UNIVERSITY OF SOUTHERN QUEENSLAND

TESTOSTERONE DEFICIENCY AND DIETARY THERAPEUTIC INTERVENTIONS IN THE RAT MODEL OF DIET-INDUCED METABOLIC SYNDROME

A Dissertation submitted by

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Abstract

Male Wistar rats fed with high carbohydrate (68%) and high fat (24%) diet (H) mimicking "Western diets" showed enhanced visceral obesity and its associated risk factors of metabolic syndrome such as impaired glucose utilisation, insulin resistance, hypertension, cardiovascular stiffness, collagen deposition, endothelial dysfunction, inflammation of heart and liver, dyslipidaemia and non-alcoholic fatty liver disease compared to low fat (8%) cornstarch diet (C) fed rats. Lowered total testosterone concentrations (2.5-5.8 ng/mL) in castrated rats induced by surgical or chemical castration (leuprolide acetate 2mg/kg body weight every four weeks), showed increased abdominal obesity and its associated metabolic risk factors in response to H diet; thus, testosterone plays a crucial role in regulating abdominal obesity associated metabolic disorders in men.

Current treatments for obesity are aimed at modifying dietary patterns, eating habits and proper workout or physical exercise to increase calorie expenditure and lowering calorie intake. I have investigated the therapeutic effects of seaweeds (*Ulva ohnoi, Derbesia tenuissima*), microalgae mixture (*Scenedesmus dimorphus* and *Schroederiella apiculata*), mineral ions (Mg^{++}, K^{+}) and dietary fibre (inulin and oligofructose) as food-based interventions in rats fed H diet. *Ulva ohnoi* and *Derbesia tenuissima* attenuated the metabolic symptoms observed in H fed rats; both seaweeds are rich in dietary polysaccharides (23.4-40.9% of dry algae). Compared to *Derbesia tenuissima*, *Ulva ohnoi* contained higher amount of soluble polysaccharides (18.1% vs <0.1 % of dry algae) and lowered total body fat mass by 24% which may be attributed to the inhibition of intestinal absorption of fatty acids. In addition higher magnesium content of UO (4.1% vs 1.3% of dry algae) suggested improving the glucose utilisation. Further, insoluble polysaccharides (19.6% of dry algae) supplementation of microalgae mixture showed increased lean mass and attenuated the visceral adiposity-induced metabolic syndrome in H fed rats, which may be due to the faecal bulking effect associated with increased hepatic and skeletal muscle β-oxidation of fatty acids.

Magnesium treatment specifically increased the faecal lipid excretion by 4.5% suggesting the ability of this divalent cation to form insoluble salt complexes with fatty acids and prevent their intestinal absorption as potential mechanism for reduction in abdominal (53%) and total body fat mass (44%) in H diet fed rats showed a complete reversal of metabolic syndrome. Treatment with 2% potassium chloride in H diet fed rats showed no significant effect on obesity but attenuated the H diet-induced metabolic risk factors which may be due to the increased (38%) circulating concentrations of magnesium. Increased faecal load (1.6 g/12h) and lipid excretion (5.4%) detected with 5% soluble fibre (inulin and oligofructose) mixture supplementation serves as the possible potential mechanism in preventing the abdominal obesity and its associated metabolic syndrome in the H diet fed metabolically sick obese rats. Thus, functional foods including seaweeds and microalgae that are rich in important mineral ions (magnesium and potassium) and fibre (dietary polysaccharides) may attenuate obesity-linked metabolic syndrome.

CERTIFICATION OF DISSERTATION

I certify that the ideas, experimental work, results, analyses, software, discussions and conclusions reported in this dissertation are entirely my own effort, except where otherwise acknowledged. I also certify that the work is original and has not been previously submitted or published for any other award, except where otherwise acknowledged.

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Signature of candidate Date

ENDORSEMENT

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Signature of Principal Supervisor Date

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Signature of Associate Supervisor Date

Contributions to thesis chapters as publications

*****Kumar SA, Brown L. Seaweeds as potential therapeutic interventions for the metabolic syndrome. Reviews in Endocrine and Metabolic Disorders 2013;14:299-308.

SAK was responsible for 60% of conception and design, 80% of analysis and review of literature, 30% of drafting and writing and 30% of final editorial input; Brown L was responsible for 40% of conception and design, 20% of analysis and review of literature, 70% of drafting and writing and 70% of final editorial input.

*****Kumar SA, Kauter K, Ward L and Brown L. Effect of low testosterone in a high-carbohydrate, high-fat diet induced rat model of metabolic syndrome. American Journal of Physiology Endocrinology and Metabolism.

SAK was responsible for 40% of conception and design, 85% collection of data, 70% of analysis and interpretation of literature, 60% of drafting and writing; KK was responsible 20% of conception and design, 5% of collection of data, 5% of analysis and interpretation of literature, 5% of drafting and writing; LW was responsible for 10% of collection of data, 5% of drafting and writing, 5% of analysis and interpretation of literature; LB was responsible for 30% of conception and design, 20% of analysis and interpretation of literature, 30% of drafting and writing.

*****Kumar SA, Magnusson M, Ward L, Paul N, Brown L. Seaweed supplements normalise metabolic, cardiovascular and liver responses in high-carbohydrate, high-fat fed rats. Marine Drugs 2014;12:doi:10.3390/md120x000x.

SAK was responsible for 25% of conception and design, 70% of collection of data, 30% of analysis and interpretation of literature, 30% of drafting and writing and 20% of final important editorial input; MM was responsible for 10% for conception and design, 20% of collection of data, 15% of analysis and interpretation of literature, 10% of drafting and writing and 20% of final editorial input; LW was responsible for 5% of collection of data; 5% of analysis and interpretation of literature, 5% of drafting and writing and 5% of final important editorial input; NP was responsible for 30% of conception and design, 5% of collection of data, 20% of analysis and interpretation of literature, 20% of drafting and writing and 25% of final important editorial input; LB, 35% of conception and design, 30% of analysis and interpretation of literature, 35% of drafting and writing and 30% of final important editorial input.

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SAK was responsible for 60% of conception and design, 90% of collection of data, 65% of analysis and interpretation of literature, 50% of drafting and writing; LCW was responsible for 10% of conception and design, 10% of collection of data, 15% of analysis and interpretation of literature, 20% of drafting and writing; LB was responsible for 30% of conception and design, 20% of analysis and interpretation of literature, 30% of drafting and writing.

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*****Poudyal H, Kumar SA, Iyer A, Waanders J, Ward L, Brown L. Responses to oleic, linoleic and α-linolenic acids in high-carbohydrate, high-fat diet induced metabolic syndrome in rats. Journal of Nutritional Biochemistry 2013;24:1381-1392.

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CHAPTER 1

CHAPTER 1.1

Introduction

1 Introduction

1.1 Obesity and metabolic syndrome

Metabolic syndrome is a cluster of metabolic disorders associated with obesity, cardiovascular disease, insulin resistance, type 2 diabetes, dyslipidaemia, fatty liver and hypertension (Osmond et al., 2009, Di Chiara et al., 2011, Reaven, 2011). Adipose tissue inflammation and hypertrophy (increased adipocyte size) cause visceral adiposity which promotes metabolic syndrome (Bjorndal et al., 2011). Obesity associated with dyslipidaemia, chronic inflammation, type 2 diabetes and hypertension serves as the major risk factor for cardiovascular disease (Bjorndal et al., 2011, Panchal et al., 2011b). Obesity prevalence in male and female adults of both developing and developed countries is increasing worldwide, varying between 3-79% to serve as the major risk factor for metabolic syndrome (Popkin and Doak, 1998, Prentice, 2006, Misra and Khurana, 2008).

