SATURATED FATTY ACIDS, LINSEED COMPONENTS AND HIGH AMYLOSE WHEAT IN ATTENUATION OF DIET-INDUCED METABOLIC SYNDROME

A thesis submitted by

Siti Raihanah binti Shafie, M.Sc

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Abstract

Metabolic syndrome is a syndrome characterised by central obesity, dyslipidaemia, hypertension, fatty liver disease and insulin resistance that ultimately raises the risk of heart disease, diabetes, stroke, cancers and osteoarthritis. In combating metabolic syndrome, lifestyle changes are considered the most important initial steps which include a healthy, well-balanced diet and increased physical activity. Enrichment of beneficial fatty acids and incorporation of functional foods and bioactive nutrients are part of healthy dietary regimes in treating metabolic syndrome. These strategies provide options other than drug therapies that may cause adverse effects. Nevertheless, the effectiveness of these foods or bioactive nutrients in treating metabolic syndrome has yet to be fully explored. Therefore, in this thesis, I examined the physiological effects of individual saturated fatty acids (lauric, myristic, palmitic and stearic acid), linseed components (lignans, raw linseed and defatted linseed) and high amylose wheat (5% and 20%) using a validated diet-induced rat model of cardiovascular, liver and metabolic changes mimicking most of the changes in the human metabolic syndrome.

Male Wistar rats fed with either diet containing 20% of lauric, myristic, palmitic, or stearic acid or corn-starch or high-carbohydrate, high-fat diet for 16 weeks showed that longer-chain saturated fatty acids (myristic, palmitic and stearic) and the mixture of stearic and *trans* fats in beef tallow produced obesity, in contrast to rats treated with lauric acid that exhibited low total fat mass, abdominal circumference and visceral adiposity index. Lauric acid supplemented rats also showed a normal cardiovascular and hepatic structure compared to other saturated fatty acids. This study suggests that replacing beef tallow with stearic and palmitic acids would show small improvements but replacement with lauric and possibly myristic acids in human diets would markedly attenuate the development of metabolic syndrome.

Linseed is a rich source of plant lignans such as secoisolariciresinol diglucoside, as well as dietary fibre. Supplementation of lignan (0.03%) and defatted linseed (3%) in a high-carbohydrate, high-fat diet for eight weeks lowered body weight gain, total fat mass, improved cardiovascular functions, reduced hepatic steatosis and altered metabolic profiles, which can be regarded as beneficial to health, whereas raw

linseed (5%) exacerbated adiposity with no changes in other metabolic biomarkers except for reduced systolic blood pressure. This study suggests that lignan and dietary fibre in defatted linseed could reduce the symptoms of metabolic syndrome. In contrast, positive physiological effects of raw linseed diminish possibly due to the properties of raw linseed that may pass through the intestine undigested, which means the nutritional benefits are unable to be realised.

Another functional food described in this thesis is high amylose wheat flour. In this study, two dosages (5% and 20%) of high amylose wheat flour were supplemented in high-carbohydrate, high-fat diet for 8 weeks. Rats fed with 5% high amylose wheat flour showed no changes in the metabolic parameters. However, high-carbohydrate, high-fat diet-fed rats fed 20% high amylose wheat flour showed reduced body fat mass and increased lean mass despite no change in the body weight. The addition of 20% high amylose wheat in the diet was also associated with better glycaemic control, decreased insulin and leptin concentrations with cardioprotective and hepatoprotective effects. These effects are probably due to the increased resistant starch content in high amylose wheat, thus ameliorating the risk of developing metabolic syndrome.

The studies in this thesis provided evidence that not all saturated fatty acids are equal, with lauric acid producing fewer pathophysiological changes in most parameters than other saturated fatty acids in this model of diet-induced metabolic syndrome. Studies from linseed components and high amylose wheat clearly indicate that these foods or food components have the potential to reverse most of the risk factors associated with metabolic syndrome. The most likely mechanisms of these food or food components are through the cardioprotective and hepatoprotective effects produced by anti-inflammatory responses.

Certification of Thesis

This thesis is entirely the work of *Siti Raihanah binti Shafie* except where otherwise acknowledged. The work is original and has not previously been submitted for any other award, except where acknowledged.

Student and supervisors signatures of endorsement are held at USQ.

Professor Lindsay Brown

Principal Supervisor

Dr Kate Kauter

Associate Supervisor

Dr Sunil. K. Panchal

Associate Supervisor

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Associated Publications

Book chapter

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Co-authored journals

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Maharshi Bhaswant, **Siti Raihanah Shafie**, Michael L Mathai, Peter Mouatt, Lindsay Brown. 2016. Anthocyanins in chokeberry and purple maize attenuate diet-induced metabolic syndrome in rats. *Article in Press, Nutrition*.

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Refereed Conference Proceedings

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List of Abbreviation

ABS	Australian Bureau of Statistics
ALA	α-linolenic acid
ALP	Alkaline phosphatase
ALT	Alanine transferase
AST	Aspartate aminotransferase
ATPIII	Adult Treatment Panel
BMI	Body mass index
COAG	Council of Australian Government
CPT-1	Carnitine palmitoyltransferase
CRP	C-reactive protein
DHA	Docosahexaenoic acid
DXA	Dual energy X-ray absorptiometry
EGIR	European Group for the study of Insulin Resistance
EPA	Eicosapentaenoic acid
EDRF	Endothelium-derived relaxing factors
FAO	Food and Agriculture Organisation
FDA	Food and Drug Administration
FFA	Free fatty acids
GLP-1	Glucagon-like peptide 1
GLUT	Glucose transporter type
GPR	G-protein coupled receptors
HDL	High-density lipoprotein

ICD	International Classification of Disease
IDF	International Diabetes Federation
IL	Interleukin
LDL	Low-density lipoproteins
MCP	Monocyte chemoattractant protein
MUFA	Monounsaturated fatty acids
NAFLD	Non-alcoholic fatty liver disease
NCD	Non-Communicable diseases
NEFA	Non-esterified fatty acids
NHANES	National Health and Nutrition Examination Survey
OGTT	Oral glucose tolerance test
PUFA	Polyunsaturated fatty acids
PYY	Peptide tyrosine tyrosine
RS	Resistant starch
RER	Respiratory exchange ratio
SBP	Systolic blood pressure
SCFA	Short-chain fatty acids
SDG	Secoisolariciresionol diglucoside
SEM	Standard error mean
SFA	Saturated fatty acids
TC	Total cholesterol
TFA	Trans fatty acids
TNF-α	Tumor necrosis factor alpha
TG/TAG	Triglycerides
VLDL	Very-low density lipoproteins
WHO	World Health Organisation

Chapter 1 Introduction

1.1 Metabolic syndrome, saturated fatty acids and functional foods

1.1.1 Background

Obesity has become one of the major public health challenges, contributing to many comorbidities worldwide (Swinburn et al., 2011) and unfortunately no country to date has reversed its obesity epidemic (Roberto et al., 2015). Thus, all countries are still far from achieving the target in the Global Action Plan from the World Health Organisation (WHO) for the Prevention and Control of Non-Communicable Diseases (NCD) 2013–2020 and its accompanying Non-Communicable Disease Global Monitoring Framework for a zero increase in prevalence from 2010 to 2025 (Roberto et al., 2015). The target to be attained is a 25% relative reduction in premature mortality from NCDs by 2025 and a halt in the rise of global obesity (Roberto et al., 2015).

The cause of obesity has been clearly established as a positive imbalance between energy intake from ingested food and energy use for daily activities; this imbalance results in excess subcutaneous and visceral fat deposition (Eknoyan, 2006). This is closely linked to the escalating prevalence of metabolic syndrome. A group of organisations including WHO, European Group for the study of Insulin Resistance (EGIR), the National Cholesterol Education Program Adult Treatment Panel (ATPIII) and International Diabetes Federation (IDF) have defined certain diagnostic criteria for metabolic syndrome, also known as syndrome X (Table 1.1).

The criteria are mainly a cluster of interconnected physiological, biochemical, clinical and metabolic factors that directly increase the risk of cardiovascular disease, type 2 diabetes mellitus, and all-cause mortality (Kaur, 2014). Central obesity, insulin resistance, atherogenic dyslipidaemia, endothelial dysfunction, genetic susceptibility, elevated blood pressure, hypercoagulable state and chronic stress are the factors which constitute the syndrome (Kaur, 2014). In 2012, the WHO International Classification of Disease (ICD-9) code (277.7) defined metabolic syndrome as a first order risk factor for atherothrombotic complications (Kaur, 2014).

Table 1.1: Diagnostic criteria and definition of metabolic syndrome (Iyer et al., 2009).

Clinical Measure	**WHO 1998**	**EGIR 1999**	**ATPIII 2004**	**IDF 2005**
Insulin Resistance	Impaired glucose tolerance (IGT), Impaired fasting glucose (IFG), Insulin resistance (IR) plus any two of the	Plasma insulin >75th percentile plus any two of the following;-	None Any three of the following five features;-	None Any three of the following five features;-
Dyslipidaemia	Triglycerides (TG)> 1.695mmol/L, High density lipoprotein cholesterol (HDL- C) < 0.9mmol/L (males)	TGs ≥150 mg/dL and/or HDL-C <39 mg/dL in men or women	TG > 1.695mmol/L, HDL-C < 40mg/dL (males) < 50mg/dL (females)	TG > 1.7mmol/L or on TG Rx, HDL-C < 1.03mmol or on HDL-C Rx
Blood pressure	> 140/90mmHg	≥140/90 mm Hg or on hypertension treatment	> 130/85mmHg	> 130/85mmHg
Plasma glucose	> 7.0mmol/L (fasting)	IGT or IFG (but not diabetes)	>5.5mmol/L	> 5.5mmol/L (includes diabetes)
Central obesity	Waist/Hip ratio (WHR) > 0.90 (males); 0.85 (females)	Waist circumference ≥94 cm in men or ≥80 cm in women	Waist circumference > 102cm (males); > 88cm (females)	Waist circumference > 94 cm
Others	Urinary albumin excretion ratio > 20mg/min			

World Health Organization, (1998), European Group for the study of Insulin Resistance (EGIR, 1999), National Cholesterol Education Programme (Adult Treatment Panel III) (2001), International Diabetes Federation (2005).

Another complication resulting from metabolic syndrome is non-alcoholic fatty liver disease that is related to the release of adipokines from adipose tissue (Pala et al., 2012). Non-alcoholic fatty liver disease ranges from simple steatosis to chronic steatohepatitis and cirrhosis (Angulo, 2002). In addition, obesity also increases the risk of reproductive complications, pulmonary abnormalities, osteoarthritis and could possibly lead to cancer (Segula, 2014, Malnick and Knobler, 2006). Based on this, further studies need to be undertaken to reduce the prevalence and suggest options to ameliorate this epidemic.

1.1.2 Obesity and metabolic syndrome prevalence

Obesity has become a common issue at every stage of life. There are many different areas of research into obesity, for example the relationship between maternal obesity and the risks to the offspring (Catalano, 2015, Desai et al., 2014, Gaillard, 2015). The global prevalence of obesity has doubled since 1980, with 1.9 billion adults being overweight and 600 million obese in 2014, accounting for 39% and 13% of total world population, respectively (World Health Organization, 2015). Results from the 2013-2014 National Health and Nutrition Examination Survey (NHANES) in the USA showed that obesity prevalence remains unchanged since 2005 among men, but increased in women (Zylke and Bauchner, 2016). Based on 2013-2014 NHANES data, 35% of men were obese (BMI ≥30) and 5.5% were morbidly obese (BMI≥40); among adult women, 40.4% were obese and 9.9% were morbidly obese (Zylke and Bauchner, 2016). In Australia, the Australian Bureau of Statistics reported that in the year 2015, 71% of Australian men and 56% of women were overweight (Australian Bereau of Statistics, 2015). The trend showed one in four adults were obese, and 27% of children were overweight or obese. Australia is not on track to meet either Council of Australian Governments (COAG) or National Preventative Health Taskforce (NPHT) targets for 2019 and 2020 (Australian Bereau of Statistics, 2015), which are to increase the proportion of children and adults with healthy body weights by 3% within 10 years (Australian Bereau of Statistics, 2015).

It is predicted that the prevalence of overweight and obesity will continue to rise and by the year 2030, 57.8% of the world's adult population (3.3 billion people) will be affected if these observed trends persist (Kelly et al., 2008). Obesity is a part of the metabolic syndrome and associated with many other chronic diseases, such as

cancer and osteoarthritis, thus leading to increased mortality. Metabolic syndrome prevalence in the world among the elderly ranges from 45-65% depending on the criteria selected in defining metabolic syndrome (Pradeepa et al., 2016). With an ageing of population, conditions in combination of multiple chronic diseases will have an enormous impact on the health system, especially on the costs and quality of care received (Vogeli et al., 2007).

The prevalence of metabolic syndrome varies considerably across the globe, with Table 1.2 showing multiple definitions of metabolic syndrome with different criteria and cut-off points. These variations of cut-off points used to identify individuals with the syndrome has led to confusion, especially among researchers and clinicians. Moreover, this also makes direct comparisons between studies more difficult. From Table 1.2, it can be summarised that metabolic syndrome occurs not only in developed countries but also in developing countries and the trend is more prevalent among women, except in Australia. This could be due to more women than men meeting the criteria for each definition of metabolic syndrome.

Table 1.2 Prevalence of metabolic syndrome per definition.

Country	Age	n	NCEP/ATPIII	IDF	WHO	Harmonized	EGIR	Reference
Australia	≥25	11247	Overall: 22.1 Men: 24.4 Women: 19.9	Overall: 30.7 Men: 34.4 Women: 27.2	Overall: 21.7 Men: 25.4 Women: 18.2	NA	Overall: 13.4 Men: 15.6 Women: 11.3	(Cameron et al., 2007)
Brazil	≥18	2130	NA	NA	NA	Overall: 32.0 Men: 30.9 Women: 33	NA	(Dutra et al., 2012)
China	≥60	2334	Overall: 30.5 Men: 17.6 Women: 39.2	Overall: 46.3 Men: 34.8 Women: 54.1	NA	NA	NA	(Keenan et al., 2006)
India (East region)	20-80	1178	NA	Overall: 33.5 Men: 24.9 Women: 42.3	NA	NA	NA	(Prasad et al., 2012)
India (South region)	≥60	222	Overall: 45.9	Overall: 37.4 Men: 31.0 Women: 45.8	Overall: 45.5	NA	NA	(Pradeepa et al., 2016)

NA: Not available.

Table 1.2 Continued.

Country	Age	n	NCEP/ATPIII	IDF	WHO	Harmonized	EGIR	Reference
Iran	25-64	5417	NA	Overall: 32.9	NA	NA	NA	(Noshad et al., 2016)
Malaysia	≥18	4341	Overall: 34.3 Men: 31.1 Women: 36.1	Overall: 37.1 Men: 32.1 Women: 39.8	Overall: 32.1 Men: 31.8 Women: 32.2	Overall: 42.5 Men: 40.2 Women: 43.7	NA	(Mohamud et al., 2012)
Romania	20-79	2681	NA	NA	NA	Overall: 38.5 Men: 43.2 Women: 34.2	NA	(Popa et al., 2016)
United States	≥20	3601	Men: 33.7 Women: 35.4	Men: 39.9 Women: 38.1	NA	NA	NA	(Ford, 2005)

NA: Not available.

1.1.3 Pathophysiology of metabolic syndrome

Positive energy balance results in excessive visceral fat accumulation which contributes to an overabundance of circulating fatty acids and further leads to reduced insulin sensitivity (Despres and Lemieux, 2006). Progressive loss of β-cell functions such as glucose-stimulated insulin secretion ultimately leading to β-cell apoptosis induced by glucotoxicity and lipotoxicity (Weir et al., 2001, Cnop et al., 2005). The consequence is hyperinsulinaemia and defects of insulin action causing glucose intolerance. Insulin resistance will then increase secretion of triglycerides (TG) and very low-density lipoproteins (VLDL) that result from increased flux of free fatty acids to the liver. The TG surplus (hypertriglyceridaemia) is then deposited at undesirable sites such as liver, skeletal muscle, heart and pancreas and this is known as ectopic fat deposition (Despres and Lemieux, 2006). Insulin resistance is also accompanied by other alterations in the metabolic profile which include increased pro-inflammatory cytokines, prothrombotic factors and non-alcoholic fatty liver disease.

Leptin, ghrelin and insulin are three “fat hormones” that affect the brain associated with changes in food intake (Korek et al., 2013). Leptin is secreted by adipocytes and other tissues and its plasma concentrations are positively associated with the amount of body fat. It promotes suppression of appetite and increased energy use and is associated with long-term energy balance (Wannamethee et al., 2007). Ghrelin on the other hand is secreted by the stomach directly before a meal, acts as a potent and fast-acting appetite stimulant and affects energy balance by decreasing glucose and fat oxidation and increasing energy storage (Cummings et al., 2001). Both leptin and ghrelin affect insulin sensitivity and glucose homeostasis (Soni et al., 2011). Impaired insulin sensitivity may both induce increased insulin concentrations and lead to raised leptin concentrations by down-regulation of the hypothalamic leptin receptors or the subsequent satiety response to leptin (Mohamed et al., 2014). Insulin is responsible for regulating body weight via down-regulation of circulating ghrelin concentrations (Erdmann et al., 2005).

Hypertension is frequently associated with the several metabolic abnormalities, of which obesity, glucose intolerance, and dyslipidemia are the most common (Ferrannini and Natali, 1991). Remodelling of cardiovascular may also be the underlying mechanism of hypertension. Cardiac remodelling is a process of change in

the size, structure and function of the heart (Reddy et al., 2005). These physiological responses lead to hypertrophy, necrosis and apoptosis of the myocyte, fibroblast proliferation and fibrosis in the interstitium (Remme, 2003). These consequently leads to abnormalities in myocardial function including impaired contractility and relaxation, diminished cardiac pump function, dilatation and increased sphericity of the heart (Remme, 2003) and ultimately progresses to systolic and diastolic cardiac dysfunction, which increase the risk of thrombosis and heart failure.

Non-alcoholic fatty liver disease (NAFLD) is regarded by researchers as the hepatic manifestation of metabolic syndrome (Yang et al., 2016). NAFLD includes a spectrum of disease that range from simple steatosis to steatohepatitis, advanced fibrosis and cirrhosis (Yang et al., 2016). Hepatic steatosis happens when there is excess deposition of free fatty acids and triglycerides in hepatocytes, in addition to protein kinase Cε activation that leads to impairment of hepatic insulin action (Yang et al., 2016). This underlying aetiology of NAFLD promotes the development and progression of cardiovascular disease and diabetes.

The gut plays a vital role in food digestion, absorption and excretion. Hormones produced by the cells lining the gastrointestinal tract affect gut health and this includes glucagon-like peptide-1 that may be involved in the regulation of obesity and diabetes (Iepsen et al., 2014). Other important factors influencing gut health are gastrointestinal motility and gut microbiota. Gastrointestinal motility is the regulation of gastric emptying that represents the most important brake against delivery of nutrients to the intestine in excess of digestive and absorptive capacity (Wisén and Hellström, 1995). Gastric emptying of solid food is accelerated in obese subjects, which may precipitate hunger and frequent eating (Xing and Chen, 2004). However, there are contradictory reports regarding gastric emptying in obese people (Wisén and Hellström, 1995). Furthermore, obese people have larger gastric volume so that satiety signals are not triggered in response to gastric distension (Wisén and Hellström, 1995). Mechanisms that are responsible for regulating gut motility include the endocrine (cholecystokinin) and enteric nervous systems and gastrointestinal regulatory peptides, are also of importance for feeding behaviour and metabolism (Wisén and Hellström, 1995). Obesity is associated with low-grade inflammation. The gut microbiome may contribute to the low-grade inflammation characterising these metabolic abnormalities

(Everard and Cani, 2013, Ding et al., 2010). Gut microflora exerts a crucial role in the development of fat mass and altered energy homeostasis (Turnbaugh et al., 2006, Bäckhed et al., 2004). In addition, the gut barrier alterations resulting from obesity and type-2 diabetes lead to disruption in the gut microbiota-host symbiotic relationship (Everard and Cani, 2013, Everard et al., 2013). This discovery provides new insight into the potential linkage between gut microbes, gut barrier function (metabolic endotoxaemia) and white adipose tissue inflammation, particularly with diet-induced obesity (Cani et al., 2007, Cani et al., 2008). However, it is not clear whether the simple sugars and saturated fats of the high energy diet change the growth patterns of the gut microbiota and hence cause the metabolic changes in the body or whether metabolic changes induced by the diet lead to the changes in the profile of gut microflora (Puupponen-Pimiä et al., 2002).

Foods high in prebiotics selectively promote the growth and activity of beneficial bacteria in the gut. Prebiotics are defined as nondigestible food ingredients that promote the growth and/or activity of one or a limited number of bacteria in the colon, thus improving host health (Gibson and Roberfroid, 1995). Later, the definition was refined to include other benefits from selective targeting of particular microorganisms (Gibson et al., 2004). The ecology system of gut microbiota comprises of balanced representations of symbionts (bacteria with health-promoting functions) and pathobionts (bacteria that potentially induce pathology) (Figure 1.1) (Boulangé et al., 2016). This crosstalk of gut microbiota in mammalian host then modulates host physiology, metabolism and inflammatory status (pro- or anti-inflammatory). A healthy gut microbiota will reduce gut permeability, endotoxemia, pro-inflammatory cytokines and increase insulin sensitivity and beneficial active molecules such as short chain fatty acids (SCFA). Meanwhile, an altered (dysbiotic) gut microbiota contributes to insulin resistance, low grade inflammation, and fat deposition through a range of molecular interactions with the host and therefore can indirectly participate in the onset of obesity and metabolic diseases (Boulangé et al., 2016).

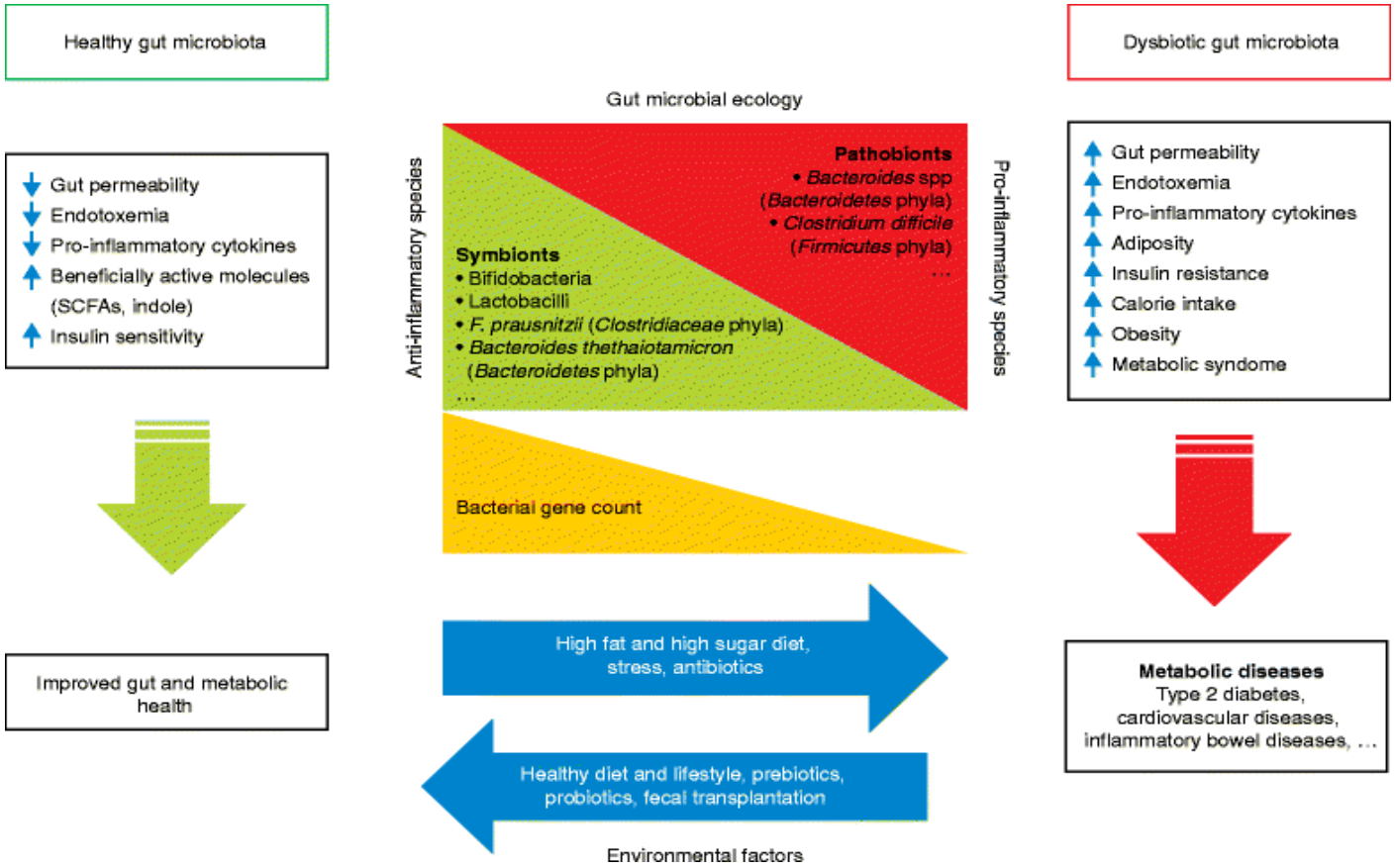

Figure 1.1: Effects of healthy gut microbiota and dysbiotic gut microbiota and the association of high fat, high sugar and other environmental factors to gut health and its metabolic consequences.

Adapted from (Boulangé et al., 2016).

1.1.4 Why are obesity and metabolic syndrome on the rise?

Obesity is most commonly caused by excessive energy intake relative to energy expenditure. However, other aetiology includes genetic, physiological, social, economic, environmental, psychological and even political factors that interact to varying degrees to promote the development of obesity (Aronne et al., 2009). Today, food and marketing industries play an important role in the obesity epidemic. Advertisement for fast, junk and processed foods can be widely seen, with advances in technology making it more convenient for everyone to overeat (Cairns et al., 2013, McClure et al., 2013). Sophisticated digital marketing techniques are used by food marketers to target youth across a host of platforms, including cell phones, video games and social media. In addition, drastic increases in sugar intake, which includes corn syrup and other sweeteners, were highlighted in a study in the United States (Bray et al., 2004). These high-caloric foods are usually available in large portions and are relatively inexpensive, which directly contributes to increased and excessive daily caloric intake (Drewnowski and Darmon, 2005). Moreover, today's fast-paced lifestyles have shifted the trend towards eating out more and cooking at home less (Smith et al., 2013). Additionally, less access to physical activity (fewer sidewalks), less physical education in schools (Gabbard, 2001), and more time spent on sedentary behaviours such as television watching, surfing the internet and playing video games worsen this scenario (Andersen et al., 1998).

1.1.5 Financial burden of obesity

Obesity-health related disorders impose substantial financial burdens on individuals, families, communities and the country as a whole. In Australia, the Australian Diabetes, Obesity, and Lifestyle (Ausdiab) study in 2005 indicated that total direct costs for overweight and obesity in 2005 were $21 billion ($6.5 billion for overweight and $14.5 billion for obesity) (Scholze et al., 2010). The number of overweight and obese adults had increased from 4.6 million in 1989–90 to 7.4 million in 2004–2005 resulting in an annual direct cost to the health system of approximately $2837 for each overweight or obese individual (Scholze et al., 2010). The main contributions to direct health care costs in those with a body mass index and waist

circumference defined as overweight were prescription medication, hospitalisation and ambulatory services, each accounting for about 32% (Colagiuri et al., 2010). Meanwhile, indirect costs for obesity including hospitalisation, prescription medication, and ambulatory services accounted for 36%, 33% and 25%, respectively. The same study estimated indirect costs of $35.6 billion per year, resulting in an overall total annual cost of $56.6 billion (Colagiuri et al., 2010). Direct medical costs refer to preventive, diagnostic and treatment services related to obesity, meanwhile indirect costs relate to morbidity and mortality costs including productivity at work (Hammond and Levine, 2010).

In the United States, the medical care costs were estimated to be $147 billion in 2008 (Finkelstein et al., 2009), and it was reported that the annual nationwide loss of production costs of obesity-related absenteeism ranged between $3.38 billion ($79 per obese individual) and $6.38 billion ($132 per obese individual) (Trogdon et al., 2008). The National Cholesterol Education Program Adult Treatment Panel III (NCEP ATPIII) reported total costs to Medicare were 20% higher among participants with metabolic syndrome ($40,873 vs. $33,010; $P < 0.001$). This study also found, after controlling for age, sex, race/ethnicity and other covariates, the abdominal obesity, low HDL cholesterol and elevated blood pressure were associated with 15% (95% CI 4.3–26.7), 16% (1.7–31.8), and 20% (10.1–31.7) higher costs, respectively (Curtis et al., 2007).

In Europe, the total annual costs attributed by metabolic syndrome in hypertension patients for 2008 was €24,427 (Germany), €1,900 (Spain) and €4,877 (Italy) million in Germany, and it was predicted to increase by 57% (€38,955), 180% (€5,329) and 157% (€12,523) in 2020 (Scholze et al., 2010). The increased incidence of metabolic syndrome is directly associated with higher cost of care (Scholze et al., 2010).

1.1.6 Strategies and prevention

The prevention and management of metabolic syndrome are complex as they involve social and cultural issues, local environment, food industry, public policies, personal attitudes and behaviour and human biology. These factors are interconnected, but we are still far from achieving sustained reduction in the prevalence of overweight and obesity. Standard behavioural treatment for obesity typically includes lifestyle modification (dietary and physical activity regimens) and it has been established that subjects who fully and continuously adhere to these regimens experience large weight losses and minimal weight regain (Klem et al., 1997). These ongoing lifestyle modifications require a set of skills involving significant planning in making healthy choices, estimating portion sizes, and diligence in monitoring caloric intake and activity. Clinical trials focusing on both food intake and exercise in obese patients showed small but significant decreases in body weight of 1.56 kg compared to control subjects at 12 months (Dombrowski et al., 2014). However, these behavioural changes may include non-adherence as maintaining the intervention needs high self-control and motivation (Foster et al., 2005). Limitations found in most research on behavioural treatment include being conducted in university-based clinic programs; such studies tell us little about the effectiveness of these approaches in settings outside of specialised clinics (Foster et al., 2005).

Diets low in carbohydrates and fats, low in glycaemic index, high in protein and high in fibre can reduce the risk of developing metabolic syndrome (Steckhan et al., 2016). This includes Mediterranean and Nordic diets (Calton et al., 2014, Kesse-Guyot et al., 2013). Complex lifestyle modification interventions can reduce metabolic syndrome-related abnormalities (Yamaoka and Tango, 2012) and a combination of a healthy dietary pattern with exercise protects patients from metabolic problems and cardiovascular events (Oh et al., 2011). In addition, lifestyle changes and physical exercise associated with a proper diet can reduce the prevalence of metabolic syndrome by 24% (Mecca et al., 2012). Figure 1.2 summaries the strategies that can be involved in reducing metabolic syndrome.

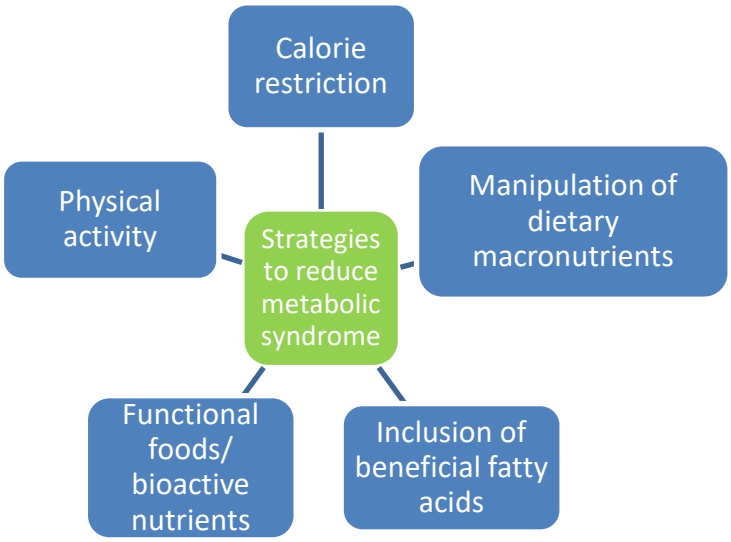

Figure 1.2: Strategies to reduce metabolic syndrome.

Omega-3 fatty acids supplementation decreased metabolic syndrome prevalence after lifestyle modification program, where metabolic syndrome patients were given long-chain polyunsaturated fatty acids (LC-PUFA) supplementation and protein meal replacement under calorie-restricted dietary conditions (de Camargo Talon et al., 2015). Omega-3 fatty acids exert beneficial effects on metabolic parameters, including reductions in blood glucose and triglyceride concentrations, HOMA-IR, circulating fatty acid profiles and the concentrations of the inflammatory cytokine IL-6 (de Camargo Talon et al., 2015). A high intake of omega-3 fatty acids including α–linolenic (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) attenuated the signs of metabolic syndrome in a rat model (Poudyal et al., 2011).

Pharmacological interventions are effective in treating severe metabolic diseases including chronic obesity. Weight-loss drugs that have been used include fenfluramine, sibutramine and rimonabant as these drugs suppress food intake (Brown et al., 2015). Other treatments include thyroid hormones and inhibitors of gut enzymes such as α-glucosidase, α-amylase and sucrase (Douglas and Munro, 1982). These hormones and inhibitors promote the loss of fat through increased metabolic rate or through decreased absorption of nutrients from the gut (Douglas and Munro, 1982). However, these drugs are not devoid of adverse effects and evidence showed a rising trend in the occurrence of undesirable complications among patients (Brown et al.,

2015). Both rimonabant and sibutramine were withdrawn from the market because of their adverse effects in 2009 and 2010, respectively (Kang and Park, 2012). Orlistat, a lipase inhibitor, is also used for weight reduction but also caused adverse effects. This includes reduction in the bioavailability of fat-soluble vitamins because of the inhibition of fat digestion (Halford, 2006). Due to these side effects, not a single prescription drug currently available will produce significant achievement in weight-loss with complete safety. In addition, there are only a few studies examining the long-term safety and efficacy of many of the drug combinations for obesity currently being prescribed (Brown et al., 2015, Yanovski and Yanovski, 2014).

This could be one reason why functional foods are in high demand by the market. The growing interest in functional foods with scientifically sustained claims include dietary phytochemicals, dietary fibre, resistant starch, prebiotics, probiotics and other bioactive compounds in foods that have high potential for health promotion and chronic disease prevention (Brown et al., 2015). Diets rich in fruits and vegetables are associated with a lower risk of all-cause mortality, particularly cardiovascular mortality (Wang et al., 2014). In addition, the intake of whole grains and cereal fibre was inversely associated with reduced total and cause-specific mortality (Huang et al., 2015b). Despite the long lists of functional foods, this thesis will focus on saturated fatty acids, linseeds and linseeds products and high amylose wheat as potential treatments for metabolic syndrome.

1.1.7 Saturated fatty acids and metabolic syndrome

Non-communicable diseases definitely impair the overall quality of life. One of the major reversible causes of non-communicable diseases is increased dietary intake. Human diets primarily consist of carbohydrates, proteins and fats as macronutrients. Nutrient-dense dietary fats and carbohydrates have been considered as causes of cardiometabolic conditions (Mozaffarian et al., 2011). Over the years, saturated fatty acids (SFA) have been generalised as raising blood low-density lipoproteins and thereby increasing the cardiovascular disease risk (Ruiz-Núñez et al., 2016). In addition, SFA increased endoplasmic reticulum stress, inflammation and liver injury to a greater extent than unsaturated fatty acids (Wang et al., 2006, Gentile et al., 2011). Recently, there were controversial findings around SFA where there was no clear evidence to support SFA links to increased risk of coronary heart disease

(Chowdhury et al., 2014, de Oliveira Otto et al., 2012). The research to date has tended to be based on generalisations on chemical structure rather than the biological actions of individual SFA (Poudyal and Brown, 2015). Moreover, a much-debated question is whether the health risk is based upon the quantity rather than the quality of the SFA. The inconsistency of findings and uncertainty on the biological effects of individual SFA, therefore, need to be explored to fill the gaps and to offer new insights into the body of knowledge.

Dietary fat also provides the essential fatty acids, linoleic and α–linolenic acids, to all tissues as these are fundamental to basic physiological functions as energy sources, elements of membrane phospholipids and main fuel molecules (Ruiz-Núñez et al., 2016). There is a broad spectrum of fatty acids of varying chain length and degree of desaturation (Mu and Hoy, 2004). Saturated fatty acids can be classified chemically into four subclasses according to their chain length (Figure 1.3): short (3 to 7 carbon atoms), medium (8 to 13 carbon atoms), long (14 to 20 carbon atom) and very long (≥ 21 carbon atoms) (FAO, 2010). The most commonly found SFA in our diets are lauric acid (C 12:0), myristic acid (C 14:0), palmitic acid (C 16:0) and stearic acid (C 18:0) (Figure 1.3). In healthy people, more than 95% of fatty acids consumed in their diet are available to the bloodstream through efficient processes of digestion and absorption (Calder, 2015a). *De novo* synthesis of fatty acids also occurs in the human body either from glucose and fructose or from other fatty acids; exceptions to this are the essential fatty acids, linoleic and α–linolenic acids, which must come from the diet (Calder, 2015b).

Stearic acid is mostly found in beef tallow and cocoa, palm oil is rich in palmitic acid, coconut oil is rich in lauric acid and myristic acid can be found in nutmeg oil and butter fat (Poudyal and Brown, 2015, Zock et al., 1994). SFA effects on lipids and lipoproteins are modulated by the food sources (Astrup et al., 2011). The majority of plant oils contain a significant amount of saturated fatty acids, particularly palmitic acid. As a result of its widespread availability in the diet, palmitic acid is the most common dietary fatty acid, with stearic acid (18:0) and myristic acid also making important contributions to the diet. Depending on the foods eaten, lauric acid (12:0) and short- and medium-chain fatty acids may also be consumed in significant quantities (Calder, 2015b).

The negative impact of saturated fatty acids was first suggested by Keys in the Seven Countries Study (Keys et al., 1984). Data from several sources highlighted the effects of SFA on total cholesterol (TC), low-density lipoprotein (LDL-C), triglyceride (TG) and high-density lipoprotein (HDL-C) (Maki et al., 2012b, Fattore et al., 2014, Cox et al., 1995). Different diets rich in palm oil, stearic acid, monounsaturated fatty acids (MUFA) or polyunsaturated fatty acids (PUFA) were compared and results showed that palm oil raised TC, LDL-C, and HDL-C as well as apoB and apoAI (Fattore et al., 2014). When compared to diets with a high content of myristic and/or lauric acid, palm oil was associated with lower TC, HDL-C and apoAI and no differences in TG, TC:HDL-C or VLDL-C (Fattore et al., 2014). Using lauric acid at the energy equivalent of butter resulted in lower TC and LDL-C, but both the butter and coconut oil diets raised TC and LDL-C compared to safflower oil (Cox et al., 1995). A follow-up study by the same investigators in a community-based intervention showed that coconut oil reduced LDL-C and apoB compared to butter-based diets (Cox et al., 1998). Apart from the findings that linked SFA with dyslipidaemia, others also emphasised the effects of SFA on insulin resistance, impaired glucose tolerance, systemic inflammation and abdominal obesity (Cascio et al., 2012).

Since then, emphasis on the restriction of SFA was applied in dietary guidelines aiming to improve cardiometabolic health. It is important that current advice has a solid scientific basis noting the weaknesses of previous studies given the large social impact of dietary advice (Calder, 2015a). This has led to the re-evaluation of dietary guidelines for intake and a re-appraisal of the effects of SFA on health (de Souza et al., 2015). The role of total fat intake vs. dietary fat quality with respect to obesity and weight gain remains controversial and this will be highlighted by this thesis as one of the study's most significant findings.

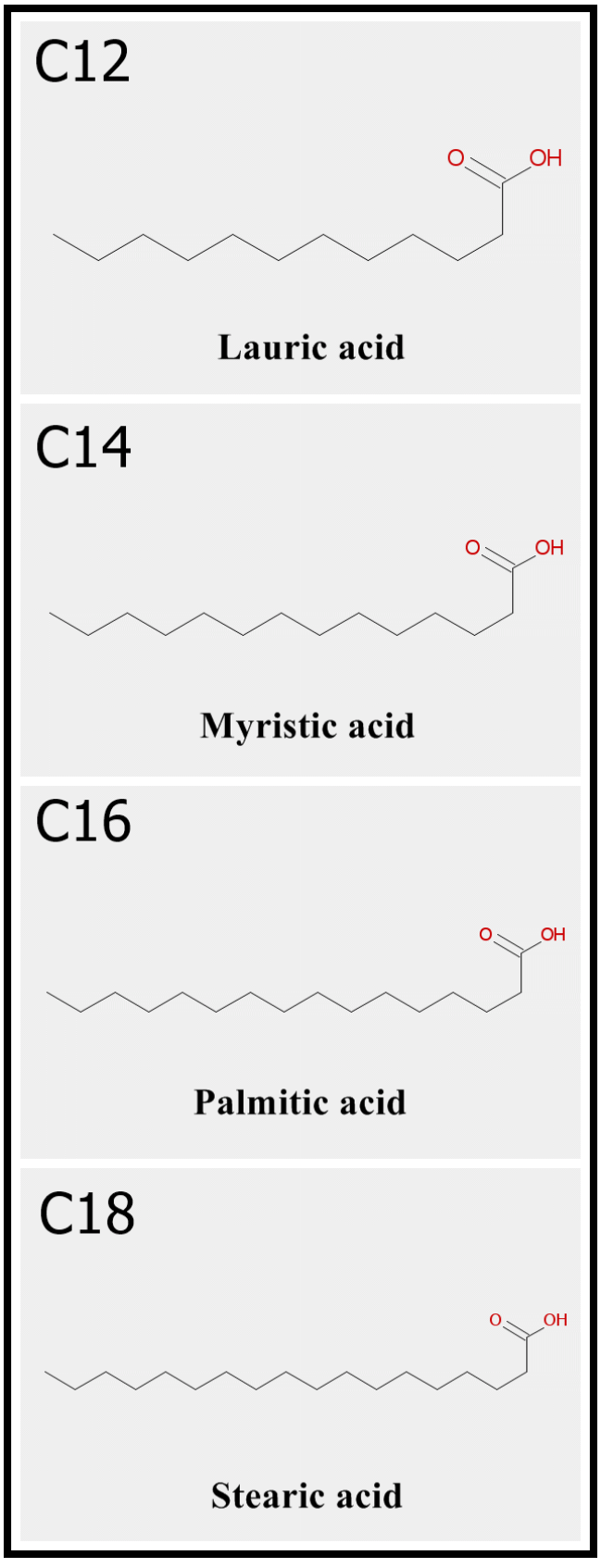

Figure 1.3: Structures of common dietary saturated fatty acids (SFA).

Several meta-analyses have found that intake of SFA was not associated with coronary heart disease mortality or other cardiovascular events (Skeaff and Miller, 2009, Siri-Tarino et al., 2010, de Souza et al., 2015). The concern is now that the health effects of reducing SFA consumption vary depending on whether the replacement nutrient is carbohydrate, MUFA or PUFA. A systematic review suggested decreased SFA consumption, but with increases in carbohydrates, rather than MUFA or PUFA, as the replacement nutrient (Micha and Mozaffarian, 2010). In essence, replacing SFA with PUFA modestly lowers coronary heart disease risk, with ~10% risk reduction for a 5% energy substitution; whereas replacing SFA with carbohydrate has no benefit and replacing SFA with MUFA has uncertain effects (Micha and Mozaffarian, 2010).

Intervention with progressively increased carbohydrate from 47 to 346 g/day with concomitant decreases in total and saturated fat, found that high intakes of saturated fat including regular consumption of whole eggs, full-fat dairy, high-fat beef and other meats do not contribute to accumulation of plasma SFA in the context of a low carbohydrate intake (Volk et al., 2014). Further, a gradual decrease in saturated fat to 32 g/day at the highest carbohydrate phase also showed that there were no significant changes in the proportion of total SFA in any plasma lipid fractions (Volk et al., 2014).

These findings contradict the popular idea that dietary SFA *per se* are harmful because the role of total fat intake vs. dietary fat quality with respect to obesity and other cardiometabolic events is still controversial.

1.1.8 Functional foods

It is important to find novel ways to treat metabolic syndrome and these novel interventions may include functional foods and nutraceuticals. Functional foods are defined as foods either natural or modified that contain biologically active components which in adequate amounts can improve health and well-being and reduce the risk of disease (Ozen et al., 2012). These foods generally contain health-promoting components beyond traditional nutrients; however, it should remain as food and not in the form of pills or capsules (Ozen et al., 2012). In the USA, functional foods are regulated by Food and Drug Administration (FDA) under the Federal, Food, Drug, and Cosmetic Act of 1938 (Sikand et al., 2015). Under this Act, food manufacturers are

limited to four categories of claims on food labels as follows: nutrient content claims; structure-function claims; health claims and qualified health claims (Sikand et al., 2015). These functional foods are seen as an alternative way of reducing the rising costs of healthcare and ultimately improving the quality of life. Moreover, due to increasing interest in health and well-being and readily available information, consumer demand for functional foods is increasing and has resulted in the need for exploration of food products with beneficial health properties. Based on previous studies, the range of readily-available functional foods includes coffee; black tea; low-fat milk; fruits and vegetables; fermented products; cereals and grains (Shiby and Mishra, 2013, Sikand et al., 2015, Brown et al., 2015). This thesis, however, will only focus on the health effects of two grains, linseed and high amylose wheat, for the management of metabolic syndrome.

1.1.8.1 Linseed as functional food

Linseed, also known as flax (*Linum privatissimum*), belonging to the Lineaceae family, is a blue flowering annual herb that produces small, flat, golden yellow or reddish brown seeds with a crispy texture and nutty taste (Figure 1.4) (Kajla et al., 2015). Linseed has been previously described as an excellent source of α–linolenic acid (ALA, 18:3n-3) (Poudyal et al., 2013a) and the lignan, secoisolariciresinol diglucoside (SDG) (Adolphe et al., 2010) that possess health-beneficial effects. More details on linseed as a functional food will be covered in Section 1.2 (Linseed as a functional food for the management of obesity).

Figure 1.4: Raw linseed, defatted linseed and lignans isolated from linseed.

1.1.8.2 High-amylose wheat as a functional food

Wheat (*Triticum spp.*) is one of the staple foods and a major grain cash crop besides rice and maize (Cooper, 2015). This crop originated in both the Levant region of the Near East and the Ethiopian Highlands probably around 9,000 years ago and is now cultivated worldwide (Cooper, 2015). Millions of tonnes of wheat are produced globally each year to meet the demands of consumers. The grain is an important source of nutrients, protein, carbohydrates, vitamins, minerals and dietary fibre in human food. The vital role of grains in the everyday diet of humans is the basis for modifying the major components in cereal grains such as fibre or resistant starch (RS). Modification of the grain crops with higher RS will increase nutritional benefits to give a profound impact on global population health and the world economy. Resistant starch plays a role similar to dietary fibre with beneficial effects on human health and provides protection from chronic diseases such as colon cancer, diabetes, obesity, osteoporosis and cardiovascular disease (Sestili et al., 2010).

The manipulation of the amylose/amylopectin ratio in wheat has been made commercially viable by the emerging evidence on health benefits of resistant starch and the elucidation of the role of enzymes involved in the starch biosynthetic pathway along with the isolation of genetic variants for these enzymes (Lafiandra et al., 2010). Wheat starch is composed of two main components: amylose and amylopectin (Figure 1.5) with amylose contributing 20%–30% of the total starch and amylopectin the remaining 70%–80% (Cauvain, 2012). Amylopectin is highly branched, easily digested to produce a larger rise in blood glucose and subsequently, a larger rise in insulin, whereas amylose is a straight chain that limits the amount of surface area exposed for digestion and decreases the rate of digestion to glucose. Characteristics of four different types of resistant starch are presented in Table 1.3.

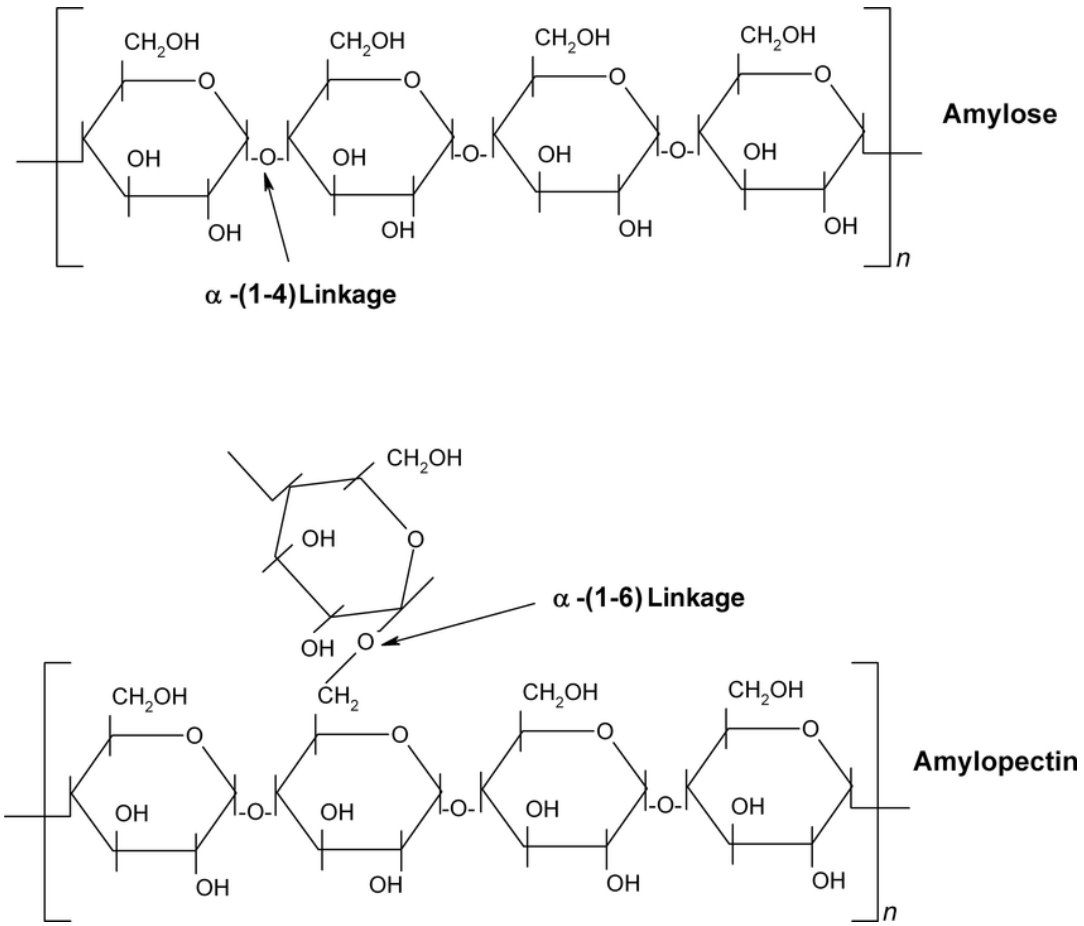

Figure 1.5: Structures of amylose and amylopectin.

Table 1.3: Characteristics of four different types of resistant starch (RS).

Type of RS	Characteristics
RS1	The starch is physically inaccessible to digestion due to intact cell walls, e.g. grains.
RS2	Native starch granules protected from digestion by the structure of the starch granule.
RS3	Retrograded starch – e.g. cooked then cooled potato, rice or pasta (forms as it cools).
RS4	Chemically modified starches – do not occur naturally but are created to be resistant to digestion.

Source: (Topping and Clifton, 2001).

Amylose predominates in resistant starch (RS) and it is defined as the fraction of starch that is not hydrolysed in the small intestine (Sharma et al., 2008). RS in the presence of heat and water will lead to gelatinisation that makes it easily digested. However, at lower temperatures, resistant starch tends to recrystallise and form retrograded amylose (Sharma et al., 2008). The retrogradation process increases enzyme resistance, hence reducing the digestion rate (Chung et al., 2006). RS is largely fermented in the large intestine by the gut bacteria, which leads to the production of short-chain fatty acids (SCFA). SCFA are reported to improve the colonic epithelial health (Cummings et al., 1996).

An elevated amylose content lowers postprandial glycaemia (Hallstrom et al., 2011, Åkerberg et al., 1998, Granfeldt et al., 1995). This is closely related to the formation of enzyme-resistant starch that influences the glycaemic response by limiting available glucose (Hallstrom et al., 2011). Another factor contributing to this is the formation of complexes between amylose and surrounding lipids and proteins that resist enzymatic degradation that may be additional factors contributing to a lower rate of starch digestion in products with elevated amylose contents (Hallstrom et al., 2011).

Dietary resistant starch is also positively associated with increased gut hormones such as serum glucagon-like peptide 1 (GLP-1) and peptide tyrosine tyrosine (PYY) (Charrier et al., 2013). These hormones are associated with increased

fermentation and increased caecal butyrate production (Keenan et al., 2006). These anorectic gut hormones also play a role in regulating the appetite which can be a potential mechanism to combat obesity (De Silva and Bloom, 2012). Accumulating evidence supports replacing rapidly digestible starch with resistant starch, thereby reducing body weight and adiposity (Birt et al., 2013). However, there is a paucity of evidence for increased amylose consumption, for example as high amylose wheat flour (Figure 6) improves the signs of metabolic syndrome in a high-carbohydrate, high-fat diet. Thus, it warrants further study to quantify the effects on health.

Figure 1.6: High amylose wheat flour.

1.1.9 Animal models of obesity and metabolic syndrome

Animal models have played a critical role in the exploration and characterization of disease pathophysiology, target identification, and in the *in vivo* evaluation of novel therapeutic agents and treatments for years. The ideal animal model should replicate, to a major extent, both a human disease phenotype and its underlying causality, the latter in terms of a mechanism of action that mimics the human disease (McGonigle and Ruggeri, 2014). In animal models, the underlying physiological, biochemical and molecular biological mechanisms can be examined in a more invasive manner that would be unethical in humans, under controlled conditions and without the confounding genetic/social factors. Various species such as rat, mouse, guinea pig, sheep, dog and pig have been used as models to study human medicine and nutritional sciences.

Pigs are an appropriate biomedical model for humans due to many genetic (Lunney, 2007), anatomical (Pracy et al., 1998) and physiological similarities to

humans (Puiman and Stoll, 2008). As an example, pigs have been used for studies on arterial restenosis because they are devoid of brown fat postnatally and because of their similar metabolic features, cardiovascular system and proportional organ sizes (Spurlock and Gabler, 2008). However, this type of model is an expensive and time-consuming option (Litten-Brown et al., 2010). Further, there are relatively few studies on cardiovascular disease models in pigs.

Rodent models have been established in biomedical research for over 100 years and have offered many advantages for studies on cardiovascular and metabolic diseases. This includes genetic models of obesity and diabetes, including *db/db* mice, *ob/ob* mice, Zucker diabetics fatty rats and Otsuka-Long Evans Tokushima fatty rats. The characteristics of these models include mutations in the leptin gene or receptor and decreased cholecystokinin-1 receptor density (Panchal and Brown, 2010). Leptin controls energy intake and expenditure, meanwhile cholecystokinin is a peptide hormone secreted from L-cells in the intestine that regulates digestion and food intake. Genetically engineered diabetic mice models, either transgenic or knockout, were also used in metabolic syndrome studies. In these models, proteins involved in development of obesity and diabetes were removed which included insulin receptor, GLUT4, IRS-1 and IRS-2 (Panchal and Brown, 2010). Other widely-used models include type 1 diabetes induced by single injections of alloxan or streptozotocin to induce selective necrosis of pancreatic β-cells in rats, mice and rabbits (Damasceno et al., 2014). These models provide useful information about particular targeted proteins, its receptor and the intracellular pathways involved in the regulation of metabolism (Panchal and Brown, 2010).

The use of hypercaloric diets as models is also a common strategy used to induce metabolic syndrome. The diets include fructose, sucrose, cholesterol, saturated fats and salt. Researchers have used different combinations of these foods to mimic metabolic syndrome. These diet-induced models differ in the contribution of carbohydrate and fat to available calories and the sources of fat. In this thesis, the high-carbohydrate, high-fat diet-induced model of the metabolic syndrome in rats was used. This diet has been used because it mimics the human diet associated with the development of metabolic syndrome (Panchal et al., 2011). More details are explained in Chapter 2.

Linseed as a Functional Food for the Management of Obesity

13

Siti Raihanah Shafie, Hemant Poudyal, Sunil K. Panchal, and Lindsay Brown

Obesity and Functional Foods

In the short term, energy from food is stored predominantly as glycogen for rapid release as glucose; in the longer term, fats are favoured for energy storage. This storage allows the energy from times of excess to be used in times of famine. Continued excess energy intake means that stores keep increasing, resulting in obesity, especially in the abdomen. Obesity is an enormous cost to the community as it greatly increases the risk of chronic diseases including diabetes, disability, depression, cardiovascular disease and some cancers, as well as mortality [1]. Food is essential for nutrition, but non-nutritive components of food can promote health and reduce the risk of disease. This combination of nutrition and health benefits defines functional foods and includes bioactive fatty acids, phenolic compounds, plant sterols, dietary calcium and dietary fibre [2].

