oil with long chain *n*−3 PUFAs from fish oil prevented the development of insulin resistance, especially in liver and skeletal muscle [113]. *n*−3 fatty acid rich fish oil-fed rats (fish oil providing 59% of total energy) showed increased rate of glycolysis and increased glycogenesis compared to an isocaloric *n*−6 fatty acid-rich sunflower oil-fed rats [114]. These effects were accompanied by decreased intramuscular TAG content and increased percent pyruvate dehydrogenase versus tricarboxylic acid cycle flux [114]. In

C57BL/6 mice fed with a 35% corn oil-based high fat diet supplemented with DHA/EPA (replacing 15% dietary lipids), rosiglitazone (10 mg/kg diet) or a combination of DHA/EPA and rosiglitazone exerted additive effects in prevention of obesity, adipocyte hypertrophy, low-grade adipose tissue inflammation, dyslipidemia, and insulin resistance, while inducing adiponectin production, suppressing hepatic lipogenesis, decreasing muscle ceramide concentrations, and improving glucose tolerance [115].

In *ob/ob* mice, dietary intake of fish oil (8% wt/wt) had insulin-sensitizing actions in adipose tissue and liver [116]. Additionally, PPAR-γ, glucose transporters (GLUT) 2 and 4, and insulin receptor substrate-1 (IRS-1)/insulin receptor substrate-2 (IRS-2) genes were up-regulated with increased adiponectin secretion and AMP kinase phosphorylation in *n*−3 fatty acid (EPA/DHA, 6% total dietary lipid)-supplemented mice [116]. In this study, lipidomic analysis showed that *n*−3 PUFA inhibited the formation of *n*−6 PUFA-derived eicosanoids, while triggering the formation of *n*−3 PUFA-derived resolvins and protectins, which mimicked the insulin-sensitizing effects of *n*−3 PUFA [116]. In rats fed 12% canola oil-based diet, supplemented with either ALA, EPA, DHA, or 1:1 EPA/DHA mixture (0.5 g/kg), reductions in fasting blood glucose concentration, fasting plasma insulin concentration, and HOMA-IR were observed with the EPA/DHA mixture being most effective and ALA having the least effect [117]. In high sucrose diet-fed rats, 7% fish oil supplementation reversed the insulin resistance and increased plasma leptin and adiponectin concentrations but not their mRNA expression in the adipose tissue [118].

PPAR-α seems to play a critical role in modulating the effects of at least the long chain *n*−3 PUFAs. Fish oil supplementation (8% of the total dietary fat) in wild-type mice restored hepatic insulin sensitivity, whereas fish oil supplementation in PPAR-α null mice failed to restore hepatic insulin sensitivity [73], suggesting that EPA/DHA in fish oil may improve insulin sensitivity in a PPAR-α-dependent manner. In high fat-fed rats (27% safflower oil), fish oil feeding (8%, 13.5%, and 27% menhaden fish oil) almost exclusively elevated plasma adiponectin concentrations and expression in epididymal adipose tissue [119]. The increase of plasma adiponectin by fish oil was completely blocked by administration of the PPAR-γ inhibitor, bisphenol-A-diglycidyl ether, in wild-type mice but not in PPAR-α null mice [119]. In mice that were given a single oral dose (400 μl) of synthetic TAG, hepatic expression of genes encoding enzymes of the lipid and carbohydrate metabolism pathways was affected most by DHA (519 out of 34000 genes studied) followed by ALA (400), LA (287), EPA (280), and oleic acid (114) [120]. The majority of hepatic genes regulated by dietary unsaturated fatty acids in wild-type mice did not show regulation in PPAR-α-null mice. These results suggest that the effects of dietary unsaturated fatty acids on hepatic gene expression are almost exclusively mediated through PPAR-α [120]. Increased IRS-1 phosphorylation is observed in insulin resistance and obesity with increased c-Jun N-terminal kinase (JNK) activity [121]. In 3xTg-AD transgenic mice, feeding of SFA (16% coconut oil) and *n*−6 PUFA (5% safflower oil)-rich diet increased activity of JNK, phosphorylated IRS-1, and tau protein [122]. Treatment with 2.4% fish oil for 4 months in these mice reduced phosphorylated JNK, IRS-1 and tau, and prevented the degradation of total IRS-1 [122]. DHA also inhibited JNK and the phosphorylation of IRS-1 and tau in cultured hippocampal neurons [122]. PPAR-α suppression induced by a high saturated fat-rich diet induced JNK-dependent IRS-1^{Ser307} phosphorylation and this effect was ameliorated by 3.3% dietary fish oil (EPA, 146 mg/g diet and DHA, 353 mg/g diet) [123].

While PPAR-α and JNK are currently the most promising targets for the *n*−3 PUFAs to decrease blood glucose and insulin resistance, data from last decade suggests that both PPAR-α and JNK are differentially regulated in rodent and human cell lines [124–126]. The primary LOX product of LA, 13-hydroperoxy-9,11-octadecadienoic acid (13-HPODE), activated PPAR-α in rat FaO hepatoma cell line but not in human HepG2 hepatoma cells [124]. PPAR-α agonists such as fenofibric acid and phenylacetic acid up-regulated PPAR-α and associated genes in rat but not in human hepatocytes [125]. Humans, in contrast to rodents, lack functional peroxisome proliferator response elements in PPAR-α regulated genes encoding enzymes such as acyl CoA oxidase [127,128]. This would invariably shift the lipid homeostasis towards β-oxidation and less towards lipogenesis in rodents but less so in humans thereby reducing lipids and lipid-induced insulin resistance in rats. These species differences demonstrate the inconsistencies in the insulin signaling and glucose handling in response to *n*−3 PUFA in rats and humans. These factors have to be carefully considered before extrapolating the results from rodent studies to human trials.

3.3. Hypertension

Essential hypertension, very common in metabolic syndrome, considerably increases the risk of cardiovascular diseases, even in the absence of overt diabetes [129]. The beneficial effects of ALA, EPA, and DHA in reducing hypertension and related cardiovascular remodeling are well-documented. Three meta-analyses of placebo-controlled trials have concluded that the *n*−3 PUFAs (3–15 g/day) in fish oil, mainly EPA and DHA, cause small but clinically relevant reductions in blood pressure (2–5 mmHg) in untreated hypertensive individuals [130–132]. An international cross-sectional epidemiologic study of 4680 men and women aged 40–59 from 17 population-based samples in China, Japan, United Kingdom, and the U.S.A. reported that *n*−3 PUFA intake was inversely related to blood pressure, in both hypertensive as well as normotensive patients [133]. EPA/DHA-rich *n*−3 capsules (4 g/day) lowered blood pressure and heart rate in a double-blind, placebo-controlled intervention in non-diabetic patients with chronic kidney disease stages 3–4 [134]. Purified fish oil *n*−3 fatty acids (180 mg EPA + 120 mg DHA capsule/day) also reduced systolic blood pressure in patients with metabolic syndrome [67]. Reductions in both systolic and diastolic blood pressure have been reported in healthy postmenopausal women, obese subjects as well as dyslipidemic patients after consuming 40 g/day of ALA-rich ground flaxseed or 15–20 g/day of flaxseed oil [135–137]. Similar effects with fish oil have also been reported, where in a double-blind, placebo-controlled, cross-over trial, supplementation with fish oil (1 g capsule/day; 38% DHA, 47% EPA) reduced seated blood pressure by 3.1 ± 1.0/1.8 ± 0.6 mm Hg [138].

A recent study has highlighted the relationship between dietary *n*−3 PUFA deficiency (particularly ALA) and hypertension as early as 24 weeks of age in female rats [139]. In conjunction with this, ALA and fish oil as a source of *n*−3 PUFAs at a large dose range of 0.44–6 g modulated vascular responses induced by angiotensin II infusion [140–142]. Furthermore, prenatal deficiency of dietary *n*−3 PUFAs induced hypertension in Sprague–Dawley rats [143–145]. This was retained in later life even if the concentrations were restored after supplementation of dietary canola oil, a rich source of ALA, as the *n*−3 PUFA source [143].

The antagonistic effects of *n*−3 PUFA on angiotensin II receptors may be responsible for modulation of hypertension (Fig. 3) [146,147]. *n*−3 PUFA play an important role in neural membrane-based receptor systems, such as photo-transduction in the retina [148] which shares common morphology with angiotensin II receptors, both being G protein–coupled receptors belonging to the 7-transmembrane domain superfamily. Increases in COX/LOX metabolites of EPA such as the vasodilative eicosanoids including the thromboxanes could also act as physiological antagonists, counteracting vasoconstriction caused by angiotensin II [149].

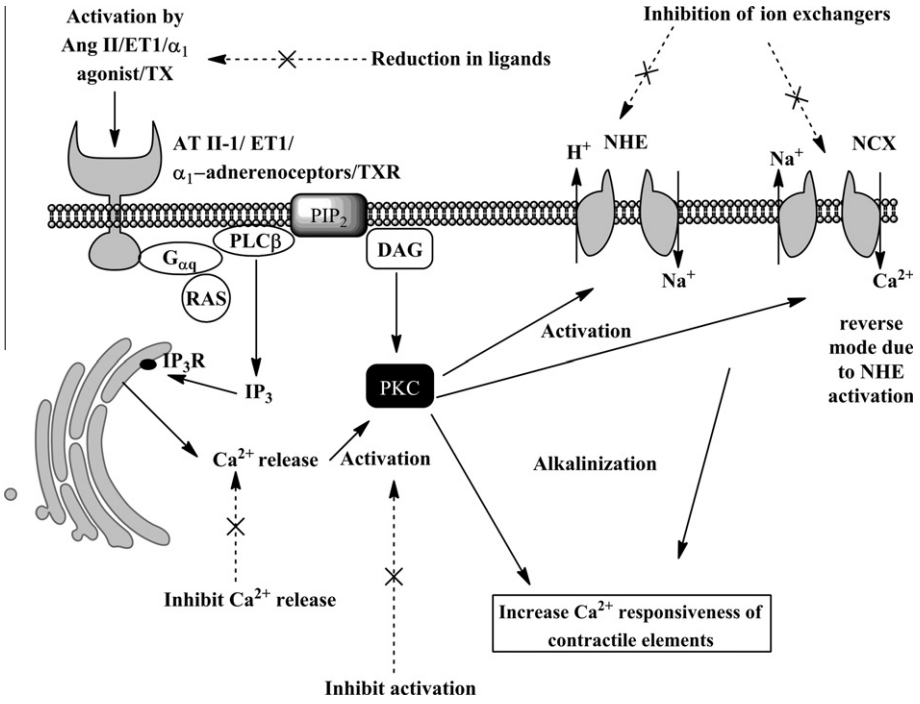

Fig. 3. Possible inhibitory effects of $n-3$ PUFA (shown by dotted arrows) on pathways leading to hypertension. Ang II, Angiotensin II; ET-1, Endothelin-1; TX, thromboxane; AT II-1, Angiotensin II receptor-1; TXR, thromboxane receptor; PLC, phospholipase C; PIP, Phosphatidylinositol phosphate; DAG, diacylglycerol; IP_3, inositol triphosphate; IP_3R, inositol triphosphate receptor; PKC, protein kinase C; NHE, Na^+-H^+ exchanger; NCX, Na^+-Ca^{2+} exchanger.

Furthermore, reasonable evidence exists for an inhibitory role of $n-3$ fatty acids on the renin-angiotensin system, specifically on renin secretion and angiotensin converting enzyme (ACE) activity. In isolated perfused rat kidneys, flaxseed oil feeding as 20% of total energy intake lowered renal venous renin secretion rates [145]. Further, reduced ACE activity as well as expression in the aorta [150] and reduction in blood pressure [151] have been reported in 6 week old Spontaneously Hypertensive Rats (SHR) fed with a 10% ALA-enriched diet. These observations are supported by *in vitro* assays where EPA most effectively inhibited purified ACE activity (EPA > ALA > DHA > GLA > LA > AA) and DHA was most effective in inhibiting cultured leukocyte ACE activity (DHA > EPA > ALA = AA > LA > GLA) [152].

Increased blood pressure may also result from damaged endothelium exhibiting dysfunctional relaxation following mechanical or biochemical insults. Incorporation of EPA and DHA into membrane phospholipids increased systemic arterial compliance [153] as the endothelium of arteries acts as a modulator of vascular tone. Dietary supplementation (10–20% of the diet) of ALA-rich flaxseed protected against the loss of endothelial-dependent vascular relaxation in SHR and cholesterol-fed rabbits [56,154]. Both DHA and EPA relaxed aortic rings isolated from SHR and normotensive Wistar–Kyoto rats in a concentration-dependent and intact endothelium-independent manner [155]. EPA and DHA possibly improved endothelial function by enhancing the release of NO [156]. This effect seems to be due to the lipid and structural modification of caveolae, plasma membrane micro-domains that act as regulators of endothelial NO synthase (eNOS) activity [157].

Early studies with long chain $n-3$ PUFA defined their involvement in the calcium signaling process in contraction of the aorta. In the presence of calcium-free medium, the $n-3$ fatty acids abolished sustained norepinephrine contractions in isolated rat aortic rings but did not reduce the phasic contractions when incubated prior to norepinephrine contraction [158]. This effect may be related to intracellular calcium mechanisms, since both EPA and DHA reversed norepinephrine-induced sustained contractions in the absence of extracellular calcium [158]. The same group also demonstrated that mechanisms of vascular relaxation, such as cyclic nucleotide elevation and calcium antagonism of potential-operated channels, are different from those induced by the $n-3$ PUFAs [159]. DHA and EPA also inhibited α_1-adrenoceptor mediated vascular contractions induced by phenylephrine which were not altered by the non-selective COX inhibitor indomethacin, the potent antioxidant nordihydroguaiaretic acid or by removal of the endothelium [160]. These results indicate the inhibition of vasoconstriction by EPA and DHA is independent of vasodilators such as prostaglandins, nitric oxide or other endothelium-derived vasodilators. It is therefore plausible that $n-3$ PUFAs prevent the increase in blood pressure by decreasing the production of vasoconstrictors.