1.2 Obesity prevalence and life-style

A high-carbohydrate and high-fat diet plays an important role in inducing obesity and its associated disorders (Ludwig, 2002). Obesity arises when the energy intake, principally stored as triglycerides, exceeds the energy expenditure (Spiegelman and Flier, 2001). Obesity is influenced by several factors such as diet, age, physical activity and genes (Friedman, 2003). The occurrence of obesity is quite common in modern societies; in the United States, nearly 68% of adults are either obese or overweight (Flegal et al., 2010). It has been estimated that by 2030, 86.3% adults in the U.S. population will be overweight or obese (Wang et al., 2008). The prevalence of obesity is increasing mainly due to consumption of diets rich in refined carbohydrates and omega-6 fatty acids (Simopoulos, 2011, Drewnowski and Specter, 2004). Further, the prevalence of obesity in developing countries has also increased due to the adoption of modern culture and changed life-styles (Prentice, 2006). In mammals, white adipose tissue functions as the major fat depot, where the fats are converted and stored in the form of triglycerides. White adipose tissue secretes signalling molecules, including proteins such as leptin, adiponectin, resistin and adipsin, that play a crucial role in lipid and glucose metabolism (Fruhbeck, 2008). Adipose tissue also serves as the key regulator of pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), nuclear transcription factor-kappa B (NF-κB), interleukin-6 (IL-6) and C-reactive protein (CRP).

1.3 Obesity and testosterone deprivation

Testosterone regulates fat mass by inhibiting adipogenesis, lipoprotein lipase activity and also by promoting lipolysis via increasing beta-adrenergic receptors and decreasing fatty acid synthesis (Marin et al., 1992, De Pergola, 2000, Singh et al., 2003). An *in vitro* study using 3T3-L1 adipocytes observed that testosterone (10nM, 30nM and 100nM) dose-dependently inhibited adipocyte differentiation by down-regulating the expression of both mRNA and protein of the key adipogenic transcription markers, CCAAT/enhancer binding protein-α &-ð (C/EBP-α and -ð) and peroxisome proliferator activated receptor-γ (PPAR-γ) (Singh et al., 2006). Similarly, another *in vitro* study in mouse pluripotent stem cells revealed that testosterone primarily promoted the differentiation of mesenchymal cells into the myogenic lineage and inhibited

their differentiation into the adipogenic lineage, and henceforth regulated both the muscle and fat mass in men (Singh et al., 2003). Twelve months observational study in prostate cancer patients (n=26) undergoing androgen deprivation therapy showed decreased serum concentrations of total testosterone (from baseline 12.8nmol/L to 0.7 nmol/L) with increased abdominal fat mass (22%) and subcutaneous abdominal fat area (13%) (Hamilton et al., 2011). Androgen deprivation therapy in prostate cancer patients by gonadotrophin releasing hormone (GnRH) agonist leuprolide acetate primarily increased the subcutaneous fat deposition followed by abdominal fat deposition producing an increase in body fat mass of 4.3-9.4 % (Smith et al., 2002, Smith et al., 2008). Furthermore, testosterone depletion in men treated with leuprolide acetate showed a decreased lean mass by 2.7% and muscle size by 3.2% (Smith et al., 2002). Central adiposity reduced testosterone concentrations by increased aromatase activity in the adipose tissue, converting more testosterone to oestrogen resulting in high concentrations of oestrogens in obese men (Vermeulen et al., 2002, Hill et al., 2009). Further, this excess aromatase activity of the adipose tissue increased the production of oestradiol, resulting in the inhibition of the gonadotrophin-mediated testosterone secretion ultimately leading to type 2 diabetes and metabolic syndrome (Hayes et al., 2001, Hill et al., 2009).

1.3.1 Testosterone deficiency and type 2 diabetes

Testosterone enhances insulin sensitivity in both fat cells and muscle cells via stimulating the insulin signalling pathway (Zitzmann, 2009). Testosterone treatment improved fasting insulin sensitivity in men detected with heart failure (Malkin et al., 2007). Men with low plasma testosterone concentrations are more likely to be type 2 diabetics (Saad and Gooren, 2011). Also, an observational study in Japanese American men (n=110) observed that diabetic subjects with decreased total testosterone concentrations (from baseline 16.8 nmol/L to 15.6 nmol/L) showed increased intra-abdominal fat (50%) (Tsai et al., 2000). Cross-sectional studies showed that men with lower total testosterone concentrations (mean difference, -76.6ng/dL) were reported with type 2 diabetes (Ding et al., 2006). Additionally, prospective studies showed that men with higher plasma testosterone concentrations (449.6-605.2 ng/dL) had low prevalence (42%) of type 2 diabetes (Ding et al., 2006). Androgen deprivation in prostate carcinoma led to decreased insulin sensitivity and increased the risk of developing type 2 diabetes (Smith et al., 2006). Similarly, androgen deprivation therapy by GnRH agonist treatment (leuprolide acetate and bicalutamide) decreased the insulin sensitivity index by 13% and increased the fasting plasma insulin concentration by 26% in men with prostate cancer (Smith et al., 2006). Men with normal testosterone concentrations exhibited a HOMA-IR measurement of 2.98 while men undergoing androgen deprivation therapy had a HOMA-IR measurement of 19.14 as a sign of marked insulin resistance with increased fasting blood glucose concentrations of 7.79nmol/L (Basaria et al., 2006).

1.3.2 Testosterone deficiency and cardiovascular complications

Testosterone deficiency associated with increased concentrations of low density lipoprotein cholesterol, triglycerides and high density lipoprotein cholesterol serves as the major risk factor of cardiovascular complications

(Whitsel et al., 2001, Isidori et al., 2005, Laughlin et al., 2008). Increased blood concentrations of total cholesterol, triglycerides and high density lipoprotein cholesterol were observed in prostate cancer patients treated with GnRH agonist, leuprolide acetate (Yannucci et al., 2006). Low testosterone concentrations in men associated with increased concentrations of total cholesterol, low density lipoprotein cholesterol and apolipoprotein-B led to cardiovascular complications (Simon et al., 1997). Testosterone concentrations positively correlated with the high density lipoprotein cholesterol concentration, playing a protective role in preventing atherosclerosis (Van Pottelbergh et al., 2003, Johnsen et al., 2005, Stanworth et al., 2011). Low testosterone concentrations associated with presence of aortic atherosclerosis (Hak et al., 2002, Jones and Saad, 2009) could aggravate the low grade systemic elevation of inflammatory cytokines and CRP concentrations thus increasing the risk of cardiovascular disease (Jones and Kennedy, 1993, Hak et al., 2002, Jones, 2010). In patients, low testosterone concentrations were correlated to the coronary heart disease (Nettleship et al., 2009) and low circulating testosterone concentrations in elderly men could also be the causative factor of stroke and ischaemic heart disease (Yeap et al., 2009). A population-based cross sectional-study in men from different age groups (25-84 years) observed lower total testosterone concentrations (12.1-13.3 nmol/L vs 13.4-15.3 nmol/L) with higher systolic blood pressure (≥140mmHg vs <140mmHg) and diastolic blood pressure (≥90 mmHg vs <90mm/Hg) and left ventricular hypertrophy (≥145.5g/m vs <145.5g/m) (Svartberg et al., 2004). Testosterone induced endothelial-independent vaso-relaxation in aortic rings isolated from rabbit and rat, showing the possible antihypertensive potential of testosterone (Yue et al., 1995). Treatment-induced hypogonadism in prostate cancer patients was directly associated with increased arterial stiffening primarily increasing the systolic blood pressure, leading to the development of left ventricular hypertrophy (O'Rourke and Kelly, 1993, Saba et al., 1993, Smith et al., 2001). Testosterone deficiency therefore plays a significant role in the development of cardiovascular disease (Jones, 2010).

1.3.3 Testosterone deficiency and inflammation

Decreased serum testosterone concentrations were associated with increased concentrations of inflammatory cytokines such as IL-1ß and IL-6 in men identified with coronary heart disease (Nettleship et al., 2007). In human cell lines, testosterone treatment down-regulated the expression of inflammatory cytokines including IL-6, interleukin-1β (IL-1β) and TNF-α (Gornstein et al., 1999, Hofbauer et al., 1999, Hatakeyama et al., 2002). In mice splenocytes, testosterone treatment stimulated the production of the anti-inflammatory marker, interleukin-10 (IL-10) (Liva and Voskuhl, 2001). Testosterone attenuated production of TNF-α and increased IL-10 production in the lipopolysaccharide (LPS)-induced murine macrophage cell line (J774) (D'Agostino et al., 1999). Testosterone treatment in hypogonadal men with type 2 diabetes attenuated the secretion of IL-1ß, IL-6 and TNF-α from peripheral blood monocytes (Corrales et al., 2006). Similarly, intramuscular testosterone treatment reduced serum IL-6 concentrations in healthy men (Zitzmann et al., 2005).