S.R. Shafie · L. Brown (✉)
School of Health and Wellbeing, University of Southern Queensland, Toowoomba, QLD 4350, Australia
e-mail: Lindsay.Brown@usq.edu.au

S.R. Shafie
e-mail: SitiRaihanah.Shafie@usq.edu.au

S.R. Shafie
Department of Nutrition and Dietetics, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

H. Poudyal
Department of Diabetes, Endocrinology and Nutrition, Graduate School of Medicine, The Hakubi Center for Advanced Research, Kyoto University, Kyoto, 606-8302, Japan
e-mail: hpoudyal@kuhp.kyoto-u.ac.jp

S.K. Panchal · L. Brown
Institute for Agriculture and the Environment, University of Southern Queensland, Toowoomba, QLD 4350, Australia
e-mail: Sunil.Panchal@usq.edu.au

History of Linseed

Wild flax fibres were possibly used for making cords by early hunter-gatherers 32,000-26,000 years ago in Dzudzuana cave, Georgia [3], but the evidence for identification as flax is argued [4]. Flax or linseed (*Linum usitatissimum* L.) is now known only as a cultivated plant with domestication in the Near East as one of the original crops in agriculture more than 8000 years ago, with pale flax as the progenitor [5–7]. Linseed is the emblem of Northern Ireland, and common linseed is the national flower of Belarus. Linseed has been widely used to make linen for cloth since ancient times. Linseed oil has been used for medicinal purposes in both ancient and modern societies and has been discussed as a modern functional food [8].

Linseed is found in two varieties with shiny yellow or dark brown seed. The seed is oblong in shape, flattened with a pointed tip, smooth glossy surface, comprising an embryo with two cotyledons surrounded by a thin endosperm [9]. Linseed is mainly grown for its oil as a major source of α-linolenic acid (ALA, Fig. 13.1), the essential n-3 fatty acid, but there are also industrial, medicinal and nutritional uses. Linseed yields vary depending on the season, weather and location, with best yields in moderate-to-cool climates [10]. Canada is the world's largest producer of linseed with 2013 production of 712,000 tons or around 32 % of the world production of 2.2 million tons; other countries producing more than 100,000 tons in 2013 were China, Russia, Kazakhstan, India and Ethiopia [11, 12]. Australia is one of many minor producers with about 7000 tons in 2013. The average yield in Canada in 2013 was 1728 kg/ha with lower average yields in Australia of 1133 kg/ha and India of 435 kg/ha [12].

M.V. Hegde et al. (eds.), *Omega-3 Fatty Acids*, DOI 10.1007/978-3-319-40458-5_13

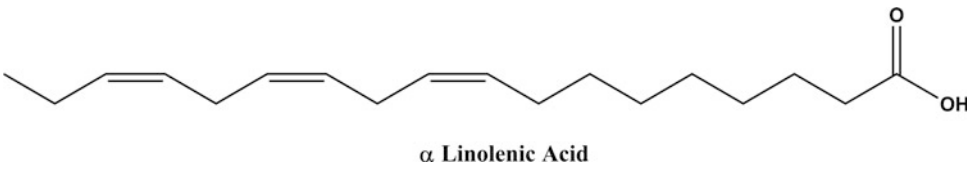

Fig. 13.1 Chemical structure of ALA

Linseed Composition

Canadian linseed contained an average of 41 % oil, 20 % protein, 29 % total carbohydrate mainly as fibre (28 %) with some sugars and starches (1 %), 7.7 % moisture and 3.4 % ash [13]. Comparisons of protein, fat and carbohydrate content of linseed, linseed meal, de-oiled linseed meal and processed fractions of linseed oil are available [14]. The 2012 survey of Canadian linseeds showed that the oil contained 56.7 % ALA, 19.2 % oleic acid, 14.7 % linoleic acid, 5.0 % palmitic acid, 3.4 % stearic acid and 0.2 % non-esterified fatty acids [14]. Linseeds contain plant lignans, especially secoisolariciresinol diglucoside (SDG), which act as precursors of mammalian lignans also known as enterolignans (Fig. 13.2), as well as vitamins and minerals (Table 13.1) [8].

Isolation of Linseed Oil

High-quality linseed oil is produced commercially by processes that include seed cleaning, flaking, cooking, pressing, solvent extraction using *n*-hexane and solvent removal [15]. These methods will influence the quality of oil because of the heat involved during the procedures. In addition, the use of *n*-hexane in defatting linseed meal enriches cyanogenic glycosides such as linustatin (Fig. 13.3), neolinustatin and linamarin which can result in goitrogenic problems in humans [16]. Cold press isolation is preferred to obtain high-quality oil but this process consumes more energy with lower yield [17]. The lack of refining processes of cold press oil is a marketing advantage by producing "first press" oil [18]. Alternative extraction methods that improve yield and maintain quality and stability of the oil include supercritical fluid extraction, accelerated solvent extraction, aqueous enzymatic extraction, ultrasound-assisted extraction and microencapsulation of oil by spray-drying techniques [17, 19]. The oil yields obtained by supercritical fluid and accelerated solvent extraction of 36.49 g and 41.90 g/100 g seeds, respectively, compared well to conventional solvent extraction of 42.40 g/100 g [15].

Pharmacology and Therapeutic Benefits of ALA

Linseed is high in the essential n-3 fatty acid, ALA. The Australian Heart Foundation recommends the consumption of at least 2 g/day of ALA to reduce the risk of heart disease [20].

ALA is highly bioavailable with oral absorption in adults of 96 % [21] while a small proportion may also be converted to conjugated linolenic acid primarily in the caecum and colon by the gut microbiota [22]. In preterm infants, absorption of [U-(13)C] labelled ALA from preterm formulae ranged from 74 to 98 % with higher absorption with increasing birth gestation [23]. Higher plasma ALA concentrations were observed after whole linseed supplementation compared to milled seed or linseed oil supplementation [24, 25]. Further, in vivo experiments in rats suggest that emulsification of linseed oil increases the rate and extent of ALA recovery in the thoracic lymph duct when compared to non-emulsified oil (C_{max} = 14 mg/ml at 3 h and 9 mg/ml at 5 h, respectively) with higher area under the curve in the emulsion group (48 mg × h/ml and 26 mg × h/ml, respectively) [26].

Once absorbed, ALA has at least three metabolic fates. It may undergo β-oxidation and carbon recycling, be deposited as ALA predominantly in adipose tissue, skin and muscle, or undergo a series of elongation and desaturation steps to produce eicosapentaenoic acid (EPA; 20:5n-3), docosapentaenoic acid (DPA; 22:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) [27–29]. In vivo tracer studies have shown that the oxidation of ALA in rats is efficient and comparable to lauric, oleic and linoleic acids, the most highly oxidisable fatty acids [30]. Of the total ingested ALA, 58.9 % was assumed to be removed by oxidation, 0.4 % was excreted in faeces, 10.6 % accumulated as ALA in adipose tissue, 9.7 % accumulated in the carcass-skin compartment, 4 % accumulated as ALA in organ compartment and 17.2 % accumulated as long-chain derivatives (14 % as DHA and 3.2 % as EPA + DPA) [31]. Similar results were obtained for disappearance, excretion and total accumulation with DHA [31].

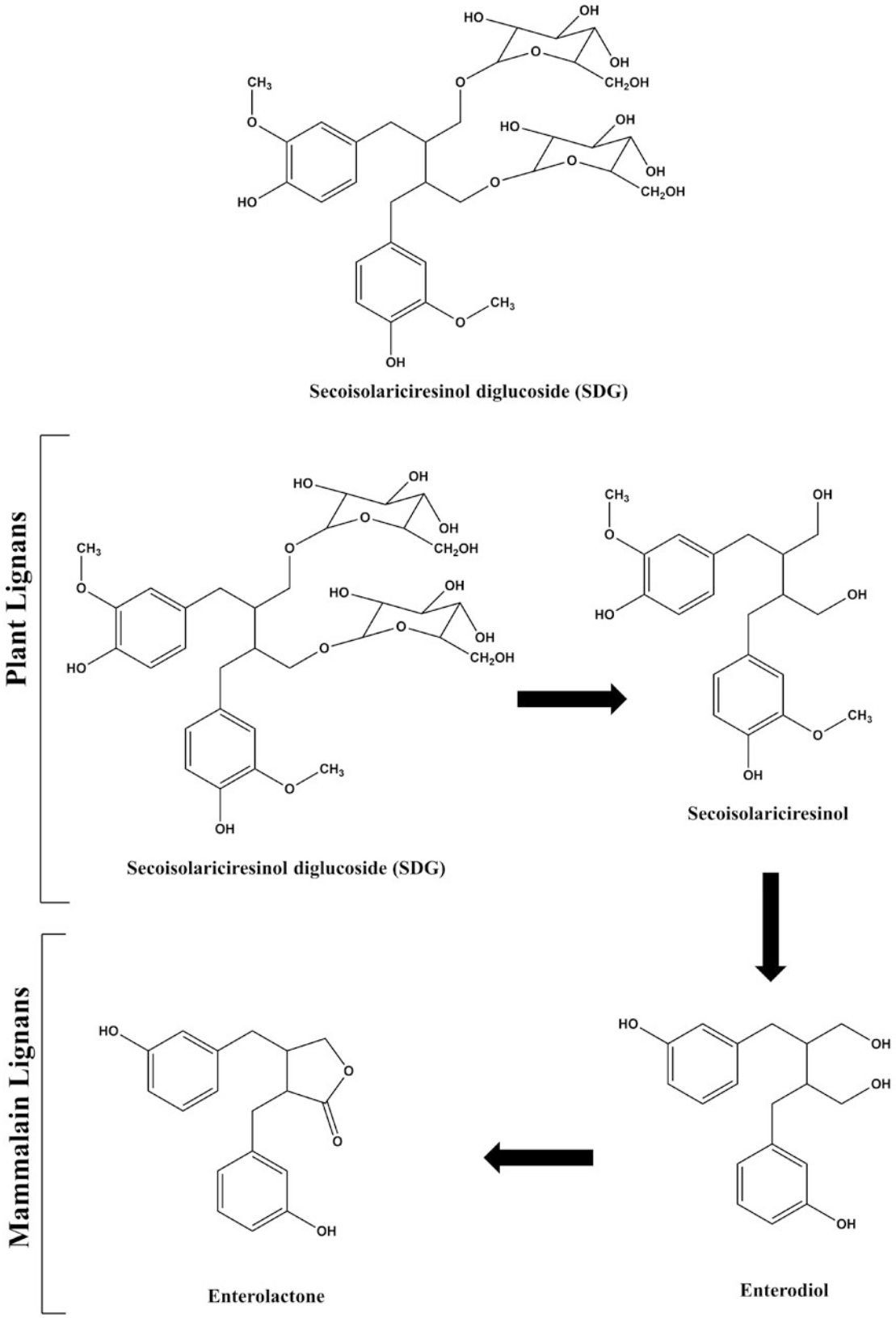

Fig. 13.2 Structures of SDG and its metabolites

Table 13.1 Vitamins and minerals in linseed

Linseed components	Quantity/100 g of seed	Proportion of recommended dietary intake (%)	
		Men	Women
Vitamins			
Ascorbic acid	0.50 mg	1.1	1.1
Thiamine	0.53 mg	44.2	48.2
Riboflavin	0.23 mg	17.7	20.9
Niacin	3.21 mg	20.1	22.9
Pyridoxine	0.61 mg	46.9	46.9
Pantothenic acid	0.57 mg	9.5	14.25
Folic acid	112 µg	28	28
Biotin	6 µg	20	24
γ-Tocopherol	8.5–39.5 mg	–	–
Minerals			
Calcium	236 mg	23.6	23.6
Copper	1 mg	58.8	83.3
Magnesium	431 mg	107.8	139
Manganese	3 mg	54.5	60
Phosphorus	622 mg	62.2	62.2
Potassium	831 mg	21.9	21.9
Sodium	27 mg	2.9–5.9	2.9–5.9
Zinc	4 mg	28.6	50

Sources Flax Council of Canada (2007) [13] and Nutrient reference values for Australia and New Zealand from National Health and Medical Research Council [135]

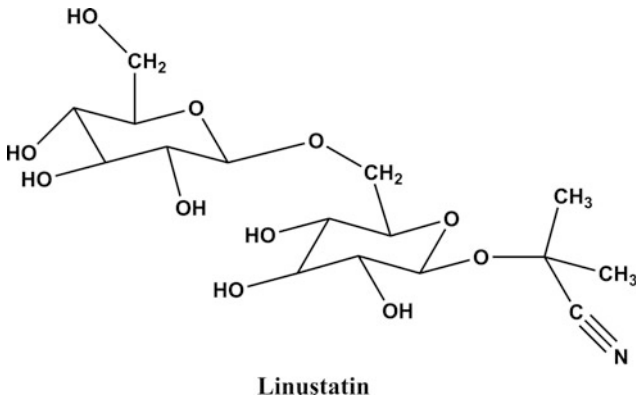

Fig. 13.3 Chemical structure of linustatin

The extent of conversion of ALA to EPA and DHA in humans has been a matter of great debate [29, 32, 33]. Healthy young men consuming a diet providing 6.3 % calories from ALA (linseed oil diet containing 28.8 % energy from fat) increased ALA concentrations in the serum and peripheral blood mononuclear cells lipids along with increase in the EPA and DHA contents of mononuclear cell lipids [34]. A physiological compartmental model of ALA metabolism derived from plasma concentration-time curves for radiolabelled ALA, EPA, DPA and DHA after consumption of 1 g oral dose of an isotope tracer ALA suggests that only about 0.2 % of the plasma ALA was destined for the synthesis of EPA, but approximately 63 % of the plasma EPA was accessible for the production of DPA and 37 % of DPA was available for the synthesis of DHA [35]. However, in plasma phospholipids, which may more closely resemble hepatic n-3 fatty acid metabolism, 7 % of dietary ALA was incorporated into plasma phospholipids, 99.8 % of which was converted into EPA and 1 % of the EPA was converted into DPA and subsequently into DHA [36]. This study suggests that the limited incorporation of dietary ALA into the hepatic phospholipid pool contributes to the low hepatic conversion of ALA into EPA [36]. A low conversion of ALA-derived EPA into DPA might be an additional obstacle for DHA synthesis [36]. However, in women, estimated net fractional conversion of ALA to EPA was 21 %, to DPA was 6 % and to DHA was 9 % with approximately 22 % undergoing β-oxidation [37]. These results suggest that women may possess greater capacity for ALA conversion than men.

Obese Patients

Whether ALA-rich linseed promotes weight loss in obese humans is controversial. Short-term (2–8 weeks) dietary intervention studies with linseed oil or flour supplementation in obese patients did not provide any evidence of weight loss [38, 39]. However, 12 weeks of linseed supplementation (30 g/day) in patients with metabolic syndrome decreased the prevalence of metabolic syndrome along with reduced central obesity, body weight, serum glucose, total- and LDL-cholesterol, apolipoprotein (Apo) B, ApoE and blood pressure [40]. However, it should be noted that the patients in the 12-week study were also undergoing lifestyle counselling using the American Heart Association guidelines [40].

Nevertheless, clinical studies consistently report beneficial effects of linseed in obese patients. In overweight or obese men and postmenopausal women with pre-diabetes, 13 g of ground linseed for 12 weeks decreased glucose and insulin concentrations and insulin sensitivity (HOMA-IR) [41]. Similar improvement in HOMA-IR was observed in obese glucose-intolerant patients [42]. Further, in middle-aged men and postmenopausal women, 40 g/day of ground linseed-containing baked products for 10 weeks improved insulin sensitivity suggesting that these effects are also observed in non-obese patients [43].

However, both short-term (3 weeks) and long-term (3 months) intervention studies with ALA from different sources including linseed oil did not improve glycaemic control in healthy or diabetic patients [44, 45]. ALA (6 g/day) from fortified rapeseed oil for 3 weeks in healthy, non-obese subjects did not affect fasting serum concentrations of glucose, insulin, fructosamine or hemoglobin A1c (HbA1c) [44]. ALA (5.5 g/day from 10 g/day linseed oil) in type 2 diabetic patients for 3 months also did not affect fasting serum glucose, insulin or HbA1c concentrations [45]. Together, these studies suggest that non-ALA components from linseed may be responsible for the improved insulin sensitivity in response to linseed supplementation.

Obesity-related inflammation is a key determinant of insulin sensitivity [46] with increased pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) and decreased anti-inflammatory adipokines such as adiponectin linked to the development of insulin resistance and type 2 diabetes [47]. It is possible that the improved insulin sensitivity in response to linseed noted above may be mediated by suppressing the pro-inflammatory state. However, this effect may be dependent on the form of linseed supplementation. A total of 13 g/day ground linseed (2.9 g/day ALA) for 12 weeks did not affect plasma C-reactive protein (CRP), adiponectin or IL-6 concentrations [41]. Markers of systemic inflammation did not change at the higher dose of 40 g/day (8.4 g/day ALA) ground linseed for 10–12 weeks [42, 43]. In contrast, linseed flour may have anti-inflammatory effects in obese patients since 30 g/day linseed flour supplementation (5 g/day ALA) for 2 weeks decreased CRP, serum amyloid A and fibronectin concentrations [39]. In morbidly obese patients, a higher dose of 60 g/day linseed flour (10 g/day ALA) for 12 weeks decreased neutrophil count and prevented further increases in fibrinogen and complement C4 concentrations and prothrombin time [48].

Improved insulin sensitivity and decreases in the pro-inflammatory state may be further assisted by reduced apparent digestibility of fat in response to dietary linseed supplementation [49]. Linseed supplementation in rye bread for 8 weeks increased faecal dry weight and excreted fat suggesting decreased fat digestibility without affecting average transit time [49]; further, linseed does not affect satiety [50]. In women in the late postoperative period following Roux-en-Y gastric bypass, whole or defatted linseed did not affect hunger, satisfaction, fullness or desire to eat for up to 180 min after ingestion of test meals [50]. However, defatted linseed increased serum leptin concentrations [50].

Obesity is also a key risk factor for breast cancer, particularly in postmenopausal women [51]. Elevated oestradiol, oestrone and testosterone concentrations are important predictors of breast cancer in postmenopausal women [52–54]. In postmenopausal women, consuming 7.5 g/day of ground linseed for the first 6 weeks and 15 g/day for an additional 6 weeks decreased oestradiol, oestrone and testosterone concentrations with pronounced effects in overweight/obese women [55]. The decreases in these hormone concentrations in response to linseed do not seem to worsen osteoporosis. A systematic review of 30 human and animal studies suggested that ALA from linseed oil, but not the lignan fraction, benefited osteoporotic bone, especially together with oestrogen therapy [56].

Further, in obese insulin-resistant patients, ALA supplementation (20 g/day for 4 weeks) from margarine products based on linseed oil increased systemic arterial compliance without changing mean arterial pressures [57]. Many animal and human studies have confirmed the blood pressure lowering effects of linseed but only after 12 weeks of treatment [58].

Other sources of ALA, such as rapeseed oil, perilla oil and chia seed, reduced blood pressure with greater reductions in diastolic blood pressure [59–62], serum inflammatory biomarkers such as CRP, plasminogen activator inhibitor-1 (PAI-1) and TNF-α, as well as YKL-40 (chitinase-3-like protein 1), an inflammatory biomarker for coronary artery disease [60, 62, 63], and reduced triglycerides [59, 63] in obese or overweight patients with metabolic syndrome but with no effect on body weight.

In summary, whole linseed improves insulin sensitivity, linseed flour but not oil attenuates the pro-inflammatory state and linseed oil but not the lignan fraction benefits osteoporotic bone in obese or overweight patients.

Obese Rodents

Studies in obese rats and mice suggest that linseed may protect from obesity-associated heart, kidney and liver damage, supporting the beneficial effects of linseed in obese patients. We have previously demonstrated that linseed and chia seed oil supplementation for 8 weeks, both containing about 59 % ALA, did not reduce total body fat in a high-carbohydrate, high-fat diet-induced model of metabolic syndrome in rats but induced lipid redistribution away from the abdominal area and improved glucose tolerance, insulin sensitivity, dyslipidaemia, hypertension and left ventricular dimensions, contractility, volumes and stiffness [64, 65]. Additionally, both linseed and chia seed oil supplementations reversed inflammation in the heart and the liver, cardiac fibrosis and hepatic steatosis [64, 65].

The obese spontaneously hypertensive/NIH-corpulent (SHR/N-cp) rat is a genetic model of obesity and type 2 diabetes that consistently develops nephropathy resembling human diabetic nephropathy [66]. Addition of 20 % linseed meal to the diets of obese SHR/N-cp rats for 6 months did not affect body weight or plasma glucose but decreased plasma insulin concentrations [66]. Measures of renal function such as plasma creatinine, creatinine clearance and urinary urea excretion also did not change but urinary protein excretion was lower in rats fed linseed meal diet [66]. Further, the percentage of abnormal glomeruli with mesangial expansion and the tubulointerstitial score as an index of severity of tubulointerstitial damage were reduced in rats fed linseed meal [66]. Linseed oil supplementation reduced concentrations of fasting serum insulin, urinary thiobarbituric acid reactive substances, hepatic triglycerides and cholesterol with higher hepatic mRNA expression of PPAR-γ as early as 4 weeks in SHR/N-cp rats fed a high-fat diet [67]. Further, linseed meal (20 % of energy) lowered plasma total, LDL- and HDL-cholesterol, and plasma triglyceride concentrations and lowered hepatic fat deposition [68]. Consistent with these findings in the liver, dietary linseed oil supplementation for 12 weeks in high-fat diet-fed rats prevented hepatic steatosis with increased EPA and DHA and lowered arachidonic acid incorporation into hepatic phospholipids [69]. These effects may be mediated, at least in part, by SDG as discussed in subsequent sections.

In Zucker fatty rats, linseed supplementation for 9 weeks did not affect obesity, oral glucose tolerance, pancreatic function or molecular markers related to insulin, glucose and lipid metabolism [70]. However, ALA-rich linseed oil supplemented diet for 8 weeks decreased adipocyte size and adipose tissue concentrations of MCP-1, IL-10 and TNF-α [71]. Linseed oil supplementation reduced T-cell infiltration into the adipose tissue but did not alter macrophage infiltration [71].

Some of the effects of linseed oil may also be neurally regulated. In a mouse model of diet-induced obesity, partial substitution of the fatty acid component of the diet by linseed oil attenuated hypothalamic inflammation, hypothalamic and whole body insulin resistance and whole body adiposity [72]. In addition, after intracerebroventricular injection in obese rats, pure ALA reduced spontaneous food intake and body mass gain [72]. These effects were accompanied by the reversal of functional and molecular hypothalamic resistance to leptin/insulin and increased pro-opiomelanocortin and cocaine- and amphetamine-mediated upregulation of transcript expressions [72]. Additionally, ALA inhibited the AMP-activated protein kinase/acetyl-CoA carboxylase (AMPK/ACC) pathway and increased carnitine palmitoyltransferase 1 (CPT1) and stearoyl-CoA desaturase-1 (SCD-1) expression in the hypothalamus [72]. Finally, acute hypothalamic injection of ALA activated signal transduction through the recently identified G protein-coupled receptor (GPR) 120 unsaturated fatty acid receptor [72].

Possible Mechanisms of Action of ALA

The mechanisms of action of ALA can be broadly classified into three categories depending on its metabolism. Firstly, ALA may act directly, as shown in studies on insulin secretion and sensitivity. ALA is considered to be the endogenous ligand of GPR40 and GPR120, and these receptors are the predominant mediators of ALA-induced insulin secretion [73]. The insulinotropic effects of ALA may be augmented by ALA-induced expression and secretion of insulin-like growth factor-I from hepatocytes [74]. Further, the effects of ALA could be mediated by reduced production of pro-inflammatory cytokines from adipose tissue [71] leading to improved insulin sensitivity in both animal and human studies as discussed in preceding sections. Further, ALA supplementation may suppress the expression and activity of SCD-1 [64, 65, 75–77]. Suppressed SCD-1 activity reduces body adiposity, increases insulin sensitivity, provides resistance to diet-induced obesity and may produce anti-inflammatory effects such as reduced macrophage inflammatory response, reduced expression of MCP-1, TNF-α, PAI-1 and vascular cell adhesion molecule 1 (VCAM-1), as well as chemokine receptor 2, colony stimulating factor 1 (CSF-1) receptor and decreased DNA-binding activity of NF-κB p65/50 [78–82].

Secondly, assuming adequate conversion, some of the effects of ALA could be mediated by EPA and DHA. EPA and DHA limit both hypertrophy and hyperplasia of adipocytes, increased lipolysis and increased fat oxidation, assisted by increased expression and activity of CPT1 and peroxisomal acyl-CoA oxidase gene that regulate the

alternate pathway of fatty acid oxidation [29]. Further, EPA and DHA can also directly act on GPR120 in the enteroendocrine L cells to secrete glucagon like peptide-1, a potent insulin secretagogue [83].

Thirdly, metabolites of EPA and DHA could also mediate the effects of ALA. 5-hydroxy-eicosapentaenoic acid (5-HEPE), a direct metabolite of EPA, is a potent agonist for GPR119, expressed in the pancreas and the intestine, and enhances glucose-dependent insulin secretion [84]. Further metabolism of EPA by the action of lipoxygenase and cyclooxygenase produces eicosanoids that induce vasodilatation and are potent anti-inflammatory and anti-thrombotic molecules [29, 85]. EPA-derived 15-hydroxy-eicosapentaenoic acid (15-HEPE) may have anti-inflammatory effects through the inhibition of 5-lipoxygenase, a major enzyme that generates pro-inflammatory lipid mediators [86]. Additionally, ω-hydroxyl fatty acids such as 7,18-epoxyeicosatetraenoic acid and 19,29-epoxy docosapentaenoic acid derived by the action of cytochrome epoxygenases on EPA and DHA, respectively, have potent anti-inflammatory effects and reduce blood pressure [86]. Effects on renal function may be mediated by ALA-derived oxylipins associated with reduced glomerulomegaly in diet-induced obese rats fed a linseed oil supplemented diet [87].

DHA metabolites including resolvins, protectins, maresins, docosatrienes and neuroprotectins are important resolution phase mediators of inflammation [88–90] that could mediate important anti-inflammatory responses to ALA. The production of DHA-derived 17-hydroxy-DHA, the precursor of resolvin D1 and protectin D1, is decreased in adipose tissue of obese mice [91]. Further, in these obese mice, 17-hydroxy-DHA decreased the expression of pro-inflammatory cytokines (TNF-α, F4/80 and IL-6), increased adiponectin expression and improved glucose tolerance and insulin sensitivity [91]. In *db*/*db* mice, treatment with resolvin D1 (2 μg/kg) improved glucose tolerance, decreased fasting blood glucose concentrations, increased insulin-stimulated Akt phosphorylation in adipose tissue, increased adiponectin, decreased IL-6 and reduced $F4/80^{+}CD11c^{+}$ macrophages in adipose tissue indicating the important role of resolvin D1 in resolution of inflammation [92]. These studies provide evidence for the role of DHA-derived resolvin D1 in attenuation of inflammation, and hence, ALA-derived DHA could produce the same responses in obesity.

Isolation and Pharmacology of SDG from Linseed

Linseed is one of the richest sources of plant lignans with SDG as the major component at 1–26 mg/g of seeds or about 75–800 times greater concentrations than in other fibre-rich plants [13, 93]. SDG content depends on the linseed cultivars, growing region and method of analysis [94]. SDG is stored in plants as a complex macromolecular structure with an ester linkage to 3-hydroxy-3-methyl glutaric acid to give a molecular weight of ~4000 Da [95]. Other lignans in linseed include isolariciresinol and pinoresinol, also found in similar concentrations in sesame seeds [96], and matairesinol [97]. The most common extraction method for SDG uses aliphatic alcohol extraction followed by alkaline hydrolysis and chromatographic separation [16]. This was less efficient than direct alkaline hydrolysis in aqueous or alcoholic solutions as an initial alcoholic extraction did not completely recover the lignan from the plant matrix [98].

The biological responses to SDG from linseed are related to the gut bacteria-mediated conversion to the enterolignans, enterodiol and enterolactone (Fig. 13.2) [99, 100]. Interactions between dominant and subdominant intestinal anaerobes were involved in lignan breakdown by deglycosylation, demethylation, dehydroxylation and dehydrogenation of SDG [101, 102]. The oral bioavailability of lignans was influenced by the microflora present in the intestine, antibiotic use, food matrix, type and form (aglycone or conjugated) of plant lignan, chronic exposure and other host-related factors such as age and gender [103]. Enterodiol and enterolactone are absorbed by the colonic epithelium either as glucuronides or sulphate conjugates or as free forms into the blood stream or directly excreted via faeces [104]. Conjugated enterolignans are absorbed from intestinal tract and transported to the liver, while free forms are conjugated before being released into the bloodstream [105]. Both forms undergo enterohepatic circulation with further metabolism in the colon and excretion in the urine in conjugated form [106]. The enterolignans are excreted via urine with conjugated enterolignans excreted mainly as monoglucuronides with only small amounts excreted as monosulphates or free aglycones [107, 108].

Pharmacokinetic studies on oral SDG have been reported in both animals and humans. SDG was administered to male and female Sprague–Dawley rats by gavaging ^{3}H-SDG (3.7 kBq/g body weight) and unlabelled SDG (5.3 μg/g body weight) daily [109]. Total lignan content was excreted primarily in faeces (40–83 %) and urine (1.2–5.2 %) in both genders. After 1–7 days dosage with SDG, most of the tissue lignans were accumulated in the liver (48–56 %) in both males and females. Prolonged ^{3}H-SDG dosage demonstrated an increased accumulation in skin and kidneys. Serum concentrations were stable after exposure for 7 days. Gender differences in lignan tissue distribution were observed, with higher concentrations in females in heart and thymus at all time points. This finding suggests the possibility for distinct treatment responses in males and females [109].

In a human study, 6 male and 6 female healthy volunteers ingested a single dose of SDG (1.31 μmol/kg body weight)

[99]. Enterodiol and enterolactone reached their maximum plasma concentrations at 14.8 ± 5.1 h and 19.7 ± 6.2 h, respectively, after ingestion. Enterodiol showed a shorter mean elimination half-life of 4.4 ± 1.3 h than enterolactone (12.6 ± 5.6 h). Enterolactone demonstrated a higher mean area under the curve (1762 ± 1117 nmol/L h) than enterodiol (966 ± 639 nmol/L h). Similarly, enterolactone (35.8 ± 10.6 h) has a longer residence time than enterodiol (20.6 ± 5.9 h). These results indicate that enterolignans accumulate in plasma when SDG is consumed 2–3 times a day before reaching a plateau state. This study also showed that, within 3 days, up to 40 % of the ingested SDG was excreted via urine as enterolignans, with 58 % as enterolactone [99].

The bioavailability and pharmacokinetics of SDG have been measured using a range of dosages and purities of the SDG [110]. Healthy postmenopausal women were given 25, 50, 75, 86 or 172 mg of SDG and purity of 28.8, 43 or 74 % SDG for one week. The sugar moiety of SDG was efficiently hydrolysed by gastrointestinal bacteria to produce secoisolariciresinol. Enterolignans appeared in the plasma after 5–7 h with a plasma elimination half-life of 4.8 h. Serum enterodiol and enterolactone concentrations peaked after 12–24 h and 24–36 h, respectively, and the half-lives were 9.4 h and 13.2 h. This study observed linear dose responses over a relatively wide range of dietary SDG intakes. In addition, the bioavailability of secoisolariciresinol was correlated ($r^2 = 0.835$) with cumulative 5-day lignan excretion. Changes in the SDG purity did not change the pharmacokinetics, and serum enterolignan concentrations reached steady state after one week of daily dosing. This study suggests that SDG is first hydrolysed and then metabolised in a time-dependent sequence in the gastrointestinal tract to secoisolariciresinol, enterodiol and ultimately enterolactone, and these metabolites are efficiently absorbed and predominantly excreted via urine [110].

Therapeutic Benefits in Humans

The effects on cardiovascular function have been investigated using different SDG doses, different purity of the compound, with interspecies differences that influence the pharmacokinetic reactions and length of study period. A better understanding of SDG metabolism is needed to verify the best model and method to determine the potential for improvements in cardiovascular health.

A study on moderately hypercholesterolaemic non-obese men given a low dose of SDG (100 mg, equivalent to 7.5 g of linseed) did not change the body weight and BMI of the subjects at 12 weeks [111]. However, the treatment decreased the ratio of LDL-/HDL-cholesterol and serum cholesterol concentrations. These effects may be mediated by a decrease in the mRNA expression levels of sterol regulatory element-binding protein-1c (SREBP-1c) in the liver. Further, this treatment regimen decreased the activities of the plasma hepatic diseases risk markers, alanine transaminase (ALT) and γ-glutamyl transferase (GGT), but not aspartate transaminase (AST) [111].

Recently, a short-term trial using 40 g dose of golden linseed for 28 days in obese subjects showed no significance difference in body weight, BMI, waist circumference or blood pressure [112]. However, the results obtained demonstrated reduced fasting blood glucose concentrations and the study suggested increased glucose elimination via increased translocation of GLUT4 on the cell membrane and increased basal glucose uptake by redistribution of GLUT1 as plausible mechanisms. Reduction in three indicators of cardiac damage, lactate dehydrogenase (LDH), total creatine kinase (CK) and CK-MB isoform, strongly suggests cardioprotective effects with SDG from golden linseed [112]. Furthermore, the lignans such as SDG were most likely to be responsible for the improved function of the liver (measured as serum activities of ALT and AST) and kidneys (measured as serum creatinine, uric acid and blood urea nitrogen). Furthermore, SDG decreased serum malondialdehyde (MDA) and protein carbonyls and increased glutathione, indicating reversal of the oxidative stress. In addition, increases in antioxidant enzyme activities (superoxide dismutase, catalase and glutathione peroxidase) were observed after 28 days of treatment [112].

Actions in Rodents and Rabbits

SDG and secoisolariciresinol demonstrated strong free radical scavenging activity and antioxidant activity in vitro [113]. The pharmacological responses to SDG and its metabolites have been measured in different animal models including rabbits, rats and mice. Different mechanisms of action leading to anti-atherogenic and cardioprotective effects have been proposed.

SDG treatment in hypercholesterolaemic rabbits showed consistent cholesterol-lowering and anti-atherosclerotic responses [114–116]. Reduction of serum cholesterol, LDL-cholesterol and lipid peroxidation products and an increase in HDL-cholesterol were seen after 8 weeks of treatment with 15 mg/kg body weight of SDG in 1 % cholesterol diet and 40 mg/kg body weight of lignan complex in 0.5 % cholesterol diet in rabbits [114, 115]. These studies suggest the decreases in serum total cholesterol and LDL-cholesterol with lignan complex could be due to the 3-hydroxy-3-methyl glutaric acid and SDG content of the lignan complex [115]. Lower serum and aortic MDA concentrations were associated with reduced development of atherosclerosis in rabbits fed the lignan complex. These effects may be mediated by antioxidant activity of SDG to prevent oxygen radical-induced endothelial cell injury [115].

A different intervention which included 2 months of regular diet following 2 months of a high cholesterol diet with supplementation of 20 mg SDG/kg body weight/day resulted in no reduction in serum lipids. However, prevention of progression of atherosclerosis was seen that may be associated with a reduction of aortic oxidative stress [116].

Lipid homoeostasis was evaluated in diet-induced hypercholesterolaemic female Wistar rats treated with either SDG or secoisolariciresinol at different dosages of 0, 3 or 6 mg SDG/kg body weight/day or 0, 1.6 or 3.2 mg secoisolariciresinol/kg body weight/day, respectively, for 4 weeks [117]. Both SDG and secoisolariciresinol showed dose-dependent reduction in the serum and hepatic cholesterol concentrations. These changes were accompanied by increased hepatic expression of acetyl-CoA acetyltransferase 2 by SDG (3 and 6 mg/kg body weight/day) and decreased hepatic expression of cytochrome P450 7a1 by secoisolariciresinol (3.2 mg/kg body weight/day). No other changes were observed in hepatic expression of lipid controlling targets in rats treated with SDG or secoisolariciresinol [117].

High-carbohydrate (10 % fructose in drinking water) diet-fed male Sprague–Dawley rats as a model of hypertriglyceridaemia were used to measure dose-dependent effects of 0, 3 and 6 mg SDG/kg body weight/day for 2 weeks [117]. In contrast to the hypercholesterolaemic rats, no changes in body and organ weights, serum triglycerides, phospholipids and non-esterified fatty acid concentrations, and hepatic steatosis relative to vehicle control fructose-fed rats were measured at any dose [117]. SDG administration did not change the hepatic expression of PPAR-α or SREBP-1c following 10 % fructose in water supplementation [117].

Acute hyperlipidaemia can be produced in rats with poloxamer-407 (1 ml of 30 %, w/v; intraperitoneally) as it inhibits lipoprotein lipase and elevates the production of triglycerides. Oral treatment with a methanol fraction of linseed lignan concentrate (MF-FLC) in poloxamer-407 hyperlipidaemic rats was evaluated at 0, 15 h and 24 h after injection [118]. The administration of MF-FLC (100 mg/kg) and n-3 fatty acid (1 ml/kg; mainly containing DHA) reduced serum cholesterol, triglycerides and VLDL-cholesterol, whereas HDL-cholesterol was increased [118]. The combination of MF-FLC and n-3 fatty acid acts mainly by blocking cholesterol synthesis.

In rats given 2 % cholesterol for 8 weeks, SDG 20 mg/kg body weight improved lipid profiles after 2 weeks treatment. Dietary supplementation of SDG reduced infarct size, improved myocardial function and enhanced neovascularisation in the infarcted myocardium. These effects were mediated through improved thickness of ventricular wall during diastole and systole as well as improved left ventricular systolic function in the rats treated with SDG. Furthermore, SDG enhanced cardiac function by increasing protein expression of endothelial NOS (eNOS), vascular endothelial growth factor (VEGF) and hemeoxygenase-1 (HO-1) in SDG-treated rats [119].

In high-fat diet-fed mice, SDG supplementation (0.5 and 1.0 % (w/w) for 4 weeks) reduced high-fat diet-induced increases in visceral and liver fat accumulation, and in plasma lipids, insulin and leptin concentrations [120]. These effects of SDG were accompanied by suppressed SREBP-1c mRNA expression in the liver, increased adiponectin mRNA expression in the white adipose tissue and increased CPT1 mRNA expression in the skeletal muscle [120]. Further, the SDG metabolite, enterodiol, induced adipogenesis-related mRNA expression including adiponectin, leptin, GLUT4 and PPAR-γ, and induced PPAR-γ DNA-binding activity in 3T3-L1 adipocytes [120].

Linseed lignans may be able to prevent obesity in rodents. Treatment with 0.02 % SDG (35 %) lignan-enriched linseed powder (LEFP) lowered body weight as compared to high-fat diet control group due to lower visceral fat weight. In addition, lower plasma concentrations of leptin and higher plasma concentrations of adiponectin reflected a low visceral fat weight in treated rats. Supplementation with LEFP for 12 weeks improved systolic blood pressure and serum triglycerides, LDL-cholesterol and HDL-cholesterol concentrations. However, a limitation of this study should be noted as LEFP also contains other bioactive compounds including coumaric acid glucoside and ferulic acid glucoside [121].

Male Wistar rats were pretreated with 500 mg/kg flax lignan concentrate (FLC) for 8 days before isoprenaline (ISO) at a dose of 5.25 mg/kg on day 9 and 8.5 mg/kg on day 10 to induce moderate lesions in the myocardium [122]. The results showed that ISO + FLC rats had lower CK-MB, LDH and AST levels indicating protection against necrotic damage of the myocardial membrane. Inflammation, necrosis and congestion were observed in a smaller area of the heart in FLC + ISO compared to the ISO rats indicating that the rat hearts were partially protected by pretreatment with FLC (500 mg/kg) against isoprenaline-induced cardiotoxicity [122].

Isolation and Therapeutic Effects of Dietary Fibre from Linseed Mucilage

Linseeds contain approximately 8 % of the exopolysaccharide, mucilage [123], that can be easily extracted from the seed coat using water as this allows swelling of cell walls and then extrusion of the content on the surface of the seeds [124]. Basic or acid treatments will also extract the mucilage but these methods lead to higher concentrations of proteins in the mucilage and their subsequent denaturing [125]. Improved techniques have been introduced using

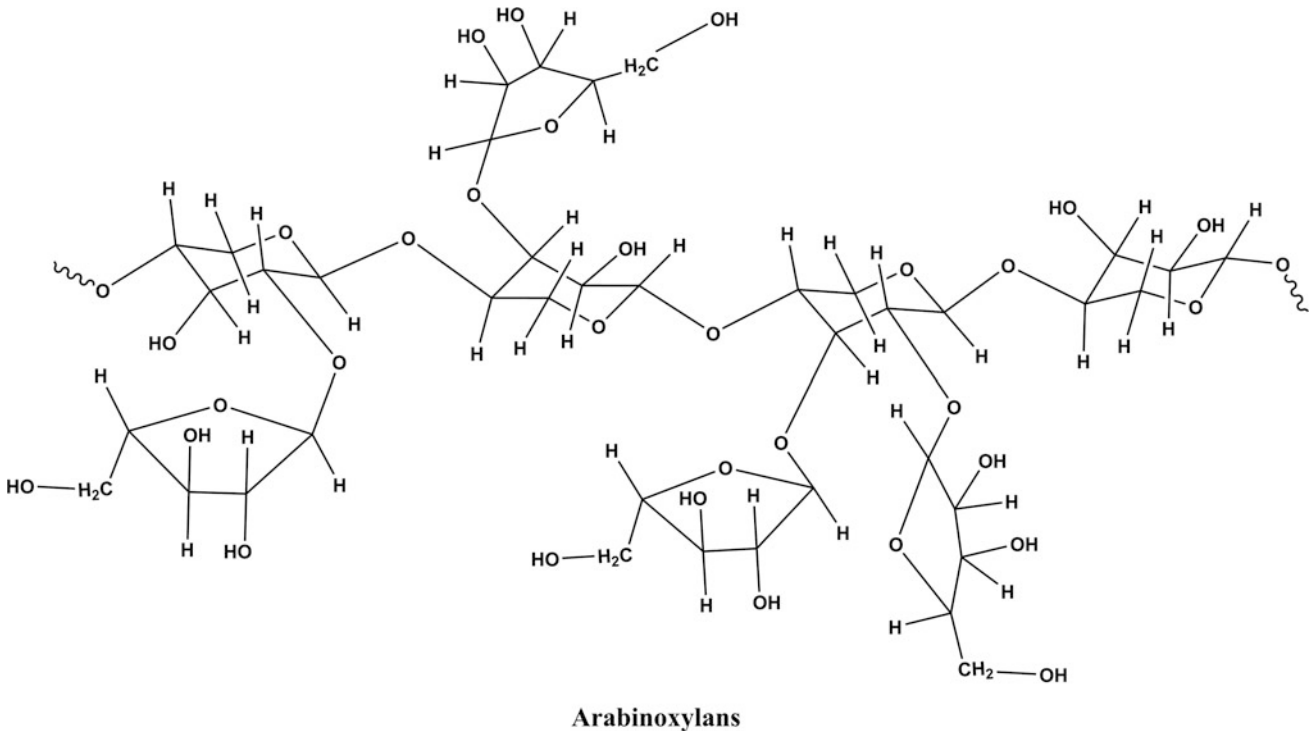

Fig. 13.4 Structure of arabinoxylans

non-classical heating modes such as microwave and ultrasound [126, 127]. However, these procedures with longer treatment times and higher temperatures can lead to degradation of the polysaccharides [127, 128]. The extraction yield of mucilage is also dependent on both the procedure and the variety of the raw material [129]. Linseeds contain approximately 28 % fibre, of which 70–80 % is water-soluble with most of these compounds present in the outermost layer of the seed hull [124]. The kernel of linseeds contains about 20 % fibre [130]. The major constituents of linseed mucilage are neutral and acidic polysaccharides, with the neutral fraction of the linseed mucilage composed mainly of arabinoxylans (Fig. 13.4) [131]. Linseed mucilage is used in the food industry to be incorporated into the daily diet in beverages, dairy products, bakery products and processed foods including thickeners, emulsifiers, stabilisers and fat replacers [130].

Human Studies

Linseed also consists of insoluble (viscous) dietary fibre that was found to play a role in cholesterol regulation. A study comparing three different diets, a low-fibre control diet, a diet with linseed fibre drink and a diet with linseed fibre bread for seven days, showed that a diet with 5 g dietary linseed fibre lowered fasting total cholesterol and LDL-cholesterol by 12 and 15 %, respectively, as compared to control group [132]. Linseed drink increased faecal excretion of energy and fat excretion by 23 and 55 %, respectively. Linseed bread ingestion was less effective than linseed drink due to the effect of differences in food matrix as baked products had less ability to induce viscosity due to processing and storage [132]. These results were consistent with a study showing that consumption of six wheat flour chapattis containing linseed gum (5 g) for three months by type 2 diabetic patients lowered fasting blood glucose, total cholesterol and LDL-cholesterol [133].

An intervention of rye bread enriched with whole linseed (10 %) was tested for 7 days in 11 healthy young men to measure the apparent digestibility of fat and the gastric transit time [49]. Linseed rye bread results in an increase in the faecal fat excretion. The possible mechanisms proposed from the results were inhibition of the pancreatic lipase activity, or binding of fat in the gastrointestinal tract by linseed. Linseed mucilage is proposed to be responsible for these effects as arabinoxylans present in linseed showed faecal bulking effects [49].

In a double-blind randomised crossover 7-h test meal study, 18- to 40-year-old males were given diets containing low dietary fibre, whole linseed, low dose mucilage and high dose mucilage [124]. This study identified the role of these interventions on the changes in plasma metabolic profile including glucose, triglycerides, non-esterified fatty acids and

insulin concentrations during 7 h. High dose mucilage meal reduced the area under the curve for triglycerides and non-esterified fatty acid concentrations during 7 h. High dose mucilage meal also provided greater feeling of satiety with less hunger and food intake compared to low-fibre meal [124]. High dose mucilage-containing meal exhibited a slower increase in ghrelin towards the end of the day indicating a delayed gastric emptying affected by a higher dietary fibre content of the meal. In addition, these effects may be due to the impaired dietary triglyceride absorption from the small intestine and increasing clearance of chylomicron particles and chylomicron remnant uptake, as well as impaired emulsification of lipids [124]. Other mechanisms may involve direct fat-binding and increased viscosity resulting in increased emulsified lipid droplet size and decreased lipolysis due to decreased insulin concentrations. Fermentation of linseed fibre in distal ileum may result in short chain fatty acids which may play a role in reducing the non-esterified fatty acids along with the reduction in the rate of glucose uptake leading to a decreased demand for insulin to match the rate of glucose uptake in peripheral tissues [124]. Another study suggests the plausible mechanism of the involvement of linseed fibre in interference with bile acid metabolism which hinders micelle formation and inhibits re-uptake of bile acids that leads to increased hepatic synthesis of bile acids, thus reducing cholesterol concentrations [132].

Rodent Studies

In male Wistar rats, dietary fibres including extracted linseed fibre and whole linseed fibre were tested for 21 days. Increased faecal fat excretion and reduced body fat and energy digestibility were seen in rats given extracted linseed fibre that in consequence suppressed body weight gain. There results were not seen in the rats that were given dietary fibre from cellulose, whole linseed or ground linseed [134]. The decreased energy availability from linseed fibre could be the reason for the decrease in the body weight. Increased content in caecum and colon was also observed in these rats, indicating increased fermentation with extracted linseed fibre [134].

Conclusion

Linseeds have been used for millennia as food and medicine, but the evidence supporting their use as a functional food for the treatment of obesity is relatively recent. Obesity is a complex disorder, so it is unlikely that a single compound will reverse the multi-organ effects. Linseeds provide ALA as an essential n-3 fatty acid, SDG as a lignan as well as fibre mainly as mucilage. These constituents have multiple biological effects to decrease the changes in obesity, probably by different mechanisms. The combination as linseeds can therefore be accurately described as a functional food. The full potential of linseeds in human obesity needs to be defined, and further studies on molecular mechanisms are also necessary.

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1.3 Objectives

The main objective of this research is to investigate the effects of saturated fatty acids and cereal-based products as functional foods on a validated rat model of metabolic syndrome. Towards achieving this primary objective, the related aims associated with it are identified as follows:

1. To determine the effects of different saturated fats together with a high intake of simple sugars such as fructose on cardio-metabolic health and on gut structure and function; and

2. To determine the effects of bioactive metabolites (lignans), raw linseed, defatted linseed, 5% high amylose wheat, and 20% high amylose wheat on cardio-metabolic health and on gut structure and function.

The hypotheses of the study are that:

1. Increased dietary saturated fats together with a high intake of fructose will induce metabolic changes and alter gut structure and function in the host but these changes will depend on the saturated fatty acid that is administered; and

2. Lignans, raw linseed, defatted linseed and high amylose wheat will produce similar improvements in attenuating the metabolic changes and on gut structure and function in rats fed a high-carbohydrate, high-fat diet.

Chapter 2 Rat model, materials and methods

2.1 Rat model

Metabolic syndrome is usually linked to poor dietary habits that are associated with western/cafeteria diet that is rich in fructose, sucrose and animal fat. To mimic metabolic syndrome in humans, an appropriate animal model fed with a similar diet producing metabolic syndrome complications is warranted. An established rat model using complex diet composition including fructose, condensed milk and beef tallow exhibits complications including hyperinsulinaemia, impaired glucose tolerance, central obesity, non-alcoholic fatty liver disease, cardiac remodelling, hypertension and endothelial dysfunction along with mild renal damage and increased pancreatic islet mass (Panchal et al., 2011). The advantage of using rat model is it shortened the period to develop the syndrome rather than years or decades as in humans. Animal study also provides the basic understanding of the intervention strategy before translating into human trials. This high-carbohydrate, high-fat diet-induced metabolic syndrome rat model has been used earlier to testing natural products for the attenuation of metabolic syndrome (Panchal et al., 2012, Poudyal et al., 2013b, Bhaswant et al., 2015). Different responses resulting from these interventions clearly suggest that this model is capable in responding to the pharmacological or nutritional interventions. Thus, utilisation of this model is the most relevant as it is close in mimicking human metabolic syndrome.

2.2 Rats and diet

Male Wistar rats (6 – 8 weeks old, weighing 250 - 300 g, n = 72) were purchased from the Animal Resource Centre in Perth, Australia. Rats were housed individually in temperature-controlled, 12-h-light/-dark conditions in the animal house facility of the University of Southern Queensland. The rats were acclimatised and were given free access to water and standard rat chow prior to diet intervention. All experimental

protocols were approved by University of Southern Queensland Animal Ethics Committee under the guidelines of the National Health and Medical Research Council of Australia. After the acclimatisation period, rats weighing 330 – 340 g were randomly allocated into different experimental groups according to studies.

2.3 Oral glucose tolerance test (OGTT)

OGTT (Figure 2.1) was performed at 0 week, 8 weeks and 16 weeks (Poudyal et al., 2012a, Panchal et al., 2011). Prior to OGTT, the rats underwent food deprivation and water *ad libitum* for 12 hours (overnight). Rats supplemented with fructose water were given normal drinking water during this period. The rats were given a dosage of 2 g/kg body weight of glucose as a 40% solution via oral gavage using a syringe. Tail vein blood samples were taken at 30, 60, 90 and 120 minutes following glucose administration. The tail vein was pricked and blood was taken to measure the basal blood glucose concentrations using Medisense Precision Q.I.D glucose meter (Abbott Laboratories, Bedford, MA, USA).

Figure 2.1: Oral gavage for oral glucose tolerance test

2.4 Systolic blood pressure (SBP) and abdominal circumference

SBP measurements were performed on all groups of rats at week 0, week 8 and week 16 (Poudyal et al., 2012a, Panchal et al., 2011). The systolic blood pressure of the rats was measured under light sedation following intraperitoneal (IP) injection of Zoletil (tiletamine 10 mg/kg, zolazepam 10 mg/kg; Virbac, Peakhurst, NSW, Australia). Blood pressure measurements were measured 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). The abdominal circumference of rats was measured using a standard measuring tape during the period of anaesthesia for SBP measurements.

2.5 Body composition by dual energy X-ray absorptiometry (DXA)

DXA (Figure 2.2) was performed to measure body composition (bone mass/fat mass/lean mass) of the rats at 16 weeks, 2 days prior to euthanasia for pathophysiological assessment. Rats were lightly anaesthetised with Zoletil (tiletamine 15mg/kg with zolazepam 15mg/kg) and Ilium Xylazil (xylaxine 6mg/kg), interperitonially. Rats were placed on the Norland XR36 DXA (Norland Corp., Fort Atkinson, WI, USA) scanner bed on a sheet of paper in the prone position (Ward LC, 2009). The scanner was operated according to manufacturer's instructions for animal scanning using manufacturer's recommended software for use with laboratory animals (Small Subject Analysis Software, version 2.5.3/1.3.1; Norland Corp) (Panchal et al., 2011). 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 (Panchal et al., 2011).

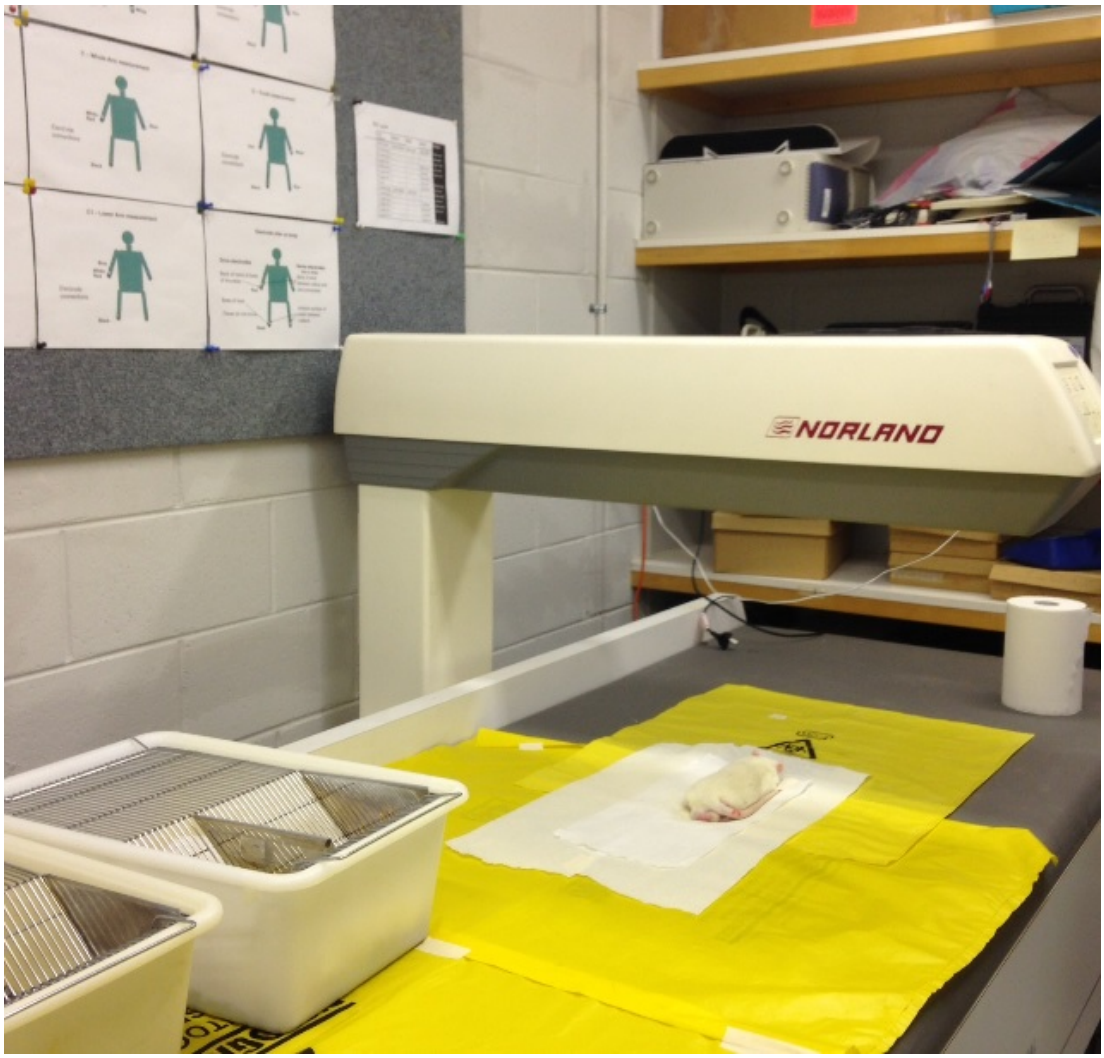

Figure 2.2: Dual energy X-ray absorptiometry (DXA) machine for scanning for body composition

2.6 Metabolic rate

Indirect calorimetry was used to measure oxygen consumption and carbon dioxide production using a 4-chamber Oxymax system (Columbus Instruments, Columbus, OH), with one rat per chamber at 16 weeks. The rat was provided with *ad libitum* food and water during the measurement. Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured individually from each chamber. The respiratory exchange ratio ($RER=VCO_2/VO_2$) was calculated by Oxymax software (v. 4.86). The interpretation of RER values is that oxidation of carbohydrate

produces a RER of 1.00, whereas fatty acid oxidation results in a RER of 0.70 (McLean, 1987). The energy expenditure was derived by assessment of the exchange of oxygen for carbon dioxide that occurs during the metabolic processing of food.