Unlike SFA, $n-3$ PUFAs reduced the formation of thromboxane A_2, a potent vasoconstrictor, and enhanced the production of PGI_3, a potent vasodilator [161,162]. In addition to this, EPA lowered the tissue concentrations of AA and enhanced those of GLA, the precursor of the endogenous vasodilator, PGE_1 [152]. Collectively, these studies disprove the role of endothelium-derived relaxing factors and cyclic nucleotide second messengers such as cAMP and cGMP in the vasoactive properties of EPA and DHA. The vasorelaxant properties of EPA and DHA may be mediated by a specific inhibition downstream of α-adrenoceptor activation (Fig. 3) [163]. KCl-induced contraction results from voltage-gated channels and norepinephrine-induced contractions through ligand-gated calcium channels. Since both DHA and EPA evoked greater relaxant responses in norepinephrine-contracted vessels than in KCl-contracted vessels [158], it is most likely that the vasorelaxant properties of at least the long chain $n-3$ PUFAs may be intracellularly mediated.

Inhibiting Na^+/K^+–ATPase, the sodium pump, leads to an increase in cytosolic Na^+ concentrations. Cellular Na^+ accumulation raises the cytosolic Ca^{2+} concentration through the involvement of the Na^+/Ca^{2+} exchanger and thereby increases contraction in

vascular smooth muscle or heart muscle. This sequence of events may lead to hypertension [164]. Rats fed a 17% menhaden oil-supplemented diet that contained DHA and EPA exhibited a decrease in cardiac sarcoplasmic reticulum Ca^{2+}-ATPase activity [165]. An indirect inhibition of Na^{+}/Ca^{2+} exchange by DHA and EPA [166] would contribute as one of the mechanisms to lower blood pressure (Fig. 3).

A growing body of evidence highlights the role of connexin 43 (Cx43), one of the major gap junction proteins in heart as well as aorta, in regulating blood pressure. Cx43 activity is down-regulated in protein kinase C (PKC)-independent hypertension models such as SHR [167,168] and nitro-L-arginine methyl ester-induced hypertension [169] and up-regulated in PKC-dependent models of hypertension mediated by angiotensin II [170–173], endothelin-1 [173,174], and α-adrenoceptor activation [175,176] thereby suggesting the vital role of PKC in connexin activation. In SHR, EPA (50 mg/day) and DHA treatment (30 mg/day EPA + DHA mixture), besides reducing the elevated blood pressure, increased Cx43 immunolabeling in endothelium and media with elevated phosphorylation and reduced stimulated aortic NOS activity [167,177]. The increase in functional Cx43 in these rats may therefore be due to increased PKC activity. Inhibiting JNK with SP600125 [anthra(1,9-*cd*)pyrazol-6(2*H*)-one 1,9-pyrazoloanthrone] or siRNA up-regulated Cx43 protein concentrations in the presence of phenylephrine [178]. Also, inhibition of reverse mode Na^{+}/Ca^{2+} exchange with KB-R7943 (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl] isothiourea mesylate) inhibited JNK activation and up-regulated Cx43 protein expression [178].

Furthermore, emerging evidence also suggests that PKC and its various isoforms directly regulate PPAR-α as well as PPAR-γ [179–181]. In COS-1 cells transfected with murine PPAR-α, inhibitors of PKC (Go6976 and HBDDE – α-isoform inhibitors; rottlerin – δ isoform inhibitor and staurosporine – non-specific inhibitor) decreased Wy-14,643-induced PPAR-α activity [180]. Additionally, mutating any of the four consensus PKC phosphorylation sites in PPAR-α (S110, T129, S142, and S179) either prevented heterodimerization of PPAR-α with retinoid X receptor-α, lowered the level of phosphorylation of PPAR-α, or prevented PPAR-α from binding to DNA [180]. Furthermore, PPAR-γ agonists such as rosiglitazone did not induce edema or weight gain in PKC-ß knockout mice [179].

Since PKC is vital for functional PPARs and strong evidence exists for the action of *n*−3 PUFAs through these receptors for lipid as well as glucose homeostasis, it is unlikely that the blood pressure lowering effects of *n*−3 fatty acids are due to inhibition of PKC signaling pathway. The blood pressure lowering effects of *n*−3 PUFAs may to a large extent be attributed to LOX/COX-mediated vasodilative eicosanoids synthesized from dietary or ALA-derived EPA. Additionally, EPA and DHA could have inhibitory effects on intracellular pathways involved in hypertension.

3.4. Oxidative stress

The potential role of oxidative stress as an early event in the pathology of metabolic syndrome and cardiovascular diseases has been widely noted [182]. Oxidative stress may play an important role in the etiology of various risk factors of metabolic syndrome-related manifestations, including atherosclerosis, hypertension, type 2 diabetes, adiposity, and insulin resistance [182]. The role of *n*−3 PUFAs in reducing oxidative damage and restoring free radical homeostasis is not completely understood. Although some studies suggest that ALA, EPA, and DHA may reduce oxidative damage in humans and animals [183–185], the data remain inconclusive.

Currently available data suggests that at least the long chain *n*−3 PUFAs have no role in reducing the biomarkers of oxidative damage. In judo athletes, 6 weeks of EPA + DHA supplementation (600 mg EPA + 400 mg DHA capsule/day) increased exercise-induced oxidative stress defined as increased malondialdehyde, conjugated dienes, and NO concentrations [186]. In exercise-trained men, daily supplementation with 2224 mg EPA + 2208 mg DHA for six months had no effect on antioxidant capacity, oxidized LDL, malondialdehyde, xanthine oxidase activity, and nitrate/nitrite ratio although blood hydrogen peroxide concentrations were reduced in response to exercise [187]. In overweight or obese men consuming a non-purified high-fat, high-fructose diet, acute fish oil (1 g capsule; 400 mg EPA + 200 mg DHA) supplementation had no effect on markers of oxidative stress (lipid hydroperoxides, oxidized-LDL, total antioxidant status) [188]. F2-isoprostanes, the products of free radical-induced peroxidation of membrane-bound arachidonic acid, are considered a reliable biomarker of oxidative stress [189]. In the LIPGENE study in subjects with the metabolic syndrome, 1.24 g/day of DHA supplementation for 12 weeks did not alter urinary concentrations of either 8-iso-PGF2α or 15-keto-dihydro-PGF2α [190]. In patients with normal or slightly elevated total blood cholesterol and TAG concentrations or both, supplementation with krill oil (3.0 g/day, EPA + DHA = 543 mg) or fish oil (1.8 g/day, EPA + DHA = 864 mg) did not change urinary 8-iso-PGF2α concentrations after 7 weeks [189].

Although no evidence exists to show definitively that either of the long chain *n*−3 PUFAs prevents oxidative damage, there is some evidence to suggest that EPA and DHA at least improve anti-oxidant enzyme status in humans. In patients on hemodialysis, EPA + DHA (3 g/day) capsule supplementation for 2 months increased circulating glutathione peroxidase and superoxide dismutase activities in addition to increased ferric reducing antioxidant power and reduced serum malondialdehyde concentrations [191]. In elderly patients chronically exposed to particulate matter, fish oil (2 g/day capsule; 52.4% DHA, 25.0% EPA, and 5.8% DPA) increased superoxide dismutase activity as well as glutathione plasma concentrations [192].

Few human studies have addressed the role of ALA in oxidative stress and therefore this role remains poorly understood. In patients with hypercholesterolemia, 2 months supplementation with wheat germ oil (8.6% ALA) down-regulated CD40L via an oxidative stress-mediated mechanism with *in vitro* experiments showing decreased platelet reactive oxygen species production, reduced activation of NADPH oxidase and phosphorylation of p38 MAP kinase, a protein implicated in the activation of NADPH oxidase [193]. However, this study did not account for the high *n*−6 PUFA or MUFA contents of wheat germ oil.

Down-regulation of the phagocytic and tissue-specific NADPH oxidase subunits (NOX-4, $gp91^{(phox)}$, $p47^{(phox)}$, $p22^{(phox)}$) has also been reported with EPA + DHA administration (0.3 g/kg/day) by gastric gavage for 12 weeks in Sprague–Dawley rats subjected to 5/6 nephrectomy, a model of chronic renal failure [184]. Parallel observations in humans with long chain *n*−3 PUFAs are yet to be made. Male Wistar rats after 30 days of dietary supplementation with fish oil (0.4 g/kg/day) increased erythrocytic catalase activity and decreased serum malondialdehyde and NO concentrations, although no effects on erythrocytic superoxide dismutase and glutathione peroxidase activities were reported [194]. In streptozotocin-induced diabetic rats, treatment with DHA reduced plasma malondialdehyde and increased glutathione concentrations with increased glutathione peroxidase activity [195]. In a rat model of uranyl nitrate-induced nephrotoxicity, dietary fish oil decreased lipid peroxidation, superoxide dismutase activity, glutathione peroxidase activity, and increased catalase activity [196]. In $ApoE^{-/-}$ mice fed on a high fat (non-purified +2% high oleic sunflower oil), high cholesterol chow diet supplemented with either 2% fish oil or DHA oil, hepatic soluble epoxide hydrolase concentrations were lowered by fish oil, but not DHA, compared with high-oleic acid sunflower seed oil-fed rats [197]. In 1% cholesterol-fed rats,

flaxseed and pumpkin seeds supplementation (33% wt/wt) decreased plasma and hepatic malondialdehyde concentrations and improved efficiency of antioxidant enzyme systems [58].

In oligodendroglial OLN-93 cells, DHA, EPA, ALA, LA, AA, oleic or stearic acids or α-tocopherol were added to the culture media to study the effects of PUFA supplements on heat shock protein (HSP) induction, altered cell survival properties and responses to oxidative stress exerted by hydrogen peroxide [198]. Fatty acid supplements caused the up-regulation of heme oxygenase-1 (HO-1) depending on the degree of desaturation (DHA having the highest effect) [198]. However, in Ea.hy 926 endothelial cells, DHA (50 μM) induced reactive oxygen species production in cells, aggravated the lysophosphatidylcholine-induced oxidative stress, and impaired the stimulation of histamine-induced eNOS activation [199]. EPA (90 μM) diminished the deleterious effect of lysophosphatidylcholine as well as the histamine-stimulated eNOS activity, although it did not modify the concentrations of reactive oxygen species produced in the presence or absence of lysophosphatidylcholine or basal eNOS activity or the stimulating effect of histamine on eNOS [199].

Although limited data are available to determine the underlying anti-oxidative mechanisms of *n*−3 PUFAs, it is most likely a consequence of reduction in AA synthesis by *n*−3 PUFAs as discussed earlier since AA has been implicated as a major component in NADPH oxidase activation [200]. Another plausible mechanism is the prevention of eNOS stimulation over the basal level thereby reducing the excess production of nitric oxide.

3.5. Inflammation

The *n*−3 PUFA such as ALA, EPA, and DHA compete with AA as substrates for the formation of pro-inflammatory mediators, such as leukotrienes, prostaglandins, and cytokines as discussed in Section 3.4. Although these pro-inflammatory mediators, in appropriate amounts, are beneficial in response to pathogenic infection, chronic production can be dangerous and is implicated in causing some of the pathological responses that occur in sub-clinical inflammatory conditions [201]. In addition to competitive inhibition of the *n*−6 fatty acid pathway, *n*−3 PUFAs may also inhibit production of inflammatory and fibrotic mediators including C-reactive protein (CRP), interleukins (IL), tumour necrosis factor-α (TNF-α), matrix metalloproteinases (MMP) 2 and 9, and tissue inhibitors of metalloproteinase (TIMPs). Although the roles of cytokines in inflammation have been well-documented [202], the exact role of MMPs in inflammatory conditions is yet to be elucidated even though MMP up-regulation is the hallmark of many inflammatory diseases such as arthritis and atherosclerosis [203].

In exercise-trained men, daily supplementation with 2224 mg EPA + 2208 mg DHA for 6 months reduced resting CRP and TNF-α concentrations and the changes were maintained throughout the exercise [187]. Patients with combined dyslipidemia treated with EPA and DHA for up to 6 months showed decreased plasminogen activator inhibitor-1 (PAI-1), fibrinogen, MMP-2, MMP-9, TIMP-1, TIMP-2, and hsCRP [66]. A study involving randomly selected Japanese employees showed inverse relationships between dietary intake of ALA and serum CRP concentrations but no statistically significant association with either EPA or DHA intake [204]. In healthy middle-aged men receiving 3.5 g/day fish oil (1.5 g/day total *n*−3 PUFA), serum concentrations of cytokines, chemokines or cell adhesion molecules were not affected as compared with placebo [205]. In subjects with metabolic syndrome, 1 g of fish oil capsule supplementation for 6 months reduced CRP and Hsp27 antibody titres [67]. In a cross-sectional study with 5677 men and women from the Multi-Ethnic Study of Atherosclerosis (MESA) cohort, fish intake was inversely associated with plasma concentrations of pro-inflammatory IL-6, MMP-3, and CRP [206]. ALA (flax oil, 14 g/day) decreased IL-6 concentrations over 12 weeks with no other changes in inflammatory cytokines in older adults with ALA supplementation during a resistance-training program [207].

Decreased concentrations of IL-18, an independent risk factor of coronary artery diseases, were positively correlated to increases in serum EPA and DHA concentrations in elderly men at high risk for atherosclerosis treated with 2.4 g/day of EPA + DHA supplement [208]. In patients with coronary heart disease, a fatty fish diet decreased the ratio of AA to EPA in cholesterol ester and phospholipid fractions and this effect was strongly correlated with the change in IL-1 mRNA levels in peripheral blood mononuclear cells [209]. In healthy Dutch elderly subjects consuming either 1.8 g EPA + DHA/day, 0.4 g EPA + DHA/day or 4.0 g high-oleic acid sunflower oil/day, high EPA + DHA intake changed the expression of 1040 genes in human blood mononuclear cells including those involved in nuclear transcription factor-B (NF-κB) signaling and eicosanoid synthesis [210]. Healthy humans placed on fish oil supplement (775 mg EPA/day) showed reduced expression of PI3Kα and PI3Kγ and the quantity of PI3Kα protein in mononuclear cells in addition to reduced capacity of stimulated neutrophils to produce LTB_4 *ex vivo* [211]. Furthermore, fish oil supplements (775 mg EPA/day) reduced the expression of many pro-inflammatory or pleiotropic interleukins including IL-1β, IL-5, IL-10, IL-17, and IL-23 [211]. DHA increased the secretion of IL-1 by cystic fibrosis neutrophils, but did not affect the secretion of other cytokines or AA in both cystic fibrosis as well as normal neutrophils [212]. It is therefore likely that the genetic effects of fish oil supplements result from the EPA fraction. However, DHA may affect the anti-inflammatory effect by inhibiting the generation of pro-inflammatory precursors [212].