1.4 Obesity and inflammation

The adipose tissue is the largest storage site for triglycerides in the body and plays an important role as an endocrine organ in energy homeostasis (Muoio and Newgard, 2006). Healthy fat cells have the potential to extract excess fatty acid from the blood and safely store it in the form of triglycerides. The adipose tissue promotes hypertrophy and adipogenesis (formation of new fat cells) to sequester the excess fatty acids in blood coming from dietary fat or metabolism of excess carbohydrates and protein that have been converted into circulating fat by the liver (Virtue and Vidal-Puig, 2010). Saturated dietary fatty acids, such as palmitic acid, induce chronic low-grade inflammation during obesity by triggering the expression of pro-inflammatory cytokines such as TNF-α, NF-κB and IL-6 (Van Gaal et al., 2006, Bradley et al., 2008). Further, palmitic acid treatment inhibited the glucose utilisation via insulin signalling cascade in primary mouse hepatocytes and pancreatic β-cells (Solinas et al., 2006). Concentrations of unsaturated fatty acids such as linolenic acid and arachidonic acid exceeding critical threshold values induce a chronic low-grade inflammation and with increased macrophages in the adipose tissue (Pompeia et al., 2003, Heilbronn and Campbell, 2008, Kurokawa et al., 2010, Calder et al., 2011). The necrosis of fat cells stimulates a migration and accumulation of macrophages into the adipose tissue (Weisberg et al., 2003, Xu et al., 2003, de Victoria et al., 2009). These newly recruited macrophages induce the secretion of additional inflammatory mediators such as IL-6, IL-1 and TNF-α (Wajchenberg, 2000, Hajer et al., 2008). Further, activation of NF-κB, a key gene transcription factor that drives the inflammatory response of the innate immune system, increases the inflammation within the adipose tissue (Weisberg et al., 2003, Xu et al., 2003, de Victoria et al., 2009). As a result, more free fatty acids are released into circulation. These excess fatty acids are deposited in other organs such as muscles, cardiac tissue, liver and pancreas causing lipotoxicity (Fukushima et al., 2005, Perman et al., 2011). Free fatty acids caused toxicity in pancreatic β-cells, resulting in an impaired insulin secretion and disruption of cell metabolism (Delarue and Magnan, 2007). Further, like the metastatic property of cancer cells, lipotoxicity will spread to various tissues, including skeletal muscle, liver, vascular endothelium and myocardium (Chinen et al., 2007). The extent of lipotoxicity will be determined by the extent of macrophage infiltration in the adipose tissue (Sears and Ricordi, 2011). This chronic low-grade inflammation triggered by excess fat deposition plays a significant role in the pathogenesis of obesity and associated metabolic disorders.

1.5 Obesity and type 2 diabetes

Skeletal muscle and liver are the major sites of insulin-stimulated glucose uptake, whereas the adipose tissue contributes relatively little to total glucose disposal (Kahn and Flier, 2000). High dietary intake of fat and refined carbohydrates increases the postprandial glycaemia (Anderson et al., 2009a, Brand-Miller and Buyken, 2012). This results in the increased secretion of insulin necessary to lower the resulting postprandial rise in blood glucose concentrations (Jellinger, 2007). With elevated insulin concentrations, the stored fat in the adipose tissue will remain sequestered due to the inhibition of hormone-sensitive lipase in healthy fat cells (Melki and Abumrad, 1993, Ailhaud, 1997). Studies carried out using 3T3-L1 adipocytes have observed that insulin stimulates adipogenesis through the AKT-TSC2-mTORC1

pathway (Zhang et al., 2009a). In rodents, palmitic acid mediated hypothalamic insulin resistance by altering protein kinase C-θ (PKC-θ) subcellular localization (Benoit et al., 2009). Increased intracellular lipid in muscle and liver cells leads to an increase in intracellular diacylglycerol (Rachek, 2014). Diacylglycerol is a potent stimulator of PKC-θ and protein kinase C-ε (PKC-ε) and further, the activation of serine threonine phosphorylation of insulin receptor substrate-1 reduced the glucose utilisation via insulin signalling cascade (Wolf, 2008). During excess fat accumulation, insulin resistance induced by chronic low-grade inflammation will keep plasma insulin concentrations constantly elevated and thereby induce excess fat deposition (Pradhan, 2007). In conclusion, during obesity/insulin resistance, the glucose utilisation via insulin signalling cascade is disrupted in both the skeletal muscle and adipose tissue that leads to hyperglycaemia (Colomiere et al., 2010). This result in the development of type 2 diabetes mellitus and obese populations become more prone to become type 2 diabetics.

1.6 Obesity and cardiovascular complications

Obese patients have elevated vascular sympathetic nerve activity and peripheral vascular resistance, which ultimately leads to the development of hypertension (Frohlich, 1991, Davy and Hall, 2004). Obese rats showed impaired baro-reflex control of sympathetic nerve function, thereby losing control over arterial pressure and becoming hypertensive (Schreihofer et al., 2007). In addition, impaired adrenoceptor-mediated constriction of the mesenteric vasculature profoundly diminished the autonomic regulation of the vasculature that contributes to the maintenance of arterial pressure (Romanko and Stepp, 2005, Ruggeri et al., 2006). Considering the coincident occurrence of hypertension and increased stroke risk in obese patients, it is possible that elevated arterial pressure will cause cerebral vascular injury in the obese population (Deutsch et al., 2009). Further changes in arterial pressure will also cause deleterious changes in cardiovascular structure. As vessels become stiffer, wall thickness can increase, and lumen diameter can decrease (Osmond et al., 2009). Obesity is a risk factor for pathological alterations in the cerebral vasculature and hence a chronic moderate increase in blood pressure may contribute to increased stroke risk and injury in the obese population (Osmond et al., 2009). Feeding rats with high fat diet increased the damage caused by a permanent ischaemic stroke. Further, the structure of the middle cerebral artery is altered in such a way that it would impede blood flow causing the vessel to become more stiff (Deutsch et al., 2009).

1.7 Obesity and endothelial cell dysfunction

Endothelial dysfunction is present in obese humans (Al Suwaidi et al., 2001, Williams et al., 2002). Endothelial-dependent vasodilatation was impaired in proportion to insulin resistance and various indices of visceral obesity (Steinberg et al., 1996). Patients with visceral obesity showed a loss of nitric oxide (NO)-mediated relaxation in the large conduit arteries and in addition, the loss of NO-independent, potassium-mediated relaxation in the small arteries (de Kreutzenberg et al., 2003). This result confirms that obese patients are more prone to premature cardiovascular disease due to endothelial dysfunction.

1.8 Obesity and gut microbiota

In obesity, the colon microbiota is densely populated with *Firmicutes* with decreased *Bacteroidetes* and *Bifidobacteria* which may play a crucial role in enhancing adiposity by suppressing the fasting induced adipocyte factor (fiaf), a circulating lipoprotein lipase inhibitor (Backhed et al., 2004, Ley et al., 2005, Million et al., 2013). Additionally, high fat diet induced changes in gut microbiota enhance adiposity via inhibiting the activation of adenosine monophosphate-activated protein kinase (AMPK) pathway that primarily regulates the fatty acid oxidation in both the liver and the skeletal muscle (Backhed et al., 2007). *Bifidobacterium* species maintain the gut barrier function via promoting a healthier microvillus environment and also by inhibiting the intestinal permeability and bacterial translocation (Ruseler-Van Embden et al., 1995, Caplan et al., 1999). *Bifidobacterium* supplementation attenuated the visceral fat accumulation, body weight gain and improved the insulin sensitivity in high fat diet fed rats (Chen et al., 2012). *In vivo* study using rodents showed that oral treatment of *Bifidobacteria* improved the mucosal barrier function by attenuating the intestinal endotoxin levels i.e. LPS concentrations (Griffiths et al., 2004, Wang et al., 2006).