2.7 Isolated Langendorff heart preparation

Rats were euthanised via intra-peritoneal (IP) injection of Lethabarb (pentobarbitone sodium, 100 mg/kg, i.p.; Virbac). Then, heparin (200 IU; Sigma-Aldrich Australia) was injected through the right femoral vein and blood (~5mL) was withdrawn from the abdominal aorta. Hearts were removed and used in isolated Langendorff heart preparations (Figure 2.3) to assess left ventricular function of the rats (Panchal et al., 2011). Hearts isolated from the rats were immediately 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 to maintain heart viability. The buffer was aerated with 95% O_2–5% CO_2 and maintained at 35°C. The 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 (k, dimensionless).

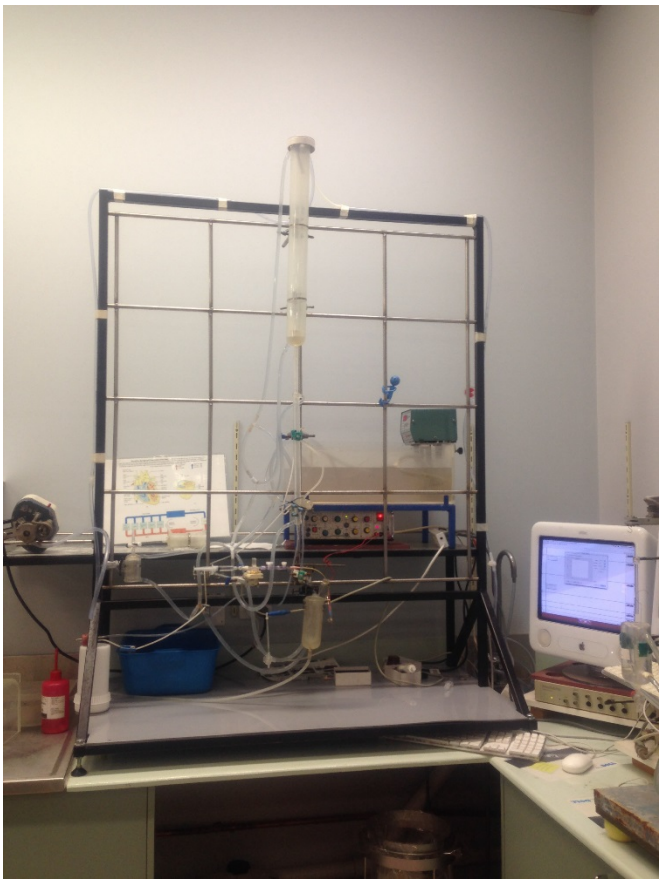

Figure 2.3: Isolated Langendorff heart apparatus

2.8 Aortic contractility

Thoracic aortic rings (~4 mm in length) were suspended in an organ bath chamber (Figure 2.4) with a resting tension of approximately 10 mN. Cumulative concentration–response (contraction) curves with concentrations ranging from 10^{-9} to 10^{-4} mol/L were measured for noradrenaline (Sigma-Aldrich Australia); concentration–response (relaxation) curves with concentrations ranging from 10^{-9} to 10^{-4} mol/L were measured for acetylcholine (Sigma-Aldrich Australia) and sodium nitroprusside (Sigma-Aldrich Australia) in the presence of a submaximal (70%) contraction to noradrenaline (Poudyal et al., 2010).

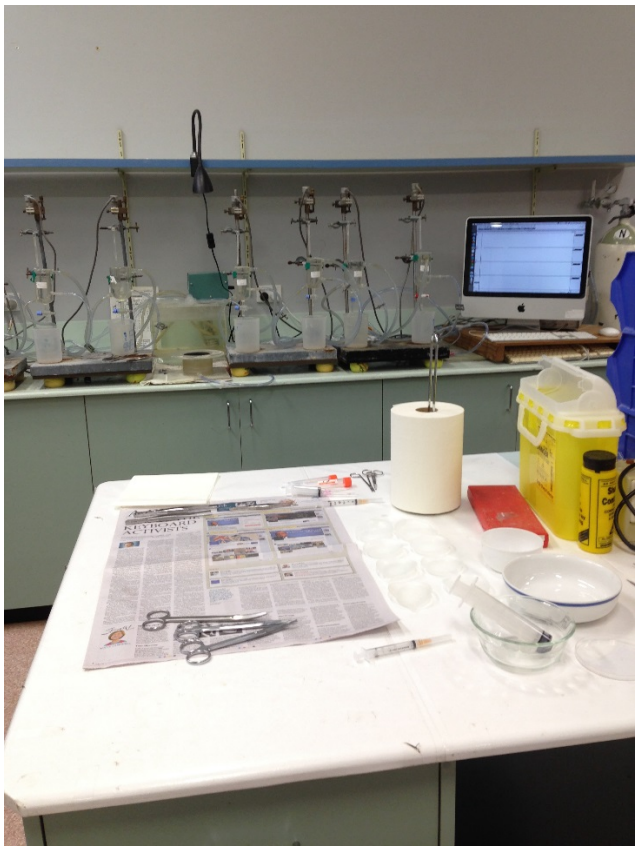

Figure 2.4: Organ bath setup

2.9 Ileum and colon contractility

Sections of distal ileum and colon of 1 cm length were isolated and immersed in a Petri dish containing Tyrode's solution to ensure tissue viability. The tissues were tied with thread and placed in the organ baths with a continuous supply of gas (95% O_2 – 5% CO_2) and were maintained at 37°C to keep the tissue alive. The tissues were stabilised at a resting tension of 10 mN. The ileum and colon reactivity as responses to acetylcholine (contraction) with a concentration ranging from 10^{-9} to 10^{-4} mol/L was measured based on dose-response on a MacLab system using Chart software (ADInstruments).

2.10 Tissue collection and histology

After performing Langendorff heart perfusion, hearts were separated into right and left ventricle (with septum) for weighing. Livers and abdominal fat pads (retroperitoneal, epididymal and omental) were isolated and weighed. Organ weights were normalised to the tibial length at the time of removal and expressed as mg of tissue/mm of tibial length. The heart, liver, small and large intestines of rats (n = 2 from each group) were exclusively used for histopathological analysis (Poudyal et al., 2012a). Thin sections (7 µm) of tissues were cut and fixed on slides. Heart sections were stained with picrosirius red to study collagen deposition and were analysed using fluorescent microscopy (EVOS® FL Cell Imaging System). Haematoxylin and eosin stain (Figure 2.5) was used to visualise infiltration of inflammatory cells in both heart and liver and then analysed using the same microscope. Periodic acid and Schiff reagent stain was used to visualise goblet cells in small and large intestines. 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.

Figure 2.5: Histology staining apparatus

2.11 Biochemical analyses

To obtain plasma, blood was centrifuged at 5000×g for 15 minutes within 30 minutes of the collection into heparinised tubes (Poudyal et al., 2012a). Plasma was separated and transferred to Eppendorf tubes for storage at −20°C before analysis. Plasma activities of alanine transferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) and plasma concentrations of total cholesterol, triacylglyceride (TG) and non-esterified fatty acids (NEFA) were determined. All these variables were measured using kits and controls supplied by Olympus using an Olympus analyser (AU 400, Tokyo, Japan) at The University of Queensland, Gatton campus.

Plasma concentrations of insulin and leptin (Laboratory Diagnostics) were measured using ELISA (ALPCO Immunoassays and ENZO) commercial kits according to manufacturer-provided standards and protocols using a Titertek Multiskan MCC/340 spectrophotometer (Flow Laboratories) and FLUOstar Omega Microplate Reader - BMG LABTECH.

2.12 Statistical analysis

All data were presented as mean ± SEM. Each group consists of 12 rats. All group data was tested for variance using Bartlett's test. Variables that were not normally distributed were transformed (using log 10 functions) prior to statistical analysis. The effects of diet, treatment and their interactions 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 non-parametric test was performed. All statistical analyses were performed using GraphPad Prism version 6 for Windows (San Diego, CA). A p value of < 0.05 was considered as statistically significant.

Chapter 3 Saturated fatty acids in metabolic syndrome in rats.

3.1 Introduction

Dietary fat is one of the lifestyle factors most often claimed to be responsible for the high prevalence of obesity. Many studies have proposed that the type of dietary fat plays a more significant role in the aetiology of obesity and metabolic syndrome than the absolute amount (Cordain et al., 2005, Agatston, 2005, Bray et al., 2002). However, it remains controversial which fatty acids promote obesity. Moreover, different dietary fatty acids, even within the same class, elicit different physiological responses (Poudyal and Brown, 2015). Saturated fat has long been generalised as the main cause of obesity, diabetes and heart diseases. The major saturated fatty acids (SFA) usually consumed in human diets are lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0) and stearic acid (C18:0) (Jonnalagadda et al., 1995) with the richest dietary sources being coconut oil, nutmeg oil, palm oil and beef tallow, respectively (Laureles et al., 2002, Piras et al., 2012, Scholtz et al., 2004, Monsma and Ney, 1993).

Epidemiological and clinical studies suggest that saturated fatty acids can have different effects on body weight regulation and accumulation of fat in the adipose tissues (Hariri et al., 2010b, Storlien et al., 2001, Ferreira et al., 2014). One large study with 810 participants demonstrated that a higher saturated fat intake for 6 months in adults was associated with a greater weight gain at 18 months ($P = 0.002$) (Lin et al., 2012). In contrast, a randomised crossover study of eight overweight or obese subjects, replacing SFA with monounsaturated fatty acids (MUFA) for 4 weeks, promoted weight loss, but SFA by itself did not promote weight gain (Piers et al., 2003). However, different nature of study, sample sizes and time frames could contribute to differences in study findings. In the USA, a substantial decline in the percentage of energy from total dietary fat and saturated fat during the last two decades has corresponded with a massive increase in obesity, and similar trends are occurring in other affluent countries (Willett, 2002). This suggests that dietary SFA itself is not a

determinant of adiposity. A review on possible roles of dietary fats in obesity using animal models show that high saturated fat diets induce obesity (Panchal et al., 2014). However, wide variations in fatty acid content and source as well as widely different micronutrient compositions (Hariri and Thibault, 2010) make it impossible to determine the effects of fat quantity or quality on obesity in animal models. Thus, it is important to have well-designed animal models in examining the effects of individual saturated fatty acids.

An established model of rats fed a high-carbohydrate, high-fat diet for 16 weeks has previously shown that this diet mimics many signs of human metabolic syndrome (Panchal et al., 2011, Poudyal et al., 2012b). Rats fed this diet developed hypertension, cardiac fibrosis, higher cardiac stiffness, endothelial dysfunction, impaired glucose tolerance, greater abdominal fat deposition, altered plasma lipid profile, hepatic steatosis with higher plasma activities of liver enzymes, higher plasma concentrations of markers of oxidative stress and inflammation as well as increased inflammatory cell infiltration in the heart and the liver (Panchal et al., 2011).

Since this model mimics most of the signs of diet-induced human metabolic syndrome, we conducted the present study to determine the cardiovascular, liver and metabolic effects of rats fed corn starch diet or the high-carbohydrate high-fat diet containing 20% beef tallow or one of four different saturated fatty acids (lauric, myristic, palmitic or stearic acids).

3.2 Methods

3.1.1 Rats and diet

All animal experiments were approved by the Animal Ethics Committees of the University of Southern Queensland and Queensland University of Technology under the guidelines of the National Health and Medical Research Council of Australia. The experimental groups consisted of 72 male Wistar rats (9–10 weeks old) weighing 332±1g purchased from Animal Resource Centre (www.arc.wa.gov.au). Rats were individually housed in a temperature-controlled, 12-h light/dark cycle environment with free access to water and food at the University of Southern

Queensland Animal House. The rats were grouped into 6 groups of 12 animals. Control (CS) diet contained 57% corn starch, 15.5% powdered rat food (Specialty Feeds, Glen Forest, Western Australia, Australia), 2.5% Hubble, Mendel and Wakeman salt mixture, and 25% water. High fat-high carbohydrate (HCHF) - based diets consisted of 17.5% fructose, 39.5% sweetened condensed milk, 20% beef tallow or SFA (20% lauric acid (HLA), 20% myristic acid (HMA), 20% palmitic acid (HPA) or 20% stearic acid (HSA)), 15.5% powdered rat food, 2.5% Hubble, Mendel and Wakeman salt mixture, and 5% water. The preparation and macronutrient and micronutrient compositions of CS and HCHF were previously described (Panchal et al., 2011, Poudyal et al., 2012a). All CS-based diets included drinking water without any additives while drinking water for H-based diets was supplemented with 25% fructose. The carbohydrate intake in both CS and H groups was approximately 68 %. 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:

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

The increase in body weight (%) was defined as body weight difference between day 56 (end of week 8) and day 112 (end of week 16); daily energy intake was defined as average daily energy intake from the end of week 8 to the end of week 16.

3.1.2 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 (Panchal et al., 2011, Poudyal et al., 2012a). Serial, *in vivo* left parasternal and left apical echocardiographic images of rats were obtained using the Hewlett Packard Sonos 5500 (12MHz frequency fetal transducer) at an image depth of 3 cm using two focal zones. Rats were anaesthetised with Zoletil (tiletamine 15mg/kg with zolazepam 15mg/kg; i.p.) together with Ilium Xylazil (xylazine 6mg/kg; i.p.) to produce general anaesthesia in rats for 2-3 hours without evidence of cardiac depression 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. Left ventricular M-mode measurements at the level of the papillary muscles included measurement of interventricular septum in diastole, diastolic posterior wall thickness (LVPWd), internal systolic dimensions, and end-diastolic dimension (LVIDd), posterior wall thicknesses and fractional shortening. Cardiac output, ejection fraction and left ventricular mass were derived from these values. Pulsed-wave Doppler analyses of mitral valve inflows were used as estimates of diastolic function. Following the scan, all rats were administered 5 mL (2.5mL each in 2 sites) of saline subcutaneously and were allowed to recover at a higher room temperature (24-25°C) to prevent dehydration and hypothermia respectively.

3.1.3 Other methods

All other methods were performed as described in Chapter 2 which included oral glucose tolerance test, systolic blood pressure, body composition, metabolic rate, isolated Langendorff heart preparation, ileum and colon contractility, tissue collections, histology and biochemical analysis.

3.1.4 Statistical analysis

All data were presented as mean ± SEM. Differences between the groups were determined by one-way analysis of variance. Statistically significant variables were treated with Tukey's *post hoc* test to compare each group of animals. All statistical analyses were performed using GraphPad Prism version 6 for Windows (San Diego, CA). P value of < 0.05 was considered as statistically significant.

3.2 Results

3.2.1 Dietary intake and body composition

The dietary intake and body composition for each group of rats is presented in Table 3.1. The food intake in the CS group was higher than in the other groups. This is because the texture of the food for CS is light and powdery. HCHF and HLA diet-fed rats had similar food intakes, with higher intakes than HMA, HPA and HSA diet-fed rats. HCHF and HMA groups showed similar water intakes which were lower than HLA, HPA and HSA groups. Stearic acid (HSA) and palmitic acid (HPA) showed highest energy intakes when compared to CS, HCHF, HLA and HMA but no difference with HPA (Table 3.1). However, HCHF diet-fed rats had increased body weight gain together with an increased feed conversion efficiency. Overall, final body weight depended on the fatty acid in the diet with HCHF > HPA = HMA = HSA > HLA groups. It was clearly shown in Figure 3.1 that HLA did not gain weight throughout the study. This can be compared with the changes in total fat mass, lean mass, abdominal circumference and visceral adiposity index (Table 3.1). The reduction in visceral adiposity index in HLA group was due to decreased total abdominal fat pads as shown in Table 3.1. For HMA, HPA and HSA rats, lean mass, fat mass, abdominal circumference and visceral adiposity index were similar. HLA rats had no difference in respiratory exchange ratio from other groups, however HLA rats had a lower heat production than other groups.

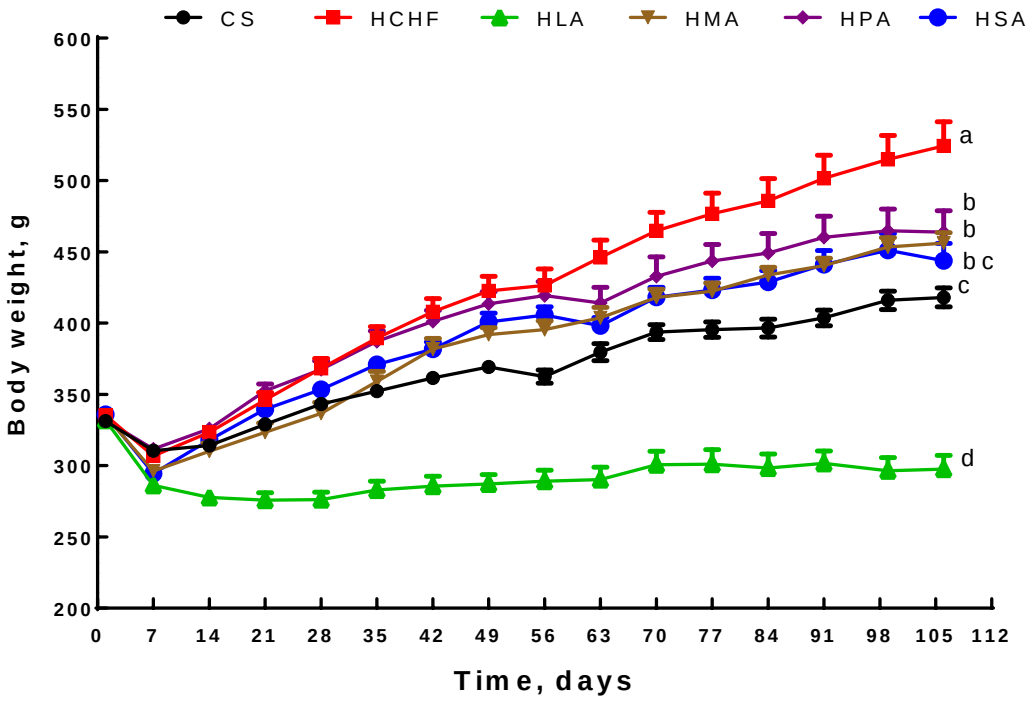

Figure 3.1: Body weight of CS, HCHF, HLA, HMA, HPA and HSA throughout 16 weeks.

Data are shown as mean ± SEM. End-point means without a common letter in each data set significantly differ at $p<0.05$ and n=12/group.

Table 3.1: Dietary intake, body composition and fat tissues proportions in CS, HCHF, HLA, HMA, HPA and HSA.

Variable	CS	HCHF	HLA	HMA	HPA	HSA
Food intake, *g/d*	40.2 ± 0.7^{a}	25.0 ± 0.6^{cd}	24.2 ± 1.0^{d}	27.7 ± 0.4^{bc}	29.7 ± 0.8^{b}	30.1 ± 0.7^{b}
Water intake, *mL/d*	26.1 ± 1.2^{b}	25.2 ± 1.0^{b}	36.5 ± 1.3^{a}	29.1 ± 0.7^{b}	34.4 ± 0.7^{a}	35.1 ± 0.8^{a}
Body weight gained, *%*	26.6 ± 1.9^{c}	55.2 ± 5.1^{a}	-11.1 ± 2.8^{d}	34.7 ± 1.9^{bc}	42.1 ± 4.6^{ab}	34.1 ± 3.4^{bc}
Energy intake, *kJ/d*	451.8 ± 7.0^{d}	543.2 ± 12.8^{c}	545.0 ± 20.1^{c}	601.2 ± 7.6^{b}	660.5 ± 11.1^{a}	671.0 ± 14.9^{a}
Feed conversion efficiency, *%*	19.5 ± 1.4^{b}	33.7 ± 2.7^{a}	-7.4 ± 2.0^{c}	19.3 ± 1.0^{b}	20.9 ± 1.9^{b}	16.9 ± 1.6^{b}
Bone mineral content, *g*	11.6 ± 0.3^{c}	15.4 ± 0.6^{a}	10.0 ± 0.2^{c}	13.5 ± 0.4^{b}	13.6 ± 0.6^{ab}	13.1 ± 0.4^{bc}
Total fat mass, *g*	79.5 ± 11.0^{bc}	207.7 ± 27.2^{a}	54.4 ± 3.2^{c}	132.4 ± 9.9^{b}	122.7 ± 12.9^{b}	122.9 ± 9.5^{b}
Total lean mass, *g*	305.0 ± 7.5^{a}	311.2 ± 16.6^{a}	229.3 ± 6.5^{b}	300.3 ± 7.3^{a}	305.9 ± 8.2^{a}	307.1 ± 7.5^{a}
Abdominal circumference, *cm*	20.2 ± 0.2^{b}	22.8 ± 0.5^{a}	17.4 ± 0.3^{c}	21.5 ± 0.3^{ab}	21.5 ± 0.2^{ab}	21.4 ± 0.3^{b}
Visceral adiposity index, *%*	4.0 ± 0.3^{b}	11.3 ± 2.0^{a}	3.6 ± 0.3^{b}	5.9 ± 0.3^{b}	5.4 ± 0.5^{b}	4.9 ± 0.4^{b}
Respiratory exchange ratio	1.01 ± 0.03^{a}	1.01 ± 0.03^{a}	0.9 ± 0.03^{a}	0.94 ± 0.02^{a}	1.01 ± 0.02^{a}	1.01 ± 0.03^{a}
Heat production, *Kcal*	3.16 ± 0.15^{b}	3.87 ± 0.09^{a}	2.46 ± 0.09^{c}	3.33 ± 0.16^{ab}	3.47 ± 0.13^{ab}	3.44 ± 0.11^{ab}
Retroperitoneal fat, *mg/mm*	155.1 ± 15.6^{b}	572.2 ± 73.0^{a}	98.0 ± 12.1^{c}	256.3 ± 15.4^{b}	248.4 ± 30.7^{b}	205.6 ± 16.8^{bc}
Epididymal fat, *mg/mm*	98.0 ± 7.0^{bc}	273.9 ± 23.2^{a}	49.0 ± 4.7^{c}	128.6 ± 7.2^{b}	128.7 ± 21.0^{b}	120.3 ± 13.2^{b}
Omental fat, *mg/mm*	88.6 ± 8.9^{c}	257.9 ± 24.6^{a}	66.6 ± 6.0^{cd}	153.1 ± 7.0^{b}	138.2 ± 17.2^{bc}	126.9 ± 9.8^{bc}
Total abdominal fat, *mg/mm*	341.8 ± 28.4^{bc}	1104.0 ± 118.1^{a}	213.6 ± 21.9^{c}	538.1 ± 26.6^{b}	515.3 ± 67.1^{b}	452.8 ± 36.6^{bc}

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

3.2.2 Hepatic structure and function

The wet weights of livers from CS rats were lower than HCHF-fed rats (Table 3.2). HCHF-fed rats had higher liver wet-weights than HLA and HSA-fed rats and no difference was found between HMA and HPA-fed rats. The livers from HLA group were the smallest compared to other H-fed rats but similar in size to the CS group. Plasma liver function in CS-fed rats showed lower ALT, AST and ALP activities compared to HCHF rats. HLA-fed rats had elevated plasma markers of liver function in comparison with CS, HCHF, HMA and HPA (Table 3.2). However, the values were no different to rats fed with HMA diet for plasma markers of ALT and ALP and not different in AST with HCHF diet. Figure 3.2 showed increased hepatic lipid deposition and inflammatory cell infiltration in all groups. Compared to CS, HCHF and HLA (Figure 3.2A, 3.2B and 3.2C) groups, HMA, HPA and HSA groups showed increased fat vacuoles (Figure 3.2D, 3.2E and 3.2F). HLA liver tissue appeared to be normal.

3.2.3 Metabolic changes

Plasma concentrations of total cholesterol (TC) for CS-fed rats showed no difference with HCHF-fed rats (Table 3.2). Similarly, there were also no differences in TC for CS-fed rats with either HPA or HSA rats. Results showed that plasma TC was higher in HLA diet–fed group compared to others, but it was no different to the HMA, HPA and HSA diet-fed groups (Table 3.2). Plasma non-esterified fatty acids (NEFA) concentrations were lower in CS rats compared to HCHF rats. The HLA group showed lower NEFA concentrations compared to HCHF and HSA rats and there was no difference with CS-fed rats. For plasma triglycerides, HCHF rats had higher concentrations than CS rats but not different from HMA, HPA and HSA rats. The HLA group had lower plasma triglyceride concentrations compared to the HCHF, HMA, HPA and HSA groups.

Plasma insulin and leptin concentrations were also measured in this study (Table 3.2). CS-fed rats had lower plasma insulin concentrations than HCHF-fed rats. The HMA, HPA, HSA and HCHF groups displayed similar plasma insulin concentrations. In contrast to other H-fed rats, the plasma insulin concentration of

HLA rats was similar to CS rats. This change in plasma insulin concentration is consistent with the findings in plasma leptin concentration, with CS rats showed lower plasma leptin concentration compared to HCHF rats and HCHF rats had the highest plasma leptin concentration compared to other H-fed rats. The lowest plasma leptin concentration was found in HLA rats and it was different from HCHF, HMA and HSA rats, but not different from CS and HPA rats.

Table 3.2: Hepatic structure and function, plasma lipid profiles and metabolic parameters in CS, HCHF, HLA, HMA, HPA and HSA groups at 16 weeks.

Variable	CS	HCHF	HLA	HMA	HPA	HSA
Liver, *mg/mm*	203.2 ± 9.3^{c}	345.9 ± 11.2^{a}	201.0 ± 9.2^{c}	303.8 ± 7.8^{ab}	322.2 ± 16.5^{ab}	295.8 ± 10.7^{b}
ALT, *U/L*	19.4 ± 1.5^{c}	40.9 ± 3.3^{b}	54.2 ± 3.8^{a}	47.3 ± 2.8^{ab}	37.7 ± 2.5^{b}	39.0 ± 2.6^{b}
AST, *U/L*	57.4 ± 3.9^{b}	66.4 ± 2.3^{ab}	75.3 ± 4.0^{a}	60.4 ± 3.4^{b}	56.2 ± 1.7^{b}	55.5 ± 2.5^{b}
ALP, *U/L*	121.7 ± 7.1^{c}	248.7 ± 17.9^{b}	326.1 ± 19.9^{a}	339.8 ± 23.2^{a}	234.9 ± 21.2^{b}	213.3 ± 10.8^{b}
Total cholesterol, *mmol/L*	1.4 ± 0.1^{c}	1.6 ± 0.0^{ab}	1.9 ± 0.1^{a}	1.8 ± 0.1^{a}	1.6 ± 0.1^{ab}	1.7 ± 0.1^{ab}
NEFA, *mmol/L*	1.1 ± 0.1^{d}	4.8 ± 0.3^{a}	2.3 ± 0.3^{cd}	3.2 ± 0.3^{bc}	3.6 ± 0.6^{abc}	4.1 ± 0.4^{ab}
TAG, *mmol/L*	0.4 ± 0.0^{c}	1.9 ± 0.2^{a}	0.7 ± 0.1^{c}	0.8 ± 0.1^{c}	1.0 ± 0.1^{bc}	1.4 ± 0.2^{ab}
Week 16 $OGTT^{AUC}$, *mmol/l. min*	687.3 ± 35.7^{a}	725.5 ± 21.1^{a}	482.0 ± 18.7^{b}	629.0 ± 19.4^{a}	670.8 ± 27.8^{a}	719.6 ± 23.6^{a}
Insulin, *µg/L*	2.4 ± 0.5^{ab}	4.1 ± 0.3^{a}	1.4 ± 0.4^{b}	3.8 ± 0.6^{a}	3.6 ± 0.4^{a}	3.6 ± 0.6^{a}
Leptin, *µg/L*	3.2 ± 0.4^{c}	11.3 ± 1.7^{a}	1.9 ± 0.3^{c}	7.9 ± 0.7^{ab}	5.5 ± 1.2^{bc}	9.7 ± 1.5^{ab}

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 ($P < 0.05$). ALT: Alanine aminotransferase, AST: Aspartate transaminase, ALP: Alkaline phosphatase, NEFA: Non-esterified fatty acids, TAG: Triglycerides, OGTT: Oral glucose tolerance test.

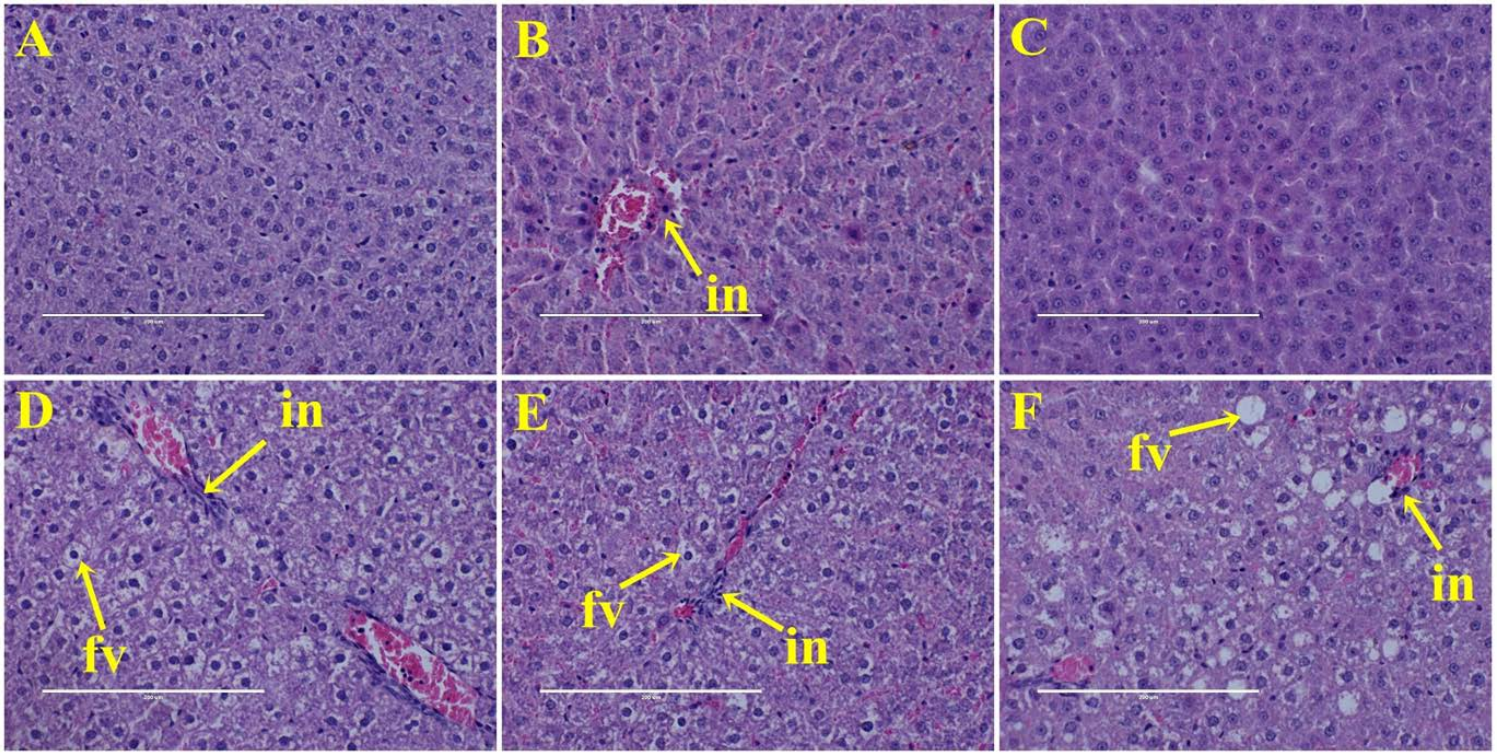

Figure 3.2: Haematoxylin and eosin staining of hepatocytes (original magnification x 20) in rats fed CS, HCHF, HLA, HMA, HPA and HSA. Figures showing inflammatory cells (marked as “in”) around the portal region in A (CS), B (HCHF), C (HLA), D (HMA), E (HPA) and F (HSA) in rats. No inflammatory cells were observed in CS and HLA livers.

3.2.4 Oral glucose tolerance test

OGTT for this study showed differences between control groups (CS and HCHF) (Figure 3.3). For CS group, blood glucose rose at 30 minutes and slowly returned to a normal concentration at 120 minutes. In contrast, the HCHF group reached the highest concentration of blood glucose at 60 minutes and then decreased but not to basal values at 120 minutes. HLA had a smaller rise in fasting blood glucose concentrations than other fatty acids and maintained the blood glucose concentrations for 2 hours. Responses in HMA, HPA and HSA did not differ with CS.

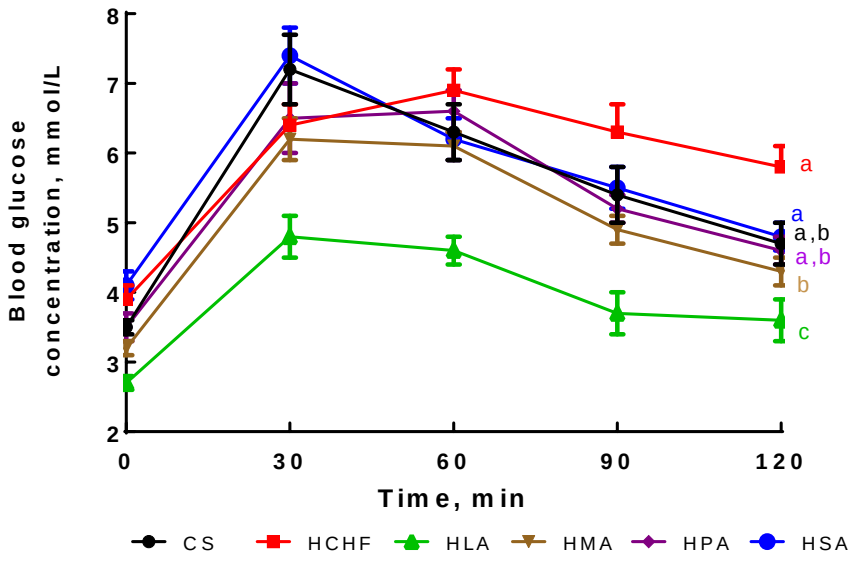

Figure 3.3: Oral glucose tolerance test in CS, HCHF, HLA, HMA, HPA and HSA diet-fed rats at week 16.

Data are shown as means±S.E.M. Means of values at 120 min without a common alphabet significantly differ. n=12/group.

3.2.5 Cardiovascular changes

Physiological changes in cardiovascular function following different diets have been presented in Table 3.3. The heart rate of all rats showed no difference between groups. The CS heart wet weight was lower than HCHF heart wet weight. However,

there was a difference in heart wet weight between CS, HLA and HPA rats, with HLA diet-fed rats showing the lowest heart wet weight (Table 3.3). Echocardiographic assessments (Table 3.3) of HLA diet–fed rats observed smaller LV internal diameter (LVIDd) and stroke volume. However, indices of relative wall thickness, fractional shortening, ejection fraction and heart rate showed no difference when compared to other groups. In addition, lauric acid reduced LV wet weight and reduced estimated LV mass. HCHF rats had higher diastolic stiffness constant than CS rats in the isolated Langendorff heart. There was no difference in diastolic stiffness between HLA diet–fed rats and CS diet–fed rats, but different from HCHF, HMA, HPA and HSA hearts. Systolic blood pressure was increased in HCHF rats compared to CS rats. The systolic blood pressure in HCHF rats was higher than in HLA and HMA rats. HLA diet–fed rats had lower systolic blood pressure compared to HCHF, HMA, HPA and HSA groups.

CS rats showed normal left ventricular tissue morphology in histological sections (Figure 3.4). HCHF showed increased infiltration of inflammatory cells (Figure 3.4B). In HLA rats, improved tissue architecture of the left ventricle was found compared to HCHF rats (Figure 3.4). Greater interstitial collagen deposition was shown in HCHF (marked “cd”; Figure 3.5B) as compared to other rats (Figure 3.5). In addition, HCHF, HPA and HSA rats showed interstitial fibrosis in Figure 3.5B, Figure 3.5E and Figure 3.5F. No major changes were observed in CS, HLA and HMA rats (Figure 3.5A, Figure 3.5C and Figure 3.5D) and the tissue morphology of the heart appeared normal.

Table 3.3: Changes in cardiovascular structure and function.

Variable	CS	HCHF	HLA	HMA	HPA	HSA
Heart rate, *bpm*	342.7±30.1	322.5±17.0	296.3±24.5	359.8±15.2	328.8±25.5	309.5±23.4
Heart weight, *mg/mm*	21.8±0.8^{b}	25.3±1.0^{ab}	16.2±0.5^{c}	23.7±0.7^{ab}	25.9±1.4^{a}	22.3±0.7^{ab}
Estimated LV mass, *g*	0.83±0.03^{c}	1.06±0.04^{a}	0.67±0.03^{d}	0.89±0.05^{bc}	1.03±0.03^{ab}	0.96±0.03^{abc}
LV+septum wet weight, *mg/mm*	18.2±0.8^{a}	20.0±0.9^{a}	13.0±0.4^{b}	19.3±0.5^{a}	20.1±1.0^{a}	18.2±0.7^{a}
Right ventricle wet weight, *mg/mm*	3.6±0.3^{c}	4.7±0.2^{b}	3.1±0.1^{c}	4.4±0.2^{b}	5.8±0.5^{a}	4.1±0.1^{bc}
LVIDd, *mm*	7.36±0.19^{b}	8.33±0.15^{a}	6.52±0.16^{c}	7.55±0.23^{b}	7.93±0.16^{ab}	7.86±0.18^{ab}
LVPWd, *mm*	1.65±0.08^{ab}	1.79±0.05^{a}	1.55±0.04^{b}	1.71±0.03^{ab}	1.83±0.04^{a}	1.72±0.06^{ab}
Relative wall thickness	0.46±0.03	0.43±0.01	0.49±0.02	0.46±0.01	0.47±0.01	0.45±0.02
Fractional shortening, *%*	53.2±3.3	43.0±1.6	50.5±4.9	50.4±3.0	42.6±2.4	41.9±2.0
LV developed pressure, *mmHg*	69.7±7.1	66.6±9.6	74.0±9.9	53.1±5.3	84.4±9.8	78.1±8.5
(+)dP/dt, $mmHg.S^{-1}$	1339.6±127.9	1265.1±171.3	1396.0±185.8	877.3±210.2	1510.4±237.5	1334.8±202.0
(-)dP/dt, $mmHg.S^{-1}$	-702.0±183.9	-849.4±151.1	-935.7±160.2	-589.0±81.1	-774.7±254.0	-942.6±126.9
Diastolic stiffness (k)	22.0±0.5^{c}	28.2±0.5^{a}	21.8±1.0^{c}	25.3±0.7^{b}	26.6±0.7^{ab}	27.0±0.4^{ab}
Ejection fraction, *%*	88.3±2.4	81.1±1.7	84.6±3.3	86.3±2.2	80.3±2.2	79.8±2.0
Ejection time, *ms*	78.2±2.6^{b}	87.0±2.3^{ab}	94.9±2.8^{a}	80.4±2.9^{b}	84.0±2.4^{ab}	85.6±3.8^{ab}

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 ($P < 0.05$). LV: left ventricle, LVIDd: left ventricular internal dimension in diastole, LVPWd: left ventricular posterior wall dimensions.

Table 3.3: *Continued.*

Variable	CS	HCHF	HLA	HMA	HPA	HSA
Stroke volume, *µL*	374.5±30.3^{b}	492.7±24.3^{a}	245.9±14.9^{c}	391.4±29.2^{ab}	420.0±22.8^{ab}	412.2±31.2^{ab}
Cardiac output, *mL/min*	133.5±19.3^{a}	159.9±12.7^{a}	71.3±5.7^{b}	139.1±10.4^{a}	137.2±12.1^{a}	125.1±11.0^{a}
Systolic wall stress, *mmHg.cm^{-1}*	75.8±9.1^{b}	131.6±9.0^{a}	101.7±18.4^{ab}	95.0±11.3^{ab}	127.9±11.2^{a}	137.8±8.4^{a}
Systolic blood pressure, *mmHg*	127.7±1.1^{d}	157.8±2.8^{a}	136.2±5.3^{cd}	141.7±1.2^{bc}	150.4±2.5^{ab}	152.9±3.5^{a}
Ascending aorta, *cm/sec*	98.6±6.0	97.4±4.6	83.4±6.8	96.3±3.9	97.8±3.5	98.9±2.7
Descending aorta, *cm/sec*	87.3±5.4	96.2±5.8	81.4±4.0	95.1±2.9	97.0±5.9	95.0±6.0

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 ($P < 0.05$).

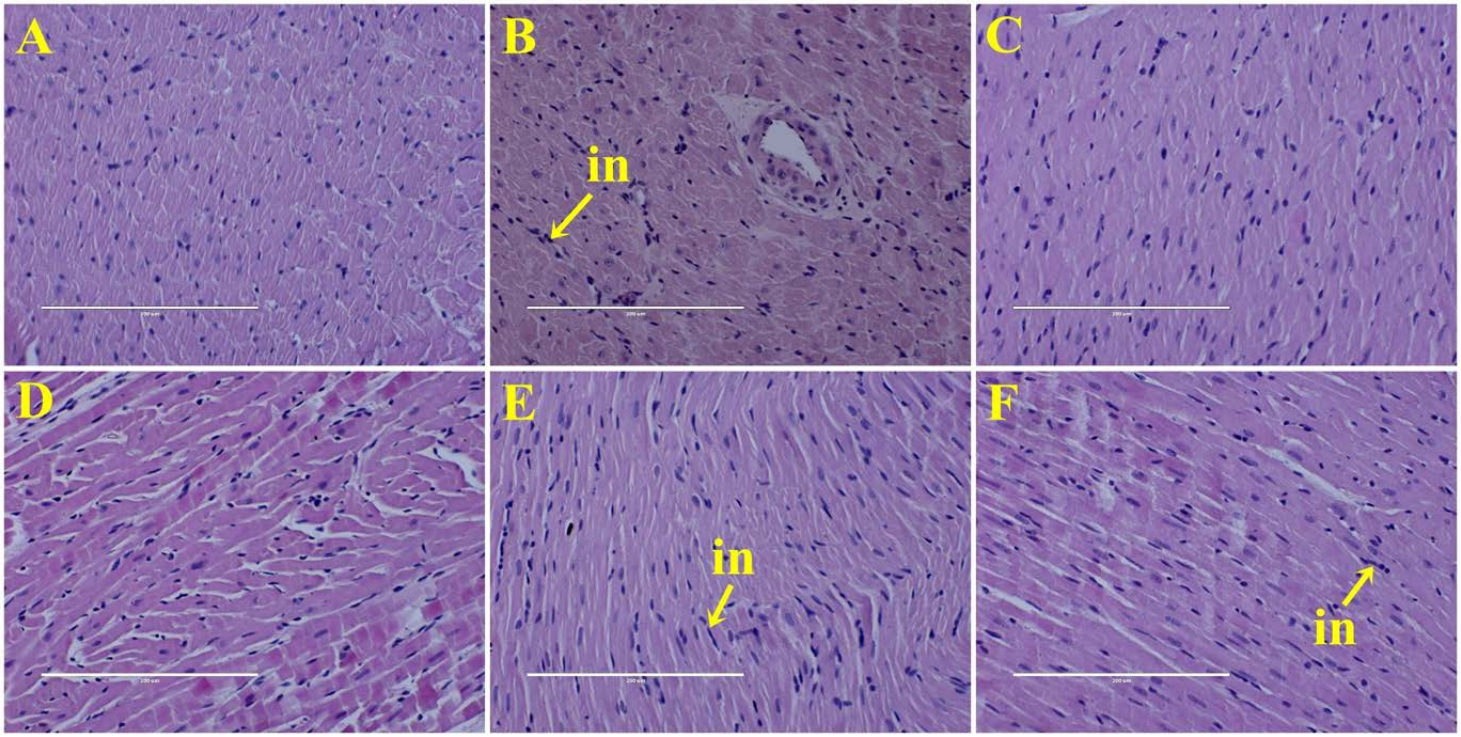

Figure 3.4 Haematoxylin and eosin staining of left ventricle (original magnification x 20) in rats fed CS, HCHF, HLA, HMA, HPA and HSA.

Figures showing inflammatory cells (marked as “in”) in A (CS), B (HCHF), C (HLA), D (HMA), E (HPA) and F (HSA) in rats. No inflammatory cells were observed in CS and HLA hearts.

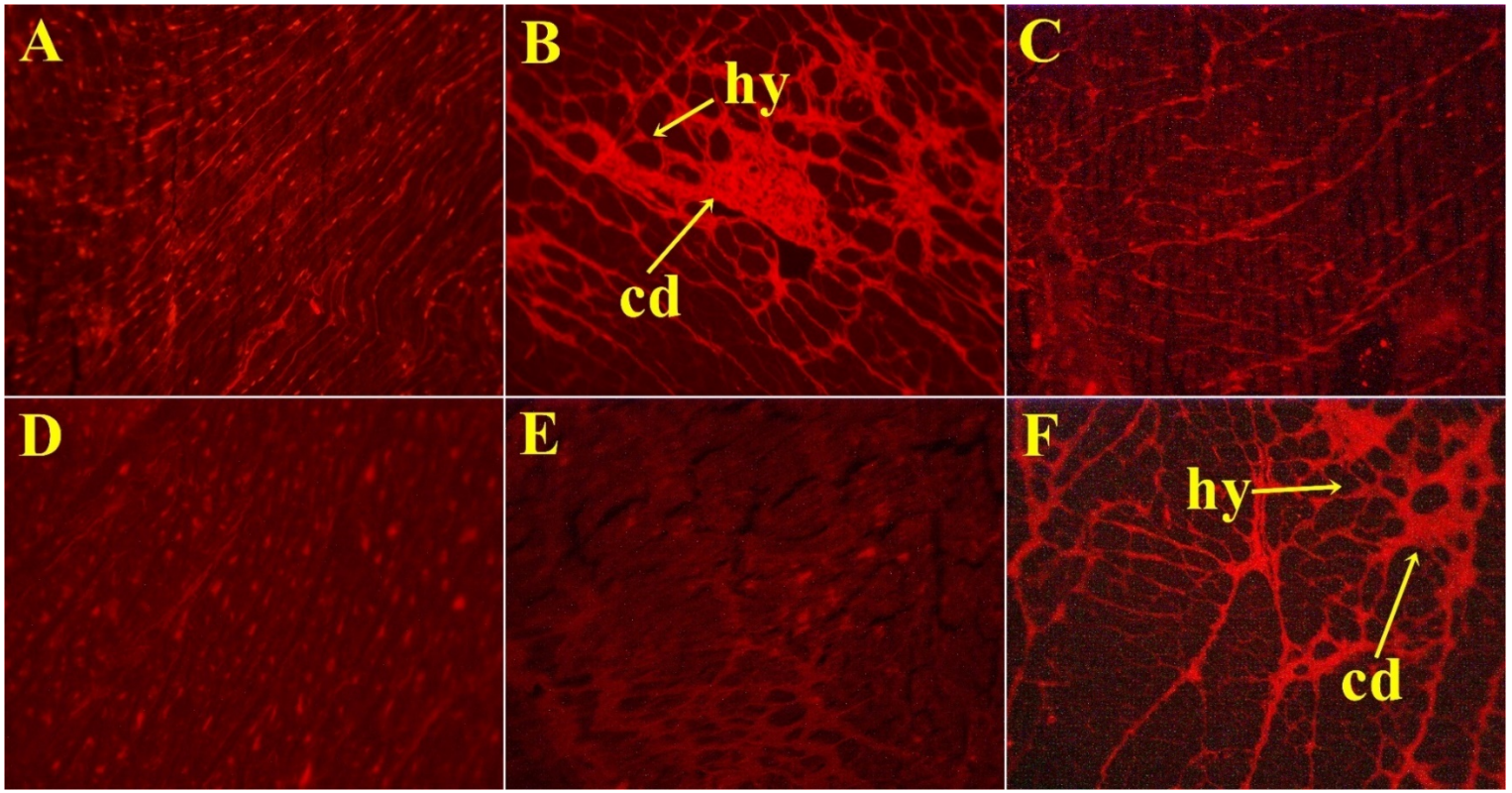

Figure 3.5: Picrosirius red staining of left ventricle (original magnification x 20) in rats fed CS, HCHF, HLA, HMA, HPA and HSA. Figures showing collagen deposition (marked as “cd”) and hypertrophied cells (marked as “hy”) in A (CS), B (HCHF), C (HLA), D (HMA), E (HPA) and F (HSA) in rats.

3.2.6 Ileum and colon contractility and histology

Organ bath studies on the ileum and colon (Figure 3.6) showed that CS, HCHF, HLA, HMA and HSA showed similar responses to acetylcholine in the ileum. Similarly, no differences were found between groups in responses of acetylcholine in the colon. Figure 3.7 shows the histological of colon tissue stained using periodic acid and Schiff (PAS) reagent. Results showed that the density of goblet cells (gc) were increased in the crypt of the colon of HCHF and HSA rats compared to other groups.

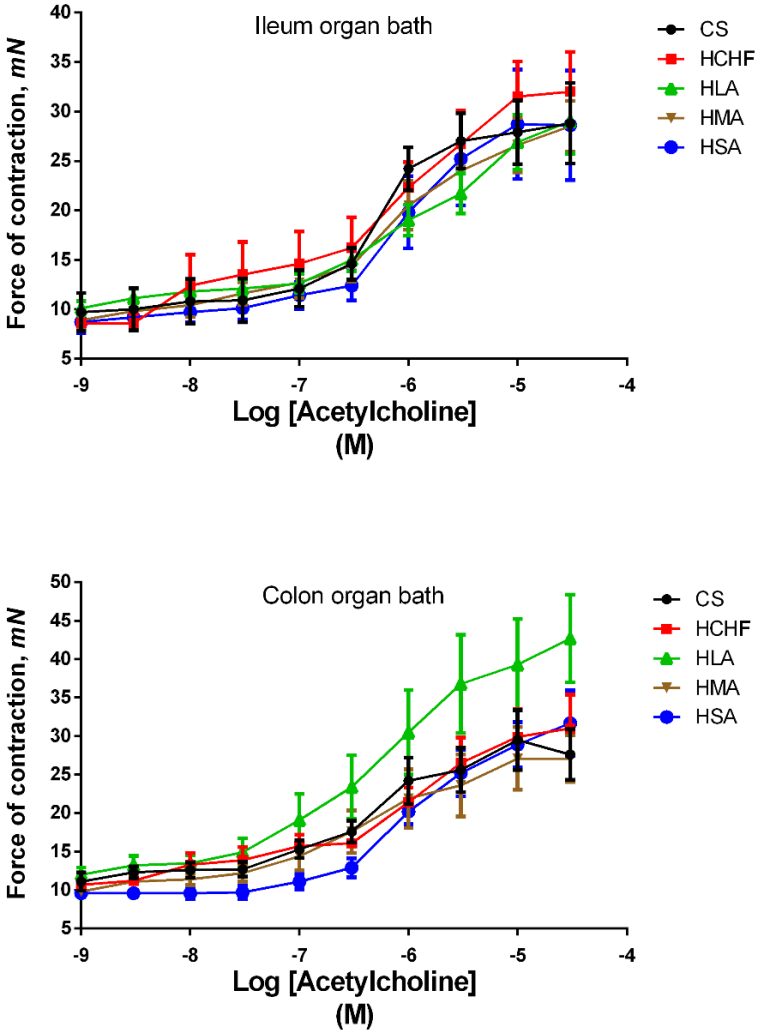

Figure 3.6: Force of contraction (mN) on ileum and colon in CS, HCHF, HLA, HMA and HSA groups.

All data are presented as mean ± SEM and n = 10 for each group.

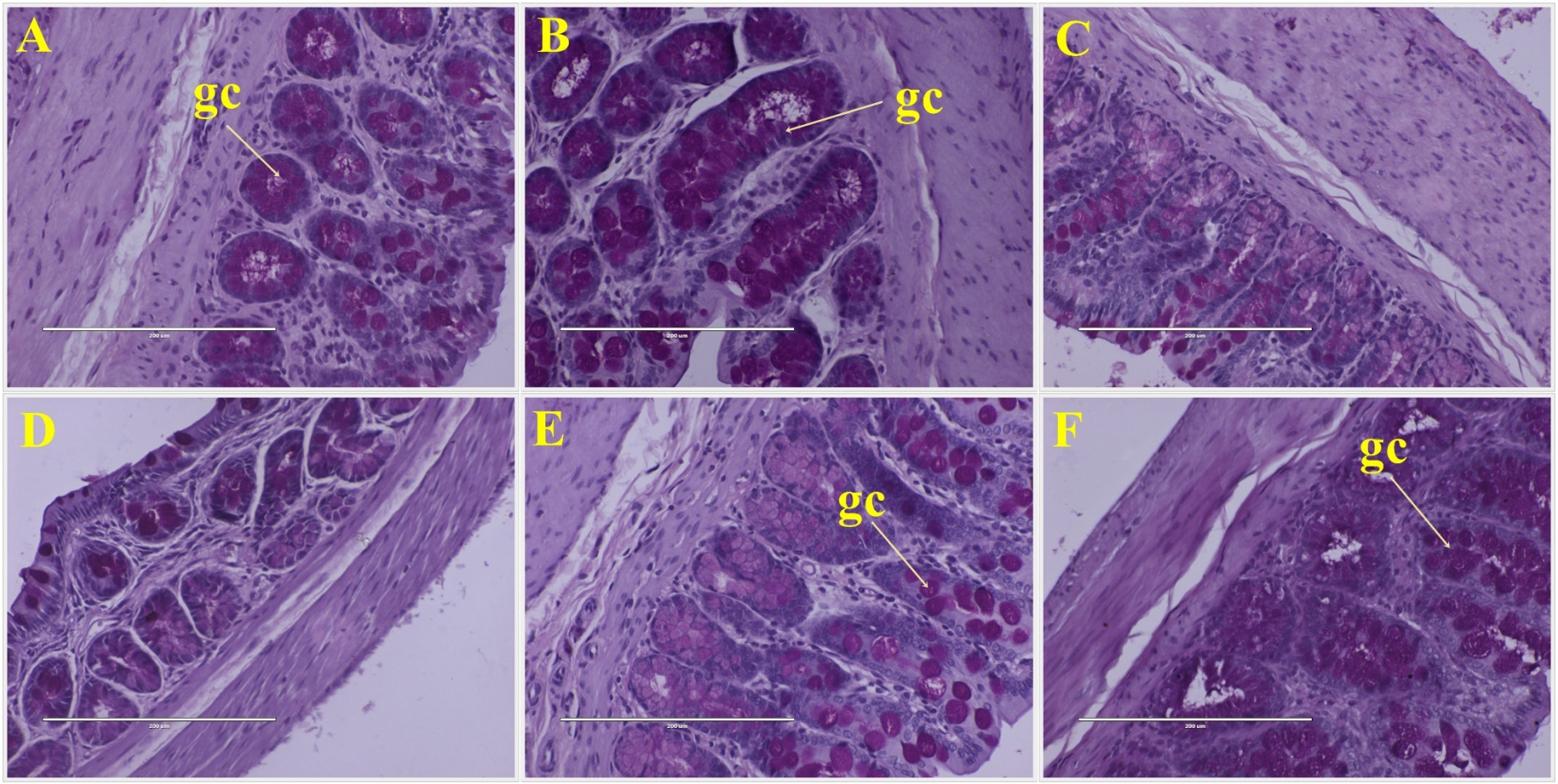

Figure 3.7: Periodic acid and Schiff reagent staining (original magnification x 20) in rats fed CS, HCHF, HLA, HMA, HPA and HSA. .

Figures showing goblet cells (gc) in the crypt of the colon in A (CS), B (HCHF), C (HLA), D (HMA), E (HPA) and F (HSA) in rats.

3.3 Discussion

Numerous studies have reported that saturated fat is one of the major nutrients that causes detrimental effects to human health if consumed in excessive amounts (Doyle, 2004, Hariri et al., 2010b). Human diets contain dietary fats which incorporate a broad spectrum of fatty acids of varying chain length (Mu and Hoy, 2004). Studies suggest that saturated fat can have dramatically different effects on human lipid metabolism, adipocytes accumulation, hepatic steatosis and alteration of gut microbiota composition (de Wit et al., 2012, Mensink et al., 2003). However, these saturated fatty acids have not been previously compared individually in the same study to differentiate the physiological responses elicited by each fatty acid in models of metabolic syndrome. In this study, we have found that SFA from C12 to C18 gave different responses with, in general, worsening of cardiovascular, liver and metabolic parameters as chain length increased, with minimal changes with lauric acid (C12) and the most obvious changes with stearic acid (C18).