In Sprague–Dawley rats subjected to 5/6 nephrectomy produced by surgical resection of the upper and lower thirds of one kidney and removal of the other kidney, EPA + DHA administration (0.3 g/kg/day) by gastric gavage for 12 weeks down-regulated COX-2, PAI-1, transforming growth factor-β (TGF-β), connective tissue growth factor, α-smooth muscle actin, fibronectin, Smad2, and monocyte chemoattractant protein-1 (MCP-1), raised Smad7, and attenuated extracellular signal-regulated kinases (ERK) 1/2 and NF-κB activation [184]. In rats subjected to aortic banding and dietary supplementation with EPA + DHA or ALA at 0.7%, 2.3%, or 7% of energy intake, ALA supplementation had little effect on left ventricular remodeling and dysfunction but EPA + DHA dose-dependently increased EPA and DHA and decreased AA concentrations in cardiac membrane phospholipids, and prevented the increase in left ventricular end-diastolic and -systolic volumes. EPA + DHA resulted in a dose-dependent increase in the anti-inflammatory adipokine, adiponectin. Supplementation with EPA + DHA had anti-aggregatory and anti-inflammatory effects shown by decreases in urinary thromboxane B_2 and serum TNF-α concentrations [213].

DHA and ALA suppressed apoptotic cell death in pancreatic acinar cells exposed to hydrogen peroxide and inhibited the expression of inflammatory cytokines, IL-1β and IL-6 [214]. Macrophages treated with EPA and DHA showed reduced expression of TNF-α, IL-6, and MCP-1 compared to control cells or those treated with SFA [215]. In addition to its anti-inflammatory effects by suppressing LTB_4 [216], ALA suppressed the synthesis of IL-1β, IL-6, and TNF-α [217] probably at transcriptional level as both IL-1 and TNF-α mRNA were reduced with ALA treatment. This effect may account for the beneficial effects of *n*−3 PUFA in models of chronic inflammatory disease [218].

Recent advances have highlighted the role of resolvins derived from *n*−3 PUFA critical to their anti-inflammatory properties [219]. These include E series resolvins (E1 or RvE1) from EPA, D series resolvins (D1 or RvD1), and the neuroprotectins/protectins (PDs) from DHA [219]. The anti-inflammatory effects of resolvins

including reduced TNF-α and IL-13 production and reduced inflammatory cell recruitment have been shown in *in vitro* studies as well as in animal models of inflammation [25,220–223]. Resolvins have been reviewed including their biosynthesis, structures and effects [25,219]. Most mechanisms linking *n*−3 PUFA with suppression of inflammation are associated either with their anti-inflammatory metabolites or the suppression of the *n*−6 fatty acid pathway.

4. Safety and nutrient-gene/nutrient-drug interactions of *n*−3 PUFAs

The *n*−3 PUFAs may interact with other dietary factors to modulate the risk factors of metabolic syndrome. *n*−3 Fatty acid deficiency for 24 weeks with higher dietary protein (30% casein supplementation) induced hypertension in female rats [139]. Co-treatment of oligodendroglial OLN-93 cells with DHA and α-tocopherol suppressed HO-1 up-regulation and restored cell survival whereas treatment with DHA alone induced HO-1 up-regulation [198]. Analysis of the lipid profile demonstrated that co-treatment of OLN-93 cells with DHA and α-tocopherol directly altered the PUFA profile of the cell membranes by increasing *n*−9 fatty acids such as mead acid at the expense of oleic acid [198].

Leptin modulates insulin action, stimulates glucose uptake and fatty acid oxidation in addition to increased insulin secretion via leptin receptors (LepR) present in pancreatic β cells, adipose tissue, and muscle [224–226]. In individuals with metabolic syndrome, low plasma *n*−3 and high *n*−6 PUFA status exacerbated the genetic risk conferred by *LEPR* rs3790433 GG homozygosity to hyperinsulinemia and insulin resistance [227]. This study also reported a negative correlation between *LepR* polymorphisms and hyperinsulinemia and insulin resistance in patients with high *n*−3 and low *n*−6 PUFA [227].

In transgenic fat-1 mice with enhanced endogenous production of *n*−3 PUFA infected with virulent H37Rv *Mycobacterium tuberculosis*, higher bacterial loads and less robust inflammatory responses in lungs increased susceptibility to tuberculosis [228]. Macrophages from fat-1 mice had reduced pro-inflammatory cytokine secretion, impaired oxidative metabolism, and diminished *M. tuberculosis*-lysotracker co-localization within phagosomes and were more readily infected with *M. tuberculosis in vitro*, compared with wild-type macrophages [228]. It has been suggested that *n*−3 PUFAs, widely regarded as possessing anti-inflammatory properties, may also lead to immunosuppression [228].

More studies will be needed to understand interactions of *n*−3 PUFAs with other nutritional factors as well as genetic factors to make informed dietary recommendations on *n*−3 PUFAs. It will also be necessary to address any possible safety and toxicological aspects of *n*−3 PUFAs.

5. Discussion and conclusions

Current evidence strongly suggests that PPARs, JNK, Na^+, K^+-ATPase, and Na^+–Ca^{2+} exchangers are early targets for *n*−3 PUFA to attenuate symptoms of metabolic syndrome (Fig. 4). Although it is unlikely that these are exclusive targets that elicit the

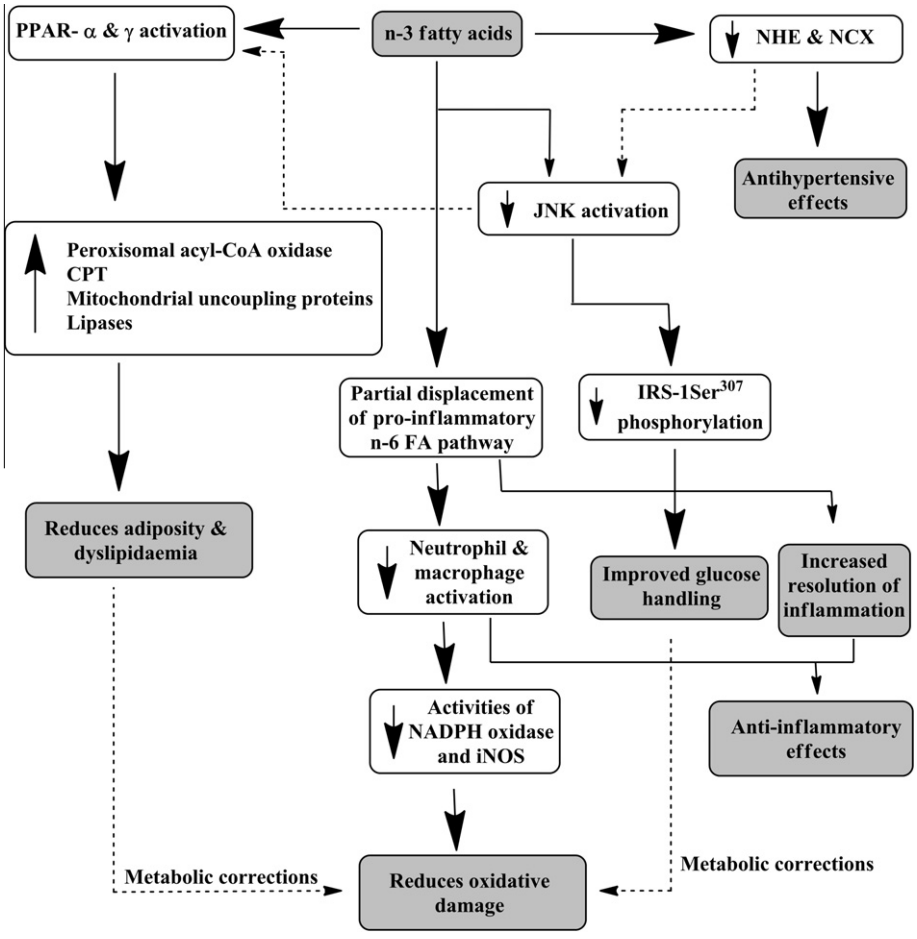

Fig. 4. Mechanism of action of *n*−3 PUFA on risk factors for metabolic syndrome. NHE, Na^+–H^+ exchanger; NCX, Na^+–Ca^{2+} exchanger; CPT, carnitine palmitoyltransferase; IRS, insulin receptor substrate; JNK, c-jun N-terminal kinase.

Table 1
Summary of responses and probable target sites of ALA, EPA and DHA.

	Physiological responses		Probable targets	
Risk Factor	ALA	EPA and DHA	ALA	EPA and DHA
Adiposity	None	• Reduces visceral adiposity • Increases mitochondrial biogenesis and oxidative metabolism	Unknown	Leptin, PPAR-α, PPAR-γ, peroxisomal acyl-CoA oxidase
Dyslipidemia	Reduces total cholesterol, LDL-cholesterol and TAG	• Increases HDL-cholesterolReduces TAG • Increases lipoprotein lipase activity • Reduces TAG lipase activity	Unknown	ApoE up-regulation
Insulin resistance	• None in humans • Animal studies show improved glucose tolerance and insulin resistance index		Unknown	None in humans. Animal studies suggest PPAR-α and JNK as the most likely targets. Inconsistency possibly due to lack of functional peroxisome proliferator response elements in some PPAR-α regulated genes in humans
Hypertension	Reduces systolic blood pressure		• Inhibits renin secretion • Reduces the formation of thromboxane A2	• Inhibits ACE • Structural modification of the blood vessel • Inhibits Na^+/K^+–ATPase and Na^+/Ca^{2+} exchange
Oxidative stress	Data inconclusive	Does not reduce oxidative damage but improves serum antioxidant enzymes levels. Animal studies show reasonable evidence of reducing oxidative damage but also show pro-oxidant responses to EPA and DHA at higher doses	Possible NADPH oxidase inhibition/reduced activation	
Inflammation	Reduces pro-inflammatory cytokines and LOX/COX metabolites of AA		Suppression of the *n*−6 fatty acid pathway as well as pro-resolving actions of resolvins, protectins and maresins derived from EPA and DHA	

therapeutic responses to *n*−3 fatty acids, there is growing consensus that the anti-inflammatory effects of *n*−3 fatty acids are mainly mediated by displacing the pro-inflammatory *n*−6 fatty acid pathway (Fig. 4). It is also possible that the effects of *n*−3 fatty acids on one of the risk factors of metabolic syndrome could mitigate the other risk factors. For example, lipid-lowering effects of *n*−3 fatty acids could also result in improved glucose handling, reduced blood pressure and oxidative stress. Serum free fatty acids upregulate gluconeogenesis in the liver [229], and elevate both insulin resistance and hypertension by inhibiting aortic NOS activity through an oxidative mechanism [230,231]. In addition to reduced NADPH oxidase and iNOS activities due to the anti-inflammatory effects of *n*−3 fatty acids, metabolic correction by reducing blood lipids and blood glucose may contribute to the anti-oxidative properties as well (Fig. 4).

Oxidative stress and inflammation are key and inter-related indications in obesity and metabolic syndrome [232,233]. Reactive oxygen species targeted L-type calcium channels on the sarcolemma and suppressed the Ca^{2+} current [234] thereby increasing the cytosolic Ca^{2+} concentration through mobilization of intracellular Ca^{2+} stores and/or through the influx of extracellular Ca^{2+} [235–237]. Both direct modulation of Ca^{2+} channels by reactive oxygen species as well as reactive oxygen species-dependent increases in vascular intracellular Ca^{2+} primarily via extracellular Ca^{2+} influx have been conclusively demonstrated [237,238]. Therefore, it is plausible that the reduction in oxidative stress by the *n*−3 fatty acids could also result in vasorelaxation. The interplay between various risk factors of metabolic syndrome has to be carefully considered to elucidate the mechanism of action of *n*−3 fatty acids.

It is clear that studies with ALA are insufficient compared to EPA and DHA (Table 1). From the limited available data, physiological responses to ALA differ from EPA and DHA primarily in lipid handling. While ALA has no effect on adiposity and body weight loss, EPA and DHA reduce adiposity, and thereby favor body weight loss. Also, ALA reduced LDL-cholesterol whereas EPA and DHA increased HDL-cholesterol concentrations with increased cardiac and hepatic lipoprotein/TAG lipases. Most of the responses to all three *n*−3 PUFA remain comparable, probably with closely associated mechanisms of action in insulin resistance, hypertension, and inflammation (Table 1). Unlike with ALA, the probable targets of EPA and DHA are seemingly well studied. However, it is still unclear whether there are different molecular targets/pathways with EPA and DHA as most studies employ EPA and DHA mixtures in purified forms as capsules or oily fish diet/supplements. Since the conversion of EPA to DHA is even less efficient than the conversion of ALA to EPA [35], EPA and DHA could, at least in theory, exert their effects through independent mediators. More studies need to be implemented with purified EPA and DHA to differentiate their effects. Considering the prominence of LOX/COX metabolites of AA in the risk factors of metabolic syndrome, we speculate that displacement of AA by EPA contributes to most, if not all, effects of oily fish diets/supplements in alleviating the symptoms of metabolic syndrome, with minimal additional effects from DHA.

Furthermore, since ALA shows higher affinity towards Δ^6 desaturase than LA [35], the physiological responses to ALA could be mediated through the derived EPA but direct responses to ALA remain possible. Evidently, more focused studies with each of the three *n*−3 fatty acids are required to draw firm conclusions on the mechanisms of action of the individual fatty acids. Also, inter-species genetic and metabolic variations have to be carefully considered for animal studies to ensure reliability and reproducibility of the results in humans.

Conflict of interest

No conflicts of interest.