The microbiota in obesity was directly associated with increased circulating LPS concentrations, showing that metabolic endotoxaemia played a significant role in the pathogenesis of non alcoholic fatty liver disease (NAFLD) via Toll-like receptor4 (TLR-4) mediated activation of hepatic stellate cells that recruits Kuppfer cells and increased hepatic TNF-α expression (Seki et al., 2007, Ruiz et al., 2007, Musso et al., 2010). High fat diet associated with lower *Bifidobacterium* counts increased the intestinal permeability that serves as the protective barrier in preventing LPS translocation, characterised by increased plasma concentrations of LPS and a low grade inflammation (Cani et al., 2007, Brun et al., 2007). Also, LPS played a significant role in the development of obesity-linked inflammatory diseases such as NAFLD and non-alcoholic steatohepatitis (Lichtman et al., 1990, Adachi et al., 1995). Metabolic endotoxaemia induced inflammation by increasing the adipose tissue concentrations of TNF-α and IL-6 are associated with the increased risk factors in the development of type 2 diabetes and obesity (Creely et al., 2007). Metabolic endotoxaemia triggered inflammation via activation of Toll-like receptor-2 (TLR-2) stimulated the inflammatory response by activating the LPS/TLR-4/CD14 signalling cascade (Cani and Delzenne, 2011). Saturated fatty acids promoted low grade inflammation by stimulating the TLR-4 dependent inflammatory signalling pathway (Shi et al., 2006, Suganami et al., 2007). The direct relationship between high fat diet and metabolic endotoxaemia serves as the major risk factor in inducing metabolic syndrome (Ghanim et al., 2009, Laugerette et al., 2011).

1.9 Treatment of obesity

Obesity is a chronic disease, for which none of the current medications provide a comprehensive cure (Bray and Greenway, 2007, Bray, 2011). In addition to pharmacotherapy and surgical treatment as long-term therapeutic interventions for treating obesity, current strategy is aimed at behavioural modifications including physical activity such as regular exercise to increase calorie expenditure and also consuming low calorie and low fat diets (Berkel et al., 2005, Munro and Garg, 2011). To improve the health of the obese population,

current treatments emphasise the importance of permanent lifestyle changes (Burke and Wang, 2011). Compared to pharmacotherapy and bariatric surgery, an effective therapeutic approach uses a combination of behavioural strategies which includes diet and exercise. Pharmacotherapy and bariatric surgery have better outcomes when improved by change in lifestyle (Burke and Wang, 2011). There is evidence that ingredients found in food play a significant role in lowering adiposity and modulating energy balance (Kim and Park, 2011). Research studies have also been carried out in screening food components against obesity and its associated disorders (Panchal et al., 2011a, Poudyal et al., 2012).

1.10 Algae as a dietary source of bioactive nutrients

Both developed and developing countries are experiencing increased rates of metabolic syndrome (7-57%) in both men and women of age groups between 20-79 years (Hu et al., 2004, Cameron et al., 2004) due to the consumption of high calorie diets and a sedentary lifestyle (Esposito et al., 2007). The prevalence of metabolic syndrome varies between 1.5-18.9% in the middle-aged and elderly Japanese population (Arai et al., 2010). Macroalgae and microalgae serve as a key dietary source of polysaccharides, mineral ions, n-3 PUFA, amino acids and carotenoids (MacArtain et al., 2007, Christaki et al., 2011). Therefore, algal intake could play an effective therapeutic role in attenuating the metabolic syndrome and promoting a healthy lifestyle (Lordan et al., 2011, Mohamed et al., 2012). The Japanese diet mainly consists of soybean products, fish, seaweeds, vegetables, fruits and green tea which indicates that these products are associated with a decreased risk of cardiovascular mortality and metabolic syndrome (Shimazu et al., 2007, Tada et al., 2011). The average seaweed intake of 3-6.9 g/day between 1950 and 1996 (Matsumura, 2001) for people in Japan, with an iodine intake of 1-3mg/day,increased longevity with health benefits such as low prevalence of cancer in the Japanese population (Zava and Zava, 2011).

Seaweeds contain polysaccharides (20-76% of dry weight), proteins (15-40% of dry weight), vitamins (20-3000 ppm), minerals (36% of dry weight) and omega-3 fatty acids, mostly eicosapentaenoic acid (EPA) as 30% of the total fatty acid content of 4-10%. Thus, seaweeds are considered as nutritionally rich foods (Smit, 2004, Rajapakse and Kim, 2011a). Seaweeds are macroalgae classified into three types: brown algae (Phaeophyta), red algae (Rhodophyta), green algae (Chlorophyta) (MacArtain et al., 2007). Microalgae occur as unicellular organisms and are able to grow in both marine and freshwater environments (Lee, 2008). Microalgae are classified into four major types: diatoms (bacillariophyta), yellow-brown flagellates (prasinophyta, prymnesiophyta, cryptophyta, chrysophyta, rhodophyta and rhaphidiophyta), dinoflagellates (dinophyta) and blue-green algae (cyanophyta) (Lordan et al., 2011).

Polysaccharides present in seaweeds are classified as either structural or storage polysaccharides (MacArtain et al., 2007). Structural polysaccharides present in seaweeds are mainly celluloses, hemicelluloses and xylans, while the storage polysaccharides are carrageenans, alginates and agar (MacArtain et al., 2007, Lordan et al., 2011). In addition to this, sulphated polysaccharides such as fucoidan (brown seaweed), porphyran (red seaweed) and ulvan (green

seaweed) are considered as bioactive ingredients (Lordan et al., 2011, Misurcova et al., 2012).

Microalgae contain extracellular and sulphated polysaccharides. The extracellular polysaccharides of microalgae such as *Porphyridium* and *Rhodella* are high molecular weight, negatively charged (anionic), sulphated heteropolysaccharides and their biological significance depends on factors such as the number of sugar residues, resistance to enzymatic digestion and viscosity (Geresh et al., 2002a, Misurcova et al., 2012). Like macroalgae, research studies are widely conducted on microalgae such as *Spirulina, Cyanobacteria* and *Scenedesmus* species for health and medical applications (Smit, 2004, Misurcova et al., 2012). As an example, 5% *Spirulina,* blue-green fresh water microalgae, improved the vascular tone by stimulating the synthesis and release of NO by endothelium in either fructose-fed obese rats or lean rats (Juarez-Oropeza et al., 2009). Additionally, *Spirulina* intake (4.5g/day) for 6 weeks by male and female hypertensive subjects (n=36) aged 18-65 years reduced blood pressure (Juarez-Oropeza et al., 2009). Type 2 diabetics (n=19) treated with *Spirulina* species (8g/d) for 12 weeks showed reduction in plasma concentrations of triglycerides (98.5mg/dl vs 128.2mg/dl), TNF-α (1.71pg/mL vs 2.09pg/mL), IL-6 (0.92 pg/mL vs 1.31 pg/mL) with increased plasma adiponectin concentrations (6.62 µg/mL vs 5.69 µg/mL) that clearly defines the biological significance of *Spirulina* species as a functional food for diabetes management (Lee et al., 2008). *Dunaliella salina* is a microalgal producer of β-carotene, mainly cultivated in Western Australia, California and Hawaii specifically for its nutrient, antioxidant and pigmentation uses in health and human supplement products (Lee, 2008).

Seaweeds as a source of dietary amino acids such as valine, isoleucine, leucine, aspartic acid, glutamic acid, threonine, valine, lysine, tryptophan and arginine have been used for animal and human nutrition (Fleurence, 1999, Bocanegra et al., 2009). Green and red algae contain higher protein content (10-47% of dry algae) compared with brown algae (3-16% of dry algae) (Fleurence, 1999, Ruperez and Saura-Calixto, 2001). Additionally, bioactive peptides from marine sources may possess therapeutic potential in the treatment or prevention of diseases (Kim and Wijesekara, 2010). *Scenedesmus acutus,* a freshwater microalgae rich in protein (47-66% of dry algae), has been considered as both a nutritional and functional food (Gross et al., 1986).