Rats consuming HCHF-diet develop hypertension, cardiac deterioration, inflammation and abdominal obesity (Panchal et al., 2011). Further, an increase in total abdominal fat pad weight from HCHF-diet fed rats were also shown in this study. An excess of energy consumption from simple sugars and saturated fats lead to fat deposition in the adipocyte and this accumulation caused hypertrophy and hyperplasia of adipocytes (Panchal et al., 2011). However, rats fed with HLA showed a marked loss of body weight and body fat although the rats still had an abundant supply of fructose and fat from the diet to meet energy requirements. In addition, the energy intake by HLA rats was no difference to HCHF-fed rats. Fatty acid absorption and oxidation are related to the chain length (DeLany et al., 2000, Ramirez et al., 2001) , with absorption of medium-chain fatty acids (MCFA) being greater than long-chain fatty acids (LCFA) and decreased oxidation with increasing carbon numbers (DeLany et al., 2000, Ramirez et al., 2001). An *in vivo* investigation of four volunteers consuming heated meals containing labelled saturated fatty acids [C12 (lauric), C16 (palmitic), or C18 (stearic)] or a C18 unsaturated fatty acid reported that lauric acid was the most highly oxidized, followed by the unsaturated fatty acids with stearic acid as the least oxidized (DeLany et al., 2000). In addition to fatty acid oxidation, there is

also a pathway that explains this which is the rate of *de novo* lipogenesis (Turner et al., 2009). MCFA such as lauric acid are more readily absorbed into the bloodstream and therefore these fatty acids are absorbed efficiently by the liver through the portal vein (Papamandjaris et al., 1998). This underlying pathway also shows that MCFA can enter the mitochondria via CPT-1–independent mechanisms for oxidation (Turner et al., 2009). These mechanisms explain the increase in energy expenditure and decreased adiposity observed with lauric acid diet-fed rats.

One of the manifestations of metabolic syndrome is non-alcoholic fatty liver disease (Vanni et al., 2010). Increased activities of ALT, AST and ALP in plasma are indications of hepatic injury and this was shown in all rats fed with HCHF, HLA, HMA, HPA and HSA. Rats fed with HCHF diet also developed dyslipidaemia where the results showed that total cholesterol concentrations in HCHF groups were higher when compared to CS group. A study on 14 men showed that lauric acid raised total and LDL cholesterol concentrations compared with oleic acid, but was not as potent for increasing cholesterol concentrations as palmitic acid (Denke and Grundy, 1992). However, a meta-analysis showed that lauric acid greatly increased total cholesterol concentrations, but much of its effect was on HDL cholesterol (Mensink et al., 2003). Furthermore, the consumption of lauric acid decreased the ratio of total to HDL cholesterol. Myristic and palmitic acids had little effect on the ratio, and stearic acid reduced the ratio slightly (Mensink et al., 2003).

Lauric acid fed-rats (HLA) had improved glucose tolerance after the glucose load of OGTT. This is in accordance with the literature which shows that a very small load of lauric acid (~80 kJ) lowered blood glucose after both breakfast and lunch when given to patients with type 2 diabetes, when compared to a placebo with similar formulation but with the omission of lauric acid (Korek et al., 2013). The authors proposed the effect was related to GLP-1 in the portal circulation where vagal afferents with nerve endings bearing receptors for GLP-1 are present in the portal venous system and activation of these results in glucose lowering through enhanced hepatic and peripheral glucose uptake, in the absence of any stimulation of insulin secretion (Korek et al., 2013). Our study findings are consistent with this human study, with HCHF diet-fed rats showing impairment of glucose clearance due to reduced insulin sensitivity. This effect may be due to increased islet mass and an increased number of α and β

cells within the islets of Langerhans in the pancreas, suggesting hyperplasia of these cell types (Panchal et al., 2011).

Physiological parameters of cardiac function in all groups showed minimal chenges in heart rate, ejection fraction and fractional shortening. However, HLA diet-fed rats showed a lower systolic blood pressure and no difference in the stiffness of heart as compared to CS group. There are only limited number of studies that have shown the effect of lauric acid on systolic blood pressure and the stiffness of the heart. However, one study determined that with a combination of fatty acids, the total proportion of lauric acid and myristic acid had a negative impact on the endothelial function index of healthy young men but interestingly did not have a similar effect in healthy young women, indicating that there may be a sex difference in cardiovascular effects of these fatty acids. This study also determined that serum fatty acid composition predicted endothelial vasodilatory dysfunction independently of serum NEFA (28). On the other hand, the inhibitory effects of saturated NEFA on endothelium-dependent relaxation have been reported (Edirisinghe et al., 2006). This study used aortic rings that were incubated for an hour with NEFA–albumin complexes derived from lauric (C12:0), myristic (C14:0), palmitic (C16:0), stearic (C18:0) and alpha-linolenic (C18:3) acids. It was observed that lauric acid had the smallest inhibitory effect (Edirisinghe et al., 2006). Increased NEFA is recognised as one of predisposing features of endothelial dysfunction. In our study, there is a consistent increase in plasma NEFA as chain length increases (lauric<myristic<palmitic<stearic acids). One of the potential mechanisms involved in this loss of endothelial function is a direct effect of NEFA in plasma on endothelium-dependent relaxation (Steinberg et al., 2000). Further, an association was reported between two individual SFA (palmitic and stearic acid) and the prevalence of peripheral artery disease (Naqvi et al., 2012).

Leptin is most commonly known as an anti-obesity hormone that regulates food intake and energy expenditure (Hariri et al., 2010a). This study shows that different dietary fatty acid compositions affect the total fat mass and plasma leptin concentrations in different ways. The plasma leptin concentrations increased with increases of chain length, however, these increases did not correlate with increases in total fat mass. Our data suggests that it may be that the leptin concentration may be

reflective of individual fat deposition and not total fat mass. In contrast to this finding, other studies found that plasma leptin was proportional to the degree of fat-mass accumulation (Enos et al., 2013, Tilg and Moschen, 2006). Clearly, additional work is needed to elucidate the mechanism in relation to leptin concentrations with individual saturated fatty acids.

The role of gut microbiota in contributing to human health is the focus of much current research with several studies of composition of gut microbiota showing association with the metabolic syndrome phenotype (de Wit et al., 2012), with lean and obese animals having different microbiota profiles, independent of genetic or diet induced obesity (Cani et al., 2007, Ley et al., 2005). Additionally, there is a positive relationship between gut dysfunction and metabolic endotoxaemia with obesity or insulin resistance (Shen et al., 2014). This suggests that diets rich in long chain fatty acids accelerate metabolic dysfunction and it also suggest that limiting total dietary fat intake, particularly from palm oil, would help promote healthy microflora, thereby improving intestinal health and reducing the risk of gut-related diseases (Shen et al., 2014). The inclusion of fats in the diet can also affect the composition of epithelial cells in the gastrointestinal tract. A 12 week HCHF diet resulted in an increased number of goblet cells in the small intestine, with a reduction in this number following 4 week on a basal diet containing nitrogen free extract (51.65%), crude protein (21.36%), crude fat (10.63%), total ash (7.41%), moisture (6.32%), crude fibre (2.63%), calcium (1.75%), phosphorus (1.1%), and water (0.23% per 100 g of the diet) (Hazarika et al., 2016). Further, consumption of palm oil with 3-fold higher concentration of palmitic acid resulted in increased mucus granule surfaces of goblet cells (>2-fold) in the rats' colon (Benoit et al., 2015a). This study proposed that subsequent effects of increased goblet cells in the colon will increase transmucosal resistance, thereby changing the colonic barrier. These findings support the present study where there is increased density of goblet cells in the colon of rats fed palmitic, stearic acid and HCHF. However, the subsequent effects of these saturated fatty acids on goblet cells were not examined in the present study. Further study is warranted to rule out the possible mechanism involved especially the changes in gut structure and function.

Overall, the metabolic changes worsened with increased chain length and this was most obvious in stearic acid with impaired cardiovascular functions and increased collagen deposition in heart tissues. However, the responses from HCHF rats were more severe than stearic acid rats. These effects could be due to the presence of *trans* fatty acids (TFA) in beef tallow as previous studies have shown that TFA triggered systemic inflammation and endothelial dysfunction (Mozaffarian, 2006). Further, TFA induced dyslipidaemia, insulin resistance and obesity (Micha and Mozaffarian, 2009, Mozaffarian, 2006). Evidence from controlled dietary studies showed that TFA markedly worsened serum lipids by increased LDL-cholesterol (Katan et al., 1995). In addition, high TFA intake is also associated with higher concentrations of inflammatory cytokines (TNF-α, IL-6), C-reactive protein (CRP) and monocyte chemoattractant protein-1 (Mozaffarian et al., 2006). Although evidence showed that TFA worsened metabolic syndrome, further studies need to be done to compare HCHF with pure *trans* fatty acids.

3.4 Conclusion

In conclusion, different types of saturated fatty acids showed different responses to the signs of metabolic syndrome. With the same amount of saturated fat given to each group, the HLA (lauric acid) group did not gain body weight throughout the study and had the lowest total fat mass, total lean mass, abdominal circumference and visceral adiposity index compared to others. The HLA group showed better responses in OGTT and blood pressure compared to the other groups. In contrast, HMA, HPA and HSA groups exhibited a worsening of disease states with increasing SFA chain length and the most obvious changes were found in rats fed with stearic acid. Palmitic and stearic acids produced lower physiological responses than beef tallow but still exhibited marked abnormalities in metabolic, cardiovascular and liver structure and function. This suggests that replacing beef tallow with stearic and palmitic acids would show small improvements, but replacement with lauric and possibly myristic acids in human diets would markedly decrease the development of metabolic syndrome.

3.5 Published article on saturated fatty acids induce development of both metabolic syndrome and osteoarthritis in rats

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Saturated fatty acids induce development of both metabolic syndrome and osteoarthritis in rats

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Sunderajhan Sekar[1], Siti Raihanah Shafie[2], Indira Prasadam[1], Ross Crawford[3], Sunil K. Panchal[2], Lindsay Brown[2] & Yin Xiao[1]

The predominant saturated fatty acids (SFA) in human diets are lauric acid (LA, C12:0), myristic acid (MA, C14:0), palmitic acid (PA, C16:0) and stearic acid (SA, C18:0). The aim of this study was to investigate whether diets containing individual SFA together with excess simple carbohydrates induce osteoarthritis (OA)-like changes in knee joints and signs of metabolic syndrome in rats. Rats were given either a corn starch diet or a diet composed of simple carbohydrates together with 20% LA, MA, PA, SA or beef tallow for 16 weeks. Rats fed beef tallow, SA, MA or PA diets developed signs of metabolic syndrome, and also exhibited cartilage degradation and subchondral bone changes similar to OA. In contrast, replacement of beef tallow with LA decreased signs of metabolic syndrome together with decreased cartilage degradation. Furthermore, PA and SA but not LA increased release of matrix sulphated proteoglycans in cultures of bovine cartilage explants or human chondrocytes. In conclusion, we have shown that longer-chain dietary SFA in rats induce both metabolic syndrome and OA-like knee changes. Thus, diets containing SFA are strongly relevant to the development or prevention of both OA and metabolic syndrome.

Obesity is a major risk factor for osteoarthritis (OA) and this has led to the description of metabolic syndrome-associated OA as a new OA phenotype[1]. In adipose tissue, adipocytes store lipids but they also produce and release many adipokines that are then responsible for the chronic low-grade inflammation in obesity[2]. Leptin, one of these adipokines, has been extensively studied in the pathophysiology of obesity[3]. Leptin may serve as the mechanism linking obesity and OA, because it is involved in cartilage and chondrocyte pathophysiology[4]. Increased intake of either mono- or poly-unsaturated fatty acids in a healthy population of middle aged adults was linked with increased possibility of developing bone marrow lesions[5]. Also, free fatty acids may be involved in inflammatory metabolic diseases such as obesity and inflammatory joint diseases[6]. Thus, dietary saturated fatty acids (SFA) could initiate the link between OA and obesity[7–9]. This is supported by the alterations in subchondral bone structure in patients with increased SFA intake[5] that may lead to OA. Further, treatment of human chondrocytes with palmitate induced apoptosis and caused articular cartilage degradation[10]. However, there are no studies that have examined the association between these diseases using individual dietary SFA, despite the importance of dietary SFA as a potential initiator of chronic human diseases including obesity and OA.

Obesity is excessive fat storage in the body which is a major component of metabolic syndrome, a constellation of hypertension, diabetes, dyslipidaemia and fatty liver disease that increases the risk of cardiovascular disease[11]. Although, it has been widely accepted that SFA as a group promotes abdominal obesity, dyslipidaemia, insulin resistance, impaired glucose tolerance and systemic inflammation[12], this role of SFA in obesity, diabetes and cardiovascular disease is now being challenged. Recent studies suggest that SFA-induced responses are dependent on chain length, with clear differences in biological responses between lauric acid and palmitic acid[13]. However, the roles of individual dietary SFA in OA have not been clearly defined.

Our previous studies reported that high-carbohydrate, high-fat diet (H; mainly containing fructose and beef tallow) for 16 weeks in young male Wistar rats mimics the symptoms of human metabolic syndrome including abdominal obesity, increased total body fat mass, elevated plasma lipids and blood pressure, impaired glucose

[1]Institute of Health and Biomedical Innovation, School of Chemistry, Physics, Mechanical Engineering, Queensland University of Technology, Brisbane, Australia. [2]Institute for Agriculture and the Environment, and School of Health and Wellbeing, University of Southern Queensland, Toowoomba, Queensland, Australia. [3]The Prince Charles Hospital, Orthopaedic Department, Brisbane, Australia. Correspondence and requests for materials should be addressed to Y.X. (email: yin.xiao@qut.edu.au)

Variables	C	HLA	HMA	HPA	HSA	H
Food intake (*g/d*)	40.2 ± 2.2^{a}	24.2 ± 3.3^{d}	27.7 ± 1.3^{c}	29.7 ± 2.6^{bc}	30.1 ± 2.4^{b}	25.0 ± 2.3^{d}
Water intake (*mL/d*)	26.1 ± 4.1^{c}	36.5 ± 4.5^{a}	29.1 ± 2.4^{b}	34.4 ± 2.2^{a}	35.1 ± 2.7^{a}	25.2 ± 3.5^{c}
Body weight gained (*%*)	26.6 ± 6.3^{c}	-11.1 ± 9.8^{d}	34.7 ± 6.8^{bc}	42.1 ± 15.3^{b}	34.1 ± 11.9^{bc}	58.6 ± 14.1^{a}
Energy intake (*kJ/d*)	452 ± 24^{d}	545 ± 70^{c}	601 ± 26^{b}	652 ± 46^{a}	671 ± 52^{a}	543 ± 44^{c}
Feed conversion efficiency (*%*)	19.3 ± 4.1^{b}	-7.4 ± 6.5^{c}	19.3 ± 3.5^{b}	21.1 ± 6.2^{b}	17.9 ± 4.4^{b}	36.2 ± 6.8^{a}
Total body fat mass (*g*)	79.5 ± 34.7^{cd}	54.4 ± 9.7^{d}	132.4 ± 31.2^{b}	122.7 ± 40.9^{bc}	122.9 ± 30.0^{bc}	207.7 ± 81.6^{a}
Total body lean mass (*g*)	305.0 ± 23.6^{a}	229.3 ± 19.5^{b}	300.3 ± 23.0^{a}	305.9 ± 25.9^{a}	307.1 ± 23.6^{a}	310.6 ± 46.9^{a}
Abdominal circumference (*cm*)	20.2 ± 0.7^{b}	17.4 ± 1.1^{c}	21.5 ± 0.9^{a}	21.5 ± 0.8^{a}	21.4 ± 1.0^{a}	22.4 ± 1.1^{a}
Visceral adiposity index (*%*)	4.01 ± 0.88^{c}	3.56 ± 0.96^{c}	5.93 ± 0.80^{b}	5.35 ± 1.45^{bc}	4.94 ± 1.26^{bc}	10.07 ± 2.59^{a}
RER	1.01 ± 0.06^{a}	0.90 ± 0.08^{a}	0.94 ± 0.06^{a}	1.01 ± 0.05^{a}	1.01 ± 0.09^{a}	1.01 ± 0.07^{a}
Heat (*Kcal*)	3.16 ± 0.38^{b}	2.46 ± 0.24^{c}	3.33 ± 0.41^{b}	3.47 ± 0.35^{ab}	3.44 ± 0.29^{ab}	3.87 ± 0.23^{a}
Retroperitoneal fat (*mg/mm*)	155.2 ± 46.7^{bc}	98.0 ± 36.5^{c}	256.3 ± 48.6^{b}	248.4 ± 92.1^{b}	205.6 ± 53.2^{bc}	572.2 ± 219.1^{a}
Epididymal fat (*mg/mm*)	98.0 ± 21.0^{b}	49.0 ± 14.0^{c}	128.6 ± 22.7^{b}	128.7 ± 63.0^{b}	120.3 ± 41.7^{b}	273.9 ± 69.5^{a}
Omental fat (*mg/mm*)	88.7 ± 26.8^{cd}	66.6 ± 18.1^{d}	153.1 ± 22.3^{b}	138.2 ± 51.5^{b}	126.9 ± 30.9^{bc}	257.9 ± 73.9^{a}
Total abdominal fat (*mg/mm*)	342 ± 85^{cd}	214 ± 66^{d}	538 ± 84^{b}	515 ± 201^{b}	453 ± 116^{bc}	1017 ± 255^{a}
Basal blood glucose concentrations (*mmol/L*)	3.59 ± 0.30^{ab}	2.64 ± 0.46^{c}	3.16 ± 0.37^{b}	3.46 ± 0.56^{ab}	3.98 ± 0.81^{a}	3.86 ± 0.51^{a}
Systolic blood pressure (*mmHg*)	126.9 ± 5.5^{d}	136.2 ± 16.9^{c}	141.8 ± 3.7^{bc}	150.4 ± 7.9^{ab}	152.9 ± 11.0^{a}	157.7 ± 10.7^{a}
Hepatic Function						
Liver (*mg/mm*)	203.2 ± 28.0^{c}	201.0 ± 27.6^{c}	303.8 ± 24.8^{b}	322.2 ± 49.5^{ab}	295.8 ± 33.9^{b}	345.9 ± 33.6^{a}
Alanine transaminase (*U/L*)	19.4 ± 5.4^{c}	54.2 ± 12.5^{a}	47.3 ± 9.6^{ab}	37.7 ± 7.6^{b}	39.0 ± 8.9^{b}	40.9 ± 11.1^{b}
Aspartate transaminase (*U/L*)	57.4 ± 13.4^{b}	75.3 ± 13.3^{a}	60.4 ± 11.6^{b}	56.2 ± 5.1^{b}	55.5 ± 8.7^{b}	66.4 ± 7.8^{ab}
Alkaline phosphatase (*U/L*)	121.7 ± 24.8^{c}	326.1 ± 66.0^{a}	339.8 ± 80.2^{a}	234.9 ± 63.7^{b}	213.3 ± 37.6^{b}	248.7 ± 59.5^{b}
Total cholesterol (*mmol/L*)	1.42 ± 0.36^{c}	1.98 ± 0.26^{a}	1.78 ± 0.29^{ab}	1.60 ± 0.18^{bc}	1.69 ± 0.41^{abc}	1.59 ± 0.11^{bc}
NEFA (*mmol/L*)	1.09 ± 0.37^{d}	2.32 ± 1.16^{c}	3.15 ± 1.05^{bc}	3.63 ± 1.69^{ab}	4.10 ± 1.55^{ab}	4.84 ± 1.13^{a}
Triglycerides (*mmol/L*)	0.36 ± 0.17^{d}	0.67 ± 0.42^{cd}	0.88 ± 0.33^{c}	1.04 ± 0.46^{bc}	1.42 ± 0.68^{b}	1.92 ± 0.67^{a}
Plasma hormone concentrations						
Insulin (*μg/L*)	2.35 ± 1.80^{ab}	1.42 ± 1.22^{b}	3.81 ± 2.25^{a}	3.60 ± 1.23^{a}	3.59 ± 2.15^{a}	4.12 ± 1.09^{a}
Leptin (*μg/L*)	3.21 ± 1.12^{cd}	1.93 ± 1.05^{d}	7.94 ± 2.35^{bc}	5.54 ± 3.71^{c}	9.67 ± 3.87^{ab}	11.37 ± 3.80^{a}

Table 1. Dietary intake and body composition in C, H, HLA, HMA, HPA and HSA rats. Measurement of dietary intake and body composition in rats fed the different diets (n = 10–12). All values are represented as mean ± SD. Mean values within a row with unlike superscript letters are significantly different ($P < 0.05$) with a > b > c > d. Abbreviations: C – corn starch diet-fed rats; H – high-carbohydrate, high-fat diet-fed rats; HLA – high-carbohydrate, high-lauric acid-fed rats; HMA – high-carbohydrate, high-myristic acid-fed rats; HPA – high-carbohydrate, high-palmitic acid-fed rats; HSA – high-carbohydrate, high-stearic acid-fed rats; RER – respiratory exchange ratio; NEFA – non-esterified fatty acids.

tolerance and insulin sensitivity, fatty liver and cardiovascular remodelling[14]. The present study has investigated the role of C12 to C18 SFA (lauric acid (LA; C12:0), myristic acid (MA; C14:0), palmitic acid (PA; C16:0) and stearic acid (SA; C18:0) as well as beef tallow (mainly SA and *trans* fatty acids) on both the signs of metabolic syndrome and OA development.

Results

SFA on metabolic syndrome. Rats given a high-carbohydrate diet together with beef tallow containing both saturated and *trans* fats (H diet) developed changes associated with human metabolic syndrome including abdominal obesity, hyperleptinaemia, hyperlipidaemia and liver dysfunction compared to a corn starch diet (C diet) (Table 1), however, H rats did not show hyperglycaemia and hyperinsulinaemia compared to C rats. C diet has low energy density and possibly for this reason, the food intake was higher in C rats than all other groups. Although the food intake was higher in C rats, energy intakes were higher in all high-fat diet groups than in C rats in the order of C < (HLA = H) < HMA < (HPA = HSA) (Table 1). With lower body weight gain, the feed conversion efficiency was lower in HLA rats than in all other groups whereas H had higher feed conversion efficiency than other groups (Table 1). No difference in feed conversion efficiency was found in C, HMA, HPA and HSA although HMA, HPA and HSA had higher energy intakes. Body fat was highest in H rats and lowest in HLA rats while C, HMA, HPA and HSA had intermediate body fat. Lean mass was lower in HLA rats with no difference in other groups. Abdominal circumference was lower in HLA rats than in C rats while other groups were higher than C rats (Table 1). Abdominal fat pads were lowest in HLA rats while highest in H rats with other groups having intermediate abdominal fat. Basal blood glucose concentrations were lowest in HLA rats with HMA slightly higher than HLA. HSA and H had higher blood glucose concentrations than other groups while C and HPA had intermediate blood glucose concentrations. HLA showed higher plasma activities of alanine transaminase, aspartate transaminase and alkaline phosphatase than C rats. Alanine transaminase and alkaline phosphatase activities were higher in other groups than C rats whereas aspartate transaminase activity was equivalent to C rats. Plasma total cholesterol was higher in HLA rats than in C rats with other groups having intermediate concentrations of

total cholesterol. Plasma NEFA was lowest in C rats with HLA NEFA higher than C rats and H group with highest plasma NEFA concentrations. Further, other groups had intermediate plasma NEFA concentrations. Plasma triglycerides were higher in HMA rats than C rats while HSA and H were further increased with H having the highest plasma triglycerides. Plasma insulin concentrations were lower in HLA rats than H, HMA, HPA and HSA with C rats having intermediate concentrations. Plasma leptin concentrations were highest in H rats while lowest in HLA rats (Table 1).

Rats given a high-carbohydrate diet together with beef tallow (H diet) showed increased systolic blood pressure and worsening cardiac function, both by echocardiography and also as an increased ventricular stiffness in isolated heart studies, without the development of heart failure, when compared to C rats. In contrast, HLA rats showed minor changes when compared with C rats. Replacement of beef tallow with HMA, HPA or HSA led to worsening cardiac function and increased systolic blood pressure compared with C or HLA rats (Table 1). Parameters such as relative wall thickness, fractional shortening, heart rate, LV developed pressure, ascending aorta flow and descending aorta flow did not show differences among the groups with different dietary fat sources (Supplementary Table 1).

SFA on histological features of the articular cartilage. The joints from C rats showed smooth articular cartilage surface, normal cellularity and exhibited strong intensity of Safranin O staining. The chondrocytes were flattened in the tangential layer and were distributed in short irregular columns in the radial layer of the articular cartilage (Fig. 1A). Mankin scores were in the range of 0–1 in the C rats indicating normal cartilage structure (Fig. 1B). In contrast, H, HPA and HSA rats showed degeneration of the articular cartilage especially at the surface zone, indicated by surface irregularity, disorganisation of the articular cartilage with apparent chondrocyte clustering in the transitional and radial zones, and loss of proteoglycans shown by the weak intensity of Safranin O staining (Fig. 1A), with markedly increased Mankin scores (Fig. 1B). HMA and HLA rats showed cartilage structures that were similar to those of the C rats, with strong intensity of Safranin O staining and similar Mankin scores (Fig. 1A,B).

OA is characterised by reduced expression of the chondrogenic marker, aggrecan (ACAN), and increased expression of the hypertrophy and degradative markers, COL10 and MMP13. C rats showed strong expression of ACAN and limited expression of COL10 and MMP13. However, H, HPA and HSA rats exhibited degradative changes in cartilage structure that were greater than in HLA or HMA rats. ACAN expression decreased and expression of MMP13 and COL10 increased in H, HPA and HSA rats. HLA and HMA rats showed the least expression for MMP13 and COL10, but showed increased expression of ACAN in the cartilage compared to other SFA groups (Fig. 1C).

The number of apoptotic cells varied markedly between groups with only 8% of cells undergoing apoptosis in the C group compared to 24% in H rats. Replacement of beef tallow with HPA (26%) or HSA (40%) led to increased apoptosis that was greater than with HLA (10%) or HMA (14%) or in H rats (Fig. 1C).

SFA on bone morphology. Abnormal subchondral bone remodelling is recognised as a characteristic feature of OA. A 3-D representation of whole rat knee joint using micro-CT showed changes to the bone architecture of H, HPA and HSA rats compared to C, HLA or HMA rats (Fig. 2A). The region of interest was set as the medial and lateral tibial subchondral bone compartment. Tibial subchondral BV/TV decreased in H rats compared to that of the C rats. Replacement of beef tallow with LA or MA in diet increased tibial BV/TV while replacement of beef tallow with PA or SA decreased tibial BV/TV compared to H rats (Fig. 2B). Similarly to BV/TV, subchondral bone mineral density (BMD) decreased in rats fed with the beef tallow diet (H) compared to the C diet. Replacement of beef tallow with LA or MA increased BMD. In contrast, replacement of beef tallow with PA or SA in diet decreased BMD compared to the H rats (Fig. 2C).

Diverse distribution of osteocytes in the subchondral bone region. In this study, the average osteocyte lacunae per mm^2 (Avg.OS.L) was increased in H, HPA and HSA rats compared to C rats, suggesting increased bone remodelling and dysregulated mineral metabolism caused by the diets. Avg.OS.L density of HLA and HMA rats was very similar to that of the C rats. The decreased presence of average osteocyte nucleus (Avg. OS.N) in H, HMA, HPA and HSA rats compared to the C rats suggests the diets induced apoptotic death of the osteocytes (Supplementary Table 2).

SFA on human chondrocytes and bovine cartilage explants. Human chondrocytes isolated from cartilage specimens (grade 0–1) were stimulated with different SFA. To induce OA-like biological changes, the human chondrocytes were stimulated with IL-1β. There was an increased sGAG release from LA, MA and PA pellets compared to the control pellet at day 3. The greatest sGAG release was seen from the pellets treated with SA in comparison to the control pellets (Fig. 3B). On day 7, the control 3D cultured chondrocyte pellets treated with BSA (carrier) showed increased presence of proteoglycans indicated by strong Safranin O staining and decreased sGAG release into the media (Fig. 3A). LA and MA-treated pellets showed staining intensity similar to control pellets. In contrast, PA and SA-treated chondrocyte pellets showed marked loss of proteoglycans and subsequent increase in sGAG release into the medium compared with the other groups (Fig. 3A,B). Treatment with IL-1β alone decreased the proteoglycan content in the chondrocyte pellet compared to the non-IL1β treated control pellet, showing that IL1β treatment promotes proteoglycan degradation. Addition of PA and SA increased the cartilage matrix degradation shown by decreased proteoglycan content in the chondrocyte pellet and increased release of sGAG into the medium compared to the control pellet, indicating the effect of IL-1β and SFA on cartilage degradation (Fig. 3A,B).

Since we observed increased sGAG release from both the PA and SA treated pellets, we performed a time course and a concentration-response experiment using these treatments. Increased concentrations of PA and

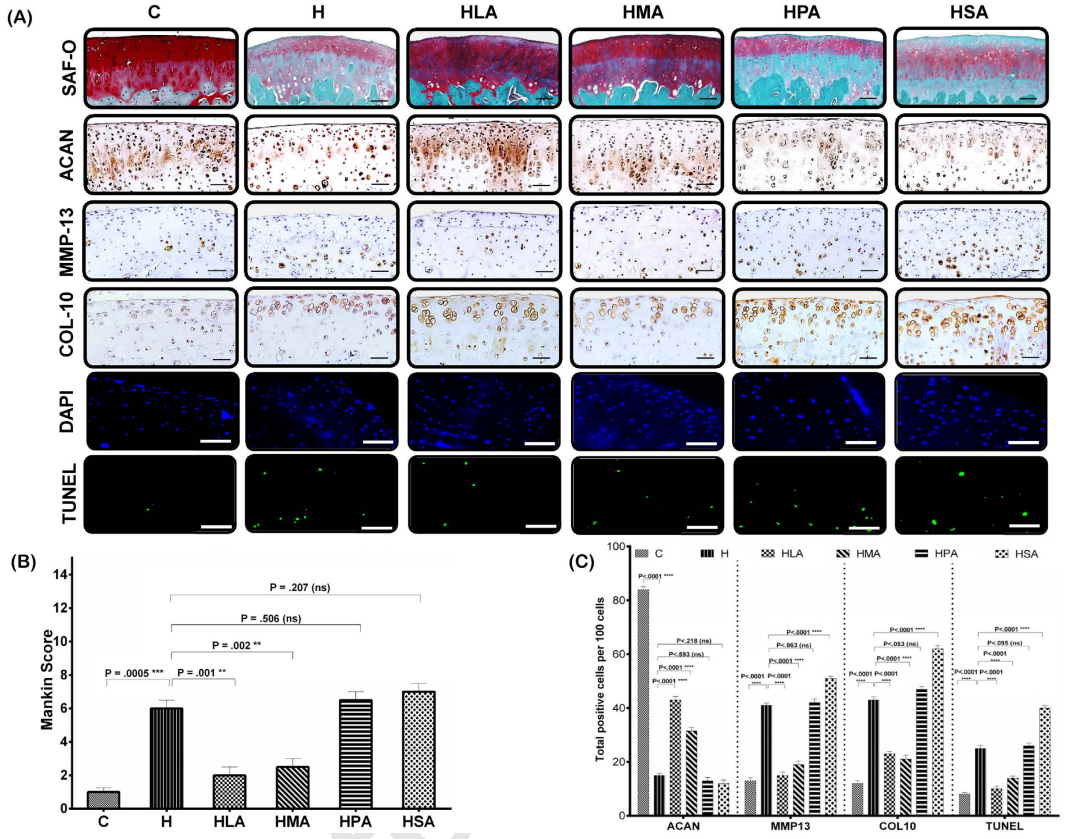

Figure 1. (**A**) Histological evaluation of the knee joints. All pictures were obtained under 20X magnification (n = 8 per group), representing the medial tibial plateau. Safranin O/Fast Green staining shows the extent of proteoglycan loss among the different diet groups. Representative immunohistochemical staining shows the number of positive cells for ACAN, MMP13, COL10 among the different diet groups. Analysis of apoptotic chondrocytes among different diets were quantified using TUNEL assay (DAPI and TUNEL images shown). The numbers of TUNEL-positive cells per section of the articular cartilage were determined under fluorescence microscopy. For each immunostaining, negative control either without primary antibody or with isotype-matched IgG instead of primary antibody was included. (**B**) Severity of articular cartilage degradation was graded using Mankin scoring system. (**C**) Quantitative histomorphometric analyses. Total positive cells per 100 cells in the cartilage were counted using ImageJ (NIH, Bethesda, MD). All values are represented as mean ± SD. Scale bar is 100 μm. ($P < 0.05$)

SA increased release of sGAG into the medium. Once again, the IL-1β treated chondrocytes exhibited increased sGAG release compared to PA and SA pellets that were not treated with it (Fig. 3C,D). To assess if the concentration of IL-1β used in this study caused significant release of sGAG, we treated the chondrocyte pellets with different concentrations of IL-1β. The day 3 and day 7 sGAG release data showed that IL-1β at 10 ng/ml did not increase the rate of sGAG release in comparison with IL-1β at 1 ng/ml.

Loss of aggrecan (ACAN) is the first sign of degeneration with increased expression of degradative (MMP13, ADAMTS4, ADAMTS5) and hypertrophic markers (COL10 and RUNX2)[15]. Therefore, these markers were assessed in normal human chondrocytes treated with SFA with or without the addition of IL-1β using quantitative RT-PCR. In comparison with control samples, SFA-treated samples had decreased expression of ACAN (Fig. 4A) both in the presence and absence of IL-1β. The expression of ACAN was markedly down-regulated in LA and MA-treated groups, and the highest down-regulation was seen in PA and SA-treated groups both in the absence and presence of IL-1β. LA and MA-treated pellets had lower expression of COL10A (Fig. 4B), MMP13 (Fig. 4C), ADAMTS-4 (Fig. 4D), ADAMTS5 (Fig. 4E) and RUNX2 (Fig. 4F), in comparison with PA and SA-treated pellets (Fig. 5) in both the absence and presence of IL-1β.

The bovine explants in the control group treated only with BSA carrier exhibited a smooth articular cartilage surface and normal cellularity. Explants treated with LA or MA showed staining intensity similar to control explants. In contrast, explants treated with PA or SA showed decreased intensity of Safranin O staining compared to control explants (Fig. 5A). Consistent with the Safranin O staining results, marked increases in the release of sGAG were observed in the explants treated with PA or SA compared to control, LA or MA-treated explants (Fig. 5B). In bovine explants treated with IL-1β, the intensity of Safranin O staining was decreased upon stimulation of different SFA. Addition of LA and MA decreased the proteoglycan content of the cartilage explants compared to the control explant and also increased the release of sGAG into the medium. Furthermore, addition

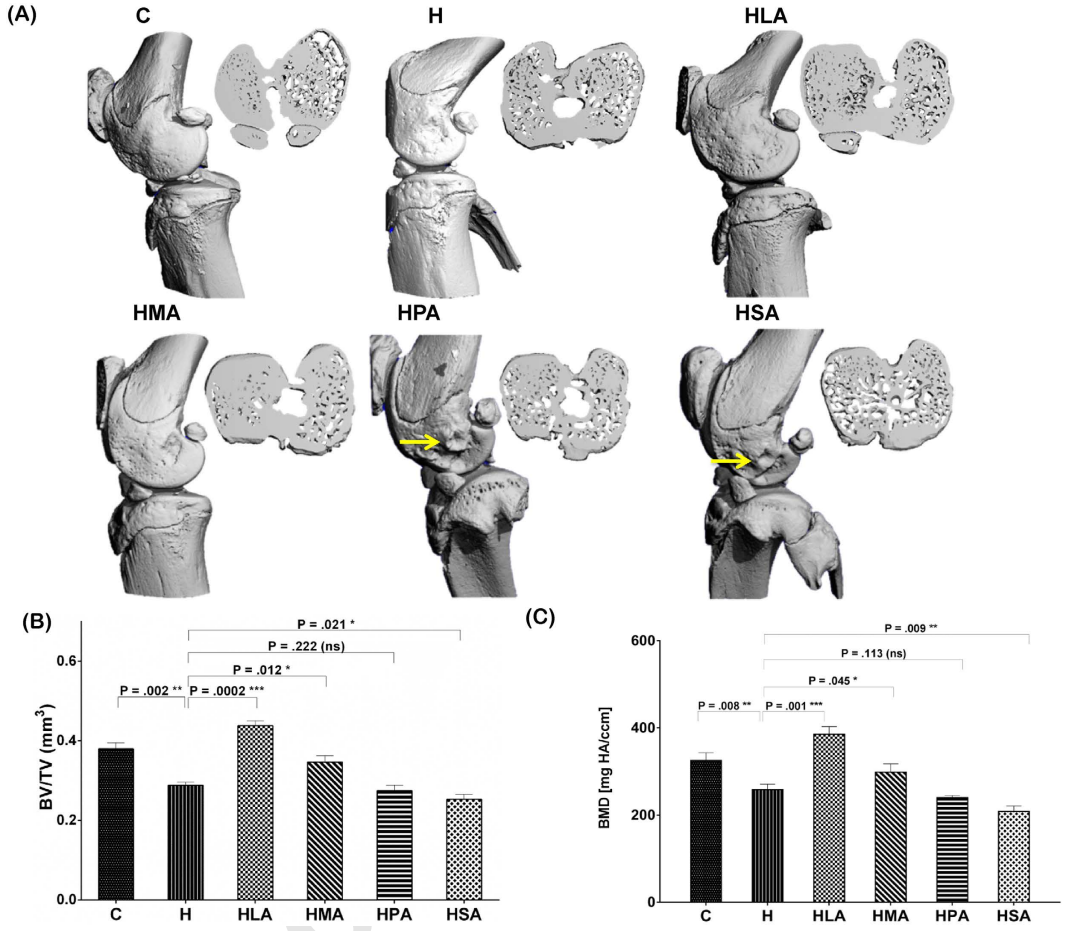

Figure 2. Micro-CT analyses of knee joints of rats fed the different diets (n = 8). (**A**) A 3-D Image of the whole knee joints of rats and the reconstructed axial micro CT cross-section images (insert) of region of interest i.e. the medial and lateral tibial plateau showing altered subchondral bone architecture (yellow arrows show abnormal bone morphological changes). For morphometric analyses, (**B**) the bone volume fraction (BV/TV) was calculated as the ratio of segmented bone volume (BV) to the total volume (TV) of the region of interest. In addition, (**C**) the bone mineral density (BMD) of the region of interest was also calculated. All values are represented as mean ± SD ($P < 0.05$).

of PA and SA decreased the proteoglycan content of the cartilage explant compared to the control explant and also increased sGAG release into the medium (Fig. 5A,B). In addition, treatment of PA and SA increased the sGAG depletion compared to the control explants both in the absence and the presence of IL-1β. In contrast, treatment of LA and MA showed sGAG depletion which was comparable to that of the control treated explants. However, when IL-1β was added to the explants, LA and MA treatment increased the sGAG depletion of the explants compared to control explants, but were not lower compared to PA and SA treatment (Fig. 5C).

Discussion

The worldwide incidence of obesity has doubled since 1980, now affecting 475–600 million people, depending on the BMI cut-off used in the estimates[16]. The incidence of OA has also markedly increased over a similar time period[17], leading to well-documented support for the concept that obesity is a modifiable risk factor for the incidence and progression of OA[18]. The prevalence of both obesity and OA are greatest in patients over 65 years of age, and this is an increasing segment of the world's population. Since both obesity and OA reduce mobility and increase cardiovascular risk, weight loss and exercise are recognised as key components for treatment of obese patients with OA[18]. Thus, understanding the common mediators for obesity and OA is clearly relevant to provide realistic and sustainable treatment options for obesity-associated OA.

Increased intake of dietary fat is one of the most important factors linking obesity and OA. Dietary fat is a key contributor to obesity and may help uncover the complex relationships between obesity and OA[19]. In obesity-related OA, SFA are relevant as they are the major dietary fatty acids. Our study strongly indicates that SFA produce parallel changes over the same time-frame in metabolic syndrome and typical OA-like lesions in the knee joint combined with increased chondrocyte death and breakdown of matrix in isolated bovine and human chondrocytes. Overall, the changes increase with SFA chain length with minimal changes following LA treatment to marked changes with SA treatment. These changes may be further increased by the presence of *trans* fatty acids as in beef tallow, but this proposal needs to be further tested by treatment with pure *trans* fatty acids. A plausible

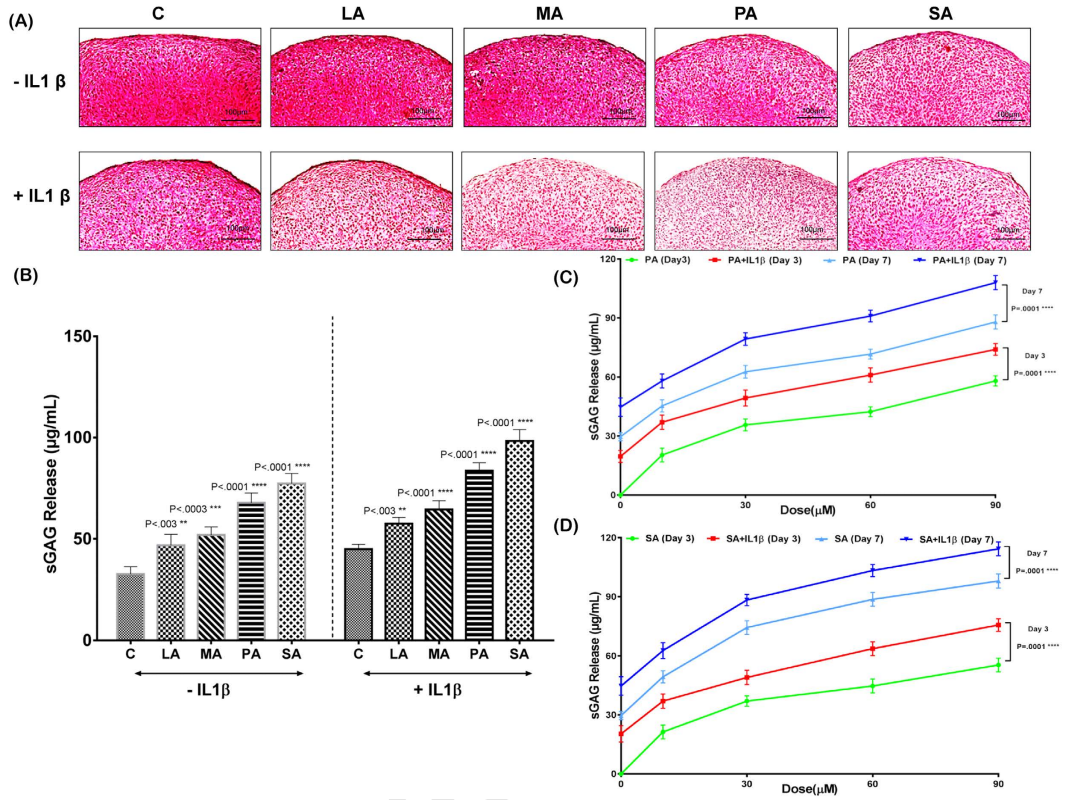

Figure 3. Proteoglycan content and sulphated glycosaminoglycan (sGAG) release of chondrocyte pellets stimulated with different SFA. All pictures were obtained under 20X magnification (n = 5 per group). (**A**) Safranin O/Fast Green staining shows the extent of proteoglycan loss among the different diet groups both in non-IL-1β treated and IL-1β treated pellets. (**B**) Quantification of sGAG release in the supernatant was performed by DMB assay, which showed that PA and SA-treated pellets exhibited a trend of decreased chondrogenesis irrespective of IL-1β treatment. (**C**,**D**) Graph showing the increase in sGAG release in response to increasing concentration of PA and SA. All values are represented as mean ± SD. Scale bar is 100 µm ($P < 0.05$).

mechanism is suggested by the correlation between obesity and OA-like changes and plasma leptin concentrations but other hormones and cytokines released from macrophages may also be involved, as discussed below. This is consistent with the reported increase in palmitate- or stearic acid-induced apoptosis in chondrocytes[10,20]. In addition, previous studies have reported that SFA-rich high-fat diet increased the severity of OA[8,21,22].

The changes in cartilage observed in this study are characteristic of OA-induced cartilage damage with increased PA and SA inducing marked depletion of proteoglycan content and increased expression of MMP13 and COL10 proteins shifting the balance of matrix homeostasis towards catabolism. MMPs are capable of degrading all components of the extracellular matrix, and their enhanced activity has been strongly implicated in cartilage degeneration[23]. Hypertrophy is known to trigger apoptosis of cartilage cells. The type of fatty acid clearly plays a role since LA did not induce these changes. PA and SA showed decreased proteoglycan content and increased release of sGAG, which confirmed their destructive properties on both cartilage explants and re-differentiated chondrocyte pellets. We further studied the effects of SFA and inflammatory cytokines such as IL-1β on chondrocytes. Co-treatment with IL-1β decreased the proteoglycan content and facilitated the increased release of sGAG in both human and bovine cartilage. Increases in gene expression of MMP13, ADAMTS 4 and 5 in PA- and SA-treated cartilage explants correlated with the increased release of sGAG. These results collectively suggest that catabolic effects of PA and SA together with IL-1β co-treatment in both human and bovine cartilage are likely to be mediated by the increased expression of cartilage-degrading enzymes that then trigger the changes similar to OA in chondrocytes. Furthermore, the number of empty lacunae is a clear indicator of osteocyte death[24]. In our recent study, the functional properties of osteocytes changed in OA patients, signifying the potential role of these cells in subchondral bone sclerosis[25]. Our results confirmed the increased presence of empty lacunae in H, HPA and HSA diet rats suggesting increased bone remodelling and unregulated mineral metabolism.

The correlation between obesity and OA is well-established, indicating that metabolic-induced inflammation in obesity[26] plays an important role in OA[27]. The initiators of the inflammatory processes in rats fed this obesogenic diet are most likely to be SFA, since SFA such as PA activate macrophages[10], and therefore increase infiltration of inflammatory cells throughout the body. Thus, obesity by itself does not cause OA, nor the reverse, but increased circulating SFA may induce infiltration of inflammatory cells throughout the body to produce parallel development of both disease states. While SFA may initiate the inflammation, there is good evidence that adipokines produced by inflammatory cells within fat pads also produce major physiological responses and

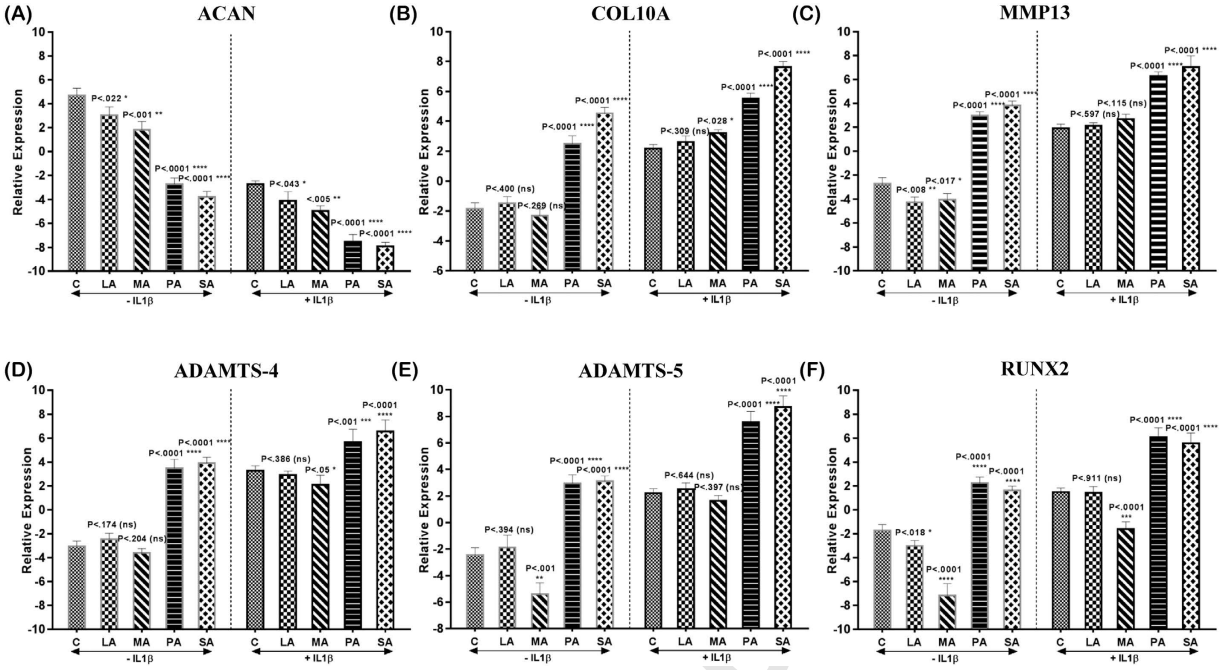

Figure 4. Extracellular matrix-related gene expression of chondrocyte pellets stimulated with different SFA (n = 5 per group). (**A**) ACAN, (**B**) COL10A, (**C**) MMP13, (**D**) ADAMTS4, (**E**) ADAMTS5 and (**F**) RUNX2 mRNA levels were assessed by RT-PCR both in non-IL-1β treated and IL-1β treated pellets. All experimental samples were performed in triplicate. All values are represented as mean ± SD ($P < 0.05$).

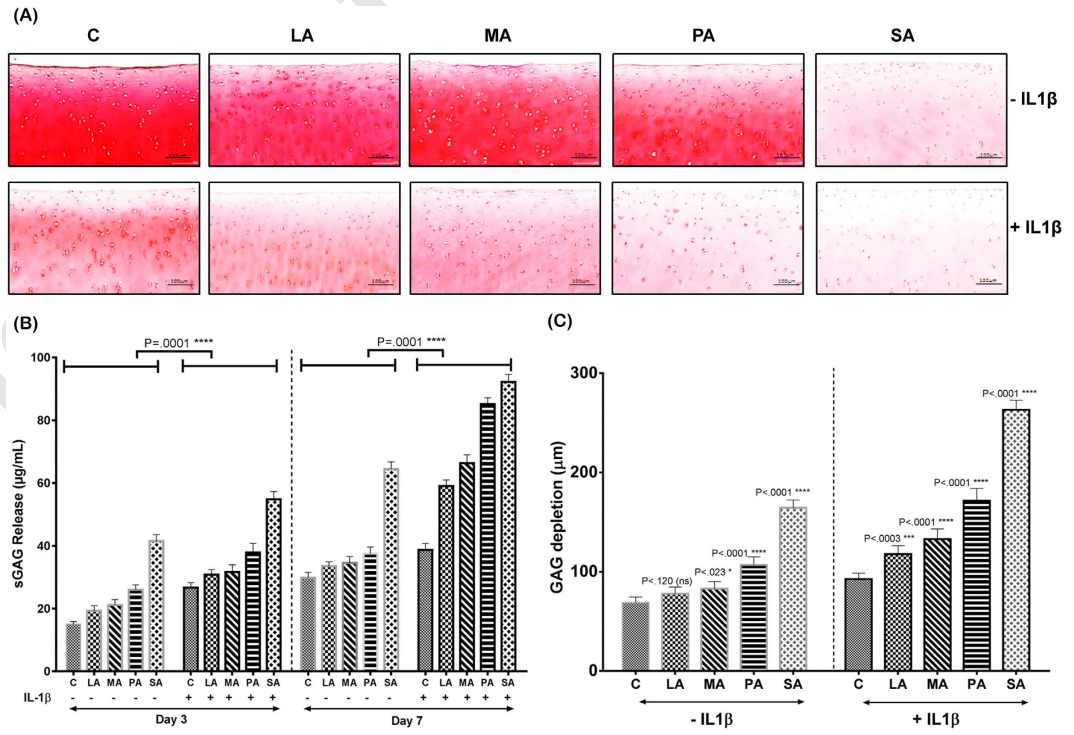

Figure 5. Proteoglycan content, sGAG release and sGAG depletion of bovine cartilage explants (n = 3) stimulated with different SFA. (**A**) Safranin O/Fast Green staining of bovine cartilage explants shows the loss of proteoglycans in the different diet groups both in non-IL-1β treated and IL-1β treated explants. (**B**) Quantification of sGAG release in the supernatant was performed by DMB assay, which showed that PA and SA-treated explants increased release of sGAG into the media on both day 3 and day 7 when compared to the other diet groups. In addition, zone of sGAG depletion (**C**) in the cartilage explants was the highest in SA-treated explants irrespective of IL-1β treatment ($P < 0.05$).

may therefore increase the inflammatory responses in OA[28]. Key cytokines are likely to include leptin and adiponectin, with possible roles of more recently discovered adipokines such as resistin and visfatin, in both obesity and OA[4,28,29]. The involvement of these cytokines in the development of obesity-induced OA could underlie the improvements following loss of abdominal obesity[30], and so decreased cytokine production, such as improved lean mass/fat mass ratio leading to increased mobility and physical activity with decreased OA-induced pain, for example in the knees. The local inflammatory reactions in obesity-associated OA may then be increased by the development of insulin resistance in type 2 diabetes[31]. Type 2 diabetes is strongly predictive of the severity of OA, independent of BMI and age[32,33], suggesting the involvement of insulin as type 2 diabetes is an insulin-resistant state. Insulin decreased autophagy in immortalised human chondrocytes and human cartilage explants, providing a mechanism by which cartilage could be damaged in an insulin-resistant state[34]. The correlation of decreased plasma insulin concentrations and decreased OA-like changes in rats fed LA, and the increased OA-like changes in rats fed PA and SA in our study strongly supports the role of insulin in OA associated with glucose intolerance and obesity. Oxidative stress as an increased biological activity of oxygen free radicals is also a key initiator of damage in both obesity[35] and OA[36]. Ageing is associated with an increased oxidative stress, and also increased prevalence of both obesity and OA, suggesting oxidative stress as a possible causative link. Further, functional foods such as omega-3 polyunsaturated fatty acids may improve oxidative status as an important therapeutic mechanism[37].

The limitations of this study include that we have not measured the morphological changes of the synovium or changes in synovial fluid cytokine concentrations. These two additional parameters may contribute additional insights to understanding local changes due to inflammation in SFA diets and so improve understanding of obesity-induced OA.

In conclusion, this study provides evidence that SFA can produce similar changes in both metabolic syndrome and OA. These changes correlate with the plasma concentrations of leptin and insulin, both involved in obesity, type 2 diabetes and OA. Our data suggest that replacement of traditional diets containing coconut-derived LA with palm oil-derived PA or animal fat-derived SA has the potential to worsen the development of both metabolic syndrome and OA. Further, human clinical trials are necessary to determine whether replacement of PA and SA in the diet with LA will attenuate or reverse the development of both OA and metabolic syndrome, especially obesity and hypertension.

Methods

Study design and diets. All animal experiments for this study were approved by the Animal Ethics Committees of the University of Southern Queensland and Queensland University of Technology while the human sample collection was approved by the Price Charles Hospital Human Ethics Committee. The procedures for using animal and human samples were performed in accordance with the guidelines of the National Health and Medical Research Council of Australia. The experimental groups consisted of 72 male Wistar rats (9–10 weeks old) weighing 330–350 g purchased from Animal Resource Centre, Perth, WA, Australia (www.arc.wa.gov.au). Rats were individually housed in a temperature-controlled, 12-hour light/dark cycle environment with free access to water and food at the University of Southern Queensland Animal House. The rats were randomly divided into 6 groups of 12 rats. Corn starch (C) diet contained 57% corn starch, 15.5% powdered rat food (Specialty Feeds, Glen Forest, WA, Australia), 2.5% HMW salt mixture and 25% water. High-carbohydrate, high-fat diet consisted of 17.5% fructose, 39.5% sweetened condensed milk, 20% beef tallow (H) or SFA (20% LA (HLA), 20% MA (HMA), 20% PA (HPA) or 20% SA (HSA)), 15.5% powdered rat food, 2.5% HMW salt mixture and 5% water. C diet included drinking water without any additives while H-based diet-fed rats were given drinking water supplemented with 25% fructose[14]. The carbohydrate intake in both C and H groups was approximately 68%. Rats had *ad libitum* access to food and water during the protocol.

Measurements of metabolic variables. Daily measurements of body weight and intakes of food and water were performed to monitor the day-to-day health of rats. Feed conversion efficiency (%) was calculated as previously described[14]. Percent body weight increase over 16 weeks was calculated as body weight difference between day 0 and day 112. Abdominal circumference was measured every 4 weeks using a standard measuring tape under light anaesthesia with Zoletil (tiletamine 10 mg/kg, zolazepam 10 mg/kg i.p; Virbac, Peakhurst, NSW, Australia).