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Appendix D

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Chronic high-carbohydrate, high-fat feeding in rats induces reversible metabolic, cardiovascular, and liver changes

AQ: 1

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AQ: 2 **Poudyal H, Panchal SK, Ward LC, Waanders J, Brown L.** Chronic high-carbohydrate, high-fat feeding in rats induces reversible metabolic, cardiovascular, and liver changes. *Am J Physiol Endocrinol Metab* 302: E000–E000, 2012. First published March 20, 2012; doi:10.1152/ajpendo.00102.2012.—Age-related physiological changes develop at the same time as the increase in metabolic syndrome in humans after young adulthood. There is a paucity of data in models mimicking chronic diet-induced changes in human middle age and interventions to reverse these changes. This study measured the changes during chronic consumption of a high-carbohydrate (as cornstarch), low-fat (C) diet and a high-carbohydrate (as fructose and sucrose), high-fat (H) diet in rats for 32 wk. C diet feeding induced changes without metabolic syndrome, such as disproportionate increases in total body lean and fat mass, reduced bone mineral content, cardiovascular remodeling with increased systolic blood pressure, left ventricular and arterial stiffness, and increased plasma markers of liver injury. H diet feeding induced visceral adiposity with reduced lean mass, increased lipid infiltration in the skeletal muscle, impaired glucose and insulin tolerance, cardiovascular remodeling, hepatic steatosis, and increased infiltration of inflammatory cells in the heart and the liver. Chia seed supplementation for 24 wk attenuated most structural and functional modifications induced by age or H diet, including increased whole body lean mass and lipid redistribution from the abdominal area, and normalized the chronic low-grade inflammation induced by H diet feeding; these effects may be mediated by increased metabolism of anti-inflammatory n-3 fatty acids from chia seed. These results suggest that chronic H diet feeding for 32 wk mimics the diet-induced cardiovascular and metabolic changes in middle age and that chia seed may serve as an alternative dietary strategy in the management of these changes.

AQ: 3 obesity; chia seed; omega-3 fatty acids; metabolic syndrome

METABOLIC SYNDROME is defined as a cluster of risk factors, including type 2 diabetes mellitus, hypertension, dyslipidemia, and central obesity, leading to cardiovascular diseases (3). The worldwide pandemic of metabolic syndrome and, particularly, central obesity has been attributed to increased consumption of lipogenic sugars, such as fructose, together with saturated fats (1, 37). The Western diet, characterized by excessive intake of saturated and *trans* fatty acids and a low n-3-to-n-6 polyunsaturated fatty acid (PUFA) ratio, has been associated with chronic diseases such as metabolic syndrome, renal diseases, and arthritis (2, 12, 24, 36).

Early onset of obesity is a key predictor of type 2 diabetes, dyslipidemia, hypertension, and obesity in young adulthood and middle age (9, 13, 19, 20). Childhood or adolescent obesity is a direct cause of cardiovascular disease in young, middle-aged, and elderly populations and increased all-cause mortality (25). The progression from childhood through middle age to old age is associated with major changes in body composition, including loss of skeletal muscle mass, disproportionate decline in muscle strength, and increased abdominal fat accumulation (18). These effects are accompanied by markedly reduced resting metabolic rate and physical activity and decreased insulin sensitivity, with a shift toward preferential oxidation of carbohydrate over fat (18). This may lead to a sharper decline in skeletal muscle mass and characteristic lipid redistribution, with loss of subcutaneous fat and increased infiltration into lean mass, in the elderly (4, 18).

The high global prevalence of obesity and metabolic syndrome in the >40-yr age group (14, 22, 23, 35), coupled with age-related physiological changes, produces an intricate pathophysiology for the management of metabolic syndrome in old-age groups. Although the etiology and pathophysiology of metabolic syndrome induced by high-carbohydrate, high-fat, or a combination of both have been widely studied in animal models mimicking the young adult human condition (27), there is a paucity of data on physiological adaptations in models mimicking the human middle age started on a high-carbohydrate, high-fat diet during adolescence or young adulthood. Understandably, such data from human studies are sparse because of the need to monitor the subjects over four decades or more.

We previously reported that the chronic changes following a high-carbohydrate (mainly fructose), high-fat (beef tallow) (H) diet for 16 wk in young male Wistar rats mimic the changes in humans, including abdominal obesity, increased total body fat mass, elevated plasma lipids and blood pressure, impaired glucose tolerance and insulin sensitivity, hepatic steatosis, and cardiovascular remodeling, compared with those following a high-carbohydrate (cornstarch), low-fat (C) diet (28). Our previous studies showed that chia seed as a source of dietary α-linolenic acid (C18:3n-3) for 8 wk attenuated most risk factors of metabolic syndrome in young H diet-fed rats (32). Chia seed supplementation induced lipid redistribution, with lipid trafficking away from the abdominal area, with increased bone mineral content, feed conversion efficiency, and total fat-free mass (32). In this study, we have investigated the changes in body composition, fatty acid profiles of major tissues, cardiovascular remodeling, and hepatic structure and function following the administration of a C or H diet for 32 wk and the effects of chronic chia seed supplementation for the final 24 wk of either diet. These rats were ~10 mo of age at the

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end of the protocol and, therefore, could be defined as middle-aged. Male Wistar rats live to ~27–30 mo of age (11), and most animal houses retire male Wistar rats as breeders at ~10–12 mo of age.

MATERIALS AND METHODS

Rats and diets. The experimental groups consisted of 48 male Wistar rats (9–10 wk old) supplied by the University of Queensland Biological Resources unit and individually housed at the University of Southern Queensland Animal House. All experimentation was approved by the Animal Experimentation Ethics Committee of the University of Southern Queensland under the guidelines of the National Health and Medical Research Council of Australia. The rats were randomly divided into four separate groups ($n = 12$ each) and fed the C diet (335 ± 1 g), the C diet + chia seed (CC; 338 ± 1 g), the H diet (336 ± 2 g), or the H diet + chia seed (HC; 333 ± 2 g). All experimental groups were housed in a temperature-controlled, 12:12-h light-dark cycle environment with ad libitum access to water and the group-specific rat diet. Body weight and food and water intakes were measured daily to monitor the day-to-day health of the rats. Feed efficiency (%) was calculated as follows: [mean body weight gain (g)/daily energy intake (kJ)] × 100 (26).

The preparation and macronutrient composition of all four diets, including the fatty acid profiles, are described elsewhere (28, 30, 32). Briefly, the H diet consisted of 175 g of fructose, 395 g of sweetened condensed milk, 200 g of beef tallow, 155 g of powdered rat food, 25 g of Hubble, Mendel-and-Wakeman salt mixture, and 50 g of water per kilogram of diet. In the C diet, fructose and sweetened condensed milk were replaced by the equivalent amount of cornstarch (570 g) and the beef tallow was replaced by equivalent amounts of water (200 g) per kilogram of diet. The chia seed-supplemented diets were prepared by addition of 5% (wt/wt) of the seed replacing an equivalent amount of water in the diet. Chia seed-supplemented diets were administered for 24 wk starting 8 wk after commencement of C or H diet feeding. Drinking water of the H and HC groups was augmented with 25% fructose for the duration of the study.

Cardiovascular measurements. Systolic blood pressure was measured at 0, 8, 16, 24, and 32 wk under light sedation following intraperitoneal injection of Zoletil [tiletamine (15 mg/kg) + zolazepam (15 mg/kg); Virbac, Peakhurst, NSW, Australia] using a piezoelectric pulse transducer (model MLT1010) and inflatable tail cuff

EQ:1 Table 1. *Dietary intake, body composition, anthropometrics, organ wet weights, tissue fatty acid composition, and plasma biochemistry in C, CC, H, and HC groups*

		Group				*P* Value		
	n	C	CC	H	HC	Diet	Treatment	Interaction
Food intake, g/day	10	39.4 ± 0.6*	38.9 ± 0.5*	28.6 ± 1.0†	25.9 ± 0.7‡	<0.0001	0.0337	0.14
Water intake, ml/day	10	24.1 ± 1.4†	36.6 ± 2.9*	23.5 ± 1.1†	30.7 ± 0.9*	0.07	<0.0001	0.14
Chia seed intake, g/day	10	0.0 ± 0.0†	1.9 ± 0.1*	0.0 ± 0.0†	1.3 ± 0.1*	<0.0001	<0.0001	<0.0001
Energy intake, kJ/day	10	442.2 ± 6.4*	475.3 ± 6.5*	593.6 ± 23.3†	583.8 ± 12.2†	<0.0001	0.41	0.13
Feed efficiency, %	10	2.5 ± 0.3‡	6.0 ± 0.4†	6.0 ± 0.8†	7.2 ± 0.6*	0.0002	0.0002	0.047
Body wt gain (8–16 wk), %		23.6 ± 2.0‡	48.7 ± 2.7*	37.0 ± 4.3†	47.3 ± 3.8*	0.08	<0.0001	0.0324
Visceral adiposity index, %	10	6.9 ± 0.8†	6.6 ± 0.4†	11.2 ± 0.8*	7.0 ± 0.4†	0.0007	0.0011	0.0039
Bone mineral content, g	8–10	15.8 ± 0.5†	19.5 ± 0.8*	19.1 ± 0.5*	19.1 ± 0.5*	0.0188	0.0034	0.0034
Total body lean mass, g	8–10	277.1 ± 9.5†	334.6 ± 12.7*	240.8 ± 7.4‡	336.5 ± 7.0*	0.08	<0.0001	0.05
Total body fat mass, g	8–10	185.9 ± 23.5†	260.5 ± 29.0*†	334.7 ± 24.4*	261.9 ± 15.8*†	0.0031	0.97	0.0036
Body fat, %	8–10	37.9 ± 3.4†	41.7 ± 3.2†	55.6 ± 2.1*	42.2 ± 1.8†	0.0025	0.09	0.004
Tissue wet wt, mg/mm tibial length	10							
Retroperitoneal fat		291.5 ± 51.2†	376.2 ± 26.3†	673.4 ± 68.3*	425.2 ± 40.4†	<0.0001	0.1	0.0017
Epididymal fat		227.7 ± 46.7†	249.6 ± 22.1*†	342.4 ± 27.0*	286.5 ± 19.6*†	0.0186	0.58	0.21
Omental fat		171.5 ± 19.9†	194.6 ± 18.1†	327.2 ± 35.7*	152.5 ± 11.3†	0.0187	0.0023	0.0001
Liver		234.6 ± 11.2‡	292.0 ± 5.1†	319.4 ± 11.3*†	337.8 ± 13.8*	<0.0001	0.0013	0.08
Heart		22.2 ± 1.0†	27.1 ± 1.1‡	27.6 ± 1.2‡	28.8 ± 1.5‡	0.006	0.0167	0.14
Tissue lipid content, mg/g	6							
Retroperitoneal fat		767.7 ± 40.9*	690.7 ± 35.9†	726.1 ± 16.2*	662.4 ± 27.1†	0.28	0.0369	0.83
Liver		59.1 ± 10.1	54.7 ± 7.7	74.3 ± 7.1	57.7 ± 2.9	0.22	0.21	0.39
Skeletal muscle		67.5 ± 3.9†	15.8 ± 2.0‡	176.1 ± 23.4*	28.1 ± 5.2‡	<0.0001	<0.0001	0.0008
Heart		23.9 ± 0.8†	32.2 ± 6.2*	23.4 ± 3.2†	45.6 ± 9.5*	0.31	0.0238	0.28
Plasma total cholesterol, mmol/l	10	1.6 ± 0.2*†	1.1 ± 0.05†	1.9 ± 0.2*	1.6 ± 0.1*†	0.0114	0.0114	0.51
Plasma triglyceride, mmol/l	10	0.6 ± 0.1†	1.0 ± 0.1†	0.7 ± 0.1†	1.4 ± 0.2*	0.07	0.0002	0.26
Plasma NEFA, mmol/l	10	2.2 ± 0.3	2.7 ± 0.3	2.5 ± 0.3	3.1 ± 0.3	0.25	0.08	0.87
Plasma fatty acid, g/100 g total fatty acid content	6							
C16:0		52.06 ± 1.36	51.32 ± 2.64	47.76 ± 2.34	42.86 ± 2.74	0.0128	0.24	0.38
C16:1n-7		0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1	1	1
C18:0		15.07 ± 0.60	14.97 ± 1.16	15.87 ± 0.35	15.51 ± 0.88	0.42	0.78	0.87
C18:1n-9		13.25 ± 0.90†	11.84 ± 1.14†	20.86 ± 1.65*	26.14 ± 3.23*	<0.0001	0.33	0.1
C18:2n-6		6.27 ± 0.50‡	12.98 ± 1.20*	7.08 ± 0.49‡	9.34 ± 0.31†	0.06	<0.0001	0.0053
C18:3n-3		0.00 ± 0.00†	3.66 ± 1.47*	0.00 ± 0.00†	2.16 ± 0.30*	0.33	0.0009	0.33
C20:4n-6		13.35 ± 1.12*	5.23 ± 0.46‡	8.44 ± 0.86†	3.99 ± 0.40‡	0.0007	<0.0001	0.027
Total SFA		67.12 ± 1.24	66.29 ± 3.05	63.62 ± 2.50	58.37 ± 3.36	0.0445	0.27	0.42
Total MUFA		13.25 ± 0.90†	11.84 ± 1.14†	20.86 ± 1.65*	26.14 ± 3.23*	<0.0001	0.33	0.1
Total PUFA		19.62 ± 1.01*†	21.87 ± 2.18*	15.52 ± 1.16†	15.49 ± 0.40†	0.0009	0.42	0.41
n-3-to-n-6 ratio		0.00 ± 0.00†	0.21 ± 0.08*	0.00 ± 0.00†	0.16 ± 0.02*	0.55	0.0002	0.55

Values are means ± SE; *n*, number of repetitive experiments. C, high-carbohydrate, low-fat; CC, C diet + chia seed; H, high-carbohydrate, high-fat; HC, H diet + chia seed; NEFA, nonesterified fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid. Means within a row with different symbols (*, †, ‡) are significantly different ($P < 0.05$).

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connected to a physiological pressure transducer (model MLT844) using a PowerLab data acquisition unit (ADInstruments, Sydney, Australia), as previously described (32).

At 32 wk, rats were anesthetized using intraperitoneal Zoletil, as previously described (6, 30), and echocardiographic examination (Hewlett Packard Sonos 5500, 12-MHz transducer) was performed, in accordance with the guidelines of the American Society of Echocardiography, using the leading-edge method (34).

Terminal anesthesia was induced via injection of pentobarbitone sodium (Lethabarb, 100 mg/kg ip). Heparin (200 IU; Sigma-Aldrich Australia, Sydney, Australia) was administered through the right femoral vein, and blood (~5 ml) was collected from the abdominal aorta. After blood withdrawal, the rate of pressure increase (+dP/d*t*) and decrease (−dP/d*t*) and the diastolic stiffness constant were assessed using the Langendorff isolated heart preparation, as previously described (5, 30, 32), and a latex balloon catheter connected to a Capto SP844 MLT844 physiological pressure transducer and Chart software on a Maclab system (ADInstruments Australia and Pacific Islands, Bella Vista, NSW, Australia).