Generally, seaweeds contain higher proportions of micronutrients than those present in terrestrial plants (Rupérez, 2002, MacArtain et al., 2007, Misurcova et al., 2011b). Sodium, calcium, magnesium, potassium, chloride, sulphur and phosphorus are present in significant amounts in seaweeds with a range of micronutrients including iodine, iron, zinc, copper, selenium, molybdenum, fluoride, manganese, boron, nickel and cobalt (Rupérez, 2002, Rajapakse and Kim, 2011b, Nakamura et al., 2012). Seaweed consumption has been recommended as a food supplement of mineral ions (Rajapakse and Kim, 2011b).

Seaweeds are a good source of water-soluble vitamins (B_1, B_2 and B_{12}) while certain seaweeds including red and green seaweeds are rich in fat-soluble vitamins such as vitamin A, vitamin E and beta (β)-carotene (Skrovankova, 2011). Vitamin C is present in higher proportions in green and brown seaweeds, compared with red seaweeds (green and brown seaweed: 185-300 mg/100g dry weight; red seaweed: 107.1 mg/100 g dry weight) (McDermid

and Stuercke, 2003, MacArtain et al., 2007, Skrovankova, 2011). Vitamins C & E have health benefits properties such as attenuating blood pressure (vitamin C), preventing cardiovascular diseases (β-carotene) and reducing the risk of cancer (vitamin E & C, carotenoids) (Skrovankova, 2011).

Seaweeds serve as the primary source of omega-3 fatty acids such as docosahexaenoic acid (DHA) for fish (Lee, 2008) Generally, lipid content of commonly consumed seaweeds does not exceed 5% of dry matter (Misurcova et al., 2011a). Brown seaweeds including *Laminaria, Undaria* and *Hizikia* species comprise EPA (14-16% of total lipids, 7-13.2% of total lipids and 42% of total lipids, respectively) (Khotimchenko, 1998, Sánchez-Machado et al., 2004, Dawczynski et al., 2007). Red seaweeds such as *Palmaria, Porphyra* and *Laurencia* species contain 6-24% EPA of total lipids while green seaweeds *Ulva* species comprise 0.8-2.4% EPA of total lipids (Sánchez-Machado et al., 2004, Dawczynski et al., 2007, Kumari et al., 2010). Brown microalgae *Schyzochytrium* species consist of 30-36% EPA/total lipids & 0.5-1.1% DHA/total lipids (Kamlangdee and Fan, 2003). Seaweeds as a rich source of EPA and DHA are considered as a significant supplement or to be the part of a balanced diet (Misurcova et al., 2011a).

On the basis of the bioactive ingredients present in seaweeds, research studies have been conducted on seaweeds for the health benefits and the prevention of metabolic syndrome (Lordan et al., 2011). An observational study in healthy Japanese children aged 3-6 years revealed that boys who had higher seaweed intake (0.58g/d vs 0.78g/d) showed reduction in diastolic blood pressure (63 mmHg to 59 mmHg) while girls who had higher seaweed intake (0.64g/d vs 0.89g/d) showed reduction in systolic blood pressure (97mmHg from 102mmHg) (Wada et al., 2011). Research study on *Undaria pinnatifida* (brown seaweed) treated 37 elderly hypertensive subjects (>150mmHg SBP/90mmHg DBP) for 4 weeks, showed a reduction in systolic blood pressure by 13±3 mmHg and diastolic blood pressure by 9±2 mmHg (Hata et al., 2001). The tropical seaweeds *Kappaphycus alvarezii* (brown seaweed), *Caulerpa lentillifera* (green seaweed) and *Sargassum polycystum* (red seaweed) as a 5% dietary supplement in rats fed with high cholesterol and high fat diet (HCF) exhibited cardiovascular protective effects by reducing the total plasma cholesterol concentration (18.5% to 11.4%) and triglycerides concentration (36.1% to 33.7%). Additionally, an anti-obesity effect was also observed in *Sargassum polycystum* treated rats and the *Kappaphycus alvarezii* treated HCF rats showed increased anti-hyperlipidaemic and antioxidant activities (Matanjun et al., 2010).

1.10.1 Algal polysaccharides

Seaweeds serve as a rich source of dietary fibre (16.4-74.6%) (Lahaye, 1991) in which seaweed supplementation increased the dietary fibre intake 2.5 times compared to the control group (Kim et al., 2008) and therefore may have the potential to prevent metabolic syndrome associated with obesity, type 2 diabetes and cardiovascular complications (Lahaye, 1991, Kim et al., 2008, Teas et al., 2009). The recommended daily intakes of dietary fibres for children and as well as for adults are 14g/1000kcal (Anderson et al., 2009b). Seaweed intake of 20g/day as a rich source of dietary fibres (75% of dry algae) (Lahaye, 1991) could meet the daily requirements of dietary fibres for both children and adults.

Algal polysaccharides are not digestible by the human gastrointestinal tract and so they are considered as dietary fibres (Dawczynski et al., 2007). Polysaccharide content in green algae (Chlorophyceae), brown algae (Phaeophyceae) and red algae (Rhodophyceae) is relatively high (33-75% of dry algae) (Lahaye, 1991, Jiménez-Escrig and Sánchez-Muniz, 2000). Polysaccharides present in seaweeds are either structural, storage or sulphated polysaccharides (Percival, 1979). Structural polysaccharides in seaweeds consist of cellulose (2-10% of dry weight), hemicelluloses (9% of dry algae) and lignin (3% of dry algae) (Holdt and Kraan, 2011). Generally, structural polysaccharides are high molecular weight polysaccharides primarily present in the cell walls of seaweeds that provide the strength and flexibility to prevent cell desiccation (O'Sullivan et al., 2010). Storage polysaccharides in seaweeds include agar, carrageenans and alginates (MacArtain et al., 2007). Sulphated hetero-polysaccharides consist of major sugar residues such as glucuronic acid, rhamnose, arabinose and galactose in which the hydroxyl groups of the sugar residues are substituted by half ester sulphate groups (Percival, 1979). Fucose, mannose, galactose and uronic acids are the main constituents of seaweed fibres (Ramnani et al., 2012). Soluble dietary polysaccharides include xyloglucans, galactomannans, hemicelluloses, β-glucans, pectins, gums and mucilages while the insoluble fibres comprise cellulose, lignins, arabinoxylans, hemicelluloses and resistant starch (Jiménez-Escrig and Sánchez-Muniz, 2000).

1.10.2 Bioactive polysaccharides of green macroalgae (Chlorophyta)

Edible green seaweeds such as *Ulva* and *Enteromorpha* species are rich in dietary polysaccharides or fibres (36-40% of dry algae) (Lahaye and Jegou, 1993). Ulvan is the major water-soluble sulphated polysaccharide made up of sulphated rhamnose linked to either glucuronic, iduronic acid or xylose and present in *Ulva, Enteromorpha, Caulerpa, Codium* and *Gayralia* species representing about 8-29% of dry algae (Lahaye and Robic, 2007, Patel, 2012). Cellulose, xylans, mannans, anionic polysaccharides containing sulphate groups, uronic acids, rhamnose and xylose, galactose and arabinose are found in green macroalgae (Chlorophyta) (Lahaye, 1991).

1.10.3 Antioxidant potential of green macroalgal polysaccharides

Hypercholesterolaemic rats treated with 1% ulvan solution (w/v) reduced the serum total cholesterol concentration (45%), and the low density lipoprotein cholesterol concentration (54.1%) by enhancing the faecal bulking effect and increasing the daily bile excretion by 1.9 fold (Pengzhan et al., 2003). This study also suggested that the cholesterol-lowering effect of ulvan may be produced by the breakdown of cholesterol into bile acids and simultaneously, the increased viscosity induced by ulvan gelled with calcium ions in the body may interfere with the bile acid absorption from the ileum and reduce the serum low density lipoprotein cholesterol concentration (Lahaye and Axelos, 1993). Syrian hamsters treated with 5% *Ulva rigida* rich in dietary fibres (40%) supplemented high cholesterol diet increased the glutathione peroxidase activity (0.69U/mg protein vs 0.43U/mg protein) and reduced the liver lipid peroxidation (thiobarbituric acid reactive substances (TBARS) concentration: 3.34ng/mg protein vs 27.05ng/mg protein) (Lahaye and Robic, 2007, Godard et

al., 2009). The plasma total cholesterol concentrations and triglycerides concentrations were also lowered by 14% and 49% by *Ulva rigida* treatment and further investigation showed that 5% *Ulva rigida* treatment reduced the cardiac superoxide anion production (41%) in high cholesterol diet-fed rats (Godard et al., 2009).