Oral glucose tolerance tests were performed on rats as previously described[14]. Briefly, rats were deprived of food for 12 hours before basal blood glucose concentration measurements followed by oral gavage of 40% aqueous glucose solution and measuring glucose concentrations again at 30, 60, 90 and 120 minutes, with calculation of AUC (area under the curve) from these measurements. Systolic blood pressure was measured every 4 weeks under light sedation with Zoletil (10 mg/kg tiletamine, 10 mg/kg zolazepam, i.p.)[14]. Echocardiography was performed to measure the cardiovascular structure and function[14]. Indirect calorimetry was used to measure oxygen consumption and carbon dioxide production using a 4-chamber Oxymax system (Columbus Instruments, Columbus, OH) with one rat per chamber. Rats had *ad libitum* access to food and water during the measurement. Oxygen consumption (V_{O2}) and carbon dioxide production (V_{CO2}) were measured individually from each chamber. The respiratory exchange ratio ($RER = V_{CO2}/V_{O2}$) was calculated by Oxymax software (v. 4.86). The oxidation of carbohydrates produces an RER of 1.00, whereas fatty acid oxidation results in an RER of about 0.70[38]. Energy expenditure was calculated by assessment of the exchange of oxygen for carbon dioxide that occurs during the metabolic processing of food.

Rats were euthanised after 16 weeks using Lethabarb® (100 mg/kg pentobarbitone sodium, i.p.). After euthanasia, blood was collected to isolate plasma and the plasma was stored at −20 °C before further analysis. Hearts were isolated to perform Langendorff heart preparation to measure diastolic stiffness constant[14]. Following this,

tissues such as liver, left ventricle (with septum), right ventricle and abdominal fat pads (including retroperitoneal, epididymal and omental) were removed for weighing and expressed as mg/mm of tibial length. Plasma concentrations of leptin, insulin, total cholesterol, triglycerides and non-esterified fatty acids (NEFA) were measured as described previously[14].

Assessment of articular cartilage. After 16 weeks of dietary interventions, rats were euthanised and the knee joints harvested and then processed for histology. Briefly, the intact joints were fixed in 4% paraformaldehyde for 48 hours, decalcified in 10% EDTA for 8 weeks, bisected laterally and embedded in paraffin. Paraffin blocks were then trimmed to expose tissue using a rotary microtome (Leica, Wetzlar, Germany). Two 5-μm sections within every consecutive six sections were cut and mounted on glass slides. Each knee yielded about 12 sections for standard haematoxylin and eosin (H&E) staining or proteoglycan staining with safranin O as previously described[39]. The severity of cartilage damage was assessed using the standard Mankin grading system[40]. The mean damage score from 5–6 samples from each animal was used to determine the mean ± standard deviation for each group. Two independent observers assessed cartilage damage in a blinded manner. Furthermore, immunostaining was performed with antibodies against matrix metalloproteinase-13 (MMP13) (1:50 Labvison, RB1681-P0, Fremont, CA, USA), aggrecan (AGAN) (1:200 Millipore, AB1031, New South Wales, Australia) and type 10 collagen (COL10) (1:200 Abcam, ab58632, New South Wales, Australia)[39]. For controls, the same procedures were carried out either without primary antibody or with isotype-matched IgG instead of primary antibody. For semi-quantitative data analysis, the positive cells from different fields of observation in the tibial medial compartment of the knee were counted and normalised to the cell number per 100 total cells in each group, using ImageJ (NIH, Bethesda, MD)[39].

TUNEL apoptosis assay. Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labelling (TUNEL) was used to identify cells that show degradation of their DNA during apoptosis. The tissue slices were first permeabilised with proteinase K for 30 minutes at 37 °C. The slides were then washed with PBS and the assay was performed using the manufacturer's protocol (Roche, Germany). The sections were incubated with DNase1 for 10 minutes at room temperature as a positive control. For semi-quantitative data analysis, the positive cells from different fields of observation were counted and normalised to the cell number per 100 total cells in each group, using ImageJ (NIH, Bethesda, MD)[39].

Assessment of subchondral bone changes. Micro-CT was performed to analyse the subchondral bone changes. Femur and tibia were scanned using a micro-CT (Scanco μCT 40, Scanco Medical, Switzerland) with isotropic voxel size of 18 μm with voltage of 55 kV and current of 145 μA with a 0.5 mm aluminium filter. The exposure time was 1180ms and Scanco's inbuilt software was used to segment the data set[39]. Manual regions of interest (ROI) were drawn around the anatomical contour in the subchondral bone region in the medial and lateral tibial plateau. The volume of interest (VOI) consisted of a stack of ROIs (25 cross-sections). The VOI started below the subchondral plate which extended distally towards the growth plate. Ratio of bone volume to the total volume (BV/TV) and bone mineral density (BMD) of the VOI were calculated.

Analysis of osteocytes. Average osteocyte empty lacunae (Avg OS.L) and average osteocyte nucleus (Avg OS.N) were counted per unit area (mm^2) in the subchondral bone region in the medial tibial plateau using ImageJ (NIH, Bethesda, MD).

Treatment of chondrocytes and cartilage explants with SFA. *Preparation of SFA.* LA, MA, PA and SA were purchased commercially at the highest purity available (Sigma-Aldrich, GC-purified, chemically synthesised). Bovine serum albumin (BSA) (fatty-acid free, Sigma Aldrich) was chosen as the carrier. The SFA-BSA complex solutions were made using a protocol described earlier[6]. Individual SFA were dissolved in 100% ethanol at 70 °C to yield a 200 mM stock solution. The stock solution was then diluted 1:10 in 10% (w/v) BSA made with Dulbecco's Modified Eagle Medium (DMEM) for 10 minutes at 55 °C. The resultant SFA solution (20 mM) was sterile-filtered (0.45 μM filter) prior to application to the cell cultures using a protocol described earlier[6]. The vehicle control (negative control) was the fatty acid free-BSA with the same concentration of ethanol.

Chondrocyte pellet and bovine explant culture. Chondrocytes were harvested using enzymatic digestion and cultured using our published protocols[39]. Briefly, biopsies of articular cartilage were obtained from primary OA patients undergoing knee replacement surgery at The Prince Charles Hospital (Brisbane, QLD, Australia). All cartilage biopsies were taken from a part of the surface of the femoral condyle considered by the surgeon to resemble intact and healthy cartilage. The five donors were all males aged 60–65 years and all provided written informed consent. The study was approved by The Prince Charles Hospital and Queensland University of Technology Human Ethics Committees. Each cartilage specimen was further characterised and scored according to Mankin score with only samples with scores of 0–1 (healthy cartilage) being used for further experiments.

The cartilage was dissected from the bone and digested with collagenase 2 solution (Invitrogen, Lakewood, NJ) overnight in the DMEM culture medium for the isolation of the articular cartilage chondrocytes (ACC). After digestion, ACCs (2.5×10^5 cells) were pelleted by centrifugation in 15 mL Falcon tubes and cultured in three-dimensional (3D) pellet culture system[39], for 14 days in chondrogenic medium in the presence or absence of different SFA.

For bovine explant studies, fresh adult bovine knee joints (n = 3) were obtained on the day of slaughter. Full thickness articular cartilage explants without the subchondral bone were then harvested from the knee joints. Cartilage discs were then aseptically dissected using a 4-mm dermal punch. The discs were cultured in DMEM, and supplemented with 10% Foetal Bovine Serum (FBS) and antibiotics at 37 °C with 5% CO_2 for 48 hours.

The pellets and discs were then stimulated with each SFA (final concentration: 30 μg/ml, derived from MTT assay – data not shown). The negative control, in which no SFA were added, was treated with BSA vehicle only. To study the effects of SFA and inflammatory cytokines, some ACC pellets and bovine cartilage discs were treated with IL-1β (10 ng/mL) in the presence or absence of different SFA. After day 3 and day 7, media were collected and stored in −80 °C for quantification of sulphated glycosaminoglycans. The cartilage discs and ACC pellets were later processed for histological analysis and quantitative real time PCR analysis (qPCR). To further assess the responses to certain SFA, different concentrations (10 μM, 30 μM, 60 μM and 90 μM) of PA and SA were added to chondrocyte pellets both in the absence and in the presence of IL-1β (10 ng/mL). After day 3 and day 7, media were collected and stored at −80 °C for quantification of sulphated glycosaminoglycans (sGAG). In addition, to assess the effects of IL-1β used in this study, chondrocyte pellets were treated with various concentrations of IL-1β (1–10 ng/ml). The day 3 and day 7 media were collected and stored at −80 °C for quantification of sGAG.

Sulfated glycosaminoglycans (sGAG) assay. To measure sGAG release, supernatants were collected from the wells containing the bovine cartilage discs or chondrocyte pellets treated with or without SFA at day 3 and day 7. The sGAG was measured using the dimethylmethylene blue method (DMB) with the kit (Blyscan™, Biocolor Ltd) following the manufacturer's instructions. Zone of sGAG depletion in cartilage explants were determined using images of Safranin O staining and were measured using ImageJ (NIH, Bethesda, MD).

RNA extraction and real-time PCR. Total RNA was extracted using TRIzol reagent (Invitrogen), treated with DNase and purified according to manufacturer's protocol using an RNeasy Mini Kit (Qiagen). cDNA was synthesised from 1 μg of the total RNA according to manufacturer's protocol using an SensiFAST cDNA Synthesis Kit. Real-time quantitative PCR[41], using SYBR Green detection chemistry, was performed on the ABI 7500 Fast Real Time PCR system (Applied Biosystems, Foster City, CA, USA). Melt curve analyses of all real-time PCR products were performed and shown to produce a single DNA duplex. All samples were measured in triplicate and the mean value of all experimental samples was considered for comparative analysis. Quantitative measurements of all primers used in this study were determined using the ($2^{-\Delta\Delta Ct}$) method, and 18 s and β-actin expression were used as the internal controls, as described previously by our group[39,41–43].

Statistics. Statistical analyses were performed using Graphpad Prism. The data are presented as mean ± standard deviation (SD) for all variables and analysed with ANOVA method. Repeated-measures analysis of variance with *post hoc* tests (Dunnett's/Bonferroni) was used to assess statistical significance. The level of significance was set at $P < 0.05$.

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Author Contributions

Y.X., L.B., I.P. and S.K.P. developed the original study aims. S.S., I.P. and S.R.S. conducted the experiments and analysed the data; R.C. provided human samples and advice on osteoarthritis. I.P. supervised and trained S.S. and has been the co-ordinating author for the manuscript. All authors have read and approved the final manuscript.

Additional Information

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Chapter 4 Linseed components on metabolic parameters in rats.

4.1 Introduction

Metabolic syndrome refers to a group of linked abnormalities including abdominal obesity, glucose homeostasis disorders including type 2 diabetes, impaired fasting glucose, glucose intolerance and insulin resistance, and dyslipidaemia with increased cardiovascular disease risk including hypertension and coagulation disorders (Kassi et al., 2011, Kaur, 2014). The prevalence of metabolic syndrome has increased, now affecting one quarter of the world's population (Thaman and Arora, 2013). The increase in prevalence reflects the transition from a traditional hunter-gatherer diet to a diet high in fats and carbohydrates with a sedentary lifestyle, changing body composition and metabolism leading to central obesity and other metabolic disorders (O'Neill and O'Driscoll, 2015).

The clinical management of metabolic syndrome is based on targeting obesity, insulin resistance and hypertension as these are fundamental risk factors of the syndrome. Modest changes in dietary intake can delay, if not prevent, the onset of metabolic disorders, for example including grains in the diet (Everitt et al., 2006). The consumption of linseed (*Linum usitatissimum L.*) has been reported to protect against metabolic syndrome and cardiovascular disease by lowering blood pressure, reducing blood glucose concentrations, delaying postprandial glucose absorption and decreasing oxidative stress and inflammation (Rodriguez-Leyva et al., 2013, Hutchins et al., 2013). These changes may be due to linseed being a rich source of bioactive compounds (Bozan and Temelli, 2008, Oomah, 2001), in particular providing an oil rich in α-linolenic acid (ALA, 18:3n-3) together with lignans, digestible proteins and mucilage from soluble fibre (Singh et al., 2011a). There is growing support for the ALA component of linseed being the active component in reducing blood triglycerides, blood pressure, platelet reactivity, neutrophil activity and increasing high-density lipoprotein (HDL) cholesterol thereby helping in lowering cardiovascular risk (Gorder et al., 1986, Cunnane et al., 1993, Li et al., 1999). Further, the

administration of 3% of linseed oil to high-carbohydrate, high-fat fed rats normalised systolic blood pressure, and improved heart function and glucose tolerance (Poudyal et al., 2013a).

Linseed is one of the richest dietary sources of plant lignans with secoisolariciresinol diglucoside (SDG) being the major component of lignans and found at a concentration 75–800 times higher than in other foods (Poluzzi et al., 2014). SDG is converted by enterolignan-producing bacteria in the mammalian colon to enterodiol and enterolactone (Muir, 2006). Animal and human studies have shown benefits of linseed lignans in chronic diseases such as the slowing of progression and regression of atherosclerosis and the amelioration of cardiovascular disease, diabetes, cancer and metabolic syndrome (Adolphe et al., 2010, Prasad and Jadhav, 2016). Visceral fat, liver triacylglyceride content, serum triacylglycerides, total cholesterol, and insulin and leptin concentrations were reduced compared with the high-fat diet without SDG in C57BL/6 mice (Fukumitsu et al., 2008).

However, the physiological responses of SDG, raw linseed and defatted linseed as separate interventions have not been evaluated in the same disease model and linseed as a much cheaper source of alpha-linolenic acid grown locally has not been tested. This is also necessary to extend the measured physiological responses of ALA from the linseed oil fraction in diet-induced obesity in rats (Poudyal et al., 2013a), while the metabolic effects of other linseed components are yet to be elucidated. The rats were fed either corn starch diet, high-carbohydrate, high-fat diet or either diet containing either 0.03% of 40% SDG to give an SDG dose approximating 6mg/kg/day, 5% raw linseed or 3% defatted linseed supplement. These rates were chosen to be similar to previous studies of SDG supplementation (Fukumitsu et al., 2008, Felmlee et al., 2009), whole seed supplementation (Poudyal et al., 2012a) and non-oil content of seeds as flour (Dugani et al., 2008). The aim of this study therefore was to determine the effects of lignans, raw linseed and defatted linseed on cardio-metabolic health parameters on high-carbohydrate, high-fat induced obesity in rats.

4.2 Materials and methods

4.2.1 SDG and raw linseed composition analysis

SDG (40% purity) was a kind gift from Archer Daniels Midland Company (Chicago, Illinois, USA). Raw linseed and defatted linseed were kind gifts from AustGrains (Moree, NSW, Australia). The analysis of SDG content was conducted by St. Boniface Hospital Research, Winnipeg, Canada and the nutritional analysis of raw linseed was provided by Agrifood Technology, Victoria, Australia.

4.2.2 Bomb calorimetry

Total gross energy content of SDG, raw linseed and defatted linseed samples were measured by bomb calorimetry (XRY-1A Oxygen Bomb calorimeter, Shanghai Changji Geological Instrument Co. Ltd) in triplicate. About 1 g of SDG, raw linseed and defatted linseed were burnt in compressed oxygen (25 kg/cm^2) in the calorimetric bomb immersed in water.

4.2.3 Rats and diet

Male Wistar rats (6 – 8 weeks old, weighing 250 - 300 g, n = 96) were purchased from Animal Resource Centre from Perth, Australia. All the rats were housed individually in temperature-controlled, 12-hour-light/-dark conditions in the animal house facility of the University of Southern Queensland. The rats were acclimatised and given free access to water and standard rat powdered food prior to diet intervention. 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.

After an acclimatisation period, rats (average weight 336±0.2 g) were randomly divided into 8 experimental groups: corn starch-rich diet-fed rats (CS; n = 12), corn starch + 40% SDG (CSLF; n = 12), corn starch + 5% raw linseed (CSRL; n = 12), corn starch + 3% defatted linseed (CSDL; n = 12), high-carbohydrate, high-fat diet-fed rats (HCHF; n = 12), high-carbohydrate, high-fat + 40% SDG (HLF; n = 12),

high-carbohydrate, high-fat + 5% raw linseed (HRL; n = 12) and high-carbohydrate, high-fat + 3% defatted linseed (HDL; n = 12). The SDG-supplemented diets were prepared by adding sufficient SDG-enriched product (0.03% of 40% SDG) to give an average daily intake of 6 mg/kg body weight SDG from the CS and HCHF diets; raw linseed supplemented diets were prepared by replacing 5% water with 5% of raw linseed in the CS and HCHF diets and defatted linseed supplemented diets were prepared by replacing 3% water with 3% defatted linseed in the CS and HCHF diets.

The dosages were chosen based on previous study using 6mg SDG/kg body weight/day with the purity of SDG at 99% (Felmlee et al., 2009). We were provided with SDG at 40% purity, so we aimed for a dose to the rats of 15 mg/kg/day to give the same SDG dose. Next, the raw linseed was the same dose as in a study which used 5% chia seeds in food (Poudyal et al., 2012a), as the oil composition of chia seed and linseed are same. Since the oil content of linseed is about 40% (Dugani et al., 2008), the non-oil component, defined here as defatted linseed, is therefore 60%. Hence, the defatted linseed flour was dosed at 3% in the food or 60% of the dose of the whole linseed.

The SDG, raw linseed and defatted linseed supplemented diets were administered for 8 weeks starting 8 weeks after the initiation of the corn starch or high carbohydrate, high-fat diet. HCHF, HLF, HRL and HDL groups were given 25% fructose in drinking water along with the diets for the 16 week duration of the study. Normal drinking water without any supplementation was given to CS, CSLF, CSRL and CSDL rats. The preparation and macronutrient and micronutrient compositions of CS and HCHF diets were previously described (Poudyal et al., 2012a, Panchal et al., 2011). Rats were monitored daily for body weight and food and water intake. Energy intake and food conversion efficiency were calculated (Poudyal et al., 2012a, Panchal et al., 2011).

4.2.4 Other methods

All other methods were performed as described in Chapter 2 which included oral glucose tolerance test, systolic blood pressure, body composition, isolated Langendorff heart preparation, aortic contractility, ileum and colon contractility, tissue collection, histology and biochemical analysis.

4.2.5 Statistical analysis

All data are presented as mean ± SEM. A total of 8 groups were analysed using two-way analysis of variance (ANOVA) with each group consisting of 12 rats. All group data were tested for variance using Bartlett's test. Variables that were not normally distributed were transformed (using log 10 function) prior to statistical analysis. The effects of diet, treatment and their interactions 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. All statistical analyses were performed using GraphPad Prism version 6 for Windows (San Diego, CA). A p value of < 0.05 was considered as statistically significant.

4.3 Results

4.3.1 Nutritional analysis of raw linseed

The proximate composition of raw linseed is presented in Table 4.1.

Table 4.1: Proximate composition of raw linseed.

Test	Result
Dry matter (%)	93.3
Moisture (%)	6.7
Ash (% of dry matter)	2.8
Crude fibre (% of dry matter)	17.9
Crude protein (% of dry matter)	25.4
Fat by acid hydrolysis (% of dry matter)	32.6
Nitrogen free extract (%)	19.9

4.3.2 Total gross energy

The total gross energy as measured by bomb calorimetry was found to be lowest in defatted linseed (16.87±0.12 kJ/g), followed by SDG with 17.48±0.07 kJ/g and the highest total gross energy was found in raw linseed (23.76±0.06 kJ/g).

4.3.3 Dietary intake

The results on food intake, water intake and energy intake that were determined after 8 weeks of lignans, raw linseed or defatted linseed supplementation are presented in Figure 4.1 and Table 4.2. Food and water intakes were higher in CS-fed rats than in HCHF-fed rats. However, energy intake was higher in HCHF rats than in CS rats. There were no differences in the food intake of CSLF, CSRL and CSDL with corn starch control group (CS). HLF, HRL and HDL also showed no differences in the food intake with high-carbohydrate, high-fat fed group (HCHF). Water intake was unchanged in CS, CSLF, CSRL and CSDL groups and there was also no difference in water intake between HCHF, HLF, HRL and HDL rats. The same results were found in energy intake, hence no change in feed conversion efficiency among high-carbohydrate, high-fat groups, whereas CSRL-fed rats showed the highest feed conversion efficiency among all corn starch fed rats.

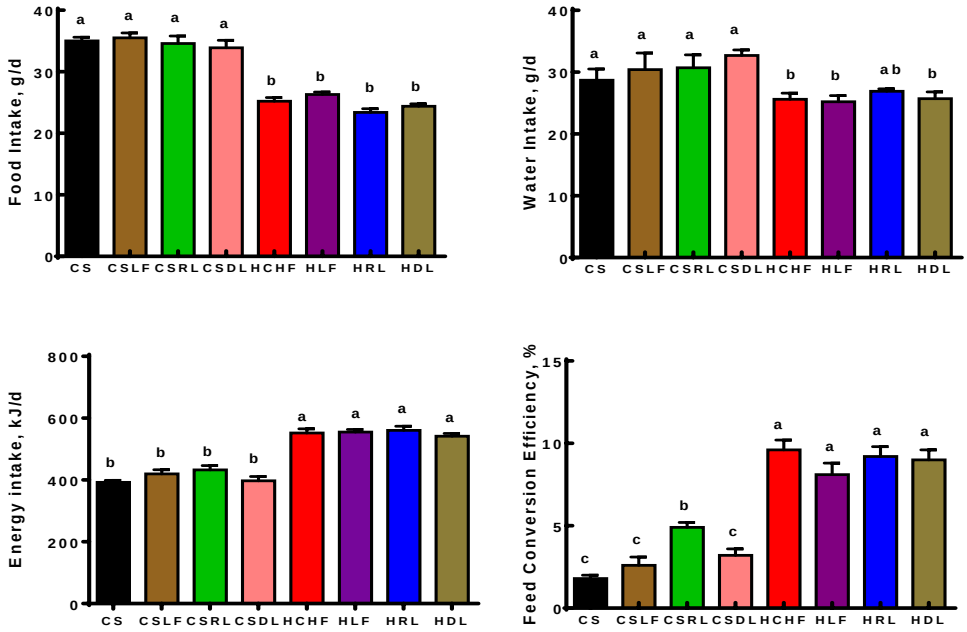


Figure 4.1: Food intake, water intake, energy intake and feed conversion efficiency in rats supplemented with lignan, raw linseed or defatted linseed at week 16.
Data are presented as mean ± SEM of. Means with unlike superscript differ; $p < 0.05$.

4.3.4 Body composition

Body weight throughout the protocol of 16 weeks are presented in Figure 4.2. All groups showed weight gains. H-fed rats had higher body weight gain than C-fed rats. Raw linseed group showed the highest weight gained as compared to control groups, while HRL showed no difference in weight gain compared to HCHF group. The contribution of bone mineral content to body composition can be seen in Table 4.2, where HRL group had higher bone mineral content compared to CS, CSRL, CSDL and HDL groups (Figure 4.3) and no difference was found with HLF and HCHF groups. CSRL supplemented rats had the highest total lean mass and this group showed no differences in total fat mass and bone mineral content with CS and CSLF groups (Figure 4.3).

Abdominal circumference and visceral adiposity index were not changed in CSLF and CSRL rats, respectively, compared to CS rats (Table 4.2). HRL showed higher abdominal circumference and higher visceral adiposity index as compared to HCHF control group and this might be the consequence of higher total fat mass in HRL rats. HLF showed no difference in total fat mass and total abdominal fat with HCHF control group. HDL supplemented rats were shown to have higher total lean mass as compared to HCHF and HLF group.

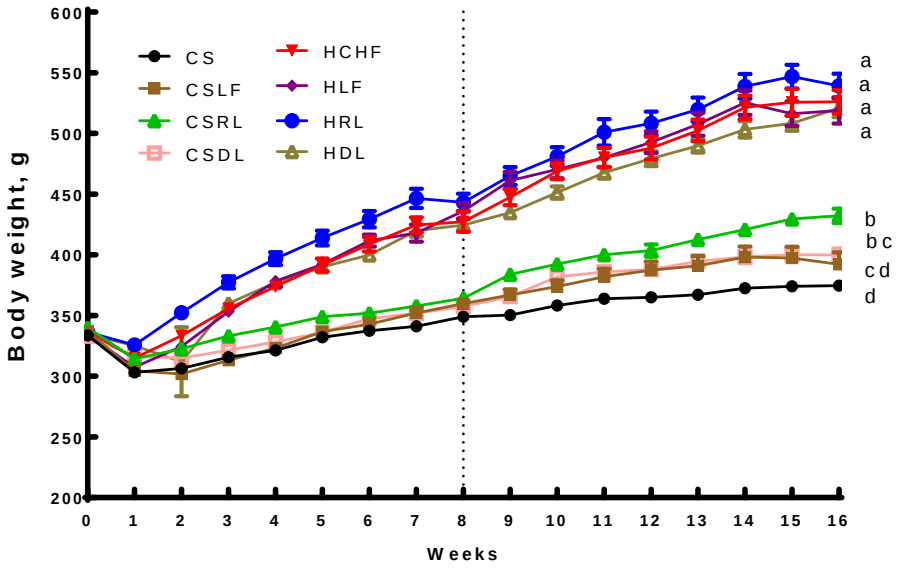


Figure 4.2: Body weight in rats from 0 to 16 week. Rats were supplemented with either lignans, raw linseed or defatted linseed starting at week 8.

Data are presented as mean ± SEM, n = 10-12. Values marked with different letters are significantly different at the level of $p < 0.05$.

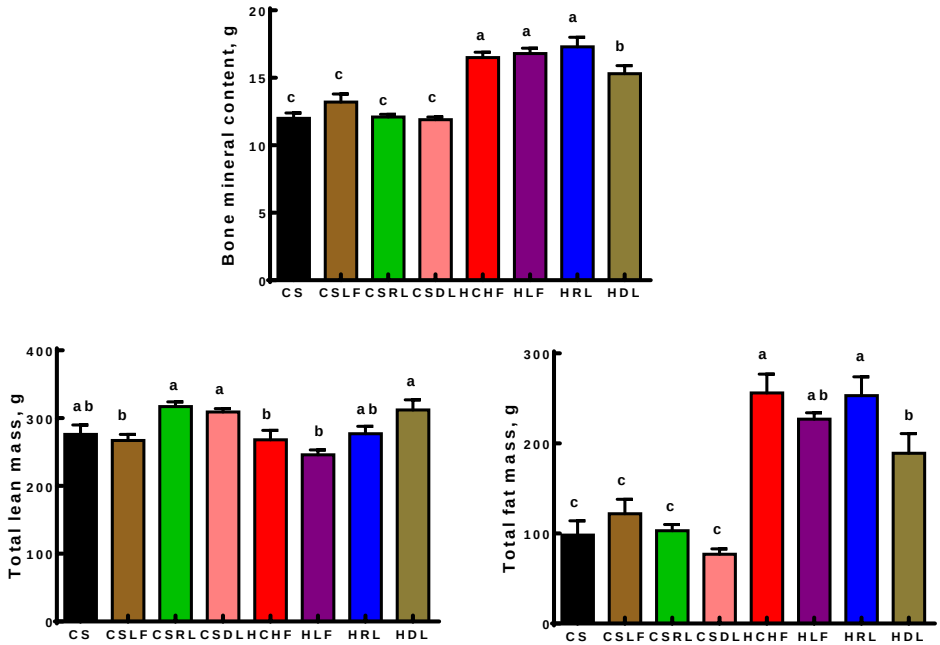


Figure 4.3: Bone mineral content, total lean mass, total fat mass (gram) in rats fed CS, CSLF, CSRL, CSDL, HCHF, HLF, HRL and HDL.

Data are presented as mean ± SEM, n = 10-12. Values marked with different letters are significantly different at 16 weeks at the level of $p<0.05$.

Table 4.2: Dietary intakes, body compositions and organ wet weights in groups fed the CS, CSLF, CSRL, CSDL, HCHF, HLF, HRL and HDL in the diet.

Variable	CS	CSLF	CSRL	CSDL	HCHF	HLF	HRL	HDL	P Value		
									Diet	Treatment	Interaction
Food intake (g/d)	35.0±0.6^{a}	35.5±0.8^{a}	34.6±1.2^{a}	33.9±1.2^{a}	25.2±0.6^{b}	26.3±0.4^{b}	23.4±0.6^{b}	24.4±0.4^{b}	<0.0001	0.0452	0.3957
Water intake (mL/d)	28.7±1.8^{a}	30.4±2.7^{a}	30.7±2.1^{a}	32.7±0.9^{a}	25.6±1^{b}	25.2±1.0^{b}	26.9±0.4^{ab}	25.7±1.1^{b}	0.0047	0.6175	0.8185
Body weight gained (%) (week 8 - 16)	7.4±1.0^{d}	9.1±1.7^{d}	18.2±0.8^{b}	13.2±1.6c	23.3±0.9^{a}	18.9±1.3^{b}	23.4±0.6^{a}	22.7±1.4^{a}	<0.0001	<0.0001	<0.0001
Energy intake (kJ/d)	392±7^{b}	420±13^{b}	433±14^{b}	397±14^{b}	552±13^{a}	555±8^{a}	561±14^{a}	542±9^{a}	<0.0001	0.1323	0.3855
Feed conversion efficiency (%)	1.8±0.2^{c}	2.6±0.5^{c}	4.9±0.3^{b}	3.2±0.4^{c}	9.6±0.6^{a}	8.1±0.7^{a}	9.2±0.6^{a}	9.0±0.6^{a}	<0.0001	0.0043	0.0048
Bone mineral content (g)	12.0±0.4^{c}	13.2±0.6^{c}	12.1±0.2^{c}	11.9±0.2^{c}	16.5±0.4^{a}	16.8±0.4^{a}	17.3±0.7^{a}	15.3±0.6^{b}	<0.0001	0.3256	0.2829

Each value is presented as mean±S.E.M, n = 8-12. Means within a row with unlike superscript differ; $p< 0.05$.

Table 4.2: Continued.

Variable	CS	CSLF	CSRL	CSDL	HCHF	HLF	HRL	HDL	P Value		
									Diet	Treatment	Interaction
Total fat mass (g)	98.3±16^{c}	122±16^{c}	103±7^{c}	76.9±6^{c}	256±21^{a}	227±7^{ab}	253±21^{a}	189±22^{b}	<0.0001	0.9756	0.23
Total lean mass (g)	276±14^{ab}	267±9^{b}	317±7^{a}	309±5^{a}	268±14^{b}	246±7^{b}	277±11^{ab}	312±15^{a}	0.0094	0.0013	0.3115
Visceral adiposity index (%)	4.9±0.4^{d}	4.8±0.6^{d}	4.7±0.3^{d}	4.2±0.1^{d}	9.6±0.7^{ab}	8.5±0.4^{bc}	10.4±0.9^{a}	7.3±0.5^{c}	<0.0001	0.2846	0.2256
Tissue wet weight (mg/mm)											
Retroperitoneal fat	189±18^{c}	194±31^{c}	179±18^{c}	151±20^{c}	531±41^{a}	469±31^{ab}	554±59^{a}	407±31^{b}	<0.0001	0.5539	0.3445
Epididymal fat	89±10^{d}	112±20^{cd}	98±8^{d}	74±5^{d}	259±17^{a}	211±11^{b}	278±28^{a}	154±16^{c}	<0.0001	0.2903	0.0374
Omental fat	114±15^{c}	102±13^{c}	124±9^{c}	102±7^{c}	240±25^{ab}	207±8^{b}	280±18^{a}	198±14^{b}	<0.0001	0.013	0.2607
Total abdominal fat	392±39^{d}	408.±63^{d}	401±35^{d}	308±36^{d}	1031±79^{ab}	887±45^{b}	1113±105^{a}	683±92^{c}	<0.0001	0.2336	0.1831
Liver	201±5^{b}	213±8^{b}	213±12^{b}	216±7^{b}	327±12^{a}	306±9^{a}	337±15^{a}	310±8^{a}	<0.0001	0.3163	0.215
Heart	22.0±1.0^{c}	21.5±0.4^{c}	25.4±0.7^{bc}	25.3±0.9^{bc}	26.8±1.1^{b}	23.6±0.9^{bc}	31.5±2.1^{a}	26.9±0.9^{b}	<0.0001	<0.0001	0.183
Kidney	45.2±1.7^{b}	44.9±0.6^{b}	46.5±1.0^{b}	49.9±1.0^{b}	57.1±2.4^{a}	55.1±1.6^{a}	56.6±2.1^{a}	56.7±0.9^{a}	<0.0001	0.6216	0.8278
Spleen	12.2±0.5^{b}	14.7±0.9^{ab}	15.9±1.1^{ab}	14.5±0.9^{ab}	17.9±1.0^{a}	16.2±0.7^{a}	17.7±1.1^{a}	17.3±1.5^{a}	0.0002	0.1364	0.0419

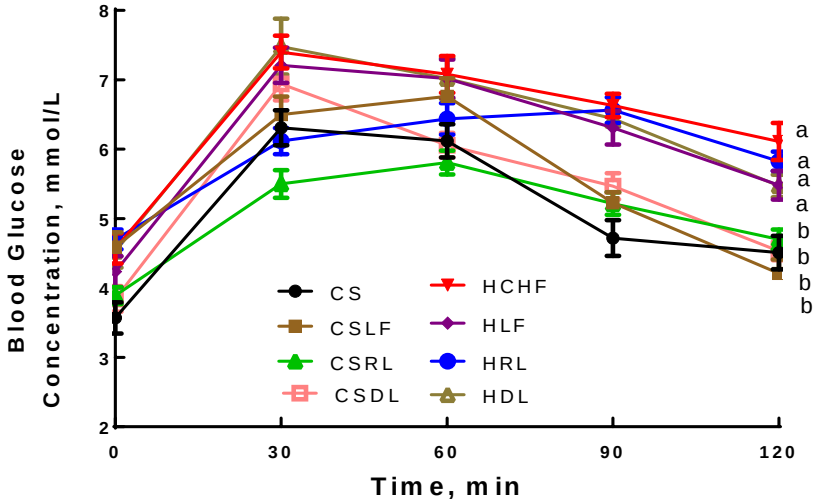
Each value is presented as mean±S.E.M, n = 8-12. Means within a row with unlike superscript differ; $p< 0.05$.

Fat depots (retroperitoneal, epididymal and omental fat) and organ wet weights (liver, heart, kidney and spleen) were measured and data are presented in Table 4.2. Retroperitoneal fat in HCHF rats was increased compared to CS rats, with the greatest weight found in HCHF and HRL fed groups. Average wet weight of epididymal and omental fats for HRL group was higher than HCHF, HLF and HDL groups. For HLF and HDL groups, the total abdominal fats were lower than HCHF group by 14% and 34%, respectively. Organ wet weight of liver, kidney and spleen showed no change among groups of high-carbohydrate, high-fat groups. However, the wet weight of heart for HRL group was higher as compared to HCHF, HLF and HDL groups. These data suggest that rats fed HRL diet gained weight particularly in the heart and abdominal fats, but rats fed HLF and HDL had lower average abdominal fats, heart, liver, kidney and spleen.

4.3.5 Plasma biochemistry analysis

Plasma total cholesterol concentrations of lignans, raw linseed and defatted linseed supplemented groups showed no difference with control groups (Table 4.3). HLF and HDL groups showed a lower concentration of plasma NEFA and TAG as compared to HCHF and HRL groups (Table 4.3). In CSLF, CSRL and CSDL rats, both plasma NEFA and TAG were unchanged as compared to CS group. Plasma insulin concentrations were lower in CSLF and CSDL groups when compared to CS and CSRL groups (Table 4.3). Compared to HCHF rats, HLF, HRL and HDL rats had lower plasma insulin concentrations. Plasma leptin concentrations were lower in HLF group as compared to HCHF, HRL and HDL groups (Table 4.3). Plasma leptin concentrations were unaffected among C-fed rats.

High-carbohydrate, high-fat rats control and supplemented groups showed higher basal glucose concentrations than corn starch control and corn starch supplemented groups (Figure 4.4). Results showed that there was no difference in total area under curve among H-fed rats and similar findings were also found in CS-fed rats.



	P-value		
Figure	Diet	Treatment	Interaction
A	<0.0001	0.0467	0.4860

Figure 4.4: Oral Glucose Tolerance Test (OGTT) at 16 weeks in rats fed CS, CSLF, CSRL, CSDL, HCHF, HLF, HRL and HDL.

Data are presented as mean ± SEM, n = 10. End-point means without a common alphabet in each data set significantly differ at $p < 0.05$.

Table 4.3: Plasma biochemistry analyses in groups fed the CS, CSLF, CSRL, CSDL, HCHF, HLF, HRL and HDL in the diet.

Variable	CS	CSLF	CSRL	CSDL	HCHF	HLF	HRL	HDL	P Value		
									Diet	Treatment	Interaction
Lipid profile											
Plasma total cholesterol (mmol/L)	1.5±0.05^{a}	1.6±0.06^{a}	1.3±0.10^{a}	1.4±0.10^{a}	1.6±0.04^{a}	1.6±0.06^{a}	1.5±0.10^{a}	1.6±0.10^{a}	0.0973	0.0226	0.3917
Plasma NEFA (mmol/L)	0.9±0.20^{b}	1.4±0.19^{b}	1.5±0.20^{b}	1.5±0.20^{b}	4.3±0.60^{a}	3.7±0.51^{a}	4.0±0.30^{a}	3.7±0.40^{a}	<0.0001	0.8585	0.3055
Plasma TAG (mmol/L)	0.4±0.06^{b}	0.4±0.06^{b}	0.4±0.10^{b}	0.5±0.01^{b}	1.6±0.30^{a}	1.3±0.20^{a}	1.5±0.20^{a}	1.3±0.20^{a}	<0.0001	0.6784	0.6784
OGTT-AUC (mmol/l x min)	636±19^{bc}	687±20^{b}	625±14^{c}	680±14^{b}	794±13^{a}	765±25^{a}	735±17^{ab}	770±17^{a}	<0.0001	0.0365	0.0940
Plasma insulin (µmol/L)	1.3±0.01^{e}	1.7±0.01^{d}	2.9±0.05^{c}	1.7±0.05^{d}	7.7±0.09^{a}	6.0±0.11^{b}	5.9±0.12^{b}	5.8±0.04^{b}	<0.0001	<0.0001	<0.0001
Plasma leptin (µmol/L)	3.2±0.40^{d}	2.1±0.62^{d}	4.2±0.06^{d}	3.9±0.03^{d}	12.3±1.54^{a}	6.3±0.97^{c}	13.1±0.03^{a}	10.2±0.08^{b}	<0.0001	<0.0001	0.0052

Each value is a mean ± S.E.M, n = 10. Means within a row with unlike superscript differ; p< 0.05. NEFA: Non-esterified fatty acids, TAG: Triglycerides, OGTT-AUC: Oral glucose tolerance test- area under curve.

4.3.6 Cardiovascular function

HLF, HRL and HDL groups showed lower systolic blood pressures as compared to HCHF group (Table 4.4). There was no difference in systolic blood pressure between CSDL and CS groups. These results with H-fed rats were reflected in Langendorff isolated heart measurement as the diastolic stiffness constant was improved in HLF and HDL groups. The heart wet weight in HLF group was same as corn-starch fed rats (Table 4.2).

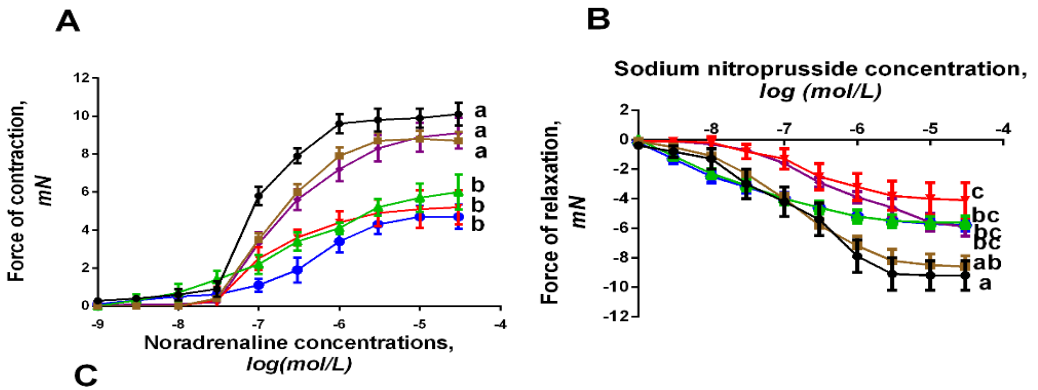
HCHF rats showed diminished α_1-adrenoceptor-mediated vascular contraction to noradrenaline in isolated thoracic aortic rings compared to CS rats (Figure 4.5A). Additionally, HCHF rats showed decreased smooth muscle-dependent relaxant responses to sodium nitroprusside (Figure 4.5B) and endothelium-dependent relaxant responses to acetylcholine (Figure 4.5C). Lignan-supplemented groups showed improved α_1-adrenoceptor-mediated vascular contraction to noradrenaline and smooth muscle-dependent and endothelium-dependent relaxant responses to sodium nitroprusside and acetylcholine, respectively (Figures 4.5A, B and C). However, raw linseed groups did not show any improvement in vascular contraction and relaxation (Figures 4.5A, B and C).

HLF rats had improved histological profile of the left ventricle after 16 weeks (Figures 4.6 and 4.7). HCHF showed greater interstitial collagen deposition (marked "cd"; Figure 4.6E) as compared to other groups (Figure 4.6) and increased infiltration of inflammatory cells (Figure 4.7E). HLF, HRL and HDL rats showed reduced ventricular collagen deposition (Figures 4.6 F, G and H). No change was observed in CSLF rats (Figures 4.6B and 4.7B) and the tissue morphology of the heart appeared normal.

Table 4.4: Systolic blood pressure and diastolic stiffness constant of CS, CSLF, CSRL, CSDL, HCHF, HLF, HRL and HDL at 16 weeks.

Variable	CS	CSLF	CSRL	CSDL	HCHF	HLF	HRL	HDL	P Value		
									Diet	Treatment	Interaction
Systolic blood pressure, mmHg	128 ± 3^{bc}	133 ± 2^{b}	129 ± 2^{bc}	120 ± 3^{c}	148 ± 2^{a}	133 ± 3^{b}	134 ± 2^{b}	129 ± 5^{bc}	<0.0001	0.0256	0.0004
Diastolic stiffness constant, κ	23.0 ± 0.4^{bc}	23.0 ± 0.8^{bc}	24.2 ± 0.7^{abc}	21.9 ± 0.3^{c}	26.6 ± 0.9^{a}	22.0 ± 0.5^{c}	25.1 ± 0.5^{ab}	23.7 ± 1.3^{bc}	0.0344	0.0012	0.0039

Data are presented as mean ± SEM, n = 10-12. Values marked with different letters are significantly different at the level of $p < 0.05$.



	P-value		
Figure	Diet	Treatment	Interaction
A	0.2773	0.7553	0.7381
B	0.3310	0.8621	0.8988
C	0.2158	0.9794	0.9794

Figure 4.5: Cumulative concentration–response curves for noradrenaline (A), sodium nitroprusside (B) and acetylcholine (C) in thoracic aortic rings from rats fed the CS, CSLF, CSRL, HCHF, HLF and HRL diet.

Data are shown as means ± S.E.M.; n=9–10/group.

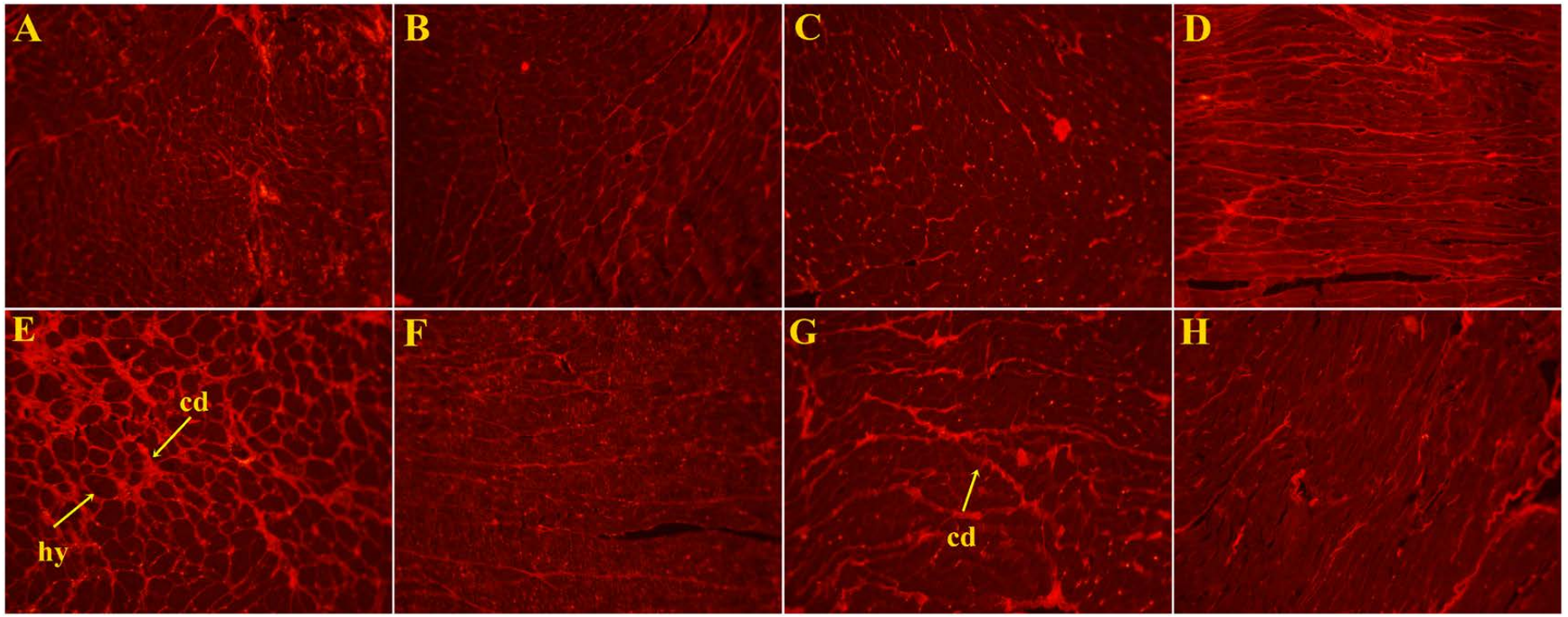


Figure 4.6: Picrosirius red staining of left ventricular interstitial collagen deposition (×20) in rats fed the CS (A), CSLF (B), CSRL (C), CSDL (D), HCHF (E), HLF (F), HRL (G) and HDL (H) diet.

Collagen deposition is marked as “cd,” and hypertrophied cardiomyocytes are marked as “hy.”

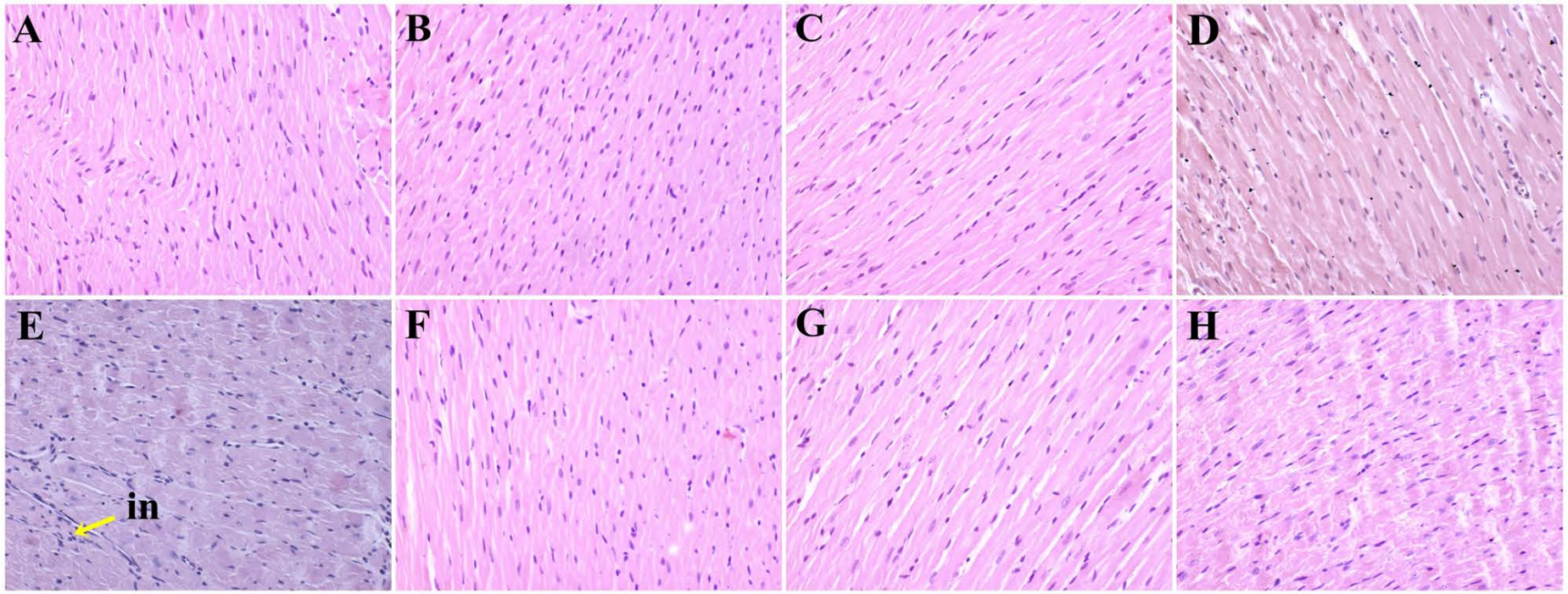


Figure 4.7: Haematoxylin and eosin staining of heart (x20) in rats fed the CS (A), CSLF (B), CSRL (C), CSDL (D), HCHF (E), HLF (F), HRL (G) and HDL (H) diet.

Inflammatory cell is marked as “in”.

4.3.7 Liver structure and function

HCHF rats had elevated plasma markers of liver function in comparison to CS rats (Table 4.5). Plasma liver function (ALT and AST) was unchanged in CSLF and HRL rats. ALT activity remained unchanged in HLF, HRL and HDL groups with HCHF rats. Plasma AST showed no difference among all H-fed groups. Figure 4.8 showed increased hepatic lipid deposition and inflammatory cell infiltration in H groups compared to C groups. HLF and HDL groups showed decreased lipid vacuoles (Figures 4.8F and 4.8H) compared to HCHF and HRL groups. CSLF and CSDL groups showed minimal hepatic steatosis and portal inflammation and tissue morphology appeared normal.

Table 4.5: Plasma liver enzymes of CS, CSLF, CSRL, CSDL, HCHF, HLF, HRL and HDL rats at week 16.

Variable	CS	CSLF	CSRL	CSDL	HCHF	HLF	HRL	HDL	P Value		
									Diet	Treatment	Interaction
Hepatic function											
Plasma ALT (U/L)	26.8±2.5^{b}	23.2±1.6^{b}	30.9±3.8^{b}	28.3±2^{b}	33.7±1.5^{a}	33.6±1.7^{a}	27.8±2.5^{ab}	33.8±1.5^{a}	0.0173	0.7369	0.0169
Plasma AST (U/L)	64.6±4.6^{a}	61.6±1.8^{a}	66.4±5.9^{a}	66.3±2.8^{a}	68.8±2.6^{a}	69.6±6.8^{a}	61.6±3.7^{a}	67.5±2.9^{a}	0.5189	0.8439	0.3753

Data are presented as mean ± SEM, n = 10-12. Values marked with different letters are significantly different at the level of $p < 0.05$.

ALT; Alanine aminotransferase, AST: Aspartate transaminase.

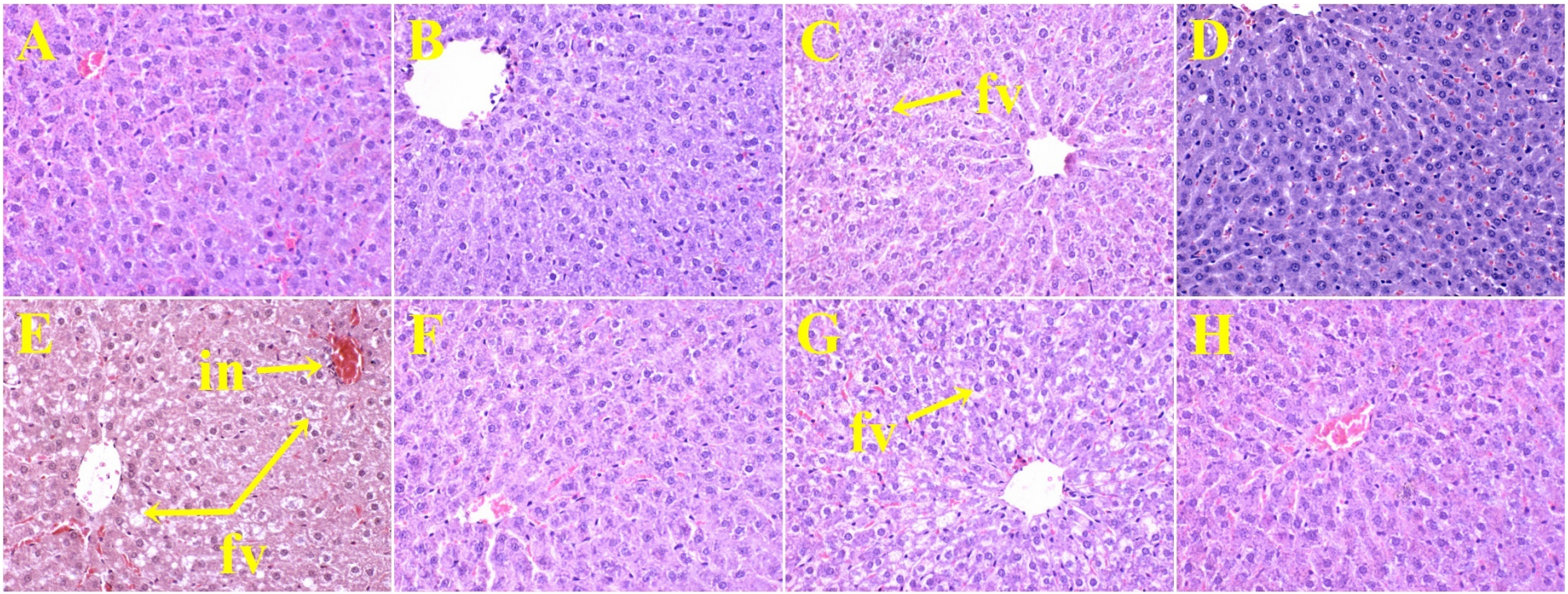


Figure 4.8: Haematoxylin and eosin staining of liver (x20) in rats fed the CS (A), CSLF (B), CSRL (C), CSDL (D), HCHF (D), HLF (E), HRL (F) and HDL (H) diet.

Figures with “fv” marked the fat vacuoles and “in” marked inflammatory cells.

4.3.8 Gut structure and function

Acetylcholine-induced contraction on ileum and colon is shown in Figure 4.9. There were no difference in ileal contractions between high fat, high carbohydrate dietary treatments at the maximum dose of acetylcholine. Similarly, there were no differences between corn starch control group and treated groups. Increased colon contractility was evident in HDL rats compared to CSRL and HRL rats, with no difference found in other groups. In histological sections, fewer goblet cells were observed in small intestines of treatment rats as compared to control rats (Figure 4.10). In the colon, fewer goblet cells were observed in HLF and HRL rats as compared to control rats, whereas the density of goblet cells was increased in the colon of HDL rats compared to control rats (Figure 4.11).

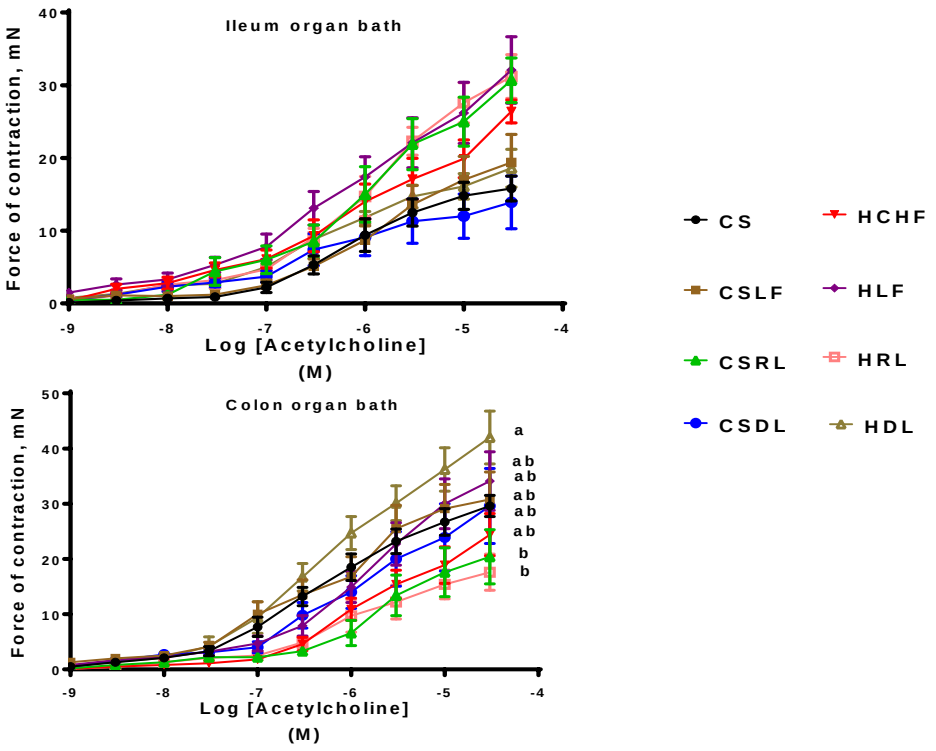


Figure 4.9: Concentration-response curves for acetylcholine in ileum and colon from rats fed the CS, CSLF, CSRL, CSDL, HCHF, HLF, HRL and HDL diet.
Data are shown as means ± S.E.M.