Thoracic aortic rings (~4 mm long) were suspended in an organ bath chamber with a resting tension of ~10 mN. Cumulative concentration-response (contraction) curves were measured for norepinephrine (Sigma-Aldrich Australia); concentration-response (relaxation) curves were measured for acetylcholine (Sigma-Aldrich Australia) and sodium nitroprusside (Sigma-Aldrich Australia) in the presence of a submaximal (70%) contraction to norepinephrine (30).

Oral glucose tolerance and insulin tolerance tests. The oral glucose tolerance test (OGTT) and the insulin tolerance test (ITT) were performed 2 days apart at 32 wk. For the OGTT, basal blood glucose concentrations were measured in blood taken from the tail vein using a Medisense Precision QID glucose meter (Abbott Laboratories, Bedford, MA) after 10–12 h of overnight food deprivation. The rats were given glucose (2 g/kg body wt) as a 40% solution via oral gavage. Tail vein blood samples were taken at 0, 30, 60, 90, and 120 min following glucose administration.

For the ITT, basal blood glucose concentrations were measured after 4–5 h of food deprivation, as described above. The rats were injected with insulin-R (0.75 IU/kg ip; Eli Lilly, West Ryde, NSW, Australia), and tail vein blood samples were taken at 0, 30, 60, 90, and 120 min. Rats were withdrawn from the test if the blood glucose concentrations dropped below 1.1 mmol/l, and 4 g/kg glucose solution was immediately administered by oral gavage to prevent hypoglycemia. For the OGTT and ITT, fructose-supplemented drinking water in the H and HC groups was replaced with normal water for the food-deprivation period.

Body composition measurements. A dual-energy X-ray absorptiometer (DXA; model XR36, Norland) was used for DXA measurements on the rats after 32 wk of feeding, 2 days before rats were euthanized for pathophysiological assessments. DXA scans were analyzed using the manufacturer's recommended software for use in laboratory animals (Small Subject Analysis Software, version 2.5.3/1.3.1, Norland), as previously described (38). The precision error

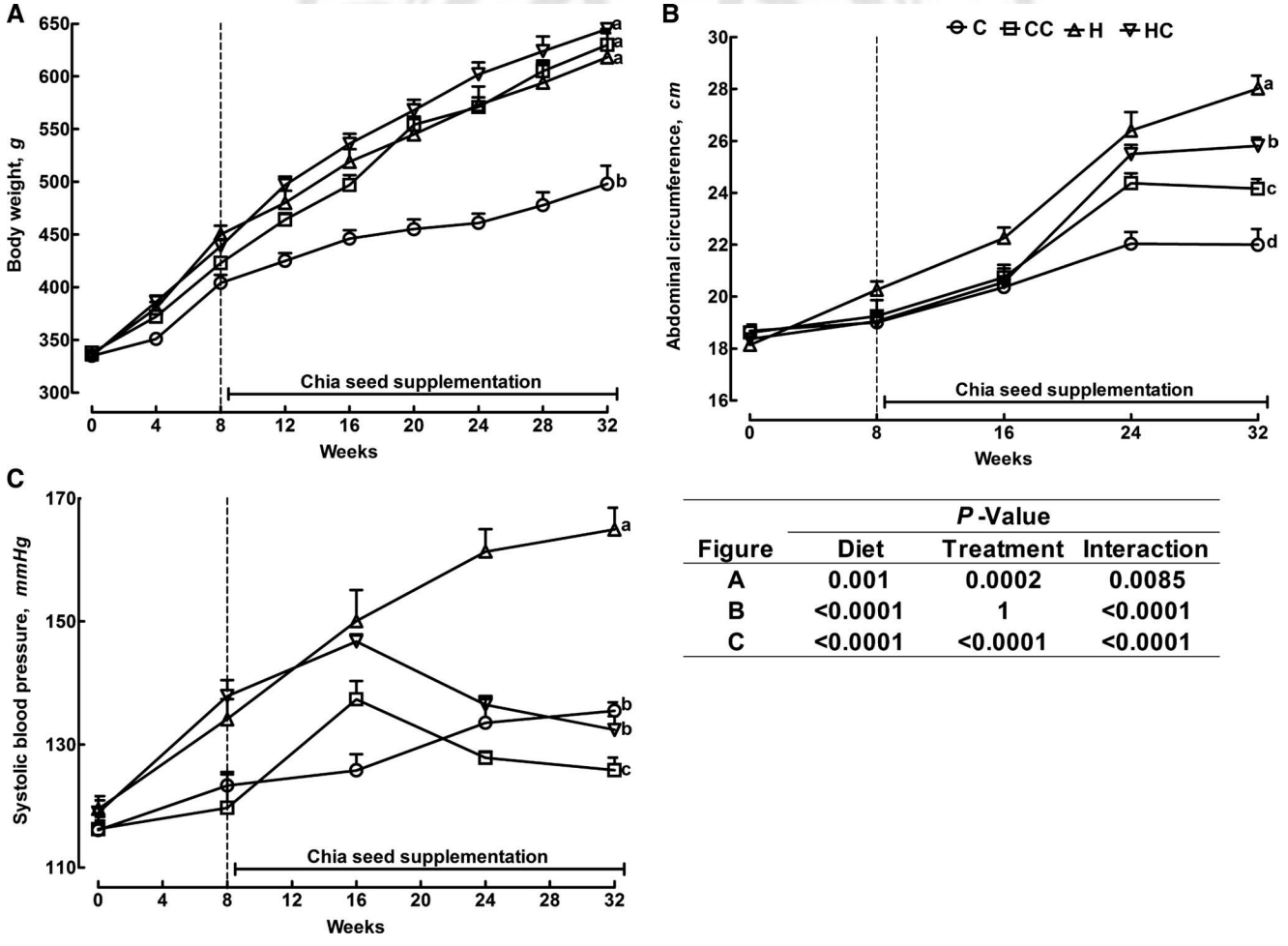

Figure	*P*-Value		
	Diet	Treatment	Interaction
A	0.001	0.0002	0.0085
B	<0.0001	1	<0.0001
C	<0.0001	<0.0001	<0.0001

Fig. 1. Changes in body weight (*A*), abdominal circumference (*B*), and systolic blood pressure (*C*) at 32 wk in rats fed the cornstarch-rich (C) diet, the C diet + chia seed (CC), the high-carbohydrate, high-fat (H) diet, and the H diet + chia seed (HC). Values are means ± SE; $n = 10$ per group. End-point means without a common lower-case letter (a–d) in each data set are significantly different.

of lean mass for replicate measurements with repositioning was 3.2%. Visceral adiposity index (%) was calculated as follows: {[retroperitoneal fat (g) + omental fat (g) + epididymal fat (g)]/body weight (g)} × 100 (17).

Organ weights. The right ventricle and left ventricle (LV, with septum) were separated after perfusion experiments and weighed. Liver, retroperitoneal fat, epididymal fat, and omental fat were removed following heart removal and blotted dry for weighing. Organ weights were normalized relative to the tibial length at the time of their removal (in mg/mm). Immediately after they were weighed, LV, liver, skeletal muscle, and retroperitoneal fat were stored at −20°C in 50-ml polypropylene centrifuge tubes for fatty acid analysis.

Fatty acid analysis. Tissue and dietary lipids were extracted by manual solvent extraction using a 2:1 chloroform-methanol mixture with 0.1% butylated hydroxytoluene as an antioxidant, as described in previous studies (16).

Approximately 15–20 mg of extracted lipid samples, with 1 mg of heptadecanoic acid (C17:0) added as an internal standard, were methylated in a clean 10-ml test tube. Saponified lipids were extracted with 2 ml of heptane and then transferred to an autosampler vial for gas chromatography. Fatty acid methyl esters were analyzed on an Agilent J&W DB-23 column (60 m × 0.25 mm × 0.25 μm; Agilent Technologies) by a gas chromatograph (model GC-17A, Shimadzu) equipped with a flame ionization detector. A multiacid standard mixture was used to check the performance of the gas chromatograph and as a recovery test for the sample preparation procedure. Quantitation of the fatty acids in all samples was based on a linear calibration equation obtained from the C17:0 standard. For identification purposes, standard containing a mixture of 28 fatty acid methyl esters (Nu-Check Prep) was used for retention time calibration. A plot of carbon number vs. logarithm of retention time for the saturated series, one degree of unsaturation, and two degrees of unsaturation allowed a relationship to be developed for identification purposes. All fatty acids are expressed as grams per 100 g of total recovered fatty acids. The n-3-to-n-6 ratio was derived using the following formula: (C18:3n-3 + C20:3n-3 + C20:5n-3 + C22:5n-3 + C22:6n-3)/(C18:2n-6 + C18:3n-6 + C20:2n-6 + C20:3n-6 + C20:4n-6 + C22:2n-6 + C22:4n-6).

Histology. Two rats per group were used exclusively for histological analysis. Two slides were prepared per tissue specimen; two random, nonoverlapping fields per slide were taken to avoid biased analysis. Organs were also collected from rats used for ex vivo studies. Immediately after removal, heart and liver tissues were fixed in 10% neutral buffered formalin for 3 days and then dehydrated and embedded in paraffin wax, as previously described (30). Thin (7-μm) sections of LV and liver were cut and stained with hematoxylin-eosin for determination of inflammatory cell infiltration. Collagen distribution in LV was determined using picrosirius red stain. The extent of collagen deposition in selected tissue sections was determined by laser confocal microscopy (Zeiss LSM 510 upright confocal microscope), with color intensity quantitatively analyzed using ImageJ software (National Institutes of Health) (30).

Plasma biochemistry. Briefly, blood was centrifuged at 5,000 *g* for 15 min within 30 min of collection into heparinized tubes. Plasma was separated and transferred to Eppendorf tubes for storage at −20°C before analysis. Activities of plasma enzymes and analyte concentrations were determined using an Olympus analyzer (model AU 400) and kits and controls supplied by Olympus, as previously described (30).

Statistical analysis. Values are means ± SE. 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. Data from C, CC, H, and HC groups were tested by two-way ANOVA. When interaction and/or the main effects were significant, means were compared using Newman-Keuls multiple-comparison post hoc test. Where transformations did not result in normality or constant variance, a Kruskal-Wallis nonparametric test was performed. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using Prism version 5.00 for Windows (GraphPad, San Diego, CA).

RESULTS

Dietary intake, body parameters, and plasma biochemistry. H rats consumed less food than C rats but equivalent volumes of water throughout the 32 wk (Table 1). Despite the lower food intake, the mean energy intake, feed efficiency, and, therefore, increases in body weight were higher in H than C rats throughout the 32-wk feeding period because of the higher energy density of the H diet (Table 1, Fig. 1*A*). Consequently, chronic H diet feeding increased measures of whole body and abdominal adiposity, such as total body fat mass, abdominal circumference, abdominal fat (retroperitoneal, epididymal, and omental), and visceral adiposity index (Table 1, Fig. 1*B*). The increase in body weight was the combination of an increase in

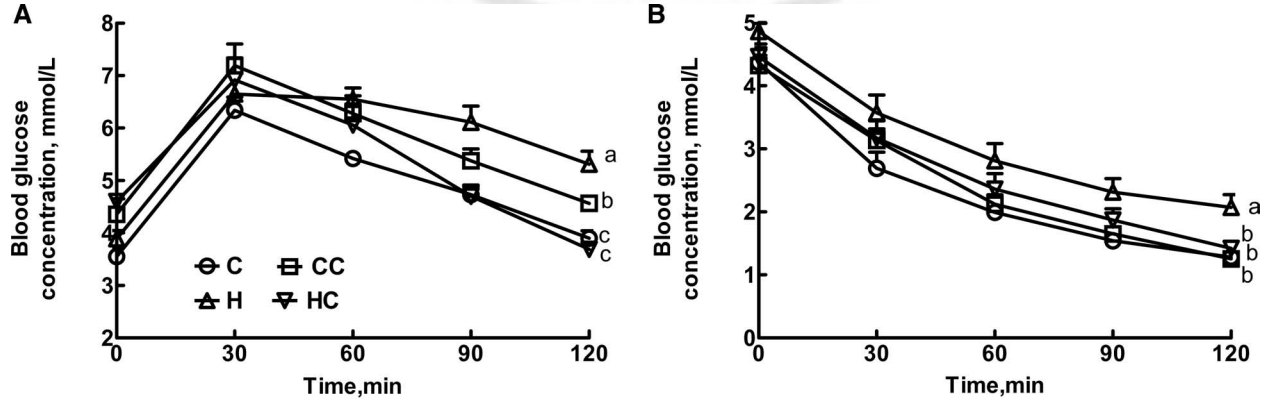

Figure	*P*-Value		
	Diet	Treatment	Interaction
A	0.11	0.0058	<0.0001
B	0.0002	0.0064	0.012

Fig. 2. Glucose (2 g/kg; *A*) and insulin (0.75 IU/kg; *B*) tolerance in C, CC, H, and HC groups. Values are means ± SE; $n = 8$–10 per group. End-point means without a common lower-case letter in each data set are significantly different.

total body fat mass and a decrease in total body lean mass induced by 32 wk of H diet feeding (Table 1). The bone mineral content was higher in H than C rats (Table 1).

Chia seed supplementation for 24 wk, starting at 8 wk of the feeding period, increased and maintained a higher water intake in the CC and HC rats but lowered food intake only in the HC rats compared with their respective controls throughout the feeding period (Table 1). Despite the added dietary energy from chia seed supplementation, the energy intake remained unchanged in CC and HC rats compared with C and H rats, respectively (Table 1). However, chia seed-supplemented rats showed increased feed efficiency and, therefore, increased body weight compared with C rats (Table 1, Fig. 1*A*). These effects were accompanied by increased lean mass in CC and HC rats but increased bone mineral content only in CC rats (Table 1). Chia seed supplementation did not change whole body adiposity but decreased the visceral adiposity index, abdominal fat, and abdominal circumference (Table 1, Fig. 1*B*).