This antioxidant potential of *Ulva* species may be due to the presence of ulvan (8-29% of dry algae), a major water-soluble sulphated polysaccharide (Lahaye and Robic, 2007). An *in vitro* study using ulvan extracts from *Ulva pertusa* showed stronger free radical scavenging activity of 90% (IC50: 9.17µg/mL) (Qi et al., 2006). Similarly another *in vitro* study using polysaccharide extracts at different concentration ranging from 43-52% of *Enteromorpha prolifera* dry powder (100g) showed higher inhibitory effects on superoxide radical at IC_{50} 0.016-0.028mg/mL showing efficient antioxidant activity compared with vitamin C, a potent antioxidant that showed maximum free radical scavenging activity at IC_{50} 1.75 mg/mL (Xing et al., 2005, Li et al., 2013).

1.10.4 Hepatoprotective effect of polysaccharides from green macroalgae

Ulva lactuca polysaccharide extracts (80% of dry algae) treatment in rats prevented the decrease of tricarboxylic acid enzymes activity of hepatocytes such as NADPH-cytochrome P450 reductase (200 U/mg/protein vs 100 U/mg/protein), ornithine transcarbamoylase (40 U/mg/protein vs 10 U/mg/protein) and arginase activity (310 U/mg/protein vs 200 U/mg/protein) compared with D-galactosamine-treated rats (Devaki et al., 2009). Additionally, increased concentrations of liver mitochondrial enzymes such as cytochrome P450 (0.90 nmoles/mg/protein vs 0.36nmoles/mg/ protein) and cytochrome b5 (0.4nmoles/mg/protein vs 0.2nmoles/mg/ protein) were observed in *Ulva lactuca* polysaccharide-treated rats. This biological effect may be due to the antioxidant activity exhibited by *Ulva lactuca* polysaccharides to protect the mitochondria from free radicals generated during D-galactosamine intoxication and therefore prevent the hepatotoxicity (Devaki et al., 2009).

A similar study (Sathivel et al., 2008) suggested that the *Ulva lactuca* polysaccharide (65% of dry algae) extracts via its antioxidant potential prevented the liver damage by attenuating the mitochondrial oxidative stress and the inhibition of energy metabolism of hepatocytes in D-galactosamine-intoxicated rats (500mg/kg body weight). In addition to this, the *Ulva lactuca* polysaccharide extracts attenuated the hyperlipidaemia associated with hepatotoxicity by preventing the increase in lipid peroxides concentration (2.8 mg/g liver tissue vs 4.3 mg/g liver tissue), low density lipoprotein cholesterol (LDL) concentration (57 mg/dl blood plasma vs 72 mg/dl blood plasma), triglyceride concentration (58 mg/dl blood plasma vs 76 mg/dl blood plasma) and free fatty acids concentration (24 mg/dl blood plasma vs 36 mg/dl blood plasma) (Sathivel et al., 2008).

1.10.5 Cholesterol-lowering effect of green macroalgal polysaccharides

Green seaweeds such as *Ulva* and *Enteromorpha* species contain higher proportions of soluble (8.0-15.8% and 14.9-15.9%) and insoluble fibres (24.2-32.6% and 21.6-28.7%). Intrinsic viscosity of the *Ulva lactuca* soluble

fractions is higher at pH 3-7.3 (147.5-175 mL/g) compared with *Enteromorpha compressa* (35.9-36.6 mL/g) (Lahaye and Jegou, 1993). Viscosity of soluble fibres plays a vital role in absorption of cholesterol and lowering of serum total cholesterol concentration, binding of lipids in the intestinal tract and altering hepatic or intestinal lipid synthesis (Edwards et al., 1987, Hunt et al., 1993). *Ulva fasciata* rich in dietary fibres (34.8 % of dry algae) attenuated the increased total serum cholesterol concentration (21%) and LDL (40%) in hypercholesterolaemic diet-fed rats (Carvalho et al., 2009). Also, compared with control rats treated with cellulose (9.7%), the increased faecal volume weight (12.73g vs 6.98g) in *Ulva fasciata* treated rats clearly suggest that the soluble fibres present in *Ulva fasciata* may exhibit the faecal bulking effect and therefore regulate the blood lipid profile (Carvalho et al., 2009). Based on this evidence, *Ulva fasciata* could play an important role in body weight control and the prevention of gastrointestinal diseases.

1.10.6 Bioactive polysaccharides of brown macroalgae (Phaeophyta)

Dietary fibres in brown seaweeds that include *Laminaria* species, *Undaria* spieces and *H.fusiform* species consist of four major polysaccharides 1) laminarans, 2) alginates 3) fucans 4) cellulose and chitin (structural) (Percival, 1979, Jiménez-Escrig and Sánchez-Muniz, 2000). Laminarans in brown algae such as *Laminaria digita* and *Eisenia bicyclis* are composed of (1,3)-β-D-glucose as a β-glucan and the other major cell wall matrix component includes gelling polyuronide, alginate, mannuronic acids, guluronic acids and uronic acids. Alginic acid is another major water-soluble polysaccharide present in brown seaweeds such as *L. gurjanovae* species (28% of dry algae) and *Sargassum* species (17-45%) (Fourest and Volesky, 1995, Shevchenko et al., 2007, O'Sullivan et al., 2010). Alginic acid mainly consists of L-guluronic acid and D-mannuronic acid (20-30 units) (Percival, 1979, Jiménez-Escrig and Sánchez-Muniz, 2000). In addition to this, fucans, a hydrophilic sulphated polysaccharide in brown algae, are classified into three major types 1) fucoidans 2) xylofucoglycuronans 3) glycorunogalactofucans (Percival, 1979, Jiménez-Escrig and Sánchez-Muniz, 2000, Gupta and Abu-Ghannam, 2011).

1.10.7 Anti-inflammatory potential of brown algal polysaccharides

Fucoidan fractions obtained from *Ecklonia cava* showed maximum inhibition of NO production (83%) at 100 µg/mL and the corresponding prostaglandin E2 (PGE_2) release (55%) at 200µg/mL in LPS-stimulated RAW macrophages cell line (Kang et al., 2011). Additionally, this study confirmed that the anti-inflammatory activity of fucoidan-purified extracts is mediated by the inhibition of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein expression in LPS-stimulated RAW macrophages cells and this protein level inhibition was observed in a dose-dependent manner at concentration (25-200µg/mL) of fucoidan extracts (Kang et al., 2011). Purified fucoidan extracts from *Laminaria japonica* at (200mg/kg body weight) attenuated the serum concentrations of inflammatory cytokines TNF-α (28.3 ng/mL vs 58.2 ng/mL) and IL-6 (160.2 ng/mL vs 289.5 ng/mL) in rats with ischaemia-reperfusion-induced myocardial damage (I/R) model (Li et al., 2011). Further, this anti-inflammatory response was exhibited via inactivation of high-mobility group box 1 (HMGB1) and NFκ-B protein expression.