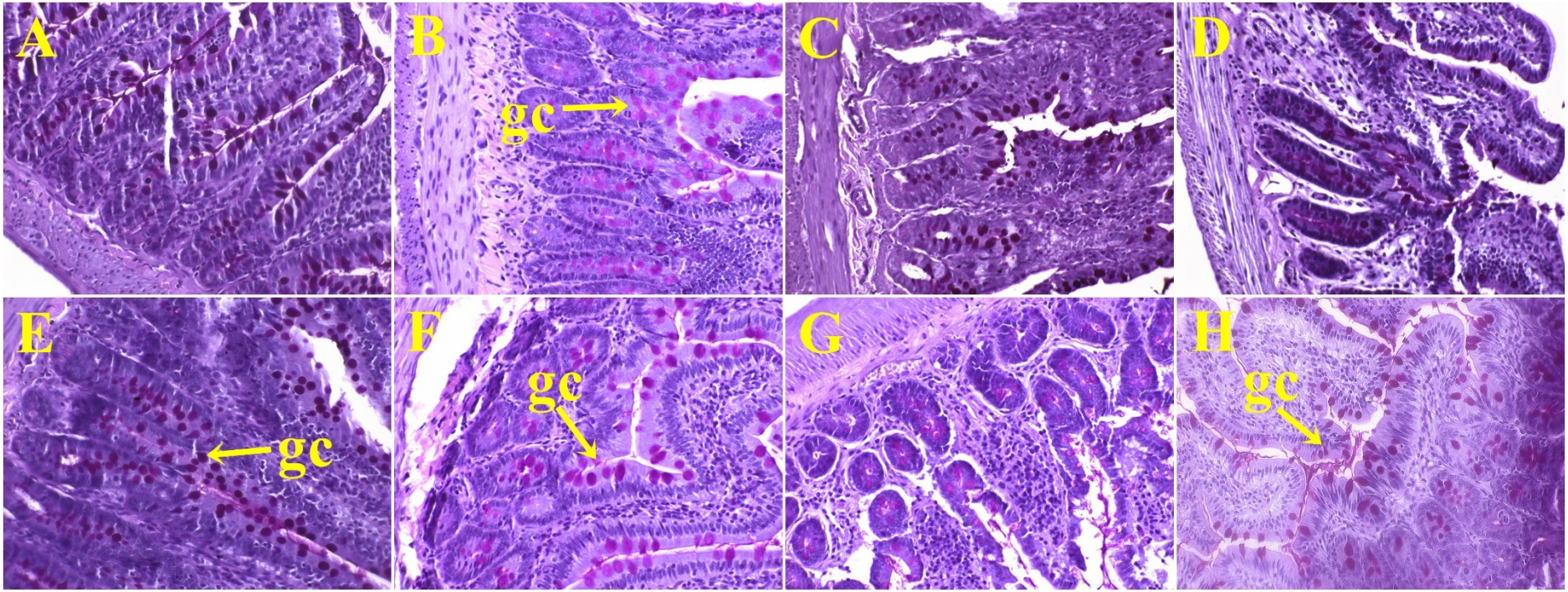


Figure 4.10: Periodic acid Schiff's staining of small intestine (x20) in rats fed the CS (A), CSLF (B), CSRL (C), CSDL (D), HCHF (E), HLF (F), HRL (G) and HDL (H) diet.

Figures with "gc" marked the goblet cells.

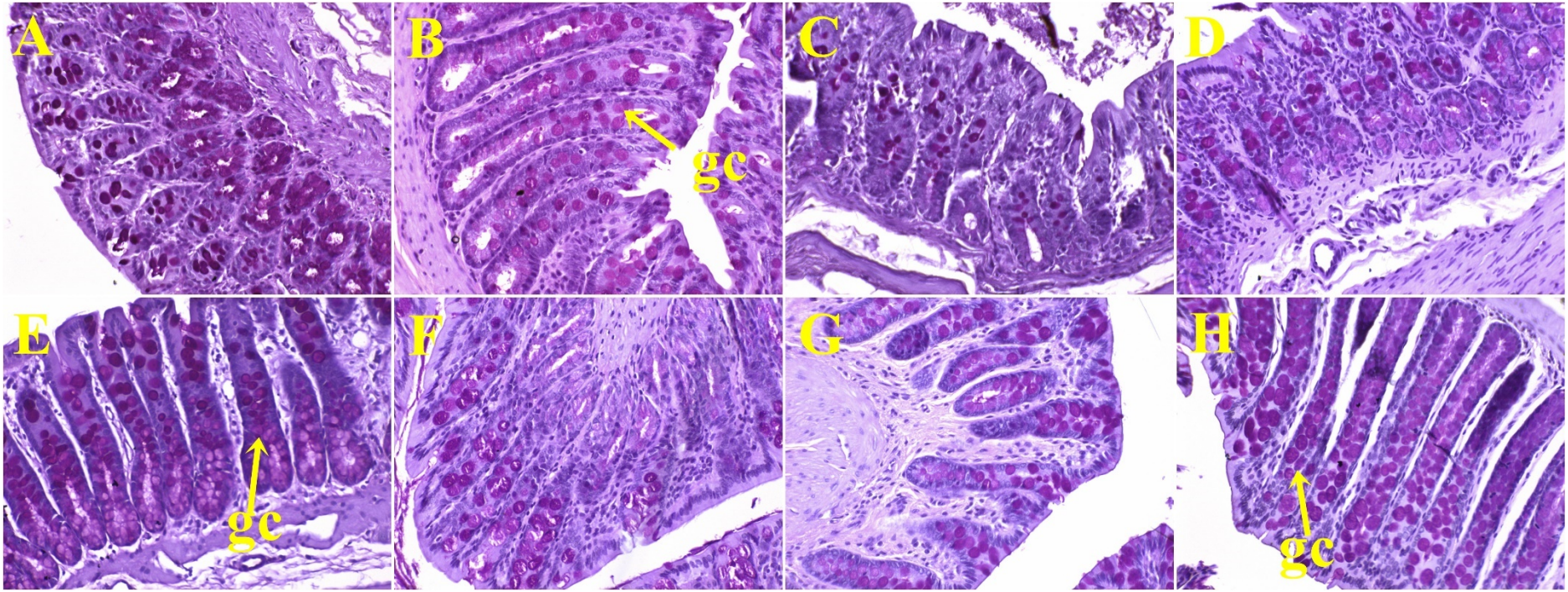


Figure 4.11: Periodic acid Schiff's staining of colon (x20) in rats fed the CS (A), CSLF (B), CSRL (C), CSDL (D), HCHF (E), HLF (F), HRL (G) and HDL (H) diet.

Figures with "gc" marked the goblet cells.

4.4 Discussion

The established metabolic syndrome rat model (Panchal et al., 2011) was used to investigate the comparative effects of 6 mg/kg/day SDG, raw linseed (5% in the diet) or defatted linseed (3% in the diet) in high-carbohydrate, high-fat rat model. The model shows progressive increases in body weight, energy intake, abdominal fat deposition, and abdominal circumference along with impaired glucose tolerance, dyslipidaemia, hyperinsulinaemia and increased plasma leptin concentrations. Moreover, after 16 weeks feeding with high-carbohydrate, high-fat diet, rats showed diminished cardiovascular function (Panchal et al., 2011). In addition, the same model was used in a previous study to determine the effect of α-linolenic acid (ALA) from linseed oil in adiposity, cardiovascular and liver structure in rats fed either a diet high in simple sugars and mainly saturated fats or a diet high in polysaccharides (corn starch) and low in fat (Poudyal et al., 2013a). This study only used linseed oil, therefore the current study explored other components of linseed for comparison with raw linseed. No studies have examined the effects of SDG, raw linseed and defatted linseed supplementation using the same model as it is crucial to see their biological actions towards reversing the metabolic syndrome. Thus, we examined the effects of 6mg/kg/day SDG, 5% of raw linseed or 3% defatted linseed diet supplementation for 8 weeks on biomarkers associated with metabolic syndrome which include glucose, leptin, insulin, blood pressure, diastolic stiffness and lipid profiles.

Rats fed with HCHF diet developed abdominal obesity, hypertension, impaired glucose, leptin and insulin tolerance, dyslipidaemia and diminished cardiac function which supports the findings of the previous study (Panchal et al., 2011). However, the addition of low dose lignans as 6 mg/kg/day SDG to the diet for only 8 weeks lowered body weight gain as compared to HCHF control group together with lower total fat mass and total abdominal fat weight. Moreover, lower plasma concentrations of leptin reflected a low visceral adiposity index in SDG-fed rats. HLF also showed improved insulin concentrations and lower blood glucose concentrations compared to HCHF. These changes were also reported with lignan-enriched linseed (35% SDG) powder fed to high-fructose, high-fat rats for 12 weeks [12]. With lower doses of SDG (0.5 and 1.0% SDG), weight changes were not as pronounced as in our study. However,

the same study found a reduction in visceral and liver fat accumulation, and improved plasma lipids, cholesterol, insulin and leptin concentrations in high-fat diet induced mice (Fukumitsu et al., 2008). These results are consistent with our study findings. An underlying mechanism of changes in adiponectin and leptin has been demonstrated to increase rates of fatty acid oxidation that mediates the insulin-sensitising effects (Dyck et al., 2006). Further, GLUT4 expression was increased in the insulin target tissue adipose, where it increased glucose uptake and enhanced insulin sensitivity (Armoni et al., 2003). Increased glucose elimination was suggested via increased translocation of GLUT4 to the cell membrane and increased basal glucose uptake by redistribution of GLUT1 (Pilar et al., 2014).

Physiological responses of CSRL and HRL supplementation showed increased body weight gain of the rats after 8 weeks. It is noted that the total lean mass of CSRL was higher than CS control group. Meanwhile, HRL had a higher total fat mass and total abdominal fat that may contribute to higher body weight gained. Raw linseed had no effect on body weight as a study using 10%, 20% and 30% raw flaxseed (linseed) by adding into mouse pellet commercial diet for 30 days showed no difference in body weight between all groups (Saman et al., 2011). Similarly, a short-term trial using a 40g aliquot of golden linseed daily for 28 days showed no differences in body weight, BMI and waist circumference in metabolic syndrome patients in both men and women (Pilar et al., 2014). In Zucker fatty rats, 10% (w/w) linseed supplementation for 9 weeks also showed no effect on obesity in this genetic model with hyperphagia (Gillam et al., 2009). These results suggest that 5 to 30% raw linseed supplemented in the diet for at least 4 to 8 weeks does not change body weight.

Removal of fat from linseed (CSDL and HDL) did result in weight changes with lower total fat mass, higher lean mass and lower abdominal circumference as seen in the CSDL and HDL groups. A clinical trial using 30g brown defatted flaxseed (linseed) flour given to obese subjects as a complement for breakfast showed that it promoted greater satiety and reduced their appetite (Monteiro et al., 2016). It appears that the higher protein and dietary fibre content from other linseed flours contain an important factor that reduced appetite and prolonged the sensation of satiety (Monteiro et al., 2016). Many possible mechanisms include homeostasis in weight maintenance that involved neuroendocrine signals that influence appetite and energy expenditure

(MacLean et al., 2015). Glucagon-like peptide-1 (GLP-1), an insulinotropic hormone released from the intestinal L cells in response to nutrient ingestion, inhibited gastric emptying and gastric acid secretion (Abu-Hamdah et al., 2009) and also association between ghrelin, satiety and cravings where a study on high protein and carbohydrate breakfast showed reduced cravings that will result in long term weight-loss (Jakubowicz et al., 2012). The present study also demonstrated that CSDL and HDL rats have lower glucose tolerance, plasma insulin and leptin concentrations. This is consistent with previous study on consumption of defatted flaxseed by obese women postoperative Roux-en-Y gastric bypass that resulted in reduced postprandial glucose and higher postprandial leptin concentrations (Cohen et al., 2013).

Raw linseed is a rich source of ALA, lignan and fibre (mucilage). In a previous study by our group, we demonstrated that linseed oil supplementation for 8 weeks containing about 59% ALA did not reduce total body fat in this high-carbohydrate, high-fat diet-induced model of metabolic syndrome in rats which is consistent with present study (Poudyal et al., 2013b). However, the previous study showed that ALA from linseed oil induced lipid redistribution away from the abdominal area and improved glucose tolerance, insulin sensitivity, dyslipidaemia, hypertension and left ventricular dimensions, contractility, volumes and stiffness (Poudyal et al., 2013b). Linseed oil supplementation also reversed inflammation in the heart and the liver, cardiac fibrosis and hepatic steatosis (Poudyal et al., 2013a, Poudyal et al., 2013b). Additionally, ALA-rich linseed oil supplemented diet for 8 weeks decreased adipocyte size and adipose tissue concentrations of MCP-1, IL-10 and TNF-α (Baranowski et al., 2012). Linseed oil supplementation reduced T-cell infiltration into the adipose tissue but did not alter macrophage infiltration (Baranowski et al., 2012). This suggests that concentrated ALA-rich linseed oil in the diet attenuated pro-inflammatory state and improved other obesity metabolic markers.

SDG, raw linseed and defatted linseed supplementation for 8 weeks decreased systolic blood pressure in HLF (~10%), HRL (~10%) and HDL (~13%) compared to HCHF rats, and these findings also showed that all treated rats had similar blood pressure to CS rats. HLF and HDL groups also showed lower left ventricular diastolic stiffness as compared to HCHF control group. The use of 20% whole flaxseed (linseed) in spontaneously hypertensive rats and normotensive Wistar-Kyoto male rats for 10 to

11 weeks showed no changes in systolic blood pressure (Talom et al., 1999). A study using 35% SDG supplementation in Sprague-Dawley male rats fed high-fat and high-fructose diet for 12 weeks reported a 45% lower blood pressure (Park and Velasquez, 2012). It is noted that there may be a difference between the rats' types and it may be their mutations that are influencing the result. However, the study findings indicate that a higher dosage of linseed and a longer duration of study may promote more reduction in systolic blood pressure.

The mechanisms of action of reduced blood pressure may be explained by the subsequent increase in ALA, EPA, DHA, and the antioxidant pool of blood (Touré and Xueming, 2010) or the gradual increase in intestinal gut flora, which may improve lignan absorption (Setchell et al., 2014) and consequently reduce blood pressure. ALA may lower blood pressure by acting as a precursor for eicosanoids, which can generate the production of prostaglandins and leukotrienes that may reduce vascular tone (Salonen et al., 1988). The other possible mechanism was ALA reduced the activity of soluble epoxide hydrolase (an enzyme that causes a loss of vasodilation and promotes inflammation) which results in decreased soluble epoxide hydrolase-derived oxylipins that subsequently reduce the systolic blood pressure (Caligiuri et al., 2014). The antioxidant effects of lignans were found to be mediated by its metabolites enterodiol and enterolactone, where enterolactone can induce phase 2 protein activation which leads to decreased oxidative stress (Caligiuri et al., 2014).

Few previous studies have measured the effects of lignans and linseed on the vascular endothelium. In the present study, HCHF rats showed impaired vascular contraction and endothelial-dependent relaxation, however with the addition of lignans to the diet the vascular contractility was identical to the CS group. This suggests that lignans improved α_1-adrenoceptor-mediated vascular contraction to noradrenaline. One previous study using 20% flaxseed diet on spontaneously hypertensive rats (SHR) for 10-11 weeks showed that flaxseed diet enhanced vascular responsiveness to acetylcholine but not sodium nitroprusside (Talom et al., 1999). This is in contrast to our findings where HRL showed a better response to sodium nitroprusside but not acetylcholine. The mechanism that was explained by the previous study was diet supplemented with flaxseed alters the release of endothelium-derived relaxing factors (EDRFs) which maybe either change the receptor affinity or the coupling of the

receptor to the effector system (Talom et al., 1999). SDG is known to possess antioxidant activity, thus another potential mechanism contributing to the beneficial effect of endothelial function could involve a protective effect of lignans on the breakdown of nitric oxide (NO).

The present study indicates that lignans, raw linseed and defatted linseed supplementation for 8 weeks did not change plasma lipid profiles and liver function. There are variations of results obtained in previous studies, such as 7.5 g/kg daily flaxseed (linseed) diet given for 2 months did not change the serum lipid concentrations in rabbits (Prasad, 1997). However, SDG isolated from flaxseed (linseed) meal lowered serum concentrations of total cholesterol by 33% in hypercholesterolaemic rabbits but had no effect in normocholesterolaemic rabbits (Prasad, 1999). This study suggested that the hypolipidaemic effect of flaxseed (linseed) resides in the SDG component of flaxseed meal that could be due to variation in the total amount of SDG in the given dose of flaxseed (linseed). The mechanism of reduced total cholesterol was suggested to be due to the 3-hydroxy-3-methyl glutaric acid and SDG content of the lignans complex (Prasad, 1999). Male Sprague-Dawley rats fed 10% fructose in drinking water supplemented with 0, 3 and 6 mg SDG/kg body weight/day for 2 weeks showed no changes in serum triglycerides (TG) and non-esterified fatty acid (NEFA) concentrations (Felmlee et al., 2009). SDG administration showed no effect on hepatic expression of PPAR-α or SREBP-1c following 10% fructose in water supplementation (Felmlee et al., 2009). Consistent with the present study, flaxseed (linseed) consumption for 4 weeks did not have deleterious effects on the liver function (Felmlee et al., 2009). Linseed also contained an important source of dietary fibre (35-45%) of which the ratio of soluble to insoluble fibre in linseed varies between 1:4 and 2:3 (Singh et al., 2011b). A study demonstrated that 3 flaxseed (linseed) fibre drink and bread per day with each drink and bread providing 5.2 g dietary fibres/10MJ when given to 17 subjects (men and women) for 7 days resulted in decreased plasma total cholesterol. This contrasts with present study findings, where no differences were found in total cholesterol of HRL and HDL groups compared to HCHF group. This differences in study findings could be attributed by higher content of dietary fibre in flax drink and bread given to the subjects.

Insoluble fibre inhibits intestinal digestive processes to result in decreased transit times within stomach and small intestine (Brownlee, 2011). The viscosity of insoluble fibre can cause chyme to have a larger bulk in the intestines (Brownlee, 2011). Partially defatted flaxseed meal (PDFM) decreased small intestinal transit time in normal mice (Xu et al., 2012). Further, PDFM increased colonic motility and stimulated colonic transit via fermentation of soluble and insoluble fibre by colonic microflora that resulted in increase of bacterial mass and short-chain fatty acids such as acetate, propionate and butyrate [44]. In addition, laxative effects after PDFM consumption may have resulted from residual oil that acts as a lubricant (Xu et al., 2012). This is consistent with the present study, where higher contractility in the colon was found in defatted linseed rats. Diet-induced obese rats demonstrated a faster gastric emptying, a weaker contraction of duodenal movement, a stronger contraction and relaxation of ileal movement but no differences in gastric emptying with control (non-obese) group (Fu et al., 2014). To support this, accelerated gastric emptying and reduced gastric distension could affect satiety and predispose healthy subjects to overeating (Cohen et al., 2013).

Previously, it has been reported that diabetes could impair the contraction and relaxation capacities of gastrointestinal smooth muscles that include adrenergic, cholinergic and serotonergic stimulation (Ozturk et al., 1996, Lincoln et al., 1984). However, conflicting results were reported with either increased, decreased or unchanged contractions of smooth muscle (Bektaş and Öztürk, 2012). In the present study, both CSDL and HDL rats showed lower contractility in small intestines and higher contractility in the large intestine, whereas HLF rats showed high contractility in ileum and colon compared to controls. Further exploration is required to explain the differences in the gut contractility especially in obese animal models.

In animal studies, a high-fat diet increased the number of goblet cells in the intestines (Benoit et al., 2015b, Hazarika et al., 2016). Increased numbers of goblet cells and mucins are indicators of intestinal disorders such as inflammatory bowel disease and cystic fibrosis (Kim and Ho, 2010). Lignans and raw linseed supplemented rats showed fewer goblet cells in both small intestine and colon. However, increased density of goblet cells were shown in the colon of HDL rats. A study done using 10% whole ground flaxseed (linseed) for 3 weeks in mice displayed goblet cell hyperplasia

(increase in goblet cells density), as well as increased colonic MUC2 expression (Power et al., 2016). This study suggests that increased colonic mucus secretion is attributed by enhanced microbial activity through increased production of caecal SCFA in flaxseed-fed mice, also increased viscosity of the luminal content, as well as faecal bulk (Power et al., 2016). In addition, a study using highly viscous soluble fibre (mucilage) produced faecal bulking effects (Dahl et al., 2005). Furthermore, dietary fibre induced higher production of goblet cells (Hedemann et al., 2006, Tanabe et al., 2006) along the small intestinal epithelium. In regards to the results of this study, particularly consumption of defatted linseed might lead to changes in the colonic microenvironment and this may be beneficial to health. However, this study is only a preliminary study and further studies comparing linseed components relating to production of goblet cells, mucin secretion or goblet cell-associated genes should be performed to decipher the mechanisms involved in the changes within the gut and assessing how these changes impact in attenuating metabolic syndrome.

4.5 Conclusion

In this study, I highlight the metabolic effects of each component from linseed using the same diet-induced rat model. Lignans and dietary fibre from the non-oil components in linseed improved physiological response especially in cardioprotection and other metabolic changes some of which can be regarded as beneficial to health. In addition, these positive effects of lignans and defatted linseed could be promoted as functional foods, however further research on the use of these components of linseed in treating some signs of metabolic syndrome, especially in relation to gut health, is warranted. A longer study duration with an increase dosage of linseed components should be considered as this may also possibly enhance the effects in attenuating metabolic syndrome.

Chapter 5 High amylose wheat on metabolic parameters.

5.1 Introduction

Wheat was the third most produced grain in 2014 after maize and rice (FAOSTAT., 2014). In 2015, an estimated 733 million tonnes of wheat were produced (FAO (UN), 2016) and this is expected to rise to 746.7 million tonnes in 2016 (FAO, 2016). Since the Green Revolution, food crop productivity had tripled with only a 30% increase in land area cultivated despite increasing land scarcity and rising land values (Pingali, 2012). The most widely cultivated wheat today is *Triticum aestivum* (bread wheat) (Fasahat et al., 2014). The varieties of wheat can be distinguished on the basis of seed coat colour, endosperm texture, dough strength and sowing season (Gooding, 2009).

Wheat grains contain approximately 60-70% starch while white flour contains 65-75% starch (Shewry, 2009). Starch content is important as it provides the main energy supply in the human diet and it has two main components: linear amylose (20-30%) and highly branched amylopectin (70-80%) (Huang et al., 2015a, Tester et al., 2004). Amylose is an essentially linear polymer of α-1,4 linked glucose units, while amylopectin is a polymer of linear α-1,4 linked D-glucose units with branching points consisting of α-1,6 linkages of D-glucose units every 20-25 D-glucose residues (Regina et al., 2015). These branch linkages are organised in clustered structures (Figure 5.1) in terms of linear A, B and C chains, where A chains do not carry any other chains, are linked to B chains which in turn are linked to a C chain containing the single reducing group (Fasahat et al., 2014).

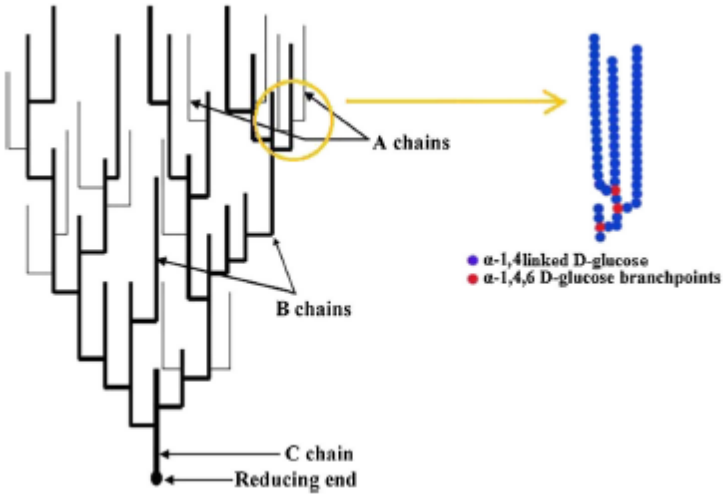


Figure 5.1: Amylopectin clustered structure.
Adapted from (Fasahat et al., 2014).

Although amylose constitutes only 20-30% of the starch component of common wheats, this polymer greatly influences the physicochemical properties, which can influence both the palatability of the grain and its health impact (Regina et al., 2015). Three genetic approaches to increasing amylose content in wheat include the alteration of the increased expression of genes involved in determining the trait via transgenic approaches, or through conventional crosses involving lines exhibiting various values of the trait or through the isolation of mutants (Fasahat et al., 2014). A variety of hexaploid wheat with unusually high levels of amylose in the starch has been developed through a normal selective breeding (non-GM) process (Collin Jenner), targeting induced local lesions in genomes (TILLING) process (Slade et al., 2012) and RNA interference by down-regulating the two different isoforms of starch-branching enzyme (SBE) II (SBEIIa and SBEIIb) in wheat endosperm to decrease amylopectin and increase amylose content (Regina et al., 2006).

The amylose content is associated with the rate at which starch is broken down in the small intestine, leading to the release of glucose in the bloodstream, but this may not explain all the factors involved as other components of the meal will also have an effect (Fasahat et al., 2014). Australian high amylose wheat contains 15.3% protein, 50.2% amylose and 3.2% fat content (Štěrbová et al., 2016). In addition, wholemeal wheat flour from this variety contains 34.4% rapidly digestible starch (RDS), 3.4%

slowly digestible starch (SDS), 16.9% resistant starch (RS) and 24.8% fibre as opposed to common white flour containing 38.4% RDS, 9.2% SDS, 6.6% RS and 12.2% fibre (Štěrbová et al., 2016).

High amylose content in food is associated with increased resistant starch. Resistant starch is one class of fibre that is inadequate in common modern diets. It is noteworthy that this type of fibre is associated with mitigating the risk of chronic diseases which include type 2 diabetes and bowel-related diseases (Jenkins and Kendall, 2000). Resistant starch is a starch that is non-digestible by enzymes in small intestine, but is then fermented in large intestine where bacteria that form the gut microbiota metabolise starch into sugars and then into short chain fatty acids and other metabolites (Nugent, 2005). Gastrointestinal microbiota play a major part in shaping host health, including immune system development, nutritional acquisition and protection against infections (Sekirov et al., 2010). Modulation of diet affects the gut microbiome (dysbiosis) which has been associated with human diseases such as obesity, diabetes, inflammatory bowel diseases and colorectal cancer (Sekirov et al., 2010). Resistant starch metabolised by human gut microflora provides a source of carbon and energy for the 400–500 species of bacteria present in this anaerobic environment (Englyst and Macfarlane, 1986). Consumption of resistant starch results in increases of *Actinobacteria* and *Bacteroidetes* and decreases of *Firmicutes* (Martínez et al., 2010). This modulation of gastrointestinal bacteria by resistant starch may lead to interactions with the host which can reduce postprandial glucose concentrations and concomitant insulin responses compared with the consumption of foods containing ordinary starch (Hasjim et al., 2010). Resistant starches also have been proposed to aid weight management, although it has yet to be adequately demonstrated (Belobrajdic et al., 2012, Aziz et al., 2009).

Replacement of common wheat with high amylose wheat to provide more resistant starch in the diet may improve gastrointestinal function and therefore general health. Therefore, the development of improved food products with increased resistant starch is important especially when wheat supplies approximately 20% of the food calories for the world population and is preferred for most cereal-based processed products, such as bread, pasta and noodles. The high resistant starch content of high amylose wheat flour could be useful as one of the best approaches to deliver functional

foods for people with high risk of diabetes and obesity. The aim of this study therefore was to determine the effects of low-dose (5%) and high-dose (20%) high amylose wheat flour on cardio-metabolic health parameters on high-carbohydrate, high-fat induced obese rats. A 5% dose of high amylose wheat was based on the same dose as a previous study from our research group using inulin which is a known prebiotic (Kumar et al., 2016). Inulin is likely to improve gut health by modulating the gut microbiota that will modulate signalling pathways to reduce metabolic diseases such as obesity and diabetes. It was hypothesised that consumption of high amylose wheat flour would reduce food intake, appetite, blood glucose and fat mass and that these effects would be dose-dependent. It is important to establish minimal levels of RS intake that elicit favourable metabolic effects in diet-induced obese rats to assist in identifying appropriate intakes for future human intervention trials.

5.2 Low-dose high amylose wheat (5%)

5.2.1 Materials and methods

High amylose wheat flour was supplied by Austgrains Pty Ltd, Moree, NSW, Australia. All animal 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.

5.2.1.1 Bomb calorimetry

Total gross energy content of high amylose wheat flour was measured by bomb calorimetry (XRY-1A Oxygen bomb calorimeter, Shanghai Changji Geological Instrument Co. Ltd) in triplicate. About 1 g of high amylose wheat flour was burnt in compressed oxygen (25 kg/cm^2) in the calorimetric bomb immersed in water.

5.2.1.2 Rats and diets

A total of 48 male Wistar rats aged 8-9 weeks old were randomly divided into 4 groups (12 rats per group). Group I was fed with corn starch diet (CS), group II was fed with corn starch diet + 5% high amylose wheat (CHAW), group III was fed with the high-carbohydrate, high-fat (HCHF) diet and group IV was fed with HCHF diet + 5% high amylose wheat (HAW). CS and HCHF rats received their diets for 16 weeks and CHAW and HAW received CS or HCHF diets for the first 8 weeks while both

diets were supplemented with 5% high amylose wheat by replacing equivalent amounts of water for a further 8 weeks. The drinking water in HCHF and HAW diet-fed groups was augmented with 25% fructose for the duration of the study. Daily measurements of food and water intake, and body weight changes were recorded. The rats had unrestricted access to food and water.

5.2.1.3 Other methods

All other methods were conducted as detailed in Chapter 2, specifically oral glucose tolerance test, systolic blood pressure, body composition, isolated Langendorff heart preparation, ileum and colon contractility, tissue collection, histology and biochemical analysis.

5.2.2 Results

5.2.2.1 Total gross energy

The total gross energy of high amylose wheat flour as measured by bomb calorimetry was 15.53±0.08 KJ/g.

5.2.2.2 Dietary intake and body composition

The food intake in CS rats was higher than HCHF rats. However, the mean energy intake, feed efficiency and body weight gains were higher in HCHF rats than in CS rats (Table 5.1). HAW and HCHF rats had similar daily food intakes. There were also no differences in the food intakes of CS and CHAW rats. Water intake for all rats was similar. Hence, there were no differences in energy intake between CS and CHAW and HCHF and HAW rats. CHAW rats gained more weight than CS rats and there was no difference between HCHF and HAW rats at 16 weeks (Figure 5.2). Table 5.1 showed more than doubled feed conversion efficiency in CHAW rats compared to CS rats and no difference in HCHF and HAW rats. This reflects the body weight gains in CHAW rats. Bone mineral content of H-fed rats was higher than C-fed rats. HAW rats revealed a higher bone mineral content as compared to other groups. There were no differences found in the bone mineral content of CS and CHAW rats.

Chronic HCHF feeding for 16 weeks resulted in increased total fat mass, abdominal circumference, visceral adiposity index, retroperitoneal fat, epididymal fat,

omental fat and liver, heart, kidney and spleen wet weights compared to CS rats. Total body lean mass was unchanged in HCHF and CS rats (Table 5.1). Treatment with either CHAW and HAW for 8 weeks showed no difference in body fat mass with CS and HCHF, respectively (Table 5.1) or in retroperitoneal, epididymal or omental fat weights (Table 5.2). Thus, no changes were found in visceral adiposity index and abdominal circumference on CHAW rats compared to CS rats and HAW rats compared to HCHF rats. Liver wet weight of HAW rats was found to be lower as compared to HCHF rats (Table 5.2). No differences were found in the liver wet weight for CS and CHAW rats. No differences were evident in heart weight of CS with CHAW rats and HCHF with HAW rats (Table 5.2). Kidney and spleen wet weight for CHAW and CS showed no differences and similar results were shown in HAW and HCHF rats (Table 5.2).

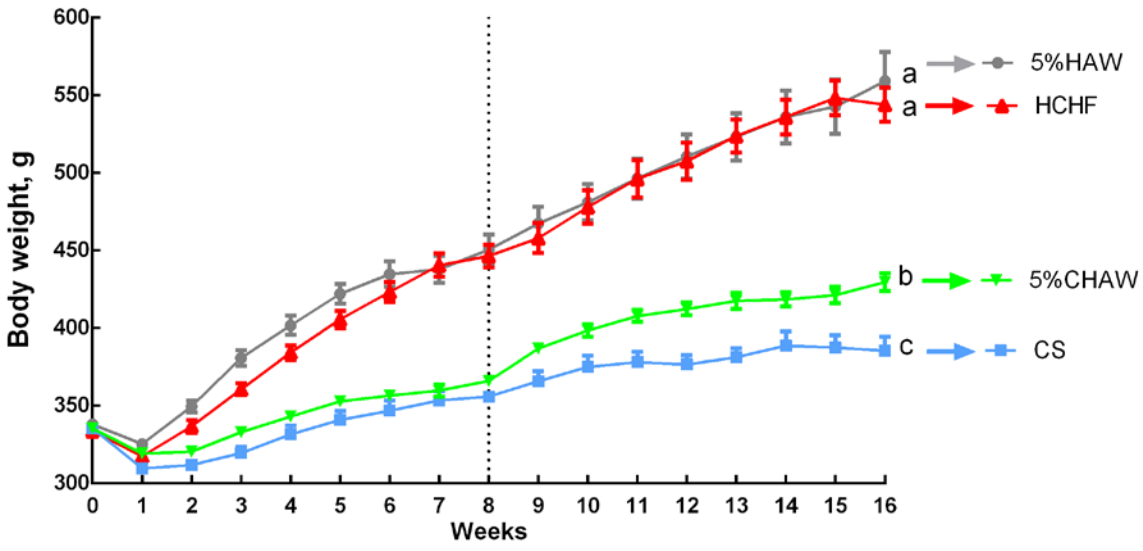


Figure 5.2: Body weight in control rats (CS and HCHF) and rats supplemented with 5% high amylose wheat flour (5% CHAW and 5% HAW) throughout 16 weeks.
Data are presented as mean ± SEM, n= 10-12. Values marked with different letters are significantly different at 16 weeks at the level of $p<0.05$.

Table 5.1: Dietary intakes and body composition at 16 weeks in groups fed CS, CHAW, HCHF and HAW diets.

Variable	CS	CHAW	HCHF	HAW	p-Value		
					Diet	Treatment	Interaction
Food intake (g/d)	35.5±0.8^{a}	36.0±0.8^{a}	25.7±0.6^{b}	23.8±0.9^{b}	< 0.0001	0.3760	0.1324
Water intake (mL/d)	27.9±1.6	27.2±1.4	27.5±1.2	27.2±1.2	0.8838	0.7149	0.8838
Body weight gained (%)	8.2±1.9^{c}	17.4±1.1^{b}	23.2±1.1^{a}	23.2±1.4^{a}	< 0.0001	0.0028	0.0028
Energy intake (kJ/d)	399±9^{b}	431±10^{b}	561±13^{a}	553±17^{a}	<0.0001	0.3612	0.1250
Feed conversion efficiency (%)	2.2±0.5^{c}	4.8±0.4^{b}	10.1±0.7^{a}	10.4±1.1^{a}	<0.0001	0.0521	0.1250
Bone mineral content (g)	11.6±0.3^{c}	12.6±0.3^{c}	15.8±0.6^{b}	17.3±0.7^{a}	< 0.0001	0.0213	0.6309
Total fat mass (g)	62.7±12.5^{b}	111.2±10.4^{b}	238.2±24.6^{a}	252.8±26.9^{a}	< 0.0001	0.1238	0.4021
Total lean mass (g)	315±7	307±5	269±20	266±17	0.0042	0.7308	0.8495
Abdominal circumference (cm)	18.8±0.3^{b}	19.2±0.1^{b}	23.4±0.4^{a}	23.2±0.2^{a}	< 0.0001	0.7576	0.3571
Visceral adiposity index (%)	4.1±0.4^{b}	5.5±0.4^{b}	8.3±0.7^{a}	8.6±1.0^{a}	< 0.0001	0.2011	0.4049

Data are presented as mean ± SEM. Values marked with different superscripts are significantly different at 16 week at the level of $p<0.05$.

Table 5.2: Organ wet weight and cardiovascular functions in groups fed CS, CHAW, HCHF and HAW in the diet at 16 weeks.

Variables	CS	CHAW	HCHF	HAW	p-Value		
					Diet	Treatment	Interaction
Organ wet weight							
Retroperitoneal fat (mg/mm)	188.8±17.8^{b}	209.1±22.7^{b}	431.7±44.6^{a}	465.8±56.5^{a}	< 0.0001	0.4833	0.8584
Epididymal fat (mg/mm)	89.1±10.3^{b}	125.3±10.3^{b}	226.9±20.8^{a}	210.7±19.9^{a}	< 0.0001	0.5414	0.1153
Omental fat (mg/mm)	113.9±14.8^{b}	125.5±11.1^{b}	226.9±23.4^{a}	219.3±24.6^{a}	< 0.0001	0.9186	0.6242
Total abdominal fat(mg/mm)	329.2±31.7^{b}	459.8±41.1^{b}	885.6±84.1^{a}	895.8±97.6^{a}	<0.0001	0.3149	0.3892
Liver (mg/mm)	210.3±5.6^{c}	204.2±7.0^{c}	330.1±11.5^{a}	300.0±6.3^{b}	< 0.0001	0.0320	0.1475
Heart (mg/mm)	23.0±0.4^{b}	25.0±1.1^{ab}	25.6±1.0^{ab}	28.3±1.3^{a}	0.0051	0.0229	0.7248
Kidney (mg/mm)	46.5±1.4^{b}	45.7±1.7^{b}	57.6±2.4^{a}	55.4±1.7^{a}	<0.0001	0.4249	0.7086
Spleen (mg/mm)	12.9±0.6^{b}	13.5±1.0^{b}	17.2±1.1^{a}	18.1±0.9^{a}	<0.0001	0.4198	0.8712
Cardiovascular function							
Systolic blood pressure (mmHg)	126±1^{c}	129±4^{c}	150±3^{a}	137±4^{b}	<0.0001	0.1019	0.0151
Diastolic stiffness constant, κ	22.7±0.3^{b}	24.1±0.9^{b}	26.4±0.8^{a}	23.3±0.9^{b}	0.0740	0.2869	0.0072

Data are presented as mean ± SEM. Values marked with different superscripts are significantly different at 16 week at the level of $p<0.05$.

5.2.2.3 Cardiovascular function and structure

Values for systolic blood pressure and diastolic stiffness of control groups and high amylose wheat flour supplemented rats are given in Table 5.2. HCHF rats showed impaired cardiovascular function with elevated systolic blood pressure and diastolic stiffness compared to CS rats (Table 5.2). HAW rats showed lower systolic blood pressure compared to HCHF rats but there was no difference between CHAW and CS groups. Reduced diastolic stiffness constant is also found in HAW rats compared to HCHF rats, whereas no difference is found in CHAW and CS rats.

Histological examinations supported the preceding results. CS-fed rats showed minimal collagen deposition, whereas HCHF-fed rats showed greater collagen deposition as well as infiltration of inflammatory cells in the left ventricle (Figures 5.3A and 5.3C). CHAW supplemented rats showed normal collagen deposition when compared to CS rats (Figures 5.3B and 5.3A). Reduced collagen deposition in HAW rats was seen (Figures 5.3C and 5.3D). The haematoxylin and eosin staining of the left ventricle of HAW rats (Figure 5.4D) also showed normalised infiltration of inflammatory cells as compared to HCHF rats. This can be seen from the infiltration of inflammatory cells in HCHF rats marked as “in” (Figure 5.4C). There were no changes observed in CS and CHAW rats as the tissue morphology appeared normal (Figures 5.4A and 5.4B).

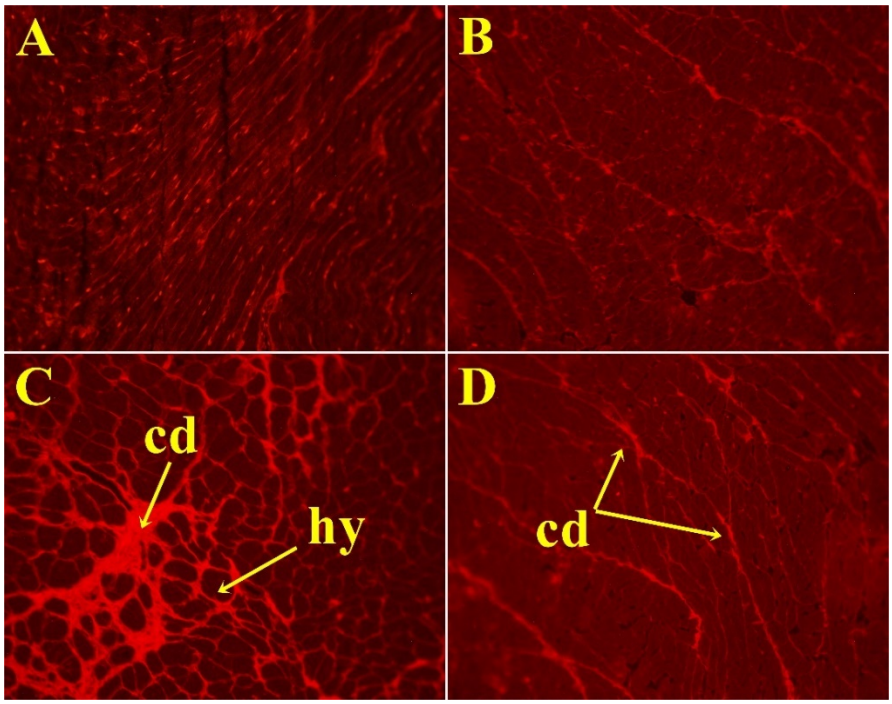


Figure 5.3: Picrosirius red staining of left ventricular interstitial collagen deposition (20x magnification) in CS (A), CHAW (B), HCHF (C) and HAW (D) rats.

Collagen deposition marked as “cd” and hypertrophied marked as “hy”.

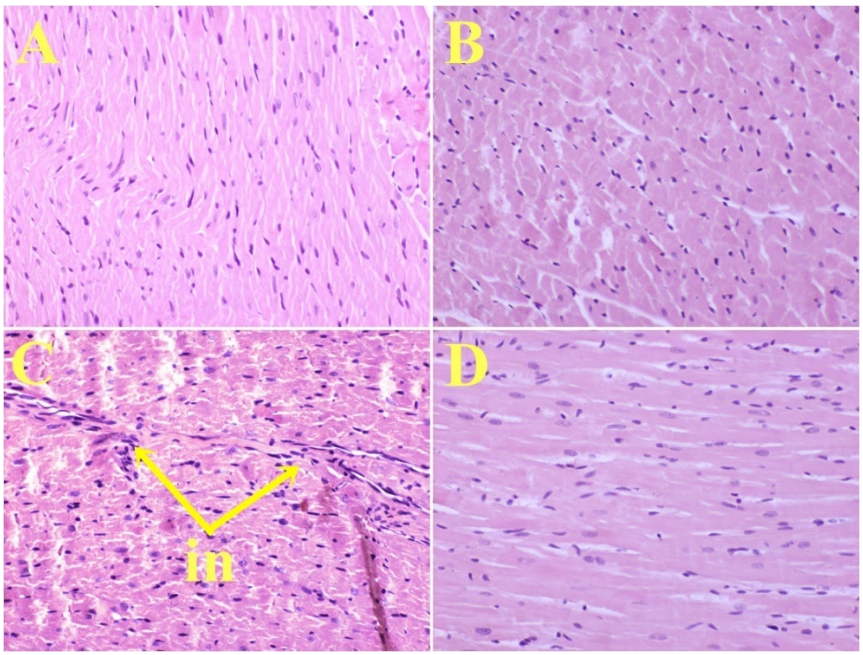


Figure 5.4: Haematoxylin and eosin staining of left ventricle (20x magnification) in CS (A), CHAW (B), HCHF (C) and HAW (D).

Inflammatory cells marked as “in”.

5.2.2.4 Liver function and structure

Elevated plasma alanine transferase (ALT) activity was found in HCHF rats, but there were no differences between CS and HAW rats (Table 5.3). CHAW rats showed a lower plasma ALT activity compared to CS rats. No changes were observed in plasma AST in all groups (Table 5.3). Histological sections of hepatic tissue were evaluated using haematoxylin and eosin staining examining the hepatic lipid deposition marked as "fv" (Figure 5.5). HCHF rats showed more fat vacuoles compared to CS rats. Liver tissues from CHAW and HAW rats showed decreased macrovesicular steatosis than HCHF rats (Figure 5.5). This is consistent with the finding of liver wet weight of HAW rats, which is lower compared to the liver weight of HCHF rats.

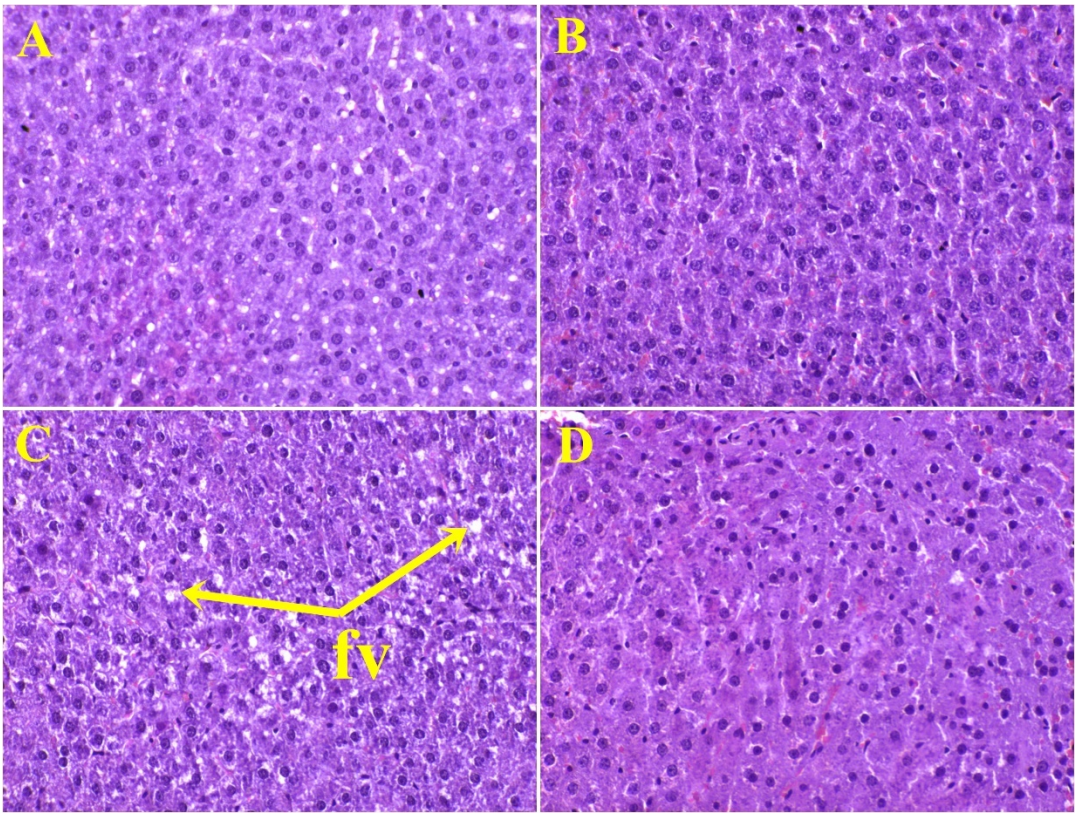


Figure 5.5: Hepatocytes stained with haematoxylin and eosin are shown in CS (A), CHAW (B), HCHF (C) and HAW (D).
Fat vacuoles marked as "fv".

5.2.2.5 Plasma metabolic parameters

Plasma lipid profiles are presented in Table 5.3. Plasma total cholesterol was found to be unaffected in CS and HCHF rats. However, plasma concentrations of triglycerides and non-esterified fatty acids (NEFA) were increased in HCHF rats compared to CS rats. No changes in total cholesterol were found after 8 weeks supplementation of high amylose wheat flour in the diet. HAW rats had higher plasma NEFA and triglycerides compared to CS and CHAW rats and there was no difference found with HCHF rats.

The blood glucose response to oral glucose loading was greater in HCHF rats than CS rats (Table 5.3). Rats supplemented with 5% high amylose wheat flour showed no difference in glucose area under the curve (AUC) with their respective controls. Plasma insulin concentrations were lower in CS rats than HCHF rats. Decreased plasma insulin concentrations were also found in HAW rats compared to HCHF rats (Table 5.3), whereas CHAW rats showed greater plasma insulin concentration than CS rats, but lower than HCHF and HAW rats. Plasma leptin concentrations were higher in HCHF and HAW rats compared to CS and CHAW (Table 5.3). No differences in plasma leptin were found in either high amylose group compared to their respective controls and this is consistent with the findings in total fat mass and total abdominal fat pads.

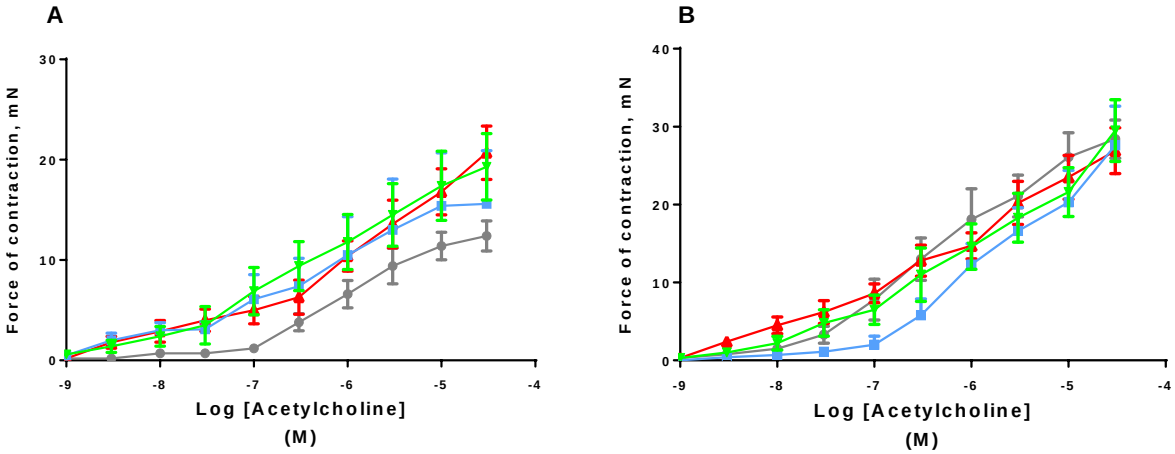
Table 5.3: Plasma biochemistry analyses in groups at 16 weeks fed CS, CHAW, HCHF and HAW diets.

Variable	CS	CHAW	HCHF	HAW	P Value		
					Diet	Treatment	Interaction
Hepatic function							
Plasma ALT (U/L)	31.6±4.2^{ab}	23.5±1.4^{b}	38.7±5.4^{a}	27.4±1.7^{ab}	0.1346	0.0105	0.6588
Plasma AST (U/L)	71.1±7.3	63.1±2.6	78.4±6.6	62.0±3.0	0.5627	0.0274	0.4338
Lipid profile							
Plasma total cholesterol (mmol/L)	1.4±0.1	1.5±0.1	1.5±0.05	1.7±0.1	0.1048	0.1048	0.5825
Plasma NEFA (mmol/L)	1.7±0.2^{b}	1.4±0.1^{b}	4.5±0.5^{a}	3.6±0.4^{a}	<0.0001	0.0853	0.3822
Plasma TAG (mmol/L)	0.5±0.1^{b}	0.4±0.05^{b}	1.7±0.3^{a}	1.2±0.2^{a}	<0.0001	0.1175	0.2922
OGTT-AUC (mmol/l x min)	669.1±20.3^{b}	618.0±16.4^{b}	752.1±21.1^{a}	730.1±16.8^{a}	<0.0001	0.0556	0.4237
Plasma insulin (µmol/L)	1.3±0.01^{d}	3.1±0.03^{c}	8.1±0.1^{a}	6.2±0.1^{b}	<0.0001	0.2969	<0.0001
Plasma leptin (µmol/L)	3.5±0.2^{b}	5.1±0.6^{b}	12.9±1.6^{a}	13.1±1.5^{a}	<0.0001	0.4609	0.5578

Each value is a mean±S.E.M. Means within a row with unlike superscript differ; P≤ 0.05. ALT: Alanine transaminase, AST: Aspartate transaminase, NEFA: Non-esterified fatty acids, TAG: Triglycerides, OGTT-AUC: Oral glucose tolerance test – Area under curve.

5.2.2.6 Gut contractility and structure

Contractility measurements for both ileum and colon are presented in Figure 5.6. There were no significant differences between dietary groups for acetylcholine-induced contraction of the ileum or colon at maximal contraction (Figures 5.6A and 5.6B). Figure 5.7 and 5.8 showed periodic acid and Schiff reagent's staining that showcases the number of goblet cells in small intestine and large intestine. The density of goblet cells was unaffected in small intestines; however, there was an increased density of goblet cells observed in CHAW and HAW as compared to CS and HCHF, respectively.



	P-Value		
Figure	Diet	Treatment	Interaction
A	0.1045	0.8009	0.5212
B	0.8099	0.9573	0.6502

Figure 5.6: Ileum contractility (Figure A) and colon contractility (Figure B) at week 16 in rats fed CS, CHAW, HCHF and HAW.

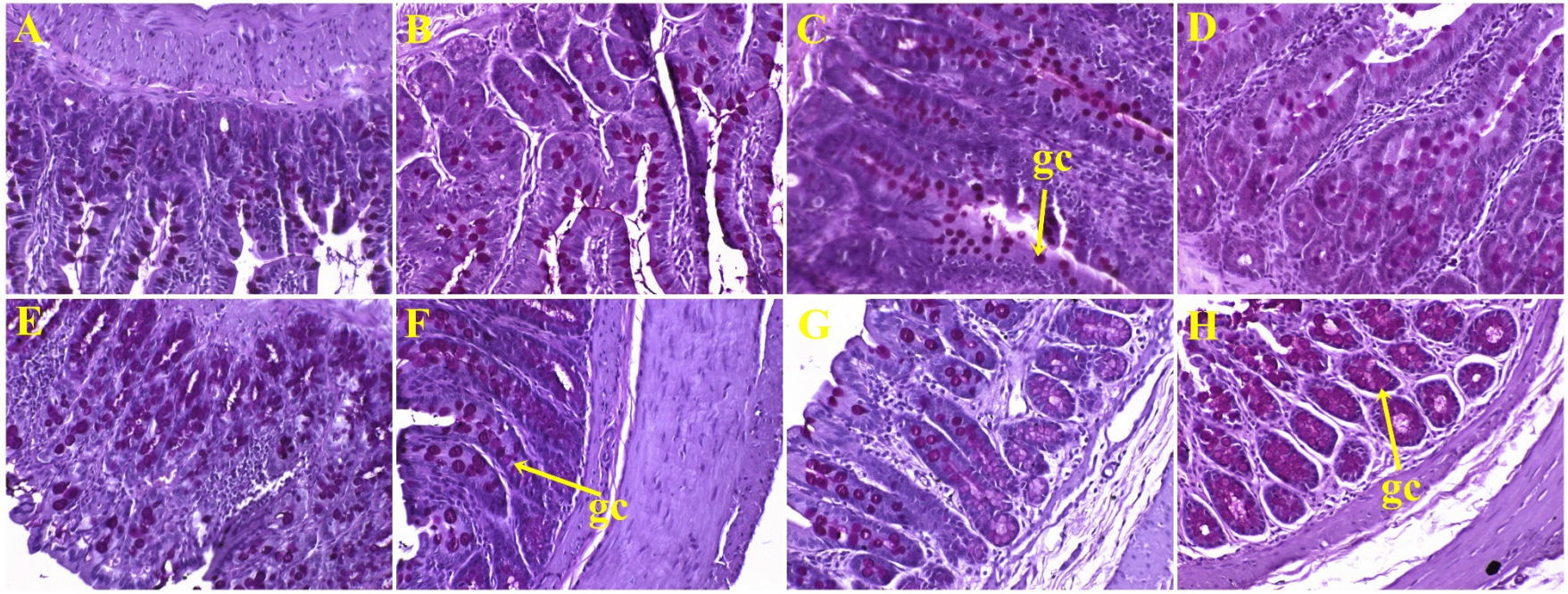


Figure 5.7: Periodic acid and Schiff reagent (PAS) staining (magnification 20x) in the small intestine and in the colon of rats fed CS, CHAW, HCHF and HAW.