Although H diet feeding for 32 wk did not alter plasma total cholesterol, triglyceride, or nonesterified fatty acid concentrations, chia seed supplementation decreased plasma total cholesterol in CC rats and increased plasma triglycerides in HC rats (Table 1). H diet feeding increased the lipid content of skeletal muscle, but not heart, liver, or retroperitoneal fat (Table 1). Chia seed supplementation decreased the lipid content in retroperitoneal fat and increased it in the heart of CC and HC rats, whereas chia seed supplementation decreased the lipid content in skeletal muscle of HC rats (Table 1). H diet feeding also decreased the tolerance to exogenous glucose and insulin, and these effects were attenuated by chia seed supplementation (Fig. 2). F2

The fatty acid profile of the plasma shows that H diet feeding increased the total plasma monounsaturated fatty acids (MUFA;

Table 2. *Changes in cardiovascular structure, function, and fatty acid composition in C, CC, H, and HC groups*

		Group				*P* Value		
	n	C	CC	H	HC	Diet	Treatment	Interaction
Heart rate, beats/min		316 ± 16	275 ± 16	305 ± 19	266 ± 26	0.61	0.0495	0.96
IVSd, mm	10	1.99 ± 0.05	2.01 ± 0.07	1.99 ± 0.09	1.99 ± 0.05	0.88	0.88	0.88
LVIDd, mm	10	7.34 ± 0.19†	7.82 ± 0.28†	8.77 ± 0.28*	7.84 ± 0.14†	0.0033	0.34	0.0042
LVPWd, mm	10	1.98 ± 0.05	1.97 ± 0.05	2.00 ± 0.05	2.00 ± 0.04	0.6	0.92	0.92
IVSs, mm	10	3.34 ± 0.10	3.14 ± 0.10	3.42 ± 0.14	3.18 ± 0.11	0.6	0.06	0.86
LVIDs, mm	10	3.48 ± 0.24	4.13 ± 0.25	3.98 ± 0.31	3.95 ± 0.21	0.53	0.23	0.19
LVPWs, mm	10	3.39 ± 0.07	3.07 ± 0.12	3.34 ± 0.10	3.25 ± 0.15	0.57	0.08	0.32
Fractional shortening, %	10	52.8 ± 2.4	47.5 ± 1.6	52.3 ± 3.3	49.7 ± 1.9	0.72	0.11	0.58
V_{max}, m/s								
Ascending aorta	10	1.01 ± 0.04†	0.82 ± 0.03‡	1.22 ± 0.06*	0.83 ± 0.05‡	0.0231	<0.0001	0.0378
Descending aorta	10	0.84 ± 0.03†	0.81 ± 0.03†	1.10 ± 0.04*	0.82 ± 0.03†	0.0002	<0.0001	0.0005
Ejection time, ms	10	80.6 ± 3.5	83.7 ± 3.5	87.1 ± 3.3	90.1 ± 3.1	0.06	0.37	0.99
Diastolic volume, μl	10	422.4 ± 32.1†	519.1 ± 56.2†	724.0 ± 61.5*	507.5 ± 27.3†	0.0037	0.21	0.0019
Systolic volume, μl	10	49.8 ± 10.7	81.0 ± 14.5	76.8 ± 18.4	69.0 ± 11.1	0.6	0.41	0.17
Stroke volume, μl	10	372.6 ± 25.8†	438.1 ± 44.4†	647.3 ± 63.9*	438.5 ± 19.1†	0.0024	0.1	0.0024
Estimated LV mass, g	10	1.02 ± 0.05†	1.12 ± 0.06†	1.31 ± 0.07*	1.12 ± 0.03†	0.0116	0.41	0.0116
Cardiac output, ml	10	116.6 ± 8.6†	120.3 ± 14.0†	201.7 ± 26.3*	114.7 ± 9.0†	0.0187	0.0141	0.008
Ejection fraction, %	10	88.8 ± 1.8	85.2 ± 1.4	88.9 ± 3.1	86.8 ± 1.5	0.68	0.18	0.72
Relative wall thickness	10	0.54 ± 0.02	0.52 ± 0.03	0.46 ± 0.02	0.51 ± 0.01	0.0408	0.48	0.11
Systolic blood pressure, mmHg	10	135.4 ± 1.1†	126.7 ± 1.7‡	167.6 ± 3.5*	134.3 ± 2.2†	<0.0001	<0.0001	<0.0001
Systolic wall stress, mmHg	10	70.7 ± 6.5†	86.9 ± 6.9†	101.7 ± 10.2*	83.9 ± 7.0†	0.08	0.92	0.0358
LV developed pressure, mmHg	10	55.9 ± 2.6*	47.6 ± 2.9*†	39.5 ± 1.9†	54.8 ± 5.0*	0.001	0.2971	0.1729
+dP/d*t*, mmHg/s	10	990.3 ± 49.4*	893.2 ± 46.5*†	716.9 ± 32.1†	1016.6 ± 118.7*	0.0077	0.1578	0.2926
−dP/d*t*, mmHg/s	10	−575.0 ± 33.3*	−409.9 ± 26.1*†	−362.3 ± 18.9†	−568.0 ± 61.7*	<0.0001	0.602	0.4838
Diastolic stiffness	10	27.8 ± 0.9†	27.8 ± 0.9†	35.9 ± 1.7*	29.4 ± 1.6†	0.0216	0.0216	0.001
LV + septum wet wt, mg/mm	10	18.8 ± 0.8†	21.5 ± 0.9*†	22.7 ± 1.2*	23.3 ± 0.9*	0.0061	0.1	0.28
RV wet wt, mg/mm	10	3.4 ± 0.3	5.5 ± 0.4	4.9 ± 1.1	5.6 ± 0.6	0.25	0.0485	0.3
Fatty acid, g/100 g of total fatty acid content	6							
C16:0		27.80 ± 1.04*	22.31 ± 0.79†	23.76 ± 1.52†	19.22 ± 0.61‡	0.0028	0.0001	0.66
C16:1n-7		1.74 ± 0.10*	0.00 ± 0.00†	0.63 ± 0.07*	0.00 ± 0.00†	<0.0001	<0.0001	<0.0001
C18:0		18.83 ± 0.50‡	22.70 ± 0.41†	21.63 ± 0.75†	24.90 ± 0.20*	<0.0001	<0.0001	0.56
C18:1n-9		10.62 ± 0.69†	8.05 ± 0.84†	14.18 ± 0.68*	10.42 ± 0.70†	0.0006	0.0003	0.42
C18:1*trans*-7		4.21 ± 0.06*	4.08 ± 0.15*	2.50 ± 0.08†	2.97 ± 0.10†	<0.0001	0.11	0.0087
C18:2n-6		11.01 ± 0.91†	18.21 ± 0.32*	9.04 ± 0.38‡	11.97 ± 0.31†	<0.0001	<0.0001	0.0008
C20:4n-6		16.96 ± 0.33*	12.83 ± 0.35§	15.81 ± 0.49†	14.42 ± 0.29‡	0.56	<0.0001	0.0015
C22:5n-3		0.78 ± 0.20†	3.63 ± 0.18*	1.92 ± 0.16†	3.77 ± 0.24*	0.0041	<0.0001	0.0197
C22:6n-3		7.45 ± 0.33§	8.19 ± 0.40‡	10.53 ± 0.50†	12.33 ± 0.53*	<0.0001	0.0101	0.25
Total SFA		47.24 ± 0.50*	45.01 ± 0.63*†	45.38 ± 0.84*†	44.12 ± 0.46†	0.0398	0.0113	0.45
Total MUFA		16.57 ± 0.75*	12.13 ± 0.88†	17.31 ± 0.73*	13.39 ± 0.73†	0.21	<0.0001	0.74
Total PUFA		36.19 ± 1.14†	42.86 ± 0.56*	37.31 ± 1.02†	42.49 ± 0.79*	0.68	<0.0001	0.42
n-3-to-n-6 ratio		0.30 ± 0.02§	0.38 ± 0.02‡	0.50 ± 0.03†	0.61 ± 0.02*	<0.0001	0.0005	0.52
Stearoyl-CoA desaturase index		0.06 ± 0.00*	0.00 ± 0.00†	0.03 ± 0.00*	0.00 ± 0.00†	<0.0001	<0.0001	<0.0001

Values are means ± SE; *n*, number of repetitive experiments. Left ventricular (LV) + septum and right ventricular (RV) wet weights were normalized against tibial length at the time of removal. IVSd and IVSs, interventricular septal wall thickness at end diastole and end systole; LVIDd and LVIDs, LV internal diameter in diastole and systole; LVPWd and LVPWs, LV posterior wall dimension in diastole and systole; V_{max}, aortic pulse wave velocity; +dP/d*t*, rate of pressure increase; −dP/d*t*, rate of pressure decrease. Means within a row with different symbols (*, †, ‡, §) are significantly different ($P < 0.05$).

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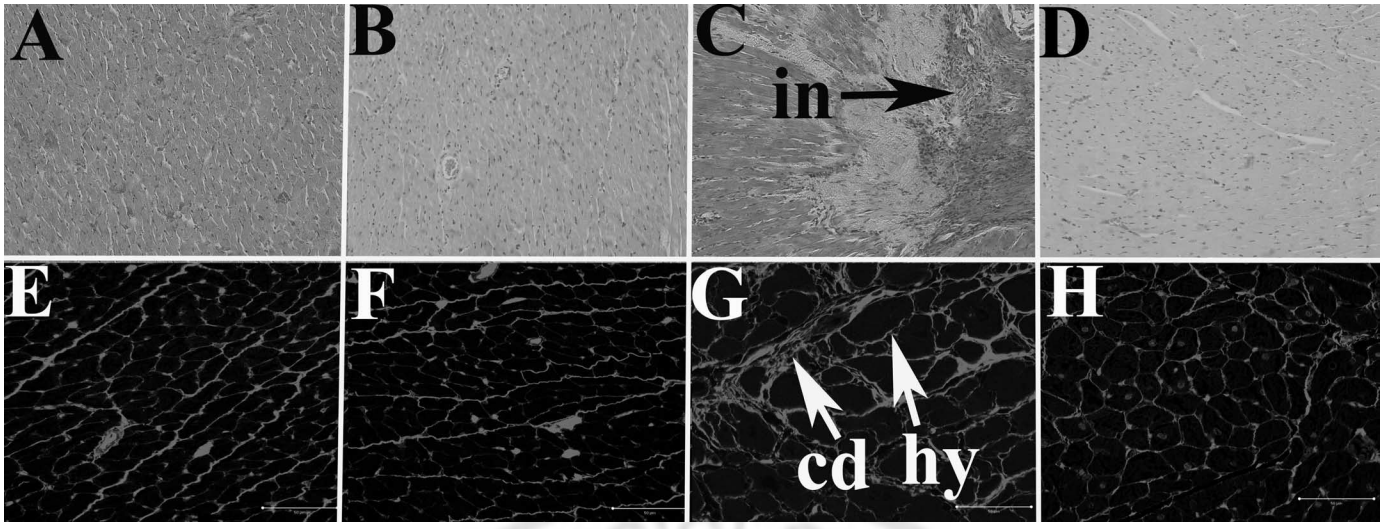

Fig. 3. *A–D*: hematoxylin-eosin-stained sections of left ventricle showing inflammatory cells (in) as dark spots outside the myocytes in C (*A*), CC (*B*), H (*C*), and HC (*D*) groups. Magnification ×20. *E–H*: picrosirius red-stained sections of left ventricle showing interstitial collagen deposition (cd) in C (*E*), CC (*F*), H (*G*), and HC (*H*) groups. Magnification ×40. hy, Hypertrophied cardiomyocytes.

mainly C18:1n-9) without changing the total saturated fatty acids (SFA) and PUFA compared with the C rats (Table 1). Chia seed supplementation increased C18:2n-6 and C18:3n-3 and decreased the proinflammatory C20:4n-6 in the plasma (Table 1).

Cardiovascular structure, function, and fatty acid composition. Compared with C rats, the H diet induced eccentric hypertrophy, characteristic of increased preload, defined as increased LV internal diameter in diastole, without changes in relative wall thickness or end-systolic dimensions (Table 2). Consequently, H rats showed impaired systolic function with increased wall stress, increased diastolic stiffness, decreased developed pressure, and decreased d*P*/d*t* compared with C rats (Table 2). However, fractional shortening, ejection time, and ejection fraction were not affected (Table 2). Additionally, diastolic and stroke volumes and, consequently, cardiac output were elevated in H rats compared with C rats, but heart rate was not affected (Table 2). The ascending and descending aortic pulse wave velocities (V_{max}) increased in H rats compared with C rats, suggesting arterial stiffening (Table 2). These changes were accompanied by increased estimated LV mass and LV wet weight. H diet feeding also increased systolic blood pressure throughout the 32-wk protocol (Fig. 1*C*).