Additionally, the fucoidan treatment (200mg/kg body weight) by its anti-inflammatory activity improved the cardiovascular dysfunction by regulating both the systolic blood pressure (132mmHg vs 98mmHg) and the end-diastolic blood pressure (5mmHg vs 8mmHg) of the left ventricle in ischaemia-reperfusion rats (Li et al., 2011). The anti-inflammatory potential of fucoidan clearly suggests that it could play an efficient therapeutic role in attenuating the inflammation-linked diseases such as myocardial infarction, rheumatoid arthritis and chronic ulcers (Senni et al., 2006). During obesity, an increase in adipose tissue macrophages plays a crucial role in activating the expression of inflammatory markers such as TNF-α, IL-6 and iNOS and serves as the major risk factor for the metabolic syndrome (Weisberg et al., 2003). Alginic acid exhibits its anti-inflammatory activity at 0.01µg/mL by inhibiting the production of inflammatory cytokines IL-1ß and TNF-α via attenuating the NFκ-B protein expression in the activated mast cells (HMG-1) (Jeong et al., 2006). Alginic acid (100mg/kg body weight) extracted from *Sargassum wightii* (Terada et al., 1995) prevented the inflammatory cell infiltration in arthritic rats by reducing the expression of CRP and the corresponding increase in enzymes linked to inflammation such as cyclooxygenase, lipooxygenase and myeloperoxidase (Sarithakumari et al., 2013). Based on the research evidence, either fucoidan or alginic acid being a potent anti-inflammatory polysaccharide could play a significant role in attenuating the inflammatory response induced by increased adipose tissue macrophages during obesity (Weisberg et al., 2003) and henceforth may prevent the metabolic syndrome induced during obesity.

Short chain carboxylic acids (SCFA) released during the fermentation of dietary fibres are more pronounced to reduce the inflammatory tone and especially butyrate exhibits its anti-inflammatory effect by inhibition of NFκ-B pathway while acetate and propionate increase the expression of the anti-inflammatory cytokine, IL-10 (Cavaglieri et al., 2003, O'Sullivan et al., 2010). Therefore, by enhancing the SCFA production, laminarin may exhibit its anti-inflammatory potential (Devillé et al., 2007, O'Sullivan et al., 2010) and henceforth could play a vital role in attenuating the inflammation associated with obesity (Iyer et al., 2010).

1.10.8 Anti obesity effect of brown algal polysaccharides

In vitro study using 3T3-L1 adipocytes showed that fucoidan at 200µg/mL concentration decreased the lipid accumulation by 52% with reduced triglyceride deposition (15%) in 3T3-L1 cells (Park et al., 2011). Simultaneously an increase in hormone sensitive lipase expression (155 arbitrary units (AU)) with decreased insulin stimulated (10µg/mL) 2-deoxy-D-glucose uptake in 3T3-L1 adipocyte cells indicates the stimulation of lipolysis activity by fucoidan (Park et al., 2011). This result clearly suggests that brown algae rich in fucoidan may play a vital role to prevent the obesity and its associated metabolic disorders by inducing lipolysis. Similar study using 3T3-L1 adipocytes observed that the fucoidan extracts from *Undaria pinnatifida* exhibited the anti-adipogenic activity in 3T3-L1 adipose cells by inhibiting the increased expression of inflammatory cytokines such as TNF-α, monocyte chemotactic protein-1 (MCP-1) and plasminogen activator inhibitor-1 (PAI-1) (Kim and Lee, 2012). In addition to this, *in vivo* study using mice revealed that fucoidan treatment (either 1%/diet or 2%/diet) reduced the body weight gain percentage, liver mass and epididymal fat mass in high fat diet-fed mice (Kim et

al., 2014). Obesity is an inflammatory-linked metabolic disorder associated with enhanced adipogenesis, and an increase in adipocyte size (hypertrophy) and number (hyperplasia) (Herberg et al., 1974, Iyer et al., 2010). Based on the research evidence, fucoidan via its anti-inflammatory potential and anti-adipogenic activity could prevent or control obesity. Sodium alginate treatment (15g in 500 mL) in normal weight human subjects (BMI 23.5±1.7 kg/m^2) reduced the energy intake (8.0%) by inducing satiety and showed a greater weight loss (6.78 ± 3.67 kg) (Georg Jensen et al., 2012). In this study, the viscosity effect of alginate fibre may promote the satiety feelings via delayed gastric emptying with decreased nutrient absorption and henceforth a decreased calorie intake and corresponding weight reduction was observed (Georg Jensen et al., 2012). After 2 weeks alginate consumption (10 g/d), healthy human subjects had increased faecal concentrations of propionic acid (130µmol/g faeces) and acetic acid (152µmol/g faeces) with corresponding increase in *Bifidobacteria* counts (log_{10} 10.27 ±0.12/ g faeces) and faecal weight (26%) (Terada et al., 1995). This study revealed the prebiotic activity of alginates. As a potential treatment for obesity in addition to its physicochemical effect, increased alginate intake may promote the production of SCFA such as acetic, propionic and butyric acids and therefore induce satiety by stimulating the expression of satiety hormones such as glucagon-like peptide (GLP-1) and peptide tyrosine tyrosine (PYY) and prevent obesity by calorie reduction (Reimer and McBurney, 1996, Delzenne et al., 2005). Additionally, laminarin, the major storage polysaccharide in brown algae such as *Laminaria digita* and *Eisenia bicyclis*, triggers the production of SCFA, especially butyrate (Devillé et al., 2007, O'Sullivan et al., 2010).

Obesity is associated with decreased *Bifidobacteria counts* in gut (Million et al., 2012). *Bifidobacteria*, a beneficial gut microflora, is indirectly associated with the reduction of inflammatory cytokines (Cani et al., 2007). Alginate fibre which specifically increase the *Bifidobacteria* counts (Terada et al., 1995) may also play an additional therapeutic effect in attenuating the inflammatory cytokines induced during obesity.

1.10.9 Anti-hypertensive and anti-hyperglycaemic potential of brown algal polysaccharides

Hypertensive subjects treated with *Unadaria pinnatifida (*UP*)* capsules (5g/d) showed a reduction in systolic blood pressure (13 mmHg) and diastolic blood pressure (8 mmHg) after 4 weeks (Yoshiya et al., 2001). This study discussed that dietary fibre (53% of dry algae) rich in alginates as an ion exchanger may increase the intestinal absorption of potassium ions by increasing the faecal excretion of sodium ions (Kloareg et al., 1987). In supporting this evidence, research conducted in spontaneously hypertensive rats (SHR) rats treated with 10% alginate supplemented diet along with 1% NaCl supplemented drinking water showed increased faecal sodium content suggesting the preventive mechanism of alginates against hypertension via inhibiting the intestinal absorption of sodium ions (Yamori et al., 1986). The study revealed the anti-hypertensive potential of alginates and therefore considered to play a crucial role in attenuating the metabolic syndrome induced by obesity and its associated disorders.

Intravenous administration of crude polysaccharide extracts from *Himanthalia elongata* brown algae showed a reduction in blood glucose concentration by 18% in normoglycaemic rabbits and 50% reduction was observed in alloxan-diabetic rabbits (Lamela et al., 1989). This study also suggested that this hypoglycaemic effect of *Himanthalia elongate* polysaccharide extracts may be due to its effect on the stimulation of insulin secretion.

1.10.10. Anti-oxidant and cholesterol lowering effect of brown algal polysaccharides

Polysaccharide fractions rich in fucoidan (209.8-263.9 µmol/g of dry algae) extracted from *Fucus vesiculosus* exhibited potent antioxidant activity by reducing 263.9 ± 4.8 µmol ferric ion Fe(II)/g (FRAP assay) (Ruperez et al., 2002). Fucoidan extracted from *Cladosiphon okamuranus* seaweed (150mg/kg/d) increased the anti-oxidant enzymes activity such as superoxide dismutase (SOD) (6.96 U/mg protein vs 3.66U/mg protein), catalase (CAT) (56.03µmol H_2O_2 decomposed/ mg protein vs 40.21µmol H_2O_2 decomposed/mg protein) and glutathione peroxidise (GSH-Px) activity (8.06 µg glutathione consumed/min/mg protein vs 6.08 µg glutathione consumed/min/mg protein) in myocardial-infarcted rats (Thomes et al., 2010). Fucoidan treatment by its anti-oxidant potential attenuated the lipid peroxide concentration by 55% in both serum and cardiac tissue and improved the myocardial damage in myocardial infarcted rats by reducing the serum total cholesterol concentration (89mg/dl vs 168mg/dl), triglyceride concentration (59 mg/dl vs 91mg/dl) and LDL concentration (7.5 mg/dl vs 21mg/dl) (Thomes et al., 2010). Fucoidan polysaccharide extracts from *Laminaria japonica* at 0.2g/kg body weight exhibited maximum anti-hyperlipidaemic activity by increasing the serum concentrations of lipoprotein lipase (LPL) (19.41 µmol/mL vs 13.32 µmol/mL) and hormone sensitive lipase concentration (10.2 µmol/mL vs 7.96 µmol/mL) in rats fed with high fat (20%) and high cholesterol diet (10%) (Huang et al., 2010). Based on the antioxidant and anti-hyperlipidaemic activity results, brown algae rich in fucoidan could play an efficient therapeutic role in attenuating the cardiovascular complications and the secondary diabetic complications induced by oxidative stress.

1.10.11 Bioactive polysaccharides of red macroalgae (Rhodophyta)

Galactans are the major sulphated polysaccharides of rhodophyceae (red algae) comprising mainly galactose units and are classified as agarans and carrageenans (Percival, 1979, Knutsen et al., 1994, Jiao et al., 2011). Carrageenans are high molecular weight sulphated D-galactans found in red algae such as *Kappaphycus alvarezii, Eucheuma spinosum, Gigartina* and *Chondrus* species (Zhou et al., 2006, Jiao et al., 2011). Carrageenans are classified into three types based on their degree of sulphation 1) kappa 2) lota 3) lambda (Percival, 1979). In addition to this porphyran, well-studied agarans (L-galactans) are found in *Porphyra capensis* and *Porphyra haitanensis* (Zhang et al., 2005, Zhang et al., 2009b).

Xylan is a water-soluble polysaccharide present in *Rhodymenia palmate, Porphyra umbilicalis, Laurencia pinnatafida* and *Chaetangium fastigiatum* (Turvey and Williams, 1970, Percival, 1979). Floridean starch is the major storage polysaccharide commonly present in red algae (Percival, 1979).

Porphyra species (Nori) rich in dietary fibres (34.7% of dry algae) are the most commonly used red algae in foods (Jiménez-Escrig and Sánchez-Muniz, 2000).

1.10.12 Anti-inflammatory and anti-hyperlipidaemic potential of red algal polysaccharides

Sulphated polysaccharide fractions extracted from red algae *Hypnea musciformis* at 10mg/kg body weight exhibited anti-inflammatory activity by reducing the concentration of inflammatory cytokine IL-1β (621 pg/mL vs 1230 pg/mL) in the peritoneal cavity and reduced the myeloperoxidase activity (87.34%) in the affected paw tissue of mice (de Brito et al., 2013). A similar study using sulphated polysaccharide extracts from the red algae *Gracilaria cornia* at 9 or 27 mg/kg body weight by its anti-inflammatory activity reduced the myeloperoxidase activity with moderate reductions in the neutrophil counts (26-32%) in the paw tissue and prevented the paw oedema in rats (Coura et al., 2012). This study also suggested that sulphated galactans in brown algae may be responsible for the anti-inflammatory action against the paw oedema induced in rats (de Sousa et al., 2013). Treatment with 5% *Kappaphycus alvarezii* (or *Eucheuma cottonii*) red algae reduced the plasma triglyceride concentration by 70% and LDL concentration by 49% in high cholesterol /high fat diet (HCF) fed rats (Matanjun et al., 2010). Viscosity effect of soluble fibres or polysaccharides such as carrageenan (18.25% of dry algae) present in *Kappaphycus alvarezii* may hinder the absorption of dietary fatty acids and therefore decrease cholesterol delivery corresponding with decreased lipoprotein secretion in the liver to exhibit the hyperlipidaemic effect in the HCF rats (Matanjun et al., 2010, de Sousa et al., 2013).

Porphyran and agar, water-soluble polysaccharides commonly present in red algae, lowered the serum total cholesterol concentration by 12% and 5% while 2% reduction in serum triglyceride concentration was observed in rats fed with agar-supplemented high cholesterol (1.5%) diet (Jiménez-Escrig and Sánchez-Muniz, 2000). The hyperlipidaemic effect exhibited by red algal polysaccharides such as agar, alginate and carrageenan (kappa-carrageenan) is directly associated with their physicochemical properties while these polysaccharides by inducing the viscous effect may bind directly to either bile acids or cholesterol or dietary fats and regulate lipid metabolism (Kiriyama et al., 1969, Yermak et al., 1999, Panlasigui et al., 2003, Brownlee et al., 2005). In addition to the anti-inflammatory activity, the physicochemical property of red algal polysaccharides may play an efficient role by regulating the lipid metabolism and therefore could prevent the excess fat deposition and its associated metabolic syndrome such as type 2 diabetes, hyperlipidaemia, cardiovascular complications, insulin resistance and hypertension induced by obesity (Smit, 2004).

1.10.13 Anti-oxidant and anti-hyperglycaemic effect of red algal polysaccharides

Intraperitoneal administration of polysaccharide fractions extracted from red algae *Porphyra haitanesis* (400mg/kg bwt) in aged mice (20 months old) reduced the lipid peroxidation at serum level by 54% and also in organs such as liver, heart, brain and spleen by 20-66% and simultaneously increased antioxidant enzymes activity including SOD, CAT and GSH-P_X (Zhang et al., 2003b). This study clearly indicates that the sulphated polysaccharides

(porphyrans) present in polysaccharide fractions of *Porphyra haitanesis* play a crucial role in scavenging the free radicals that include superoxide anion (O_2^-), hydroxyl radical (OH^-) and peroxynitrite ($ONOO^-$) and prevents the risk of lipid peroxidation in the aged mice (Zhang et al., 2003a). *In vitro* study using carrageenans of different structural forms (kappa, lambda and jota) isolated from *Gigartinaceae* and *Tichocarpaceae* red algae revealed that the lambda-carrageenans showed maximal anti-oxidant activity towards inhibition of 35-55% superoxide anion radicals (O_2^-) compared to vitamin C which showed 30% inhibition. In addition, an *ex vivo* study revealed that the kappa-carrageenans at 100µg/mL showed increased erythrocyte SOD activity (35%) compared with the lambda-carrageenans (27%) (Sokolova et al., 2011).
Porphyrans, water soluble sulphated polysaccharides isolated from the red algae *Porphyra yezoensis* compared with cellulose, with 5% supplementation had improved the insulin resistance (HOMA IR: 17.6 vs HOMA IR: 40.8) and the glucose metabolism by decreasing fasting blood glucose concentration (273 mg/dl vs 401mg/dl) in the diabetic KK-Ay mice (a model for type 2 diabetes). This hypoglycaemic effect of porphyrans was exhibited via increased plasma adiponectin concentration (11µg/dL vs 5 µg/dL) (Kitano et al., 2012). The prebiotic effects of porphyrans were confirmed in this study by the increased caecum weight (3.59 m/g bwt vs 2.19 mg/g bwt), increased propionate production (1.6 µmol/g caecum vs 0.4 µmol/g) and the increased *Bacteroides* species population counts (11.6 log copies/ g cecum vs 10.4 log copies/ g cecum), a beneficial gut microbiota in the diabetic KK-Ay mice. Among the SCFA, propionate is directly associated with the expression of glucose-dependent insulinotropic polypeptide (GIP), the key modulator of energy homeostasis, insulin secretion and glucose metabolism while butyrate is directly linked to the expression of satiety gut hormones such as glucagon like peptide-1 (GLP-1), peptide YY (PYY) protein that regulate weight management by inhibiting food intake (Lin et al., 2012). Therefore, in addition to the direct effect of adiponection secretion in attenuating the insulin resistance (Kitano et al., 2012), increased propionic acid production with 5% porphyran treatment in the caecum may have also influenced the improvement in insulin resistance and glucose metabolism in the diabetic KK-Ay mice. Porphyran via its potent anti-oxidant and anti-hyperglycaemic activity may play an efficient therapeutic role in the treatment and prevention of type 2 diabetes.

1.10.14 Bioactive polysaccharides of microalgae

Marine microalgae polysaccharides are classified into two types, exo- or extra-cellular polysaccharides and sulphated polysaccharides (Raposo et al., 2013). The sulphated polysaccharides are present in all groups of microalgae that include diatoms, phylum chlorophyta, phylum rhodophyta and cyanophyta. Among the