Top row: Periodic acid and Schiff reagent (PAS) staining (magnification 20x) in the small intestine of CS (A), CHAW (B), HCHF (C) and HAW (D). Bottom row: Periodic acid and Schiff reagent (PAS) staining (magnification 20x) in the colon of CS (E), CHAW (F), HCHF (G) and HAW (H).

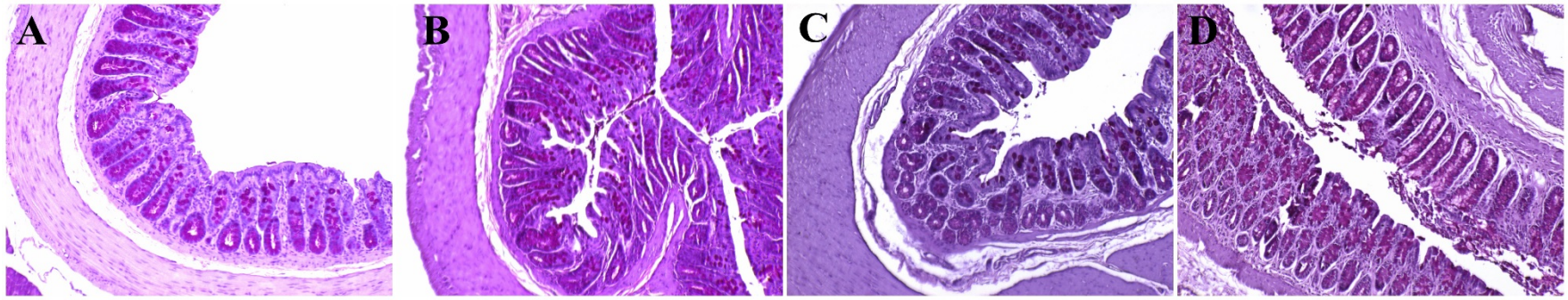


Figure 5.8: Periodic acid and Schiff reagent (PAS) staining (magnification 10x) in the colon of CS (A), CHAW (B), HCHF (C) and HAW (D).

5.3 Intervention with increased dose of high amylose wheat

5.3.1 Materials and methods

5.3.1.1 Rats and diets

A total of 48 male Wistar rats aged 8-9 weeks old were randomly divided into 4 groups (12 rats per group). For this study, we were using a reformulation of CS and HCHF diet to allow an increased dose of 20% high amylose wheat. A 20% dose of wheat translates to around 5g wheat intake/rat/day as average rat food intake is around 25g food/day for rats weighing 450g. This corresponds to about 11.11 g/kg/day for rats and this equates to around 220g/day for an adult human (~70Kg) using the accepted scaling equation (Bachmann et al., 1996b). Modified corn starch (MCS) diet contained 57% corn starch, 2.5% Hubble, Mendel and Wakeman salt mixture, 0.5% canola oil, 4.5% skim milk powder, 0.5% vitamin mixture and 35% water (Figure 5.9). Modified high fat-high, carbohydrate (MHCHF) - based diets consisted of 17.5% fructose, 39.5% sweetened condensed milk, 15% beef tallow, 2.5% Hubble, Mendel and Wakeman salt mixture, 0.5% canola oil, 4.5% skim milk powder, 0.5% vitamin mixture and 20% water (Figure 5.9). Group I was fed with modified corn starch diet (MCS) for 16 weeks. Group II was fed with MCS + 20% high amylose wheat for 8 weeks (20%CHAW). Group III was fed with the modified high-carbohydrate, high-fat (MHCHF) diet for 16 weeks. In group IV, rats were fed with MHCHF diet + 20% high amylose wheat for 8 weeks (20%HAW). MCS and MHCHF rats received their diets for 16 weeks and 20%CHAW and 20%HAW received MCS or MHCHF diets for the first 8 weeks while both diets were supplemented with 20% high amylose wheat by replacing equivalent amounts of water for a further 8 weeks. The drinking water in MHCHF and 20%HAW diet-fed groups was augmented with 25% fructose for the duration of the study. Daily measurements of food and water intake, and body weight changes were recorded. The rats had unrestricted access to food and water.

5.3.1.2 Other methods

All other methods were conducted as detailed in Chapter 2, specifically oral glucose tolerance test, systolic blood pressure, body composition, isolated

Langendorff heart preparation, ileum and colon contractility, tissue collections, histology and biochemical analysis.

Figure 5.9: Ingredients used to make modified diet supplemented with 20% high amylose wheat flour.

5.3.2 Results

5.3.2.1 Dietary intake and body composition

The average food intake was the same for both modified high-carbohydrate, high-fat (MHCHF) rats and modified corn starch (MCS) rats (Table 5.4). 20%HAW rats had lower average food intake as compared to MHCHF rats. There was no difference in food intake between MCS rats and 20%CHAW rats (Table 5.4). A lower water intake was found in MHCHF rats compared to MCS, 20%CHAW and 20%HAW rats (Table 5.4). Despite the lower water intake in MHCHF rats, the mean energy intake and feed efficiency were higher in MHCHF rats than in MCS rats (Table 5.4).

The body weight gains were found to be in this sequence; 20%CHAW > 20%HAW > MHCHF > MCS (Table 5.4). Figure 5.10 showed that 20%HAW had a consistent body weight gain from week 8 until week 14 when the rats started to reduce body weight. 20%CHAW rats showed a consistent body weight gain until week 16.

Similar trend was found in MHCHF but it was only a gradual increase throughout the study. In contrast with MCS rats, their body weight had a slight decrease but not significant starting at week 8 until week 16. This reflects in the feed conversion efficiency where MCS showed negative feed conversion efficiency and the highest is from 20% HAW rats.

Body composition of rats is presented in Table 5.4. Compared to MCS rats, MHCHF rats had higher bone mineral content, total lean mass and total fat mass. Supplementation with 20%CHAW for 8 weeks showed a higher bone mineral content compared to MCS rats. However, it is lower compared to MHCHF and 20%HAW rats. There is no difference in total fat mass between 20%CHAW, MCS and 20%HAW. MHCHF had the highest total fat mass and it is higher from MCS and 20%CHAW, but not with 20%HAW. Total lean mass increased in the 20% high amylose wheat flour supplemented rats compared to MHCHF and MCS rats.

The abdominal circumference was found to be higher in MHCHF rats than MCS rats. Similarly, 20%HAW rats also had higher abdominal circumference compared to 20%CHAW and MCS rats and no difference with MHCHF rats. MHCHF rats had highest visceral adiposity index than 20%CHAW and MCS rats. There was no difference in visceral adiposity index between MHCHF and 20%HAW rats (Table 5.4). The retroperitoneal, epididymal and omental fat tissues were found to be higher in MHCHF than MCS rats (Table 5.5). 20% CHAW rats had higher wet weight of retroperitoneal, epididymal and omental fat tissues compared to MCS rats, but lower than MHCHF and 20%HAW rats. Lower omental fat was found in 20%HAW rats compared to MHCHF rats. These results are then consistent with total abdominal fat results.

MHCHF rats had higher liver wet weight than MCS rats (Table 5.5). There is no change observed in liver wet weight of 20%HAW with 20%CHAW rats. However, the liver wet weight of 20%CHAW and 20%HAW were found to be lower than MHCHF rats and higher than MCS rats. Treatment with 20% high amylose wheat in either diet for 8 weeks did not change heart wet weights (Table 5.5). Kidney wet weight and spleen weight were reduced in MCS rats compared to 20%CHAW, 20%HAW and MHCHF (Table 5.5).

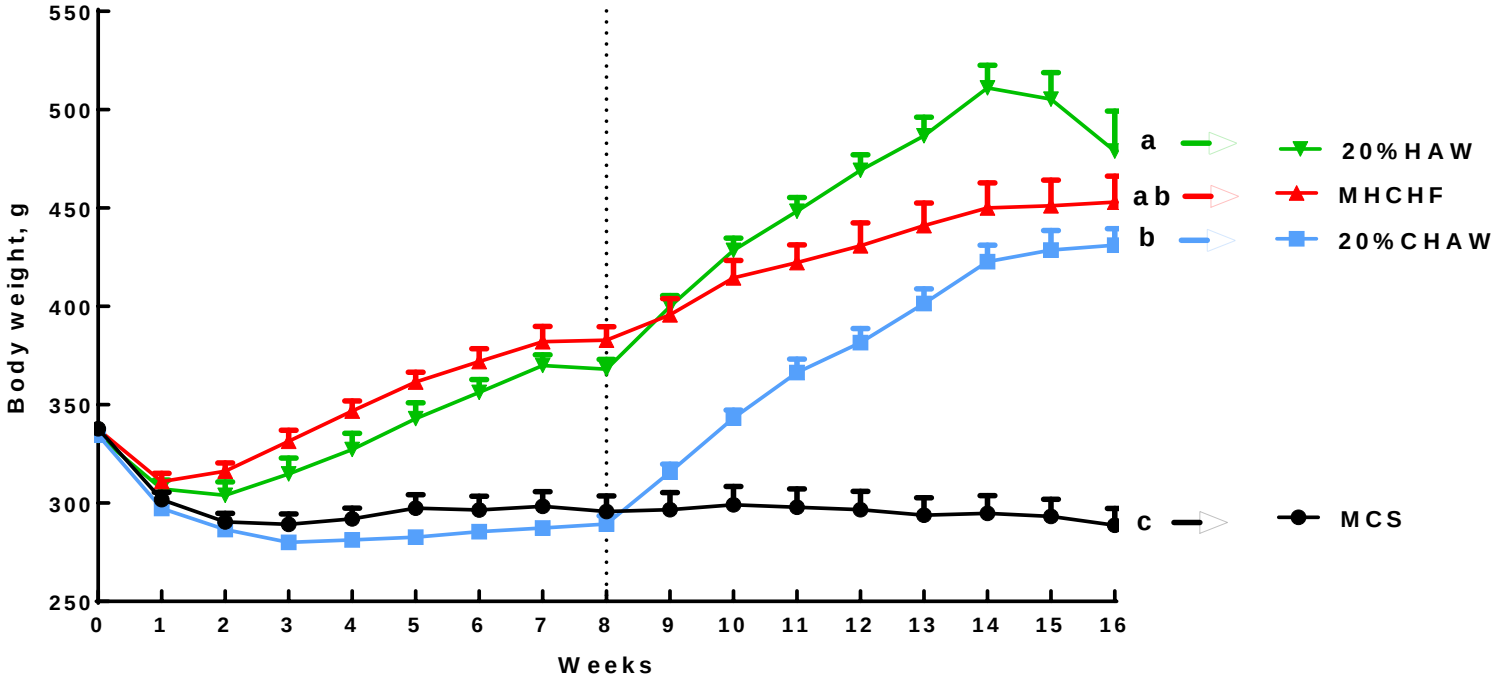


Figure 5.10: Body weights in control rats (MCS and MHCHF) and rats supplemented with 20% high amylose wheat flour (20% CHAW and 20% HAW) throughout 16 weeks.

Data are presented as mean ± SEM, n= 10-12. Values marked with different letters are significantly different at 16 weeks at the level of $p<0.05$.

Table 5.4: Dietary intakes, body composition and organ wet weights in groups fed MCS, 20%CHAW, MHCHF or 20%HAW diets.

Variable	MCS	20%CHAW	MHCHF	20%HAW	p-Value		
					Diet	Treatment	Interaction
Food intake (g/d)	32.6 ± 1.3^{ab}	34.5 ± 1.1^{a}	29.8 ± 1.3^{b}	22.0 ± 1.0^{c}	<0.0001	0.0162	0.0002
Water intake (mL/d)	33.0 ± 3.2^{a}	35.3 ± 1.9^{a}	21.5 ± 1.4^{b}	29.0 ± 1.1^{a}	<0.0001	0.0189	0.2029
Body weight gains (%)	-2.4 ± 1.4^{d}	49.2 ± 3.4^{a}	18.1 ± 1.5^{c}	38.8 ± 3.6^{b}	0.0890	<0.0001	<0.0001
Energy intake (kJ/d)	321 ± 13^{c}	446 ± 14^{b}	517 ± 24^{a}	503 ± 19^{a}	<0.0001	0.0046	0.0006
Feed conversion efficiency (%)	-0.3 ± 0.2^{c}	11.2 ± 0.9^{b}	6.5 ± 0.8^{b}	19.6 ± 2.5^{a}	<0.0001	<0.0001	0.6177
Bone mineral content (g)	9.1 ± 0.3^{c}	11.1 ± 0.2^{b}	12.9 ± 0.5^{a}	12.8 ± 0.3^{a}	<0.0001	0.0086	0.0041
Total fat mass (g)	34.2 ± 7.4^{b}	32.7 ± 6.8^{b}	110.1 ± 18.9^{a}	75.8 ± 13.8^{ab}	<0.0001	0.1583	0.1950
Total lean mass (g)	256 ± 9^{c}	385 ± 8^{a}	328 ± 11^{b}	399 ± 7^{a}	<0.0001	<0.0001	0.0023
Abdominal circumference (cm)	16.5 ± 0.2^{c}	19.4 ± 0.2^{b}	20.7 ± 0.4^{a}	20.9 ± 0.2^{a}	<0.0001	<0.0001	<0.0001
Visceral adiposity index (%)	4.0 ± 0.3^{b}	4.6 ± 0.4^{b}	6.7 ± 0.4^{a}	6.1 ± 0.5^{a}	<0.0001	>0.9999	0.1483

Data are presented as mean ± SEM. Values marked with different superscripts are significantly different at 16 weeks at the level of $p<0.05$.

Table 5.5: Organ wet weight and cardiovascular functions in groups fed CS, CHAW, HCHF and HAW in the diet at 16 weeks.

Variables	MCS	20%CHAW	MHCHF	20%HAW	p-Value		
					Diet	Treatment	Interaction
Organ wet weight							
Retroperitoneal fat (mg/mm)	128.3 ± 15.8^{b}	197.9 ± 25.5^{b}	378.1 ± 43.2^{a}	295.2 ± 21.9^{a}	<0.0001	0.8213	0.0139
Epididymal fat (mg/mm)	47.2 ± 5.9^{c}	89.2 ± 12.0^{b}	134.3 ± 15.6^{a}	129.7 ± 16.3^{a}	<0.0001	0.1487	0.0747
Omental fat (mg/mm)	81.8 ± 8.4^{d}	121.9 ± 11.3^{c}	217.5 ± 17.7^{a}	172.9 ± 17.3^{b}	<0.0001	0.8728	0.0049
Total abdominal fat (mg/mm)	257.4 ± 3.0^{d}	408.9 ± 4.6^{c}	730.0 ± 8.9^{a}	597.7 ± 1.7^{b}	<0.0001	0.0960	<0.0001
Liver (mg/mm)	180.2 ± 9.6^{c}	254.5 ± 6.0^{b}	347.6 ± 25.7^{a}	300.6 ± 12.3^{ab}	<0.0001	0.4006	0.0007
Heart (mg/mm)	19.2±2.6	25.7±1.0	28.4±4.3	26.0±1.1	0.1069	0.4788	0.1300
Kidney (mg/mm)	38.5 ± 1.1^{c}	48.8 ± 0.7^{b}	66.6 ± 5.3^{a}	54.0 ± 1.1^{b}	<0.0001	0.7013	0.0006
Spleen	11.0 ± 1.0^{b}	16.0 ± 1.0^{a}	18.3 ± 1.4^{a}	18.9 ± 1.9^{a}	0.0006	0.0428	0.1068
Cardiovascular functions							
Systolic blood pressure (mmHg)	137 ± 2^{a}	130 ± 2^{a}	141 ± 3^{a}	120 ± 3^{b}	0.2644	<0.0001	0.0114
Diastolic stiffness constant, κ	25.1 ± 0.5^{a}	20.4 ± 0.9^{b}	25.2 ± 0.7^{a}	23.7 ± 0.8^{a}	0.0245	0.0002	0.0334

Data are presented as mean ± SEM. Values marked with different superscripts are significantly different at 16 week at the level of $p<0.05$.

5.3.2.2 Cardiovascular structure and function

Systolic blood pressure and diastolic stiffness are given in Table 5.5. Rats supplemented with 20%HAW showed lower systolic blood pressure compared to MHCHF, 20%CHAW and MCS rats. There is no difference in diastolic stiffness constant in 20%HAW rats with MHCHF rats. 20%CHAW rats showed lower diastolic stiffness constant as compared to all other groups.

The results above were supported by histological evaluation. Normal collagen deposition was found in both MCS and 20%CHAW rats (Figures 5.11A and 5.11B). MHCHF rats showed striking collagen deposition in the left ventricle, meanwhile 20%HAW rats showed reduced collagen deposition (Figures 5.11C and 5.11D). The haematoxylin and eosin staining of the left ventricle sections of 20%CHAW was observed to be normal (Figure 5.12B). Infiltration of inflammatory cells in MHCHF rats were seen in Figure 5.12C. 20%HAW fed rats (Figure 5.12D) showed an improvement in the left ventricle as compared to MHCHF rats.

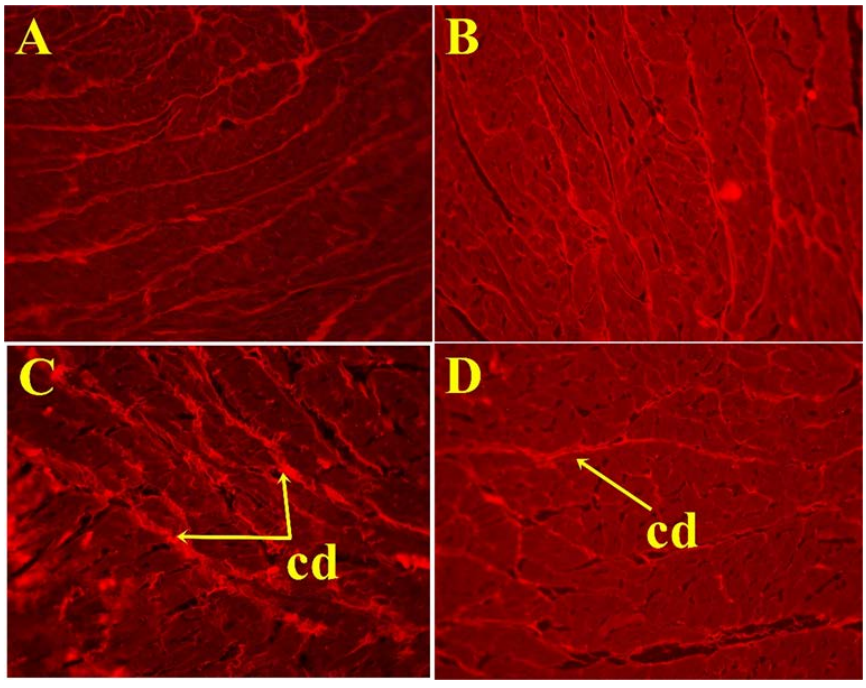


Figure 5.11: Picrosirius red staining of left ventricular interstitial collagen deposition (20x magnification) in MCS (A), 20%CHAW (B), MHCHF (C) and 20%HAW (D) rats.

Collagen deposition marked as “cd”.

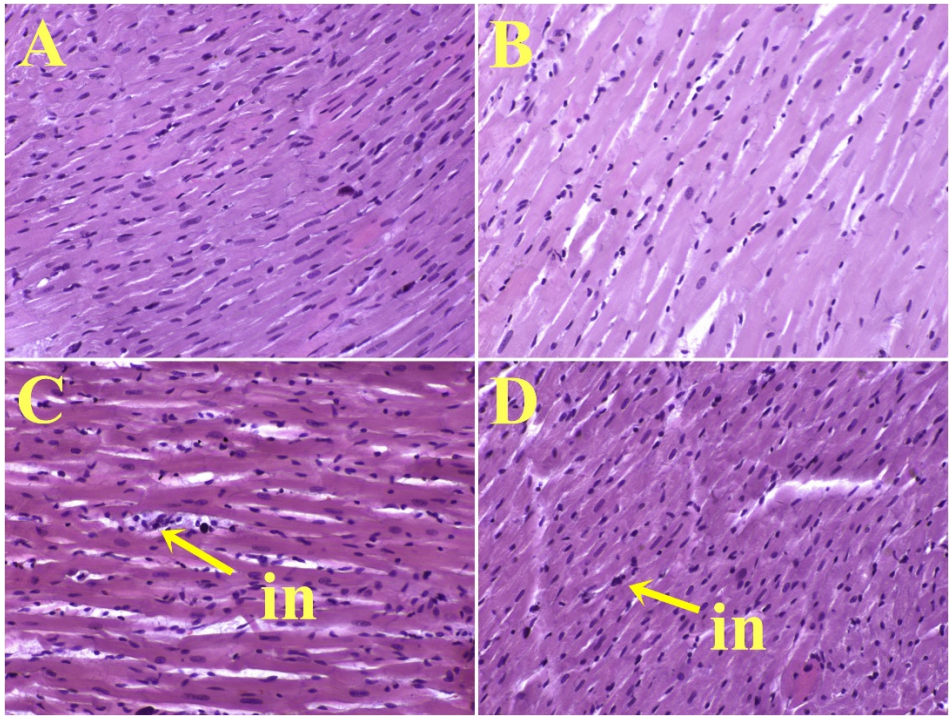


Figure 5.12: Haematoxylin and eosin staining of left ventricle (20x magnification) in MCS (A), 20%CHAW (B), MHCHF (C) and 20%HAW (D).
Figures with “in” showing inflammatory cells.

5.3.2.3 Liver structure and function

Plasma alanine transferase (ALT) activity was similar in all groups (Table 5.6). 20%HAW rats had decreased plasma AST activity compared to MCS rats, but no difference was observed with 20%CHAW and MHCHF groups. Liver histological sections were evaluated using haematoxylin and eosin staining looking at the fat vacuoles deposition marked as “fv” (Figure 5.13). MHCHF rats showed more fat vacuoles compared to other groups. 20%CHAW and 20%HAW rats had smaller fat vacuoles in liver tissues and appeared to be normal as can be seen in MCS rats (Figure 5.13). This is consistent with the finding of liver wet weight of 20%HAW rats, where it had lower liver wet weight compared to MHCHF rats.

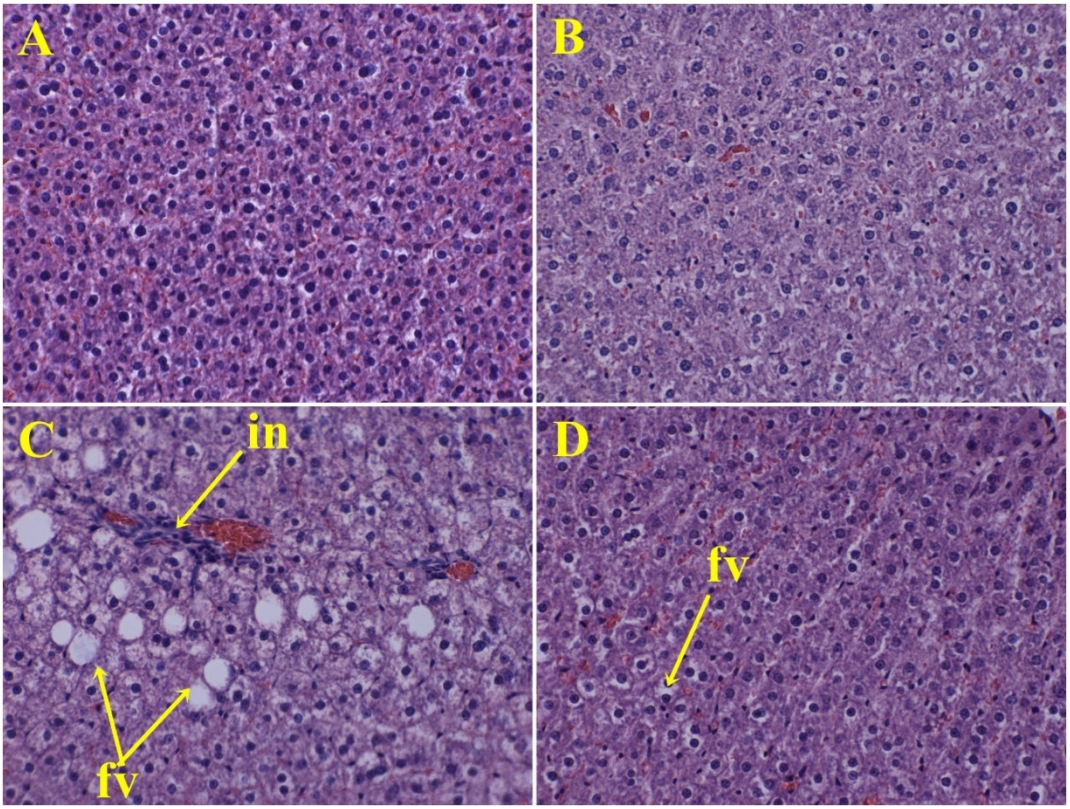


Figure 5.13: Hepatocytes stained with haematoxylin and eosin were shown in MCS (E), 20%CHAW (F), MHCHF (G) and 20%HAW (H).
Figures with “fv” marked the fat vacuoles and “in” marked the infiltration cells.

5.3.2.4 Plasma biochemistry analysis

Plasma concentrations of total cholesterol, non-esterified fatty acids (NEFA) and triglycerides (TAG) are presented in Table 5.6. Plasma total cholesterol remained unchanged in MCS and MHCHF rats, and in rats supplemented with 20% high amylose wheat flour. In contrast, MHCHF group had increased plasma NEFA and TAG concentrations at week 16. It was also found that 20%HAW rats had higher NEFA and TAG concentrations than 20%CHAW and MCS rats. There was no difference found between 20%HAW and MHCHF rats in plasma NEFA and TAG.

Compared to MCS rats, MHCHF rats showed higher glucose area under the curve (AUC). Rats supplemented with 20%HAW had lower glucose AUC compared to MHCHF and it was no difference from 20%CHAW and MCS rats (Table 5.6). Consistent results were found in plasma insulin concentrations (Table 5.6). 20%HAW rats had lower plasma insulin concentration than MHCHF rats. 20%CHAW rats showed greater plasma insulin concentration than MCS rats, but lower than HCHF and HAW rats. Both MHCHF and 20%HAW rats had higher plasma leptin concentrations compared to 20%CHAW and MCS rats (Table 5.6). No difference was observed in plasma leptin concentrations between MCS and 20%CHAW rats, but it was found that MHCHF rats had higher plasma leptin concentrations than 20%HAW rats.

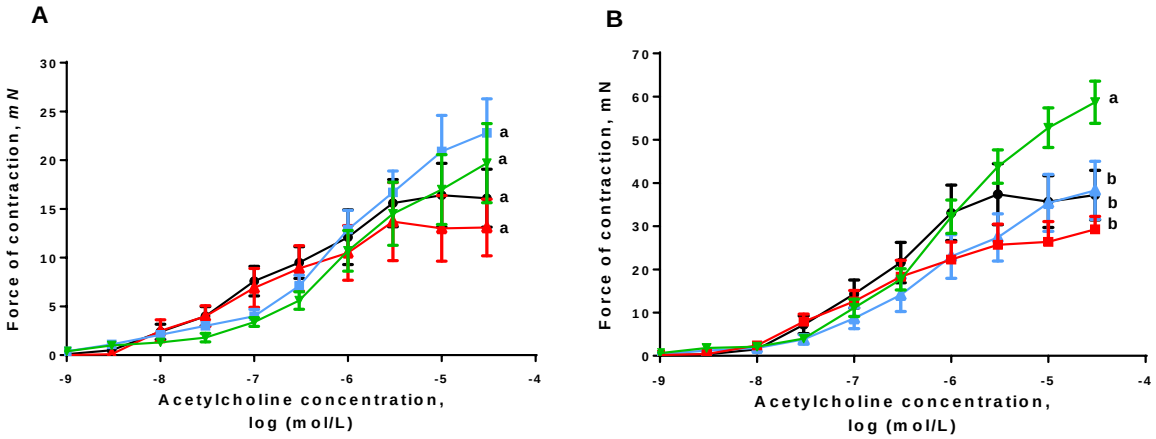
Table 5.6: Plasma biochemistry analyses in groups fed the MCS, 20%CHAW, MHCHF and 20%HAW in rats at week 16.

Variable	MCS	20%CHAW	MHCHF	20%HAW	P Value		
					Diet	Treatment	Interaction
Hepatic function							
Plasma ALT (U/L)	26.9±1.0	32.1±2.8	30.8±2.8	30.6±2.2	0.6213	0.3063	0.2699
Plasma AST (U/L)	86.0±6.1^{a}	70.7±4.2^{ab}	69.1±5.8^{ab}	65.1±2.9^{b}	0.0213	0.0459	0.2332
Lipid profile							
Plasma total cholesterol (mmol/L)	1.6±0.1	1.7±0.1	1.6±0.1	1.6±0.1	0.6270	0.6270	0.6270
Plasma NEFA (mmol/L)	1.6±0.3^{b}	2.0±0.2^{b}	4.4±0.3^{a}	3.4±0.5^{a}	<0.0001	0.3888	0.0500
Plasma TAG (mmol/L)	0.7±0.1^{b}	0.5±0.1^{b}	1.6±0.2^{a}	1.3±0.2^{a}	<0.0001	0.1201	0.7514
OGTT-AUC (mmol/l x min)	703.8±16.8^{c}	735.3±15.6^{c}	844.3±28.8^{a}	732.3±11.1^{bc}	0.0010	0.0434	0.0007
Plasma insulin (µmol/L)	1.1±0.1^{d}	3.8±0.1^{c}	6.7±0.2^{a}	5.1±0.1^{b}	<0.0001	0.0005	<0.0001
Plasma leptin (µmol/L)	2.6±0.4^{c}	3.1±0.7^{c}	13.2±2.1^{a}	9.1±1.0^{b}	<0.0001	0.1137	0.0473

Data are presented as mean ± SEM. Values marked with different supercripts are significantly different at 16 weeks at the level of $p<0.05$. ALT: alanine transaminase, AST: aspartate transaminase, NEFA: non-esterified fatty acids, TAG: triglycerides, OGTT-AUC: oral glucose tolerance test – area under curve.

5.3.2.5 Gut function and structure

Contractility measurements for both ileum and colon are presented in Figure 5.14. There were no significant differences between dietary groups for acetylcholine-induced contraction of the ileum at maximal contraction (Figure 5.14A). For the colon, maximal contractility induced by acetylcholine showed a higher contractility between 20%HAW supplemented rats and other groups of rats (Figure 5.14B). Figure 5.15 showed periodic acid and Schiff reagent's staining that showcase the density of goblet cells in small intestine and large intestine. There were increases in the density of goblet cells in small intestine from both 20%CHAW and 20%HAW compared to their respective control groups (Figure 5.15B and 5.15D). However, the density of goblet cells in 20%CHAW and 20%HAW appeared to be similar to MCS and MHCHF,respectively.



	P-Value		
Figure	Diet	Treatment	Interaction
A	0.4090	0.0791	0.9891
B	0.2827	0.0126	0.0196

Figure 5.14: Ileum contractility (Figure A) and colon contractility (Figure B) at week 16 in rats fed MCS, 20%CHAW, MHCHF and 20%HAW.

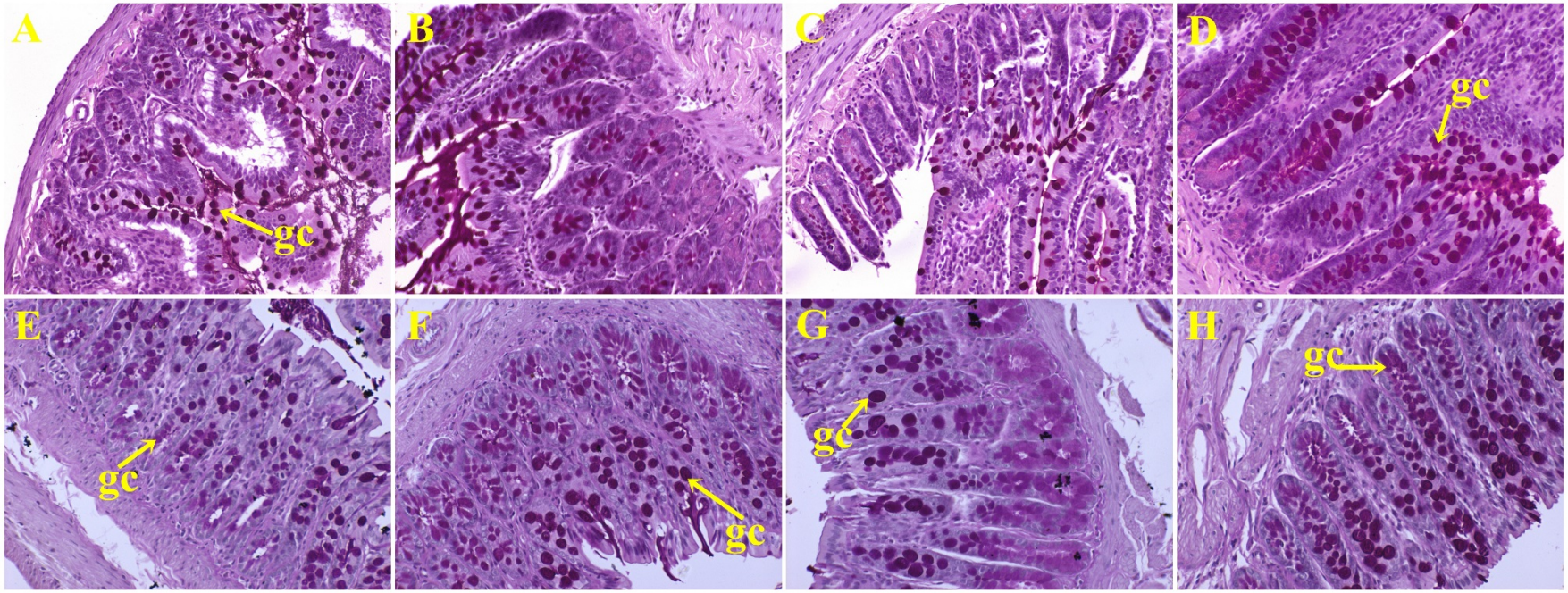


Figure 5.15: Periodic acid and Schiff reagent (PAS) staining (magnification 20x) in small intestine and colon of rats fed MCS, 20%CHAW, MHCHF and 20%HAW.

Periodic acid and Schiff reagent (PAS) staining (magnification 20x) in small intestine of MCS (A), 20%CHAW (B), MHCHF (C) and 20%HAW (D). Similar staining in colon of MCS (E), 20%CHAW (F), MHCHF (G) and 20%HAW (H). Goblet cells marked as “gc”.

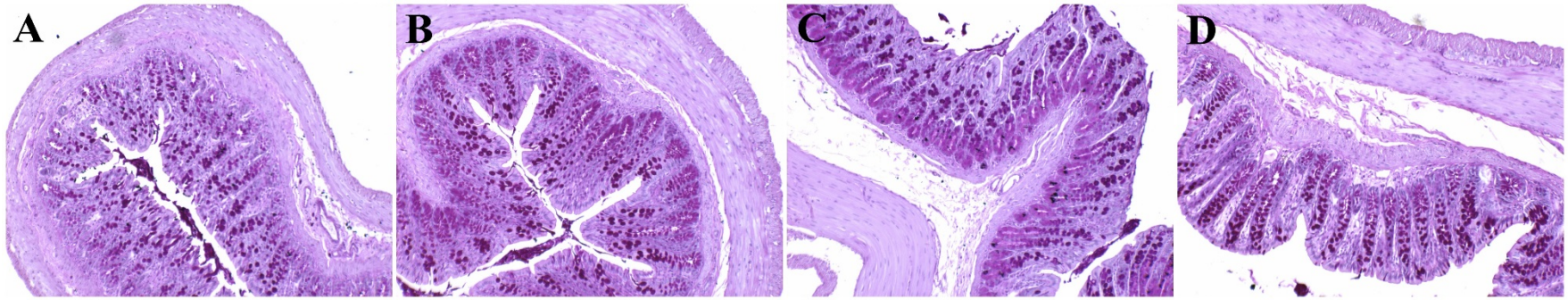


Figure 5.16: Periodic acid and Schiff reagent (PAS) staining (magnification 10x) in colon of MCS (A), 20%CHAW (B), MHCHF (C) and 20%HAW (D).

5.4 Discussion

One of the most consistent factors in development of metabolic syndrome is a high intake of fats and simple sugars. However, the multi-faceted nature of metabolic syndrome is complex and more diverse studies should be conducted to better define causation and amelioration of this issue. This present study evaluated the effect of high amylose wheat flour (5% and 20%) when supplemented in a high-carbohydrate and high-fat diet using a rat model which mimics metabolic syndrome in humans. High amylose wheat flour contains increased resistant starch that is characterised as an increased ratio of amylose to amylopectin content. Unlike regular starch, resistant starch acts as functional fibre that is beneficial to health. However, there is insufficient evidence for the beneficial effects of high amylose wheat in metabolic syndrome to date. Most studies used healthy animals or humans rather than models of metabolic syndrome. Therefore, the current study was warranted as it examined the physiological effects of high amylose wheat flour supplementation using a diet-induced metabolic syndrome rat model.

In this study, the corn starch and high-carbohydrate, high-fat diet were supplemented with 5% or 20% of high amylose wheat flour. These amounts translate to approximately 55g/day and 220g/day in the average 70kg adult human using a relevant scaling equation (Bachmann et al., 1996a). There is approximately 17% resistant starch in high amylose wheat (Štěrbová et al., 2016) so this would equal to 9.35g and 37g of resistant starch from 5% and 20% high amylose wheat when incorporated in the human diet. An adequate intake (AI) for dietary fibre was set by National Health and Medical Research Council (Council, 2006) at 30g/day for men and 25g/day for women. A suggested dietary intake for dietary fibre set by Food Standards Australia New Zealand (FSANZ) is 38g/day for men and 28g/day for women (Zealand., 2012). To date, there is still no Nutrient Reference Value (NRV) specifically set for resistant starch. Previously, 20g/day resistant starch was suggested as necessary to obtain bowel-related health benefits (Baghurst P.A et al., 1996).

In this study, high-carbohydrate and high-fat model was used to induce metabolic syndrome in rats because it showed the associated complications including

central obesity, dyslipidaemia, hypertension and impaired glucose tolerance. In addition, there are also deficits in cardiovascular function that include cardiac hyperthrophy, fibrosis and increased diastolic stiffness. The hepatic changes included steatosis, infiltration of inflammatory cells and portal fibrosis along with increased plasma activities of liver functions. These are consistent with previous study findings using the established HCHF diet (Panchal et al., 2011, Panchal et al., 2013).

The addition of the high amylose wheat flour to the HCHF and MHCHF diets resulted in a decrease in food intake by 7% and 26%, respectively. Previous studies in humans indicate resistant starch may play a role in enhancing both short-term (Willis et al., 2009, Anderson et al., 2010) and long-term satiety (Nilsson et al., 2008, Bodinham et al., 2010). This is also supported by a study in young men using 66% RS2 high amylose maize starch (HAMS) that found a reduction in cumulative food intake by 15% (Anderson et al., 2010). Animal models also suggested a reduction in food intake is associated with increased gene expression and circulating concentrations of PYY and GLP-1 after consumption of dietary resistant starch and these satiety hormones remained elevated for 24 hours (Keenan et al., 2006, Zhou et al., 2006, Zhou et al., 2008). The authors postulated that fermentation of resistant starch and the production of short chain fatty acids in the colon was responsible in regulating the hormones and the gene expression of GLP-1, PYY and proglucagon (Zhou et al., 2008).

Rats supplemented with high amylose wheat flour both in corn starch and high-carbohydrate and high-fat (5% and 20%) for 8 weeks showed an increased body weight. In 5% high amylose wheat flour fed rats, no changes in body compositions were seen compared to HCHF rats. However, 20% high amylose wheat flour supplemented rats showed that the body weight gains are mainly increases in body lean mass rather than body fat mass. This is consistent with previous findings that found no change in body weight, but also found lower fat mass and/or higher lean body mass with resistant starch ingestion (Shen et al., 2009, So et al., 2007, Shimotoyodome et al., 2010). These data suggest that resistant starch causes a change in metabolic flux that increased fat oxidation with a concomitant decrease in carbohydrate and protein oxidation (Higgins, 2014). This leads to increased protein accretion (lean body mass) and reduced fat deposition without changing total energy expenditure (Higgins, 2014).

The mechanisms suggested that there is a link between the consumption of fermentable resistant starch and PYY. PYY was effective in reducing food intake in obese humans (Batterham et al., 2003) and adiposity in obese mice (Adams et al., 2006). Studies also demonstrated that obesity is "a peptide YY-deficient, but not peptide YY-resistant, state" (le Roux et al., 2006, Young, 2006). The metabolic effects were also postulated to be through pro-opiomelanocortin gene expression in the arcuate nucleus of the hypothalamus (Shen et al., 2009) and oxidation of fat as demonstrated by reduced respiratory quotient (Zhou et al., 2009).

Interesting observations from this study are the lower glucose area under the curve (AUC) and plasma insulin concentrations in both 5% and 20% high amylose wheat flour supplemented rats in high-carbohydrate, high-fat diet compared to HCHF and MHCHF rats. Food starch is normally rapidly digested and absorbed as glucose, resulting in a hyperglycaemic response (Birt et al., 2013). It also triggers insulin secretion and tissue-specific intracellular uptake of glucose that can then result in hypoglycaemia. Repetition of this cycle results in insulin resistance and type 2 diabetes, thereby contributing to obesity (Birt et al., 2013). In contrast, enzyme-resistant starches improve glycaemic control. A few human studies reported that the ingestion of whole grain high amylose maize flour produced low blood glucose areas under the curve (Anderson et al., 2010, Luhovyy et al., 2014) and improved insulin sensitivity (Maki et al., 2012a, Robertson et al., 2003). One of the potential mechanisms for the insulin-sensitising effects of fermentable fibre is the absorption of short chain fatty acids generated in the large intestine due to fermentation by gut microbes. Increased butyrate production was associated with higher insulin sensitivity and improved glycaemic control (Gao et al., 2009).

The fermentation and production of short chain fatty acids may also trigger a reduction in release of free fatty acids (FFA) and glycerol from adipose depots, presumably through direct or indirect inhibition of hormone-sensitive lipase (Higgins, 2004). Many reports in the literature indicate that the consumption of RS markedly reduces NEFA and triglycerides concentrations (Metzler-Zebeli et al., 2015, Brites et al., 2011). However, the present study showed no difference between rats fed with 5% and 20% high amylose wheat with respective control groups, but decreased fat vacuoles in liver tissues were found in the high amylose wheat group. The present

study also did not find any changes in total cholesterol and a study fed high amylose rice to diabetic Zucker fatty rats also showed similar findings, where an increased resistant starch does not exert positive effects on the rats' lipid metabolism (Zhu et al., 2012). In contrast, *Himalaya 292* a high amylose barley cultivar given to pigs in the diet for 3 weeks resulted in reduction of plasma total and non-HDL cholesterol (Bird et al., 2004). However, this study proposed that this effect is largely through β-glucan or low non-starch polysaccharide content in the barley (Bird et al., 2004).

Rats from 20% high amylose wheat flour in high-carbohydrate, high-fat diet reduced plasma leptin concentrations compared to MHCHF rats despite higher body weight. This highlights the observation that changes in circulating leptin concentrations are driven by the lipid content rather than the overall body weight (Skurk et al., 2007). Readily digestible starch increased leptin concentrations, with larger adipocytes and generally higher insulin:glucose ratio as compared to high resistant starch (So et al., 2007). Mechanisms behind lowering of leptin after ingestion of 20% high amylose wheat flour could possibly include the binding of short chain fatty acids to the G protein–coupled receptors GPR41 and GPR43. This G protein–coupled receptors trigger leptin release from adipocytes (Robertson et al., 2005). To support this, high-fat diet induced rats fed with sorghum resistant starch also showed low leptin concentrations compared to control group and it was proportional to body fat loss (Shen et al., 2015). This low leptin concentration was suggested to be related to increased activity of the hypothalamic orexigenic signals, which stimulated appetite and suppressed energy expenditure (Valassi et al., 2008).

Cardiovascular risk factors are among the cluster of metabolic dysfunction. This includes the associations of the systolic and diastolic blood pressure parameters. This study has demonstrated that 5% and 20% high amylose wheat supplementation for 8 weeks to rats had no effect on systolic blood pressure and diastolic stiffness compared to their controls. Although values in the supplemented rats were lower, this was not statistically different. An increased feeding time in this trial may have shown significance. This can be visualised from the histological sections, where improved left ventricular structures were found in high amylose wheat-treated rats. There is lack of information available regarding the effect of resistant starch on blood pressure or heart functions. Significant difference in response of resistant starch Type 4 (RS4) was

reported in diastolic blood pressure but there was no difference in systolic blood pressure (Upadhyaya et al., 2016). The authors reported that this was correlated with changes in fasting glucose and IL-6 concentrations (Upadhyaya et al., 2016). In contrast, there were no differences in blood pressure, carotid–femoral pulse wave velocity or any of the clinical markers of vascular function following type 2 resistant starch (HAM-RS2) in type 2 diabetic patients (Bodinham et al., 2014).

Resistant starch undergoes fermentation and produces short chain fatty acids; acetate, propionate and butyrate which substantially contribute to gastrointestinal function and health (Patten et al., 2015). To our knowledge, there are limited data reported on ileal and colonic muscular contractility using metabolic syndrome induced rats, particularly testing high amylose wheat. The current data show that there were no changes in the contractility of the ileum and colon in 5% CHAW and 5% HAW compared to control groups. Similarly, 20% CHAW showed no change in contractility. However, we found an increased colonic contractility in 20% HAW compared to other rats. Previous reports on butyrylated low-amylose maize starch demonstrated that elevation of butyrate concentrations in the large bowel of the rat reduced the contractile activity of colonic musculature (Bajka et al., 2010). They suggested that butyrate stimulated Na^+ and fluid absorption, thus reduced colonic contractility and colonic motility. This is in contrast with our findings and this is probably due to the differences in nature of experiment being done which include the agonists used, cumulative or non-cumulative addition of agonist, the composition of experimental diet, the duration of the experiment and strain of rats used. Another study testing high amylose wheat in rats fed a western diet found no changes to the genes or colonic contractility relative to the effect of low-amylose maize starch (Patten et al., 2015). Inconsistent results were also shown in the findings for dietary supplementation of fish oil rich in the long-chain n-3 polyunsaturated fatty acids, which increased muscarinic-induced ileal contractility in healthy rats (Patten et al., 2002) and both ileal and colonic contractility in hypertensive rats (Patten et al., 2005).

In addition to the physiological effects on the gut, changes on the intestinal sections can be assessed and more details can be provided in term of the capacity for the mucosa to produce mucins. Dietary fibre increased the number of goblet cells found in the mucosa in comparison to low fibre or fibre-free controls (Hino et al., 2013,

Hino et al., 2012). There was no change in the density of goblet cells in the 5% high amylose wheat, however supplementation with 20% high amylose wheat showed increased density of goblet cells compared to non-supplemented rats. Certain types of fibre, such as isolated cellulose, appear to have minor or no effects on goblet cell numbers, while other fibre types such as arabinoxylans doubled goblet cell coverage in the villus with modest impacts of around 25% increase in the intestinal crypts (Chen et al., 2015). Furthermore, diet high in fibre or resistant starch altered gastrointestinal health through the production of short chain fatty acids, which in turn will affect the intestinal morphology (Bird et al., 2004). Intravenous infusion of short chain fatty acids in rats reverses colonic atrophy linked to butyrate production (Topçu et al., 2002). Hence, inclusion of the increased fibre or resistant starch in the diet may contribute to positive changes in gut and this could probably affect systemic responses.

5.5 Conclusion

The present study shows that a daily consumption for a period of 8 weeks of 20% high amylose wheat flour supplemented in the diet elicited more favourable effects on cardio-metabolic health than a daily consumption of 5% high amylose wheat flour. Addition of 20% high amylose wheat in the diet reduced food intake, increased lean mass and lowered total fat pads in obese rats, although there was no effect on body weight. Supplementation of 20% high amylose wheat flour is also associated with a better glycaemic control, improved insulin and leptin metabolism with cardioprotective and hepatoprotective effects. These effects are probably due to the increased resistant starch in high amylose wheat. Therefore, high amylose wheat flour can be used as a value-added ingredient to replace flour containing higher amounts of rapidly digestible starch and thereby aimed to attenuate the risk of having metabolic syndrome. However, further investigation comparing high amylose wheat with a low amylose wheat as a baseline of normal wheat will give a better understanding on the evaluation of the effects. Translation of these animal studies to human is also warranted to show reproducibility of findings, however caution is warranted before extrapolating these results to dosage in humans.

Chapter 6 General conclusion and future directions.

6.1 Conclusions

It is well established that obesity, particularly an excessive visceral adiposity, is associated with insulin resistance, hyperglycaemia, dyslipidaemia and hypertension, which together are termed “metabolic syndrome” (as discussed in Chapter 1). These clusters of metabolic abnormalities increase the risk of cardiovascular disease and type 2 diabetes which contribute to increased morbidity and mortality (Alberti et al., 2009). Environmental factors especially physical inactivity and unhealthy dietary lifestyle play strong roles in driving the increase in obesity prevalence. Thus, the promotion of healthy diets, nutrition and lifestyles to reduce the global burden of these diseases is continually being advocated. One possible solution is an increased consumption of functional foods and value-added food products with disease-preventing characteristics (as discussed in Chapter 1). Therefore, research is warranted in evaluating these functional foods using appropriate animal model as a foundation before translation to human clinical trials.

Research in metabolic syndrome testing possible interventions requires a well-established and well-designed animal model mimicking human pathogenesis to provide better understanding of physiology, biochemistry and pathophysiology. Thus, my study utilised the most relevant model that mimics the diet responsible for human metabolic syndrome and cardiovascular complications. Young male Wistar rats fed with a combination of simple sugars and saturated/*trans* fats in the diet for sixteen weeks developed central obesity, hypertension, impaired glucose tolerance, dyslipidaemia, hyperinsulinemia, hyperleptinemia, cardiovascular complications and hepatic steatosis (Panchal et al., 2011). The physiological effects of saturated fatty acids (Chapter 3), linseed components (Chapter 4) and high amylose wheat (Chapter 5) were determined in this diet-induced rat model of metabolic syndrome.

Saturated fatty acid has long been generalised as the main culprit in developing obesity and the associated morbidities. Previous research also highlighted the effects of saturated fatty acids as a whole rather than of individual fatty acids. Since effects of

individual saturated fatty acids remain to be fully elucidated, Chapter 3 investigated the physiological effects of lauric, myristic, palmitic and stearic acids in response to the metabolic syndrome biomarkers. It shows that not all saturated fatty acids gave undesirable effects. The longer-chain saturated fatty acids (myristic, palmitic and stearic) and the mixture of stearic and *trans* fats in beef tallow produced obesity, in contrast to rats treated with lauric acid with low total fat mass, abdominal circumference and visceral adiposity index. This study found that increased chain length is associated with worsening of disease state and the most obvious changes in rats fed the individual saturated fatty acids were found with stearic acid. However, stearic acid exhibited less abnormalities compared to the combination diet of high-carbohydrate and high-fat diet which contained 20% beef tallow with similar amounts of stearic acid and *trans* fats, suggesting that the *trans* fats worsen the responses to stearic acid. From this chapter, it is clear that individual saturated fatty acids produce distinctly different responses. This study suggests that replacing beef tallow with palmitic and stearic acids would show small improvements but replacement with lauric and possibly myristic acids in human diets would markedly decrease the development of metabolic syndrome. Reductions in the prevalence and incidence of metabolic syndrome would lead to significant improvements in the health and wellbeing of individuals and families, and this indirectly leads to substantial savings to the healthcare system and to overall workplace productivity.

In Chapter 4, supplementation of lignan or defatted linseed in the high-carbohydrate, high-fat diet improved cardiovascular function, reduced hepatic steatosis and altered metabolic profiles, some of which can be regarded as beneficial to health, whereas raw linseed exacerbated adiposity with no changes in other metabolic biomarkers except for reduced systolic blood pressure. This study suggests that lignan (SDG) and dietary fibre in defatted linseed could reduce the symptoms of metabolic syndrome. However, raw linseed diminishes the nutritional or positive physiological effects of the seeds and induced an increased intake of fat and energy. There could be a possibility that ground linseed is easier to digest compared to whole raw linseed. Whole linseed may pass through the intestine undigested, which means the nutritional benefits are unable to be realised. Thus, to unlock the benefits of linseed, lignan powder and defatted linseed powder would be better choices to be included in

the diet. Lignan powder and defatted linseed powder could be used in many ways such as adding these components into our breakfast cereals, yoghurt or salad, or including lignan and defatted linseed powder in baked goods such as muffins, cookies, bagels and bread. It would not only taste good, but also provide health benefits when consumed daily at an appropriate dosage. This could be one dietary approach for subjects with metabolic syndrome, hence reducing the health-care costs in the community.

Another functional food described in this thesis is high amylose wheat flour (Chapter 5). Two dosages (5% and 20%) high amylose wheat were given to diet-induced obese rats for 8 weeks. The addition of 20% high amylose wheat in the diet reduced food intake, increased lean mass and lowered total fat pads in obese rats, with no changes in body weight. Supplementation of 20% high amylose wheat flour was also associated with a better glycaemic control, improved insulin and leptin metabolism with cardioprotective and hepatoprotective effects. These effects are probably due to the increased resistant starch content in high amylose wheat. Resistant starch is broken down by resident colonic bacteria thus generating a range of beneficial changes that include increased stool bulk which give a mild laxative effect (Phillips et al., 1995), encouraging the growth of healthy bacteria in the bowel (Topping et al., 2003) and producing short chain fatty acids which promote intestinal health (Weaver et al., 1992) and overall health. These findings will need further research to be translated to humans and one possible study would be to determine the effects of high amylose wheat in food products such as bread, pasta and noodles. As wheat supplies approximately 20% of the food calories for the world population, it could possibly attract policy-makers to replace normal wheat with high amylose wheat flour. This will benefit farmers, food industry and economy as well as the health of everyone especially in ameliorating metabolic syndrome and risk of colon cancer.

This thesis emphasises that generalisation of saturated fatty acids based on the chemical class rather than on their biological actions may give misleading information to consumers. A classification of fatty acids based on their pharmacological or therapeutic effects may be more relevant for clinicians and nutritionists when formulating therapeutic and dietary guidelines (Poudyal and Brown, 2015). Lauric acid could be classified with other bioactive fatty acids that elicit physiological

responses that may reduce one or more cardiometabolic risk factors (Poudyal and Brown, 2015). This thesis also highlights that inclusion of lignans, defatted linseed and high amylose wheat in the diet may be value-added ingredients to a range of convenience foods to consumers. These foods will be the most practical source for everyday consumption as these foods are natural and staples for most societies. It is also advisable for diabetics or obese patients to consume at least the amount of fibre and whole grains recommended for the general public. Inclusion of defatted linseed and high amylose wheat in the diet could indirectly increase the daily intake of lignans, dietary fibre and resistant starch since the present study have shown that these linseed components and high amylose wheat give better glycaemic controls and have potential in reversing most of the risk factors associated with metabolic syndrome. This could be achieved without making a major change to the dietary intake of linseed or cereal grains.

In overview, the study findings highlight that saturated fatty acids are not equal and linseed components and high amylose wheat have potential as functional foods ingredients that could ameliorate metabolic syndrome. Further, the study findings suggest that improving the nutrient profile of foods through incorporation of these functional foods ingredients will then increase the availability of healthy food choices and indirectly improve the health and well-being of individuals and families. Emphasis on functional foods and bioactive components could also attract policy makers to strategise new action plans especially in making decisions regarding methods to encourage the development and consumption of healthier foods. Innovations in the agriculture and food sector could also lead to the development and increased production of foods that provide additional health benefits to consumers. Food products that confer health benefits can result in wider social benefits through reducing health-care costs and the associated cost of illness, such as losses in productivity and long-term disability.

Overall, this study had achieved the aims set in Chapter 1, where effects of different saturated fats, lignans, raw linseed, defatted linseed and high amylose wheat on cardio-metabolic health and on gut structure and function were hypothesised. The first hypothesis is proven that saturated fats together with a high intake of fructose induced metabolic changes in the host and these changes depended on the saturated

fatty acid that was administered. The second hypothesis is proven for lignans, defatted linseed and high amylose wheat except for raw linseed, as raw linseed exacerbated most of the cardio-metabolic parameters.

6.2 Observed limitations and future directions

There are clear indications that lauric acid as a saturated fatty acid, linseed components and high amylose wheat improved the markers of metabolic syndrome in these obese rat model. However, the mechanisms of action of these foods and their bioactive components remain unclear, as does their ability to be translated to humans. It is acknowledged that there is generally not a direct numerical equivalence between animal and human experiments but responses to treatments are, on the whole, qualitatively similar allowing responses of animals to more extreme treatments to be used to predict the anticipated response in humans (Litten-Brown et al., 2010). Although the dosages used in these studies corresponds to human dose given accepted translation equations, the translation of results from rodent studies to human trials remains a problem.