CC and HC rats showed normalized LV volumes and eccentric hypertrophy and, hence, the consequent abnormalities induced by H diet feeding (Table 2). CC and HC rats showed

Table 3. *Changes in hepatic structure, function, and fatty acid composition in C, CC, H, and HC groups*

		Group				*P* Value		
	n	C	CC	H	HC	Diet	Treatment	Interaction
Plasma ALT, U/l	10	24.8 ± 1.0†	19.2 ± 1.6§	34.8 ± 2.7*	29.8 ± 1.0†	<0.0001	0.004	0.86
Plasma AST, U/l	10	109.5 ± 9.3*	67.0 ± 2.3†	78.2 ± 4.9†	75.9 ± 3.9†	0.06	0.0004	0.0012
Plasma LDH, U/l	10	497.2 ± 82.4	317.7 ± 27.3	328.5 ± 46.2	345.1 ± 39.2	0.17	0.12	0.06
Plasma ALP, U/l	10	114.6 ± 6.0*	111.8 ± 5.5*	153.0 ± 16.5†	183.6 ± 1.8‡	<0.0001	0.14	0.08
Bilirubin, μmol/l	10	2.3 ± 0.2*	1.7 ± 0.1†	2.1 ± 0.1*	1.8 ± 0.2†	0.75	0.0073	0.35
Fatty acid, g/100 g total fatty acid content	6							
C16:0		26.08 ± 0.86*	22.71 ± 0.63†	25.80 ± 0.45*	22.89 ± 0.67†	0.94	0.0001	0.73
C16:1n-7		5.11 ± 0.81*	4.82 ± 0.80*	1.66 ± 0.25†	1.64 ± 0.18†	<0.0001	0.8	0.82
C18:0		10.83 ± 1.42	8.92 ± 1.34	8.50 ± 1.05	10.95 ± 0.39	0.9	0.81	0.07
C18:1n-9		31.31 ± 2.02‡	21.56 ± 1.07§	50.08 ± 2.84*	39.03 ± 1.05†	<0.0001	<0.0001	0.74
C18:1*trans*-7		4.19 ± 0.12*	3.81 ± 0.32*	1.58 ± 0.22†	1.87 ± 0.10†	<0.0001	0.83	0.13
C18:2n-6		7.91 ± 0.62†	13.06 ± 0.74*	4.44 ± 0.38‡	7.64 ± 0.47†	<0.0001	<0.0001	0.1
C18:3n-3		0.00 ± 0.00‡	8.73 ± 0.93*	0.00 ± 0.00‡	1.66 ± 0.10†	<0.0001	<0.0001	<0.0001
C20:4n-6		10.67 ± 1.43*	4.83 ± 0.45†	4.86 ± 1.13†	5.05 ± 0.37†	0.0084	0.0079	0.005
C20:5n-3		0.00 ± 0.00‡	3.29 ± 0.13*	0.00 ± 0.00‡	1.65 ± 0.14†	<0.0001	<0.0001	<0.0001
C22:5n-3		0.00 ± 0.00‡	3.35 ± 0.23*	0.00 ± 0.00‡	1.41 ± 0.12†	<0.0001	<0.0001	<0.0001
C22:6n-3		3.09 ± 0.41†	4.76 ± 0.42*	1.69 ± 0.77‡	4.55 ± 0.27*	0.12	0.0002	0.25
Total SFA		37.73 ± 1.16*	31.81 ± 1.82†	35.68 ± 0.74*†	34.78 ± 0.95*†	0.71	0.0121	0.06
Total MUFA		40.61 ± 2.76†	30.18 ± 1.95‡	53.32 ± 2.83*	42.53 ± 1.06†	<0.0001	0.0001	0.94
Total PUFA		21.67 ± 2.21†	38.01 ± 1.39*	10.99 ± 2.17‡	22.69 ± 1.08†	<0.0001	<0.0001	0.21
n-3-to-n-6 ratio		0.16 ± 0.01‡	1.14 ± 0.06*	0.15 ± 0.06‡	0.70 ± 0.02†	<0.0001	<0.0001	<0.0001
Stearoyl-CoA desaturase index		0.19 ± 0.03*	0.21 ± 0.04*	0.07 ± 0.01†	0.07 ± 0.01†	0.0002	0.73	0.73

Values are means ± SE; *n*, number of repetitive experiments. ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase. Means within a row with different symbols are significantly different ($P < 0.05$).

normalized diastolic stiffness and systolic wall stress. Chia seed supplementation normalized systolic blood pressure after 12 wk of treatment (Fig. 1*C*) compared with H rats and decreased V_{max} measured at the end of the protocol (Table 2).

After 32 wk, H rats showed greater infiltration of inflammatory cells into the LV (Fig. 3*C*), as well as increased interstitial collagen deposition (Fig. 3*G*), compared with C rats (Fig. 3, *A* and *E*). Chia seed supplementation normalized inflammatory state (Fig. 3*D*) and markedly reduced collagen deposition in HC rats (Fig. 3*H*). Minimal changes were seen in CC rats (Fig. 3, *B* and *F*). The reduction in LV fibrosis and inflammation was consistent with the reduced diastolic stiffness in HC rats (Fig. 3, *D* and *H*, Table 3).

Furthermore, H diet feeding diminished endothelium-dependent relaxation to acetylcholine (Fig. 4*C*) but did not affect the endothelium-independent relaxation to sodium nitroprusside (Fig. 4*B*) or the α_1-adrenoceptor-mediated vascular contraction to norepinephrine in isolated thoracic aortic rings compared with C rats (Fig. 4*A*). Although chia seed supplementation did not change α_1-adrenoceptor responses, it improved endothelium-dependent and -independent relaxation in isolated thoracic aortic rings (Fig. 4). This effect may contribute to the blood pressure-lowering response to chia seeds.

Similar to the plasma fatty acid profile, H rats showed increases in the proportion of total MUFA compared with C rats but maintained the proportions of SFA and PUFA in the heart (Table 2). Chia seed supplementation decreased C16:0, C16:1n-7, and C18:1n-9 but increased C18:0 (Table 2). CC and HC rats accumulated C18:2n-6 and depleted its elongation product, C20:4n-6, and increased elongation products of C18:3n-3 (C20:5n-3 and C22:6n-3). These results strongly suggest the preferential metabolism of the n-3 PUFA by Δ^6- and Δ^5-desaturases, thereby inhibiting the proinflammatory n-6 metabolism pathway.

Hepatic structure, function, and fatty acid composition. H rats showed elevated plasma alanine transaminase and alkaline phosphatase activities compared with C rats (Table 3). However, aspartate transaminase activity was elevated in the C rats, and plasma lactate dehydrogenase activity and total bilirubin concentration remained unchanged (Table 3). Chia seed supplementation normalized plasma activities of alanine transaminase, aspartate transaminase, and lactate dehydrogenase, while alkaline phosphatase activity was increased in HC rats, and CC and HC rats showed reduced plasma bilirubin concentrations (Table 3). Additionally, H rats showed increased inflammatory cell infiltration (Fig. 5*C*) and lipid deposition (Fig. 5*G*) com-

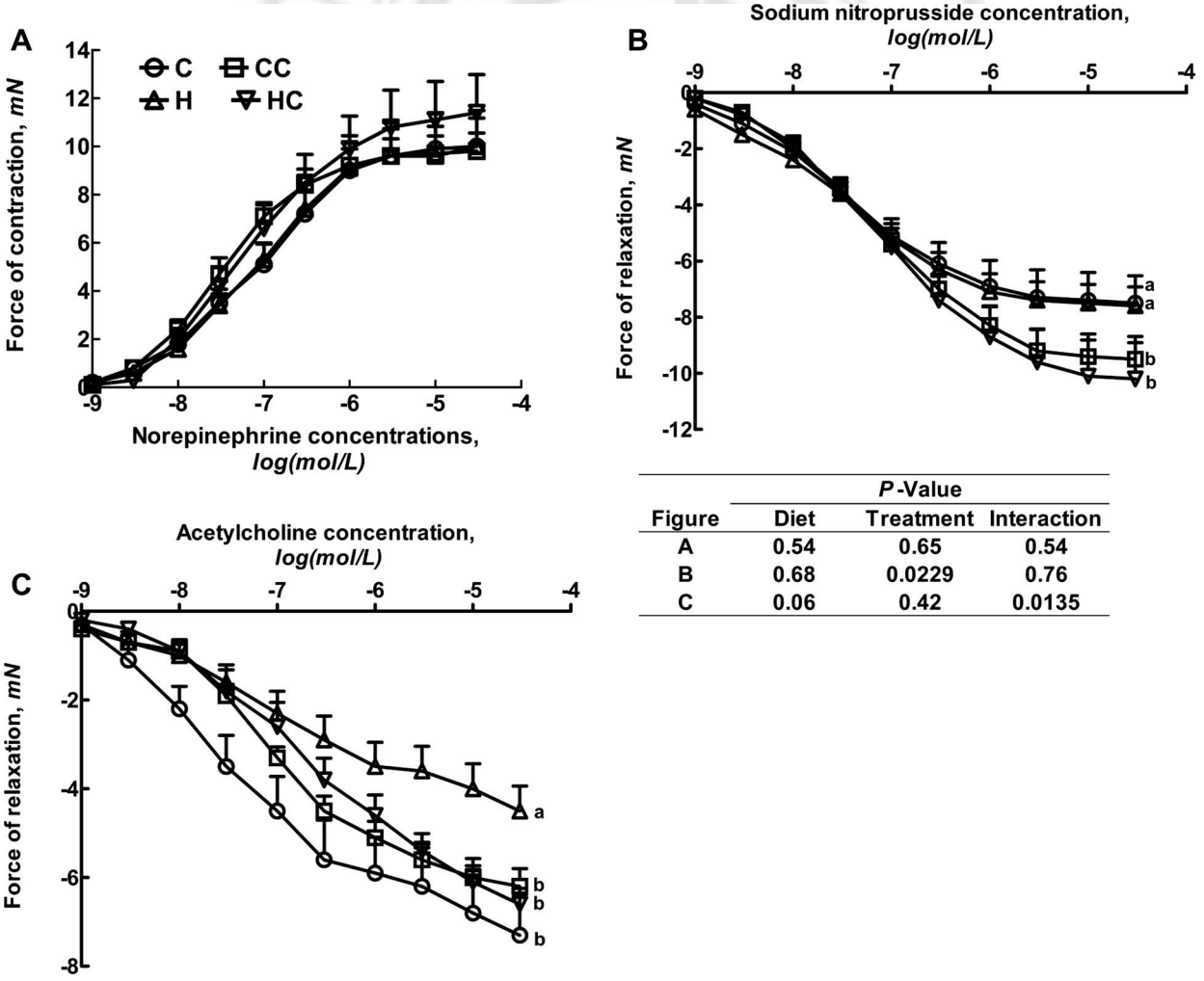

Figure	*P*-Value		
	Diet	Treatment	Interaction
A	0.54	0.65	0.54
B	0.68	0.0229	0.76
C	0.06	0.42	0.0135

Fig. 4. Cumulative concentration-response curves for norepinephrine (*A*), sodium nitroprusside (*B*), and acetylcholine (*C*) in thoracic aortic rings from C, CC, H, and HC groups. Values are means ± SE; n = 8–10/group. End-point means without a common lower-case letter in each data set are significantly different.

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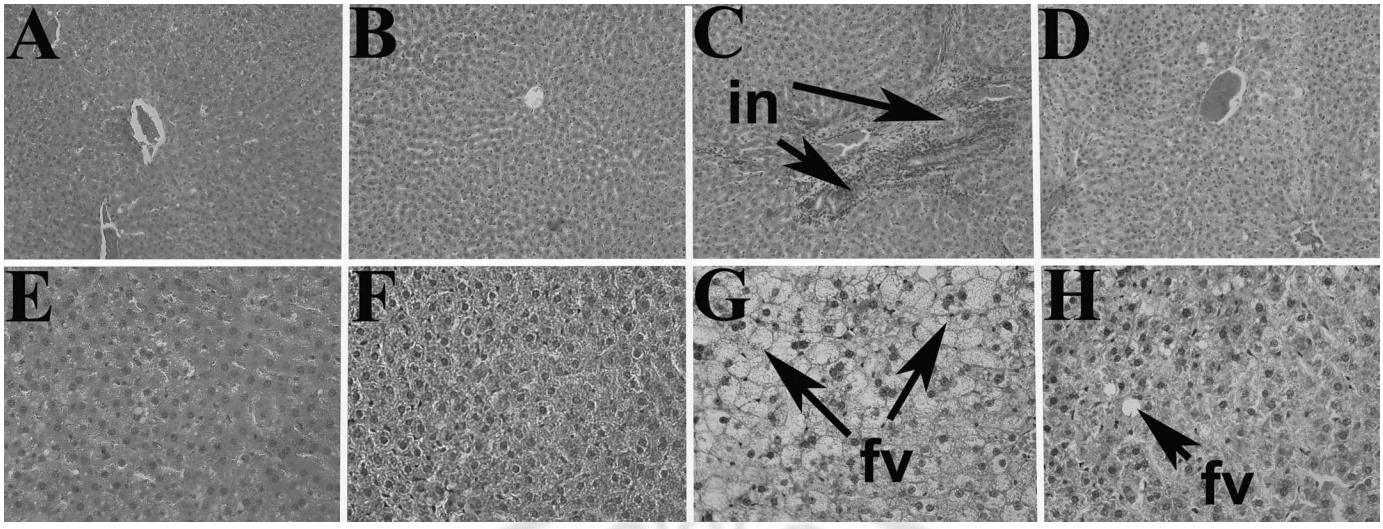

Fig. 5. Hematoxylin-eosin staining of hepatocytes showing inflammatory cells (in) around the portal region in C (*A*), CC (*B*), H (*C*), and HC (*D*) groups and hepatocytes with enlarged fat vacuole (fv) in C (*E*), CC (*F*), H (*G*), and HC (*H*) groups. Magnification ×20.

pared with C rats (Fig. 5, *A* and *E*). CC (Fig. 5, *B* and *F*) and HC (Fig. 5, *D* and *H*) rats showed normalized macrovesicular steatosis and portal inflammation.

H diet feeding increased the total MUFA (mainly C18:1n-9) content and decreased the total PUFA (C18:2n-6 and C20:4n-6) content in liver compared with C rats (Table 3). Chia seed supplementation decreased the MUFA content and increased C18:2n-6, C18:3n-3, C20:5n-3, C22:5n-3, and C22:6n-3, consequently increasing the n-3-to-n-6 ratio (Table 3). C20:4n-6 decreased only in the CC rats (Table 3).

Adipose tissue and skeletal muscle fatty acid composition. Similar to the fatty acid profile in liver and heart, H diet feeding increased C18:0 and C18:1n-9 and decreased C16:1n-7 and C18:2n-6 content in adipose tissue compared with C rats (Table 4). Chia seed supplementation decreased C16:0 and C18:0 in HC rats and C16:1n-7 and C18:1n-9 in CC rats. These effects were accompanied by increased proportions of C18:2n-6 and C18:3n-3 in adipose tissue (Table 4).

Similarly, the skeletal muscle of H rats had increased proportions of C18:1n-9 and decreased proportions of C18:2n-6 (Table 5). Chia seed supplementation increased SFA (C16:0 and C18:0) and n-6 fatty acids (C18:2n-6 and C20:4n-6) and decreased C18:1n-9 in skeletal muscle. C18:3n-3, C22:5n-3, and C22:6n-3 were increased in the chia seed-supplemented groups (Table 5).

DISCUSSION

We have shown that long-term C or H diet feeding in male Wistar rats closely mimics the changes in normal middle-aged humans and the pathophysiology of middle-aged humans with metabolic syndrome, respectively. Compared with our previous studies of young rats fed a C diet for 16 wk (28, 32), chronic C diet feeding for 32 wk induced age-dependent physiological changes without metabolic syndrome, such as disproportionate increases in total body lean and fat mass, reduced bone mineral content, cardiovascular remodeling with increases in systolic blood pressure, LV, and arterial stiffness, and increased plasma markers of liver injury. Compared with C diet feeding, H diet feeding for 32 wk induced increases in visceral adiposity with reduced lean mass, impaired glucose tolerance and insulin sensitivity, and increased LV and arterial

Table 4. *Fatty acid profiles of retroperitoneal adipose tissue and desaturase index in C, CC, H, and HC groups*

	Group				*P* Value		
	C	CC	H	HC	Diet	Treatment	Interaction
C14:0	1.56 ± 0.04‡	1.36 ± 0.08§	2.20 ± 0.02*	1.95 ± 0.06†	<0.0001	0.0005	0.65
C14:1n5	0.15 ± 0.00*	0.00 ± 0.00†	0.14 ± 0.03*	0.00 ± 0.00†	0.74	<0.0001	0.74
C16:0	24.57 ± 0.46*	23.71 ± 0.73*	21.01 ± 0.24†	18.67 ± 0.22‡	<0.0001	0.0024	0.12
C16:1n-7	9.37 ± 0.43*	5.77 ± 1.10†	2.18 ± 0.15‡	2.23 ± 0.15‡	<0.0001	0.0078	0.0064
C18:0	2.29 ± 0.11‡	2.56 ± 0.08‡	7.07 ± 0.32*	5.96 ± 0.30†	<0.0001	0.08	0.007
C18:1n-9	46.92 ± 0.40†	33.98 ± 0.54‡	63.04 ± 0.44*	62.91 ± 0.72*	<0.0001	<0.0001	<0.0001
C18:1*trans*-7	5.41 ± 0.14*	4.15 ± 0.10†	0.86 ± 0.28‡	0.60 ± 0.29‡	<0.0001	0.0024	0.0336
C18:2n-6	9.23 ± 0.40*	16.54 ± 0.59*	3.00 ± 0.11§	5.50 ± 0.14‡	<0.0001	<0.0001	<0.0001
C18:3n-3	0.44 ± 0.03‡	11.94 ± 0.29*	0.00 ± 0.00§	2.19 ± 0.05†	<0.0001	<0.0001	<0.0001
Total SFA	28.42 ± 0.55†	27.63 ± 0.84†	30.78 ± 0.50*	26.57 ± 0.49†	0.3	0.0006	0.0112
Total MUFA	61.85 ± 0.29†	43.90 ± 0.75‡	66.22 ± 0.49*	65.73 ± 0.59*	<0.0001	<0.0001	<0.0001
Total PUFA	9.73 ± 0.46*	28.47 ± 0.70*	3.00 ± 0.11§	7.69 ± 0.13‡	<0.0001	<0.0001	<0.0001
n-3-to-n-6 ratio	0.05 ± 0.00‡	0.73 ± 0.03*	0.00 ± 0.00‡	0.40 ± 0.02†	<0.0001	<0.0001	<0.0001
Stearoyl-CoA desaturase index	0.39 ± 0.02*	0.25 ± 0.06†	0.10 ± 0.01‡	0.12 ± 0.01‡	<0.0001	0.08	0.0227

Values are means ± SE. Means within a row with different symbols are significantly different ($P < 0.05$).

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Table 5. *Fatty acid profiles of skeletal muscle and desaturase index in C, CC, H, and HC groups*

	Group				*P* Value		
	C	CC	H	HC	Diet	Treatment	Interaction
C14:0	1.73 ± 0.06†	1.41 ± 0.10‡	2.43 ± 0.10*	1.95 ± 0.06†	<0.0001	<0.0001	0.34
C14:1n-5	0.00 ± 0.00†	0.00 ± 0.00†	0.20 ± 0.02*	0.00 ± 0.00†	<0.0001	<0.0001	<0.0001
C16:0	25.44 ± 0.35*†	27.33 ± 0.33*	21.74 ± 0.84‡	24.04 ± 1.05†	<0.0001	0.0082	0.78
C16:1n-7	8.73 ± 0.69*	6.60 ± 0.80†	2.91 ± 0.30‡	2.50 ± 0.22‡	<0.0001	0.0346	0.14
C18:0	3.32 ± 0.21‡	8.38 ± 0.67†	7.13 ± 0.66†	10.26 ± 0.62*	<0.0001	<0.0001	0.11
C18:1n-9	44.42 ± 0.28†	22.97 ± 0.83‡	57.99 ± 2.07*	42.14 ± 2.79†	<0.0001	<0.0001	0.13
C18:1*trans*-7	5.50 ± 0.09*	3.88 ± 0.07†	1.23 ± 0.33§	2.17 ± 0.04‡	<0.0001	0.07	<0.0001
C18:2n-6	9.65 ± 0.35†	14.28 ± 0.62*	4.01 ± 0.37§	8.19 ± 0.38‡	<0.0001	<0.0001	0.62
C18:3n-3	0.00 ± 0.00‡	5.48 ± 0.29*	0.28 ± 0.03‡	1.91 ± 0.14†	<0.0001	<0.0001	<0.0001
C20:4n-6	1.22 ± 0.14†	2.81 ± 0.38*	0.82 ± 0.42†	2.32 ± 0.37*	0.21	0.0002	0.9
C22:5n-3	0.00 ± 0.00‡	2.18 ± 0.19*	0.00 ± 0.00‡	1.24 ± 0.17†	0.0015	<0.0001	0.0015
C22:6n-3	0.00 ± 0.00†	4.12 ± 0.37*	0.00 ± 0.00†	3.27 ± 0.60*	0.24	<0.0001	0.24
Total SFA	30.48 ± 0.38†	37.12 ± 0.39*	31.80 ± 1.43†	36.26 ± 1.52*	0.83	<0.0001	0.32
Total MUFA	58.64 ± 0.53*	33.45 ± 1.58‡	63.09 ± 2.15*	46.80 ± 2.84†	0.0002	<0.0001	0.0349
Total PUFA	10.88 ± 0.46‡	29.43 ± 1.25*	5.11 ± 0.73§	16.94 ± 1.39†	<0.0001	<0.0001	0.0039
n-3-to-n-6 ratio	0.00 ± 0.00§	0.73 ± 0.03*	0.07 ± 0.01‡	0.60 ± 0.03†	0.18	<0.0001	0.0002
Stearoyl-CoA desaturase index	0.34 ± 0.03*	0.24 ± 0.03†	0.14 ± 0.02‡	0.11 ± 0.01‡	<0.0001	0.0135	0.16

Values are means ± SE. Means within a row with different symbols are significantly different ($P < 0.05$).

stiffening, cardiac fibrosis, hypertension, and hepatic steatosis. These results are consistent with the effects of 16 wk of H diet feeding (28), except 32 wk of H diet feeding additionally induced arterial stiffening and loss of lean mass and increased fat storage typical of the aging process (18).

The cardiovascular remodeling with the H diet aggravates the typical characteristics of a middle-aged human heart. Age-dependent structural deterioration of the heart includes LV, as well as cardiomyocyte, hypertrophy, increased collagen deposition, and stiffening of the ventricular wall (15). The functional manifestation of these changes includes impaired diastolic function without alterations of systolic function, with preserved fractional shortening, ejection fraction, stroke volume, cardiac output, and resting heart rate (15). In addition to these changes, H diet feeding increased diastolic and stroke volume while maintaining resting heart rate and systolic volume, thereby increasing cardiac output. These increases in diastolic volume and stroke volume are due to the increased preload and consequent eccentric hypertrophy characteristic of H diet feeding (28).

The shift from young adulthood to middle age is associated with changes in arterial function, such as impaired distensibility with increased V_{max}, a noninvasive clinical measure of aortic stiffness (10, 15). Age and blood pressure are independently associated with increased V_{max} (10). However, increased arterial stiffness is not exclusively the result of age-dependent deterioration of arterial structure but may also be affected by the endothelial regulation of vascular smooth muscle tone. Aging arteries have increased endothelial permeability and reduced endothelium-dependent relaxation responses to acetylcholine (15, 21). Consistent with the literature, H diet feeding increased V_{max}, elevated systolic blood pressure, and reduced vasodilator response to acetylcholine in isolated thoracic aorta. However, neither α_1-adrenoceptor-mediated vascular contraction to norepinephrine nor endothelium-independent relaxation response to sodium nitroprusside was affected. This suggests that the H diet selectively reduced production or availability of nitric oxide from the endothelium while preserving the contractility of vascular smooth muscle and nitric oxide-mediated vasodilation pathways.

In the absence of the traditional cardiovascular risk factors, obesity and type 2 diabetes are associated with increased arterial stiffness (10, 33). H diet feeding produced increased whole body and visceral adiposity, with the loss of lean mass and increased accumulation of fat in the skeletal muscle. These effects may have been mediated by reduced skeletal muscle mitochondrial function and generalized decline in muscle protein synthesis with aging (18).

The loss of muscle mass is coupled with gradual loss of subcutaneous fat after young adulthood (7). This is where dietary supplementation with chia seed may be an important strategy in improving the quality of life in an aging population. Consistent with our previous report (32), 24 wk of chia seed supplementation attenuated and maintained most structural and functional modifications induced by age or the H diet. More importantly, chia seed supplementation increased whole body lean mass and reduced abdominal fat, but not whole body fat, therefore suggesting lipid redistribution away from the abdominal area. As the total lipid content in major tissues, such as skeletal muscle and liver, decreased or remained unchanged, it is plausible that most of the fat from the viscera was redistributed as subcutaneous fat. Additionally, chia seed supplementation normalized the chronic low-grade inflammation induced by H diet feeding. This effect is most likely caused by the decrease in the proinflammatory n-6 fatty acid metabolism seen as increased precursor (C18:2n-6) and decreased or unchanged product (C20:4n-6), together with increases in the anti-inflammatory fatty acids of the n-3 series (C18:3n-3, C20:5n-3, C22:5n-3, and C22:6n-3) in all tissues. The n-3 series fatty acids have been well studied for their role in maintaining cardiovascular structure and function, as well as reducing the risk factors of metabolic syndrome (31). Although the high α-linolenic acid content may play an important role in mitigating the responses to chronic chia seed supplementation, chia seed is also a rich source of proteins [~25% (wt/wt)] and minerals (mainly calcium and phosphorus) (8), which may also contribute to some of the observed effects, especially on body composition. More importantly, the response to chia seed supplementation was maintained from our previously reported 16-wk study, and no major adverse effects were observed (32).

In addition, while 8 wk of chia seed supplementation failed to attenuate increased blood pressure as a result of H diet feeding for 16 wk (32), blood pressure was normalized with chronic chia seed supplementation.

However, there are striking differences in the handling of SFA, MUFA, and *trans* fatty acids with both H diet and chia seed supplementation compared with our 16-wk study (32). We found that 32 wk of C or H diet feeding decreased the net proportion of *trans* fats, as well as the desaturation index, therefore decreasing MUFA and increasing SFA content compared with our previous study (32). Also, H diet feeding for 32 wk induced preferential storage of C18:1n-9 over C16:1n-7 as the primary MUFA compared with the C rats. Because of this change in fatty acid content, the stearoyl-CoA desaturase index was normalized with 32 wk of H diet feeding, as the index is calculated as a ratio of C16:0 to C16:1n-7. In contrast to the 16-wk study (32), chia seed supplementation in this study only affected the n-6 and n-3 PUFA metabolism, and not SFA, MUFA, or *trans* fatty acids. Since mammals can produce fatty acids up to C18:1 in situ following a series of elongation and desaturation reactions (29), these results strongly suggest a transition in fatty acid metabolism with age toward increased lipogenesis, as the complete pathway would result in the formation of C18:1n-9 or C16:1n-7 as the final products irrespective of the starting substrate (29). In heart, liver, and skeletal muscle, the proportions of C18:1n-9 were reduced in rats supplemented with chia seed compared with H rats and remained comparable to or lower than proportions of C18:1n-9 in C rats, suggesting a possible inhibitory role of chia seed in lipogenesis.

These results validate our hypothesis that long-term consumption of the C diet mimics the process of aging, while H diet feeding exacerbates this process, with cardiovascular remodeling, obesity, loss of lean mass, and liver injury. Chia seeds may serve as a complementary dietary strategy to manage age-related morbidity due to the unique lipid redistribution, increased lean mass, and other cardiovascular and hepatic protective responses.

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DISCLOSURES

AQ: 4 No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

H.P. and L.B. developed the original study aims and analyzed and interpreted the data; H.P. and S.K.P conducted the experiments; L.W. performed dual-energy X-ray absorptiometry and provided nutritional advice; J.W. assisted in gas chromatographic techniques; H.P. and L.B. prepared manuscript drafts, with all authors contributing to the final version; L.B. has been the corresponding author throughout the writing process.

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Appendix E: Conference presentations during the candidature

1. Oral presentation on "*Ibuprofen attenuates metabolic and cardiovascular symptoms in rats fed a high carbohydrate/high fat (HCHF) diet*" in International Conference of Australian Health and Medical Research Congress on November 20, 2008 at Brisbane Convention and Exhibition Centre, Brisbane, Australia.
2. Oral presentation on "*Does a high carbohydrate/high fat (HCHF) diet in rats induce oxidative stress-related metabolic and cardiovascular symptoms?* in International Conference of Society for Free Radical Research (Australia) on November 30, 2008 at Bio21, The University of Melbourne, Melbourne, Australia.
3. Poster presentation on "*Rutin attenuates symptoms of metabolic syndrome*" in International Conference of Society for Free Radical Research (Malaysia/Asia) on Chemoprevention and Translational Research held during July 9 to 12, 2009 at Langkawi, Malaysia.
4. Poster presentation on "*Attenuation of non-alcoholic steatohepatitis by rutin*" in International Conference on Polyphenols held during August 24 to 27, 2010 at Montpellier, France.
5. Oral presentation on "*Rutin attenuates non-alcoholic steatohepatitis in high-carbohydrate, high-fat diet-fed rats*" in International Liver Congress organised by The European Association for the Study of the Liver held during March 30 to April 3, 2011 at Berlin, Germany.
6. Oral presentation on "*Differential effects of rutin and quercetin in diet-induced metabolic syndrome in rats*" in National level conference on Natural Products in Healthcare organised by Gurunanak College of Pharmacy, Nagpur, India held during November 24 to 26, 2011 at Nagpur, India.