Relevant human doses have been estimated from rodent doses, but these doses assume high oral bioavailability given in the food while the metabolism may vary between rats and humans. Bioavailability is an important factor to be consider in determining the biological effects of any functional foods or bioactive compounds. In addition, animal studies conditions are usually optimized to demonstrate the hypothesized effects, and the doses used are usually higher than the levels of human consumption. Effects of confounding factors related to lifestyle, such as smoking, physical activity, and dietary intake of calories, fat, and fiber, make it difficult to interpret human data (Yang and Hong, 2013). Further, the food matrix is a well-known variable affecting absorption of food components. The oral absorption of small molecules can be extremely variable between rodents and humans due to interspecies differences in organ structure and drug-metabolizing enzymes in the small intestines (Cao et al., 2006). Moreover, the length of dosing is a factor to consider too, with the average life-span of laboratory rats of about 2 years being much less than the average human life-span of 75-80 years in many countries. Thus, 8 weeks interventions in rats are approximately 6 years interventions in humans, based on life-span. Longer interventional studies in humans, at higher doses, may be necessary to show

therapeutic benefits. However, caution should be applied in using high doses as it may impose the risk of hepatotoxicity and other safety issues in the use of new interventions in humans.

The investigations in this thesis also raised several valid topics for further research. These topics include:

- Determining the molecular mechanism for lauric acid improving glucose metabolism and reducing inflammation.
- Quantifying the effects of lauric acid on the diversity of gut microbiota.
- Extending the fatty acid trials to include the effects of trans fatty acids in the obese rat model used in the studies described in this thesis.
- Elucidating the effects of linseed and linseed components on mucin production at the genetic and the protein level.
- Ascertaining the impact of low amylose wheat in the current obese rat model.
- Compiling data to determine the most appropriate avenue for translation of these results to humans to ameliorate the obesity epidemic.

These studies will provide novel insights to the knowledge gap and a better understanding of molecular mechanisms will then strengthen the results from this current study. These further studies could also improve individual health and thus impact on the whole community by providing functional foods to reduce the financial costs of the metabolic syndrome.

"*Nutrition has often been the subject of conjectures and ingenious hypotheses—but our actual knowledge is so insufficient that their only use is to try to satisfy our imagination. If we could arrive at some more exact facts they could well have applications in medicine*" Francois Magendie (1783–1855).

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Appendices

Appendix I

Background/Aims: Insulin is one of the most potent anabolic agents involved in the macronutrients synthesis and storage. It plays a pivotal role in the development of obesity while within the central nervous system acts as a growth factor in synaptogenesis and nerve growth. Therefore the lack of insulin or presence of insulin resistance (IR) could potentially lead to cognitive decline and impaired learning. This review focuses on animal and human studies that assess the relationship between the IR and regulation of cognitive function and mood.
Methods: Literature searches were conducted on electronic databases examining the association between insulin resistance, glucose regulation, obesity, cognitive function and mood.
Results: In animal models, the central focus is on effects to the hypothalamic-pituitary-adrenal axis (HPA-axis), an area identified as the most likely area to influence mood and stress. Damage to the insulin receptors in this region was found to be associated with the increase in food intake and occurrence of adiposity. In humans, these areas play a central role in motivation and decision making. Furthermore, IR and major depressive illness share several pathologies, including disorders of the HPA-axis, the autonomic nervous system, platelets and endothelial function.
Conclusions: There is increasing evidence suggesting a close association between the obesity and mood disorders such as depression, anxiety, panic and bipolar disorders. However, there is no clear evidence of individual aspects that can be ascribed as pathological drivers of the problem.
Funding source(s): N/A.

IMPAIRED CEREBROVASCULAR RESPONSIVENESS TO A WORKING MEMORY TASK IN OLDER ADULTS WITH TYPE 2 DIABETES MELLITUS (T2DM)

R.S. Nealon, P.R.C. Howe, L. Jansen, M.L. Garg, R.H.X. Wong. *Clinical Nutrition Research Centre, School of Biomedical Sciences and Pharmacy, University of Newcastle, NSW, Australia*
E-mail address: rhenan.nealon@uon.edu.au (R.S. Nealon)

Background/Aims: Impairments in specific cognitive domains in T2DM may be partly attributable to stiffness in cerebral arteries, resulting in poor cerebral perfusion. This cross-sectional study investigated whether impairments in the ability of the cerebrovasculature to supply blood in response to a battery of cognitive tests could predict poorer cognition in T2DM.
Methods: Forty nine T2DM and 28 non-T2DM adults underwent transcranial Doppler ultrasound measurements of basal mean cerebral blood flow velocity (MBFV) and pulsatility index, a measure of arterial stiffness, in the left and right middle cerebral arteries (MCA). A battery of cognitive tasks assessing domains of working memory, executive function and information processing speed was then administered whilst MBFV was recorded. Cerebrovascular responsiveness (CVR) to cognitive tasks was calculated as a percentage increase in MBFV from the basal level.
Results: Using *t*-tests, we found no differences in basal MBFV; however, cerebral vessels were 14 percent stiffer in T2DM ($p < 0.05$). As expected, T2DM performed poorer in tasks relating to working memory (i.e. N-back, Digit-Symbol Coding, Symbol-Digit Coding), executive function (Concept Shifting Task) and information processing speed. Importantly, CVR to the N-back task was reduced by 53 percent in T2DM ($p < 0.05$) but was independent of task performance.
Conclusions: We have shown for the first time that impaired cerebral perfusion during a working memory task is accompanied by poor task performance. We plan to evaluate the ability of selected *vasoactive* nutrients to enhance cerebrovascular function and see whether this improves cognition in at-risk populations.
Funding source(s): N/A.

INCIDENCE AND RISK FACTORS OF TYPE 2 DIABETES: RESULTS FROM THE THAI COHORT STUDY

K. Papier [1], S. Jordan [2], C. D'est [1], C. Banwell [1], C. Bain [2], V. Yiengprugsawan [1], S. Seubsman [3], A. Sleigh [1]. [1] *National Centre for Epidemiology and Population Health, Australian National Uni., ACT, Australia;* [2] *Population Health, Queensland Institute of Medical Research QLD, Australia;* [3] *School of Human Ecology, Sukhothai Thammathirat Open Uni., Nonthaburi, Thailand*
E-mail address: keren.papier@anu.edu.au (K. Papier)

Background/Aims: The global prevalence of type 2 diabetes mellitus (T2DM) is high and increasing rapidly in countries undergoing a nutrition transition like Thailand. This study aimed to assess the relationship between T2DM and factors associated with the nutrition transition among Thai adults.
Methods: Data were from Thai Cohort Study participants surveyed in 2005, 2009 and 2013 ($n = 39{,}519$). Cumulative incidence of diabetes was calculated and multivariable analyses were conducted using logistic regression.
Results: T2DM incidence (per 1000) was higher in males (24.9 *vs.* 11.9). The factors most strongly associated with T2DM in both sexes were increasing age and BMI but, amongst males, smoking [Odds Ratio (OR) =1.70, 95%CI: 1.29-2.24] and alcohol intake (OR = 1.67, 95%CI: 1.00-2.82) were also associated with increased risk. Infrequent gardening, low vegetable intake, and urban childhood residence were also related to T2DM risk however these associations attenuated after adjusting for BMI. Among females, high income was associated with T2DM (OR = 1.72, 95%CI: 1.03-2.89). Urban childhood residence and education were also associated with T2DM however these associations were attenuated after adjusting for BMI.
Conclusions: The factors associated with T2DM risk in our study are consistent with findings from previous studies conducted in countries undergoing a nutrition transition. With the prevalence of these factors projected to increase it is likely that the incidence of T2DM will keep rising. This may be of particular concern for Thai men who appear to be in the earlier stages of the nutrition transition. Our study suggests that females are at a more advanced stage of the nutrition transition.
Funding source(s): NHMRC.

LAURIC ACID DIFFERS FROM OTHER SATURATED FATTY ACIDS IN METABOLIC SYNDROME IN RATS

S. Shafie [1,2], L. Brown [1]. [1] *School of Health & Wellbeing, Faculty of Health, Engineering & Science, Uni. of Southern Queensland, QLD, Australia;* [2] *Dept. of Nutrition & Dietetics, Faculty of Medicine & Health Sciences, Universiti Putra Malaysia, Malaysia*
E-mail address: sitiraihanah.shafie@usq.edu.au (S. Shafie)

Background/Aims: The aim of this study is to evaluate different saturated fatty acids on cardio-vascular, liver and metabolic responses in rats.
Methods: Rats were fed 20% lauric (HLA), myristic (HMA), palmitic (HPA) or stearic (HSA) acids or beef tallow (HCHF) for 16 weeks with increased fructose and condensed milk. Control rats were fed a corn starch (C) diet. Food and water consumption, body weight, body composition, heart stiffness, blood pressure, blood glucose, lipid profiles and liver function of rats were measured.
Results: Final body weight ranked HLA < C < HMA = HPA = HSA < HCHF rats with HLA rats showing 11.1% decrease. Total fat mass reflected changes in body weight with HLA (54.4 ± 3.2 g) < C (79.5 ± 11.0 g) < HPA (122.7 ± 12.9 g) = HSA (122.9 ± 9.5 g) = HMA (132.4 ± 9.9 g) < HCHF rats (207.7 ± 27.2 g). Left ventricular diastolic stiffness (κ) was similar in control (22.0 ± 0.5) and HLA rats (21.8 ± 1.0) but less than HMA (25.3 ± 0.7), HPA (26.6 ± 0.7), HSA (27.0 ± 0.4) and HCHF rats (28.2 ± 0.5). Systolic blood pressure increased with C (127.7 ± 1.1 mmHg) < HLA (136.2 ± 5.3 mmHg) = HMA (141.7 ± 1.2 mmHg) < HPA (150.4 ± 2.5 mmHg) = HSA (152.9 ± 3.5 mmHg) = HCHF rats (157.8 ± 2.8 mmHg). HLA rats showed improved glucose tolerance, insulin sensitivity and attenuated dyslipidaemia compared to HMA, HPA, HSA and HCHF rats. Plasma liver enzymes increased in HLA, HMA, HPA, HSA and HCHF rats compared to C rats.
Conclusions: For most parameters, lauric acid produced less pathophysiological changes than other saturated fatty acids in this model of diet-induced metabolic syndrome.
Funding source(s): University of Southern Queensland.

HIGHER VITAMIN D STATUS IS INVERSELY ASSOCIATED WITH THE METABOLIC SYNDROME AND RISK OF T2DM IN VICTORIAN ADULTS

Appendix II

Accepted Manuscript

Anthocyanins in chokeberry and purple maize attenuate diet-induced metabolic syndrome in rats

Maharshi Bhaswant, Siti Raihanah Shafie, Michael L. Mathai, Peter Mouatt, Lindsay Brown

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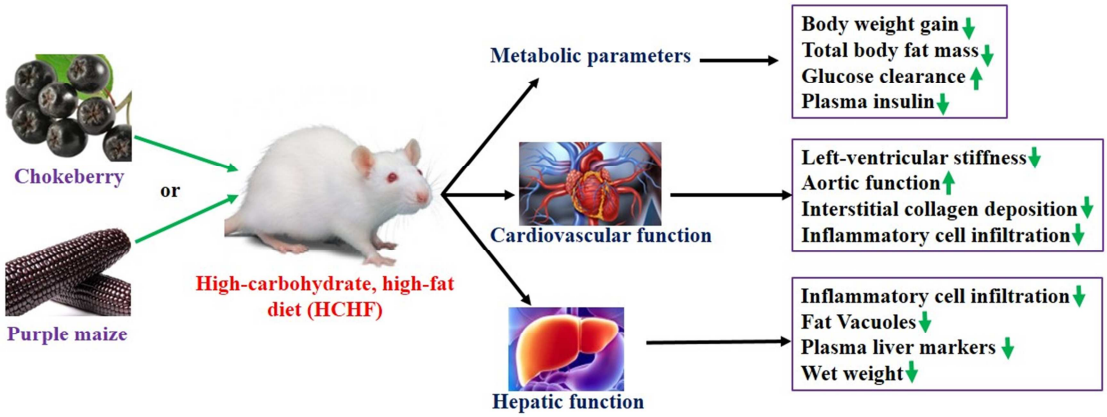

Nutrition

Anthocyanins in chokeberry and purple maize attenuate diet-induced metabolic syndrome in rats

Maharshi Bhaswant[1,2], Siti Raihanah Shafie[2], Michael L Mathai[1], Peter Mouatt[3], Lindsay Brown[2]

[1]*Centre for Chronic Disease, College of Health and Biomedicine, Victoria University, Melbourne VIC 3021, Australia*

[2]*School of Health and Wellbeing, University of Southern Queensland, Toowoomba QLD 4350, Australia*

[3]*Centre for Phytochemistry and Pharmacology, Southern Cross University, East Lismore NSW 2480, Australia*

Running Title: Chokeberry and purple maize in metabolic syndrome

Address for correspondence:

Professor Lindsay Brown, School of Health and Wellbeing, University of Southern Queensland, QLD 4350, Australia; Tel.: +61-74631-1319.

E-mail: Lindsay.Brown@usq.edu.au

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Abstract

Objective: Increased consumption of fruits and vegetables as functional foods leads to the reduction of signs of metabolic syndrome. In this study, we have compared and measured cardiovascular, liver and metabolic parameters following chronic administration of the same dose of anthocyanins either from chokeberry (CB) or purple maize (PM) in rats with diet-induced metabolic syndrome. *Research methods and procedures*: Male Wistar rats were fed with cornstarch diet (C) or high-carbohydrate, high-fat diet (H) diet and divided into six groups for 16 week feeding with C, C with CB or PM for last 8 weeks (CCB or CPM), H, H with CB or PM for last 8 weeks (HCB or HPM); CB and PM rats received ~8 mg anthocyanins/kg/day. The rats were monitored for changes in blood pressure, cardiovascular and hepatic structure and function, glucose tolerance and adipose tissue mass. *Results:* HCB and HPM rats showed reduced visceral adiposity index, total body fat mass and systolic blood pressure, improved glucose tolerance, liver and cardiovascular structure and function, decreased plasma triglycerides and total cholesterol compared to H rats. Inflammatory cell infiltration was reduced in heart and liver. *Conclusion:* CB and PM interventions gave similar responses, suggesting that anthocyanins are the bioactive molecules in the attenuation or reversal of metabolic syndrome by prevention of inflammation-induced damage.

Keywords: anthocyanins; chokeberry; *Aronia melanocarpa*; purple maize; *Zea mays*; metabolic syndrome

Introduction

Fruits and vegetables rich in antioxidant phytochemicals such as flavonoids and polyphenols are effective in attenuating the signs of metabolic syndrome [1, 2]. An increased intake of fruits and vegetables has been associated with a decrease in cardiovascular diseases [3], and better control of type 2 diabetes [4] and non-alcoholic fatty liver disease [5]. Anthocyanins are bioactive flavonoids that give red to purple colours to a wide range of fruits and vegetables including rhubarb, cabbage, berries, cherries and red grapes [6]. Regular consumption of anthocyanins has been credited with reducing risk of chronic diseases such as obesity, non-alcoholic fatty liver, diabetes and cardiovascular diseases [7-9]. It is estimated that ~1000 mg of polyphenols, including up to 215 mg of anthocyanins, are consumed daily by an average adult in the USA [9, 10].

This study focuses on black chokeberry and purple maize as two rich dietary sources of anthocyanins similar to purple carrots and Queen Garnet plums [11, 12]. Black chokeberry (*Aronia melanocarpa*) is described as an attractive garden plant, native to eastern North America but now grown widely in northern Europe, primarily Poland, where the sour fruit is eaten raw or processed for incorporation into foods. Black chokeberries are rich in cyanidin anthocyanins, chlorogenic acids and proanthocyanidins, and also contain quercetin flavonols [13]. While these components indicate that black chokeberries may be an effective functional food, more rigorous studies are needed to support popular indications for heart disease, hypertension, hyperlipidaemia and urinary tract infections, as well as actions against bacteria and viruses, and to strengthen memory and digestion [14, 15]. In metabolic syndrome subjects, chokeberry extract decreased blood pressure and plasma lipid concentrations with no change in body weight [16]. Chokeberry attenuated body weight gain and insulin resistance in rats fed with fructose-rich diet [17].

Purple maize has been cultivated in the Andean region, especially Peru and Bolivia, for centuries where it is used as a food and a colourant in a drink believed to improve health [18, 19]. Treatment with purple maize (*Zea mays*) decreased abdominal adiposity [20], improved glucose metabolism [21] and decreased blood pressure in healthy humans [22]. However, there is no clear evidence that intervention with these anthocyanin-containing traditional functional foods will improve the widespread organ dysfunction observed in patients with metabolic syndrome, despite improvements in individual signs.

This study has compared the cardiovascular, liver and metabolic responses of two dietary sources of anthocyanins, chokeberry and purple maize, at the same daily anthocyanin dose as in rats fed cyanidin 3-glucoside or Queen Garnet plums [12] using the same high-carbohydrate, high-fat diet as a model of human metabolic syndrome [23]. These measurements included systolic blood pressure, echocardiography, vascular reactivity, collagen deposition and stiffness of heart, plasma biochemistry and histology for structural changes on heart and liver. Our results suggest that an adequate intake of foods containing cyanidin-type anthocyanins can normalise the metabolic, cardiovascular and liver changes induced by a high-carbohydrate, high-fat diet by decreasing infiltration of inflammatory cells in the organs.

Materials and methods

Analysis of chokeberry juice and purple maize flour

Chokeberry juice (CB) was supplied by Fasbay Pty Ltd, Sydney, Australia and purple maize flour (PM) was supplied by Spectrum Ingredients Pte Ltd, Singapore. The anthocyanin contents were determined by HPLC based on the method outlined in the British Pharmacopoeia 2014 (Eur. Pharm 2394) using an Agilent 100 series HPLC system. Briefly, samples were prepared by extraction by 2% v/v HCl in methanol, using sonication for 15

minutes in volumetric flasks, then made up to volume and diluted as required to be within the standard calibration. Analysis was performed using a gradient of mobile phases A (water and formic acid, 91.5:8.5) and B (acetonitrile, methanol, water and formic acid, 22.5:22.5:41.5:8.5) over 56 minutes. The gradient ran from 7 to 25% B in 35 minutes, to 65% at 45 minutes followed by 100% B to 50 minutes and return to 7%. The column used was a Phenomenex 250mm C18 5µm column with a flow rate of 1 mL per minute and temperature 30°C. Detection and quantification were performed using a diode array detector (DAD) at 535nm with cyanidin chloride (PhytoLab, CAS No. 528-58-5, B# 80022 5368) as the calibrating standard. Total anthocyanins were calculated as cyanidin chloride and cyanidin 3-glucoside by mass correction.

Rats and diets

The experimental groups consisted of 72 male Wistar rats (8-9 weeks old; weighing 335 ± 3 g) purchased from Animal Resource Centre, Murdoch, WA, Australia and individually housed in a temperature-controlled (20±2°C), 12-hour light/dark cycle environment with *ad libitum* access to water and rat diet at the University of Southern Queensland Animal House. All experimentation was pre-approved by the Animal Ethics Committee of the University of Southern Queensland under the guidelines of the National Health and Medical Research Council of Australia. The rats were randomly divided into six separate groups (n = 12 each) and fed with maize starch (C), maize starch + chokeberry juice (CCB), maize starch + purple maize flour (CPM), high-carbohydrate, high-fat (H), high-carbohydrate, high-fat + chokeberry juice (HCB) and high-carbohydrate, high-fat + purple maize flour (HPM).

The preparation and macronutrient composition of basal diets, including the dietary fatty acid profiles, have been described [23-25]. C and H rats received their diets for 16 weeks and CCB, CPM, HCB and HPM rats received C or H diets for the first 8 weeks while

both diets were supplemented with chokeberry juice 50 ml/kg or purple maize flour 50 g/kg by replacing equivalent amounts of water for a further 8 weeks. The drinking water in all H diet-fed groups was augmented with 25% fructose for the duration of the study. Body weight and food and water intakes were measured daily and feed efficiency (%) was calculated [24] using the following equation:

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

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

Oral glucose tolerance test

For the oral glucose tolerance testing, the glucose concentrations in blood collected by tail prick on tail vein of overnight food-deprived rats were measured using Medisense Precision Q.I.D glucose meter (Abbott Laboratories, Bedford, USA). Fructose-supplemented drinking water in H, HCB and HPM rats 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% aqueous solution via oral gavage. Tail vein blood samples were taken at 30, 60, 90 and 120 minutes following glucose administration.

Body composition measurements

Dual energy X-ray absorptiometric (DXA) measurements were performed on 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) [24]. The precision error of lean mass for replicate measurements, with repositioning, was 3.2%. Visceral adiposity index (%) was calculated [23].

Cardiovascular measurements

Systolic blood pressure was measured under light sedation following intraperitoneal (i.p.) injection of Zoletil (tiletamine 10 mg/kg, zolazepam 10 mg/kg; Virbac, Peakhurst, NSW, Australia) using a MLT1010 Piezo-Electric Pulse Transducer and inflatable tail-cuff connected to a MLT844 Physiological Pressure Transducer using PowerLab data acquisition unit (ADInstruments, Sydney, Australia) [24].

Anaesthesia using Zoletil (tiletamine 10 mg/kg and zolazepam 10 mg/kg i.p.) and Ileum xylazil (xylazine 6 mg/kg; Troy Laboratories, Smithfield, NSW, Australia) was used for echocardiographic examination (Hewlett Packard Sonos 5500, 12 MHz transducer) performed at 16 week [23-25], in accordance with the guidelines of the American Society of Echocardiography using the leading-edge method [26].

The left ventricular (LV) function of the rats in all groups was assessed using the Langendorff heart preparation [23-25]. Terminal anaesthesia was induced via i.p. injection of pentobarbitone sodium (Lethabarb®, 100 mg/kg). After heparin (200 IU; Sigma-Aldrich Australia, Sydney, Australia) administration 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 left ventricle 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 during pacing of the heart at 250 beats per minute using an electrical stimulator. End-diastolic pressures were obtained starting from 0 mmHg up to 30 mmHg.

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, Sydney, Australia); concentration-response (relaxation) curves were measured for acetylcholine (Sigma-Aldrich Australia, Sydney, Australia) and sodium nitroprusside (Sigma-Aldrich Australia, Sydney, Australia) in the presence of a submaximal (70%) contraction to noradrenaline [25].

Organ weights

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

Histology

Two rats per group were taken exclusively for histological analysis. Two slides were prepared per tissue specimen and two random, non-overlapping 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% neutral buffered formalin for 3 days and then dehydrated and embedded in paraffin wax [23-25]. Thin sections (~5 μm) of left ventricle and the liver were cut and stained with haematoxylin and eosin stain for determination of inflammatory cell infiltration with a 20× objective using a Olympus BX51 microscope (Olympus, Melville, NY). Left ventricular sections were stained with picrosirius red to determine collagen distribution. Laser confocal microscopy (Nikon A1R+ upright Confocal Microscope) was used to determine collagen distribution in left ventricular sections.

Plasma biochemistry

Blood was centrifuged at 5,000*g* for 15 minutes within 30 minutes of collection into heparinised tubes. Plasma was separated and transferred to microcentrifuge 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 analyser (AU 400, Tokyo, Japan) [23-25]. Plasma insulin and leptin concentrations (ALPMO, USA) were estimated using commercial ELISA kits according to manufacturer-provided standards and protocols.

Statistical analysis

Data are presented as mean ± SEM. Results were tested for variance using Bartlett's test and variables that were not normally distributed were transformed (using log 10 function) prior to statistical analyses. Data from C, CCB, CPM, H, HCB and HPM 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 a Newman-Keuls multiple comparison *post hoc* test. Where transformations did not result in normality or constant variance, a Kruskal-Wallis non-parametric test was performed. A *P*-value of <0.05 was considered as statistically significant. All statistical analyses were performed using GraphPad Prism version 6.00 for Windows (San Diego, California, USA).

Results

Diet and body composition

CB and PM contained similar concentrations of total anthocyanins with cyanidin 3-glucoside as the major anthocyanin (Table 1). The average daily intake of anthocyanins was higher in CCB and CPM rats compared to HCB and HPM rats, as the food intake was higher in CCB and CPM rats (Table 2). Compared to C rats, H rats consumed less food but a similar

213 amount of water (Table 2). Despite the lower food intake, the mean energy intake, feed
214 efficiency and the increases in body weight were higher in H rats than in C rats (Table 2).
215 Chronic H diet feeding for 16 weeks increased abdominal circumference, body mass index,
216 total body fat mass and the individual abdominal fat pads, and increased the visceral
217 adiposity index (Table 2). No change in total body lean mass was measured (Table 2). The
218 bone mineral content was higher in H rats compared to C rats (Table 2).

Table 1. *Chokeberry juice and purple maize flour analysis*

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

Values are represented as mean of duplicate assays.

[#] Analysed by authors.

[$] Total anthocyanins calculated as cyanidin 3-glucoside.

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

220

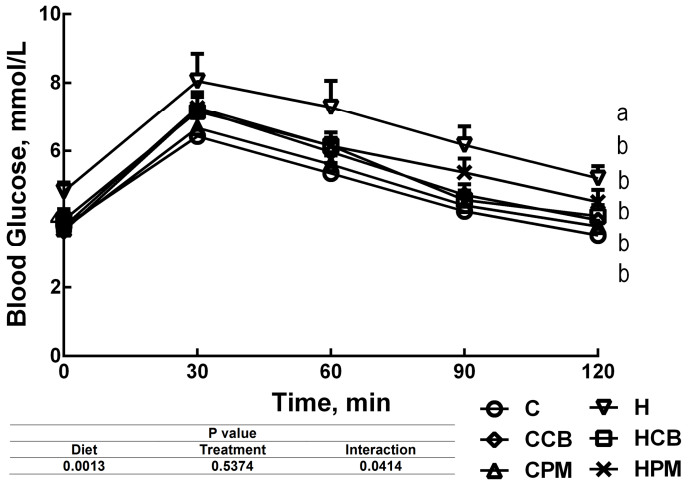
221 Treatment with either CB or PM for 8 weeks, starting at 8 weeks of the feeding
222 period, did not change food or water intake (Table 2). Compared to controls, CB treatment
223 groups had similar energy intake while PM treatment groups had an increased energy intake.
224 Lower feed conversion efficiency and body weight gain were observed in HCB and HPM rats
225 (Table 2). Both treatments decreased total body fat mass, except CPM rats had higher total
226 body fat mass compared to C rats (Table 2). Abdominal fat (retroperitoneal, epididymal and
227 omental fat pads), body mass index and visceral adiposity index decreased in both HCB and

HPM rats (Table 2). Total lean mass was unchanged in both HCB and HPM rats, and HCB rats had decreased bone mineral content compared to H rats (Table 2).

Plasma biochemistry and glucose handling

Plasma concentrations of total cholesterol, triglycerides and non-esterified fatty acids (NEFA) were increased in H rats compared to C rats (Table 2). HCB and HPM rats showed decreased plasma lipid concentrations, compared to H rats. However, HPM rats had increased concentrations of triglycerides and NEFA than HCB rats (Table 2). Plasma leptin concentrations were doubled in H rats compared to C rats. HCB and HPM showed normalised leptin concentrations, consistent with the changes in total fat mass and abdominal fat pads (Table 2).

H rats had increased fasting blood glucose concentrations compared to C rats; HCB and HPM rats showed similar concentrations to C rats (Table 2). The plasma glucose response to oral glucose loading was greater in H rats than C rats (Figure 1). At 120 min, HCB and HPM rats along with C rats had lower plasma glucose concentrations compared to H rats (Figure 1). Plasma insulin concentrations almost doubled in H rats compared to C, HCB and HPM rats (Table 2). This change is consistent with glucose tolerance area under the curve (Table 2); similarly, HCB and HPM rats had improved glucose clearance compared to H rats (Table 2).



246

247 ***Figure 1.*** *Effect of CB and PM on oral glucose tolerance in C, CCB, CPM, H, HCB and*
248 *HPM rats. Data are shown as mean ± SEM. End-point means without a common letter in*
249 *each data set significantly differ, P<0.05 and n=10/group.*
250

251 *Cardiovascular structure and function*

252 Compared to C rats, H rats showed eccentric hypertrophy measured as increased left
253 ventricular internal diameter in diastole (LVIDd) without any changes in relative wall
254 thickness or end systolic dimensions (Table 3). H rats showed impaired systolic function with
255 decreased fractional shortening and increased wall stress compared to C rats (Table 3).
256 However, ejection time and ejection fraction were not affected (Table 3). Diastolic and stroke
257 volumes and consequently the cardiac output were increased in H rats compared to C rats.
258 These effects were seen with increased heart rate in H rats compared to C rats (Table 3).
259 These changes in H rats were accompanied by increased LV wet weight and elevated systolic
260 blood pressure (Table 3).

261 Both CB and PM treatment improved LV function by decreasing LVIDd and normalising
262 developed pressure (Table 3). Ejection time, ejection fraction, fractional shortening and
263 LVIDs were unaffected in both treatment groups (Table 3). Systolic wall stress, cardiac
264 output, diastolic and stroke volumes and heart rate were normalised with CB and PM

265 treatments. However, systolic volumes were elevated in both HCB and HPM rats with no
266 change in relative wall thickness (Table 3). In the isolated Langendorff heart, LV stiffness
267 was increased while LV dP/dt was decreased in H rats; these changes were normalised in
268 HCB and HPM. These effects were accompanied by decreased LV wet weight and systolic
269 blood pressure in both HCB and HPM rats (Table 3).

270 Histological evaluation of the left ventricle after 16 weeks showed greater infiltration
271 of inflammatory cells into the LV with H diet feeding (Figure 2D), as well as increased
272 interstitial collagen deposition (Figure 2J) compared to C rats (Figure 2A and G). HCB and
273 HPM rats showed normalised inflammatory cell numbers (Figure 2E and F) and markedly
274 reduced ventricular collagen deposition (Figure 2K and L). The reduction in LV fibrosis and
275 inflammation was consistent with the reduced diastolic stiffness in HCB and HPM rats (Table
276 3), while CCB (Figure 2B and H) and CPM (Figure 2C and I) rats showed minimal changes.
277 No other changes were observed and tissue morphology appeared normal.

278

279 ***Table 2.*** *Dietary intakes, body composition and anthropometrics, organ wet weights, changes in glucose tolerance test, plasma insulin and*
280 *plasma biochemistry in C, CCB, CPM, H, HCB and HPM diet-fed rats (n=10 rats/group)*

Variable	C	CCB	CPM	H	HCB	HPM	P values		
							Diet	Treatment	Interaction
Food intake (g/d)	32.9±1.1^{a}	33.2±0.6^{a}	34.1±0.4^{a}	27.1±0.8^{b}	26.4±0.8^{b}	28.5±0.7^{b}	<0.0001	0.11	0.7
Water intake (ml/d)	27.4±2.1	24.3±1.5	27.2±1.8	26.6±1.4	26.5±1.6	29.7±1.5	0.34	0.2	0.55
Chokeberry juice intake (ml/d)	0.0±0.0	1.7±0.0^{a}	0.0±0.0	0.0±0.0	1.4±0.0^{b}	0.0±0.0	<0.0001	<0.0001	<0.0001
Purple maize powder intake (g/d)	0.0±0.0	0.0±0.0	1.8±0.0^{a}	0.0±0.0	0.0±0.0	1.5±0.0^{b}	<0.0001	<0.0001	<0.0001
Anthocyanins intake (mg/kg/d)	0.0±0.0	9.4±0.0^{a}	9.1±0.0^{a}	0.0±0.0	7.8±0.0^{b}	7.4±0.0^{b}	<0.0001	<0.0001	<0.0001
Energy intake (kJ/d)	369±12^{d}	378±7^{d}	409±5^{c}	580±17^{b}	570±19^{b}	640±17^{a}	<0.0001	0.0005	0.39
Feed conversion efficiency (%)	2.4±0.3^{b}	2.1±0.3^{b}	2.9±0.3^{b}	7.1±0.9^{a}	4.4±1.3^{b}	5.5±0.9^{ab}	<0.0001	0.16	0.25
Body weight gained (8-16 weeks) (%)	8.2±1.4^{b}	7.7±1.4^{b}	8.3±1.3^{b}	21.6±2.6^{a}	9.9±2.8^{b}	10.8±1.6^{b}	0.0004	0.0051	0.0075
Visceral adiposity index (%)	4.9±0.4^{b}	4.4±0.3^{b}	4.8±0.2^{b}	8.9±0.9^{a}	6.1±0.5^{b}	6.0±0.3^{b}	<0.0001	0.0021	0.0137
Abdominal circumference (cm)	19.2±0.1^{c}	18.2±0.2^{d}	19.1±0.1^{cd}	22.3±0.3^{a}	19.7±0.4^{c}	20.7±0.2^{b}	<0.0001	<0.0001	0.0022
Body mass index (kg/m^{2})	5.0±0.2^{d}	4.8±0.1^{d}	4.7±0.1^{d}	7.3±0.2^{a}	5.7±0.1^{c}	6.4±0.2^{b}	<0.0001	<0.0001	0.0002
Bone mineral content (g)	11.3±0.3^{c}	11.4±0.3^{c}	11.5±0.3^{c}	15.8±0.4^{a}	13.5±0.5^{b}	14.8±0.4^{a}	<0.0001	0.0166	0.009
Total body lean mass (g)	309±12	303±12	307±11	311±14	305±11	304±8	0.98	0.87	0.98
Total body fat mass (g)	74.3±7.6^{d}	94.4±8.4^{d}	113.7±8.5^{cd}	224.6±12.6^{a}	144.4±17.2^{c}	177.3±10.4^{b}	<0.0001	0.0203	<0.0001
Tissue wet weight (mg/mm)									
Retroperitoneal adipose tissue	179.7±15.3^{c}	141.9±13.7^{c}	173.0±12.6^{c}	521.7±45.6^{a}	279.2±38.2^{b}	311.8±16.7^{b}	<0.0001	<0.0001	0.0003
Epididymal adipose tissue	93.4±7.9^{c}	83.3±6.9^{c}	95.4±5.2^{c}	278.5±22.5^{a}	155.4±15.2^{b}	156.8±11.7^{b}	<0.0001	<0.0001	<0.0001
Omental adipose tissue	106.6±8.1^{d}	91.8±8.0^{d}	101.1±5.5^{d}	261.1±21.3^{a}	141.0±12.3^{cd}	193.1±9.9^{b}	<0.0001	<0.0001	0.0002
Liver	214.2±6.7^{c}	188.8±6.2^{c}	204.1±5.5^{c}	319.6±8.9^{a}	250.7±11.1^{b}	268.6±7.0^{b}	<0.0001	<0.0001	0.0111
Glucose metabolism and plasma biochemistry									
Fasting blood glucose (mmol/L)	3.8±0.2	3.7±0.2	3.7±0.3	4.8±0.3	3.9±0.3	3.8±0.3	0.0321	0.17	0.08
OGTT-AUC (mmol/L min)	591±12^{d}	645±9^{c}	613±6^{d}	786±18^{a}	658±7^{c}	694±6^{b}	<0.0001	0.001	<0.0001
Plasma insulin (μmol/L)	1.4±0.3^{b}	1.1±0.2^{b}	1.7±0.3^{b}	4.1±0.5^{a}	2.3±0.4^{b}	2.6±0.6^{b}	<0.0001	0.042	0.07
Plasma leptin (μmol/L)	5.3±0.7^{b}	4.9±0.6^{b}	6.1±0.5^{b}	11.1±0.9^{a}	7.0±1.5^{b}	7.9±1.0^{b}	<0.0001	0.06	0.06
ALP (U/L)	181±12^{c}	214±16^{c}	166±12^{c}	312±18^{a}	252±20^{bc}	265±18^{bc}	<0.0001	0.17	0.0224

ALT (U/L)	29.6±2.1^{b}	28.1±2.2^{b}	29.7±1.5^{b}	43.2±2.8^{a}	34.6±3.9^{ab}	38.0±3.1^{ab}	<0.0001	0.19	0.4
AST (U/L)	60.4±1.9^{b}	60.9±2.7^{b}	64.0±1.6^{b}	83.5±3.1^{a}	63.1±7.9^{b}	64.6±2.9^{b}	0.0099	0.0383	0.0099
Total cholesterol (mmol/L)	1.5±0.2^{b}	1.5±0.1^{b}	1.6±0.1^{b}	2.2±0.0^{a}	1.6±0.1^{b}	1.6±0.0^{b}	0.0038	0.0166	0.0039
Triglycerides (mmol/L)	0.5±0.0^{c}	0.4±0.0^{c}	0.5±0.0^{c}	1.6±0.2^{a}	0.7±0.1^{c}	1.0±0.1^{b}	<0.0001	<0.0001	0.0005
NEFA (mmol/L)	1.3 ±0.2^{c}	1.2 ±0.1^{c}	1.4 ±0.1^{c}	3.4 ±0.2^{a}	1.8 ±0.4^{c}	2.3 ±0.3^{b}	<0.0001	0.0036	0.0073

Each value is a mean ± SEM. Means within a row with unlike superscripts differ, P<0.05.
* In all groups body-weight gained calculated as percentage of body weight increase from 8 weeks to 16 weeks. OGTT-AUC, oral glucose tolerance test-area under the curve; ALP, alkaline phosphatase; ALT, aspartate transaminase; AST, aspartate transaminase; NEFA, non-esterified fatty acids.

Insert Figure 2 here.

Figure 2. *Haematoxylin and eosin staining of the left ventricle (original magnification ×20) showing inflammatory cells (marked as "in") as dark spots outside the myocytes in C (A), CCB (B), CPM (C), H (D), HCB (E) and HPM (F) rats. Picrosirius red staining of left-ventricular interstitial collagen deposition (original magnification ×20) in rats fed the C (G), CCB (H), CPM (I), H (J), HCB (K) and HPM (L) diet. Collagen deposition is marked as "cd" and hypertrophied cardiomyocytes are marked as "hy".*

Table 3. *Changes in cardiovascular structure and function in C, CCB, CPM, H, HCB and HPM diet-fed rats (n=10-8 rats/group)*

							P values		
							Diet	**Treatment**	**Interaction**
Variable	**C**	**CCB**	**CPM**	**H**	**HCB**	**HPM**			
Heart rate (bpm)	277±18^{b}	246±9^{b}	256±15^{b}	335±16^{a}	243±9^{b}	265±11^{b}	0.06	0.0001	0.06
IVSd (mm)	1.9±0.1^{ab}	1.8±0.0^{b}	1.9±0.0^{ab}	2.1±0.1^{a}	1.9±0.1^{ab}	1.9±0.0^{ab}	0.09	0.11	0.38
LVIDd (mm)	6.4±0.2^{b}	6.7±0.3^{b}	7.0±0.1^{b}	7.9±0.3^{a}	7.1±0.2^{b}	7.2±0.2^{b}	0.0005	0.51	0.0139
LVPWd (mm)	1.8±0.1^{b}	1.7±0.0^{b}	1.8±0.0^{b}	2.1±0.0^{a}	1.9±0.1^{b}	1.9±0.0^{b}	0.0001	0.0393	0.23
IVSs (mm)	3.2±0.2^{b}	3.0±0.1^{b}	2.9±0.0^{b}	3.8±0.1^{a}	3.2±0.1^{b}	3.3±0.1^{b}	<0.0001	0.0022	0.42
LVIDs (mm)	3.7±0.2	4.0±0.2	3.7±0.2	4.5±0.2	3.7±0.3	4.3±0.3	0.07	0.58	0.06
LVPWs (mm)	2.9±0.1^{b}	2.6±0.1^{b}	2.7±0.1^{b}	3.4±0.1^{a}	3.2±0.1^{ab}	3.0±0.1^{ab}	<0.0001	0.0099	0.32

Fractional shortening (%)	50.9±2.1^{a}	53.7±0.8^{a}	53.4±0.9^{a}	47.5±2.1^{ab}	58.0±1.2^{a}	55.7±1.4^{a}	0.39	0.0002	0.0392
Ejection time (ms)	79.6±2.3	93.3±3.4^{ab}	86.0±2.7	92.8±2.9^{ab}	86.4±3.9	95.0±3.0^{ab}	0.0486	0.33	0.0053
Ejection fraction (%)	87.3±1.4	83.3±1.3	84.6±1.6	89.0±2.2	84.1±4.0	83.6±2.9	0.8	0.14	0.85
Diastolic volume (µL)	353±34^{b}	365±33^{b}	364±22^{b}	515±39^{a}	386±28^{b}	395±37^{b}	0.01	0.15	0.07
Systolic volume (µL)	45±10^{b}	57±8^{a}	56±8^{a}	90±10^{a}	65±23^{a}	63±12^{a}	0.0203	0.72	0.11
Stroke volume (µL)	268±18^{b}	298±20^{b}	307±18^{b}	425±29^{a}	306±26^{b}	322±19^{b}	0.0018	0.13	0.002
Cardiac output (mL/min)	92.3±11.2^{b}	70.4±13.6^{b}	78.6±6.6^{b}	144.8±21.7^{a}	93.0±7.7^{b}	100.3±10.7^{b}	0.0038	0.0165	0.41
LV developed pressure (mmHg)	69.6±3.5^{a}	71.4±4.1^{a}	64.8±5.6^{a}	43.7±3.6^{b}	63.7±5.7^{a}	60.2±3.8^{a}	0.001	0.06	0.0444
(+)dP/dt (mmHg/S)	1147±61^{a}	1285±53^{a}	1195±65^{a}	784±66^{c}	1089±63^{a}	1002±76^{ab}	<0.0001	0.0044	0.33
(-)dP/dt (mmHg/S)	-782±51^{a}	-804±49^{a}	-795±43^{a}	-489±57^{b}	-700±44^{a}	-711±52^{a}	0.0002	0.0316	0.08
Diastolic stiffness (k)	22.9±0.8^{b}	22.3±0.4^{b}	23.1±0.6^{b}	28.6±0.6^{a}	23.9±0.7^{b}	24.2±0.5^{b}	<0.0001	0.0002	0.0006
Estimated LV mass, Litwin (g)	0.93±0.06^{b}	0.79±0.08^{b}	0.88±0.02^{b}	1.14±0.05^{a}	1.01±0.06^{ab}	1.10±0.04^{ab}	<0.0001	0.05	0.99
LV+septum wet weight (mg/mm tibial length)	16.1±0.5^{c}	15.3±0.5^{c}	15.0±0.5^{c}	19.5±0.8^{a}	16.9±0.8^{c}	18.5±0.5^{b}	<0.0001	0.0268	0.23
Right ventricle wet weight (mg/mm tibial length)	3.8±0.2	3.8±0.2	4.0±0.2	4.4±0.2	4.3±0.3	5.0±0.4^{a}	0.0018	0.18	0.6
Relative wall thickness	0.50±0.03	0.57±0.09	0.53±0.02	0.56±0.03	0.51±0.01	0.48±0.01	0.63	0.69	0.29
Systolic blood pressure (mmHg)	130±2^{b}	130±3^{b}	132±2^{b}	152±2^{a}	120±1^{b}	134±3^{b}	<0.0001	<0.0001	<0.0001
Systolic wall stress (mmHg)	83.0±4.0^{b}	79.1±4.3^{b}	75.3±11.8^{b}	119.6±7.6^{a}	76.8±7.5^{b}	89.5±9.7^{b}	0.0171	0.0129	0.06

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

H diet feeding diminished α_1-adrenoceptor-mediated vascular contraction to noradrenaline (Figure 3A), endothelium-independent relaxation to sodium nitroprusside (Figure 3B) and endothelium-dependent relaxation to acetylcholine (Figure 3C) in isolated thoracic aortic rings compared to C rats. Isolated thoracic aortic rings from HCB and HPM rats showed increased responses to noradrenaline (Figure 3A), sodium nitroprusside (Figure 3B) and acetylcholine (Figure. 3C).

Insert Figure 3 here.

Figure 3. *Cumulative concentration-response curves for noradrenaline (A), sodium nitroprusside (B) and acetylcholine (C) in thoracic aortic rings from C, CCB, CPM, H, HCB and HPM rats. Data are shown as mean ± SEM. End-point means without a common letter in each data set significantly differ, P<0.05 and n=10/group.*

Liver structure and function

Plasma alkaline phosphatase (ALP), alanine transaminase (ALT) and aspartate transaminase (AST) activities were increased in H rats compared to C rats, indicating liver damage (Table 2). HCB and HPM showed lowered but not normalised ALP, ALT and AST activities (Table 2). H rats showed increased inflammatory cell infiltration and lipid deposition as fat vacuoles in the liver (Figure 4D) compared to C rats (Figure 4A). In HCB and HPM rats, macrovesicular steatosis and portal inflammation were decreased compared to H rats (Figure 4E and F). Livers from CCB and CPM rats showed normal tissue architecture (Figure 4B and C).

Insert Figure 4 here

Figure 4. Haematoxylin and eosin staining of hepatocytes (original magnification ×20) showing inflammatory cells (marked as “in”) and hepatocytes with fat vacuoles (marked as “fv”) in C (A), CCB (B), CPM (C), H (D), HCB (E) and HPM (F) rats.

Discussion

Rats fed with H diet developed abdominal obesity, hypertension with endothelial dysfunction, cardiac fibrosis together with increased left-ventricular stiffness, dyslipidaemia, inflammation in heart and liver, increased plasma liver enzyme concentrations and impaired glucose tolerance [23]. Excess fat deposition in the abdomen increases chronic low-grade inflammation, oxidative stress, dyslipidaemia, non-alcoholic fatty liver disease, cardiovascular diseases, type 2 diabetes and insulin resistance [27-32]. In this study, we showed that intervention with the same dose of anthocyanins as cyanidins from either chokeberry or purple maize attenuated the metabolic, cardiovascular and liver changes in rats fed a H diet. We suggest that these improvements derive from decreased inflammatory cell infiltration into the tissues.

Recent studies suggest that modification of the gut microbiome by anthocyanins could be important in mediating the reduced inflammation throughout the body. Anthocyanins are extensively metabolised by gut microbiota to protocatechuic acid [33, 34], one of a group of phenolic acids produced by gut bacteria that may act as potential systemic bioactive compounds to produce the positive responses to anthocyanins [35, 36]. Further, treatment diet-induced obesity in mice with anthocyanins decreased intestinal inflammation and increased gut bacteria especially *Akkermansia* spp. [37]. Increased *Bifidobacteria* in faeces together with increased urinary concentrations of anthocyanin metabolites confirm the important role of anthocyanins as bacterial substrates [38]. Moreover, anthocyanins may act like prebiotics to increase the growth of beneficial gut bacteria [35]. Thus, anthocyanins may improve gut health, possibly decreasing the access of bacterial components into the body, and also by producing metabolites that improve health when absorbed. These gastrointestinal responses may decrease the systemic inflammatory stimulus that increases lipid uptake by

adipocytes and so trigger further changes in the body, especially modifying the production and release of adipokines.

Abdominal adipose tissue is a dynamic organ producing adipokines that have pro-inflammatory or anti-inflammatory responses. Dysregulated production of these adipokines leads to obesity and also induces low-grade inflammation and insulin resistance [39]. In the current study, plasma insulin concentrations were normalised in rats fed chokeberry and purple maize, consistent with a previous study with the same dose of cyanidin 3-glucoside [12] and in HFD-fed mice treated with purified mulberry anthocyanins [40]. In our diet-induced obese rats, plasma leptin concentrations were increased; further, treatment with either chokeberry or purple maize reduced both plasma leptin concentrations and abdominal fat mass. Similar results were shown with chokeberry extract [17], cyanidin 3-glucoside and Queen Garnet plums [12]. All these interventions contain cyanidin 3-glucoside as the major anthocyanin and this compound is the likely bioactive component. Since leptin is pro-inflammatory, reduced leptin concentrations should reduce inflammation throughout the body, as we have shown in the heart and liver. Thus, we suggest that normalisation of adipokine production may be the key systemic change as fat pads reduce in response to changes by the gut microbiota, thus further reducing inflammation throughout the body and also reducing organ damage. Our results showing fat pad reduction following anthocyanin intervention are consistent with studies with purple maize extract in C57BL/6J mice [20]. Further, KK-*Ay* mice treated with anthocyanins showed similar changes together with decreases in mean diameter of the visceral and subcutaneous adipocytes, suggesting that anthocyanin supplementation inhibits lipid accumulation [41].

Our study suggests that anthocyanin treatment decreased triglycerides and non-esterified free fatty acids which attenuated the liver steatosis. Cyanidin 3-glucoside showed increased phosphorylated AMP-activated protein kinase (pAMPK) and decreased lipoprotein

lipase activity in skeletal muscle and adipocytes [41]. In addition, purple sweet potato treatment in diet-induced obese mice and HepG2 hepatocytes showed similar phosphorylation of AMPK [42]. AMP-activated protein kinase (AMPK) regulates and monitors cellular energy balance [43] and pAMPK stimulates free fatty acid oxidation via activation of acetyl coenzyme–A carboxylase in skeletal muscle [44] and regulates lipolysis and lipogenesis by converting adipocytes into lipid oxidising cells [45]. Anthocyanins also down-regulated lipid metabolism proteins, sterol regulatory element-binding protein-1c and fatty acid synthase via AMPK inhibitor compound C [42, 46]. Additionally, in C57BL/KsJ db/db mice treated with purple maize extract also increased pAMPK and decreased phosphoenolpyruvate carboxykinase and glucose 6-phosphatase gene expression in liver and glucose transporter 4 expression in skeletal muscle [47]. Therefore, these changes may decrease adipose storage leading to decrease in body weight gain and improved liver function and glucose metabolism.

Cardiovascular structure and function was improved by anthocyanins in the CB and PM interventions together with decreased plasma concentrations of non-esterified free fatty acids (NEFA). Increased plasma NEFA inhibited aortic endothelia nitric oxide synthase via oxidative mechanism and caused hypertension [48]. In subjects with metabolic syndrome, supplementation with CB extract and PM extract powder decreased blood pressure and plasma lipids [16, 22]. Similarly, many epidemiological studies suggest that increased dietary intake of anthocyanin-containing strawberries, blueberries and moderate intake of red wine is associated with a reduction in cardiovascular disease [9]. However, there is no direct evidence that anthocyanins are helpful in decreasing cardiovascular disease in humans. In cultured bovine artery endothelial cells, cyanidin 3-glucoside increased the expression of endothelial nitric oxide synthase (eNOS) [49]. Increased expression of eNOS enhanced nitric oxide release to improve endothelial function [50]. Similarly, chokeberry juice treatment

showed improved endothelial function in porcine coronary arteries by redox-sensitive activation [51]. Anthocyanin treatment with CB and PM also showed consistent improvement in endothelial function and decreased blood pressure.

In this study, we have treated rats either with 3.2 mg/kg BW of CB juice or 3.1 mg/kg BW of PM powder, corresponding to ~46 ml/day CB or ~50 g/day PM to achieve a dose of ~110 mg of anthocyanins in a 70 kg human, based on body surface area comparisons between rats and humans [52]. Similar results to the same dose of anthocyanins either from CB and PM indicate that anthocyanins are the major bioactive compound in CB and PM in reversing or attenuating the signs of metabolic syndrome.

Conclusion

The effects of CB and PM in a diet-induced rat model of human metabolic syndrome are consistent with the reported effects of anthocyanins as they are the only polyphenolic compounds present in sufficient dose in both the treatments. These findings suggest that anthocyanin interventions using chokeberry or purple maize might be beneficial in attenuating obesity and metabolic syndrome in humans. However, further investigations on anthocyanin-containing foods are necessary to understand the mechanism of action, especially to determine the changes in the gut microbiota. Similar responses in CB- and PM-treated rats suggest a clinical trial corresponding to the same dose as in this study is necessary to determine if these positive effects can be translated to humans with metabolic syndrome.

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Author contributions

M.B. and L.B. developed the original study aims and analysed and interpreted the data; M.B. and S.R.S conducted the experiments. M.M. provided nutritional advice in the design of the study. P.M. assisted in HPLC techniques. M.B. 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. All authors have read and approved the final manuscript.

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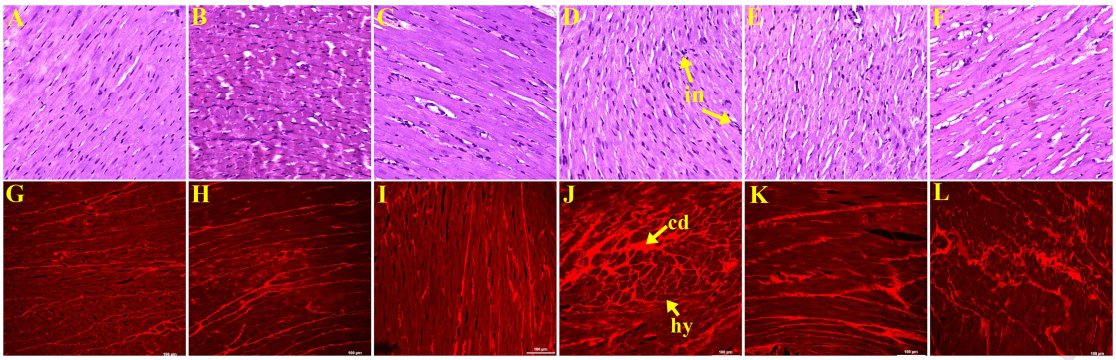


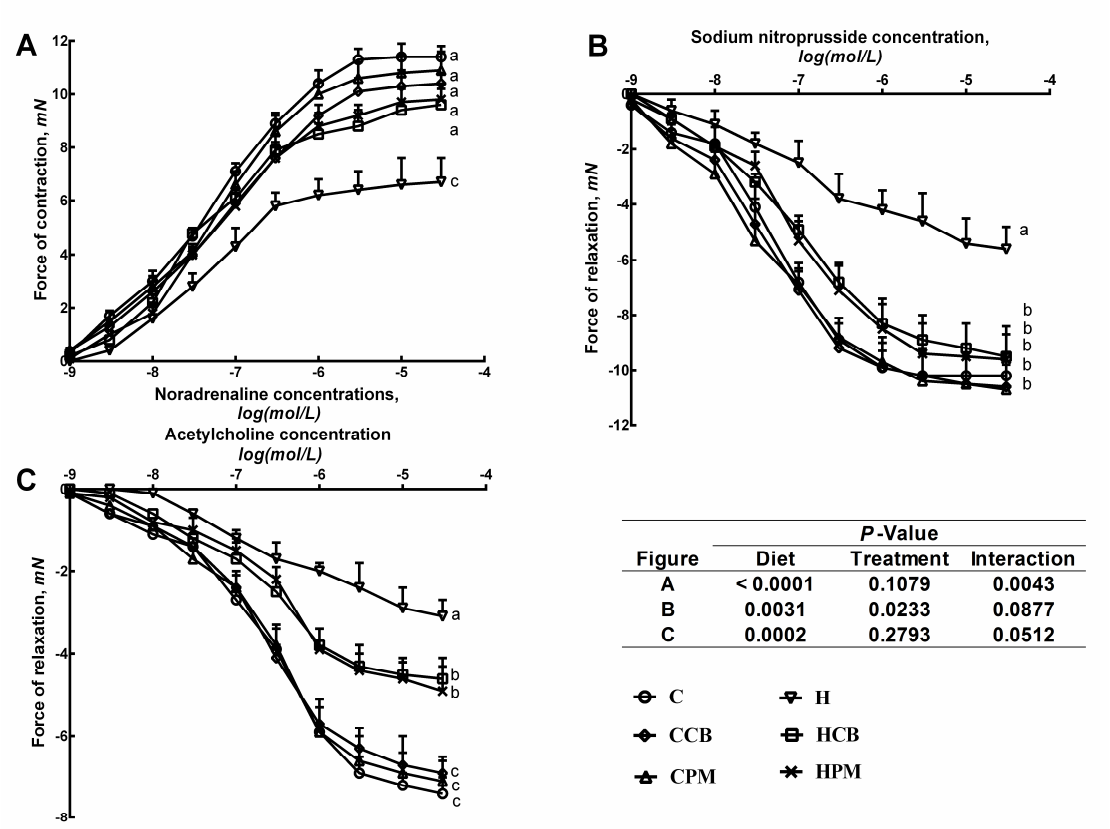
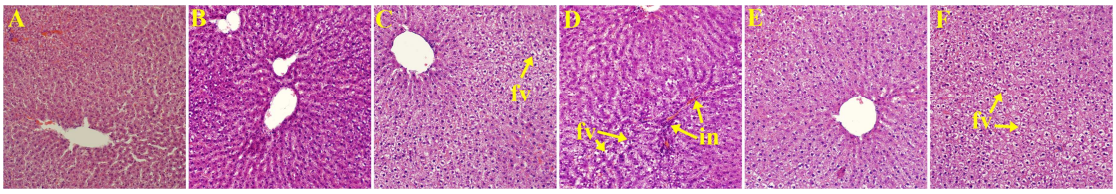


Figure	*P*-Value		
	Diet	Treatment	Interaction
A	< 0.0001	0.1079	0.0043
B	0.0031	0.0233	0.0877
C	0.0002	0.2793	0.0512



Highlights

- Chokeberry and purple maize are sources of cyanidin 3-glucoside, an anthocyanin
- Both interventions reverse diet-induced symptoms of metabolic syndrome in rats
- The mechanism is likely to be prevention of infiltration of inflammatory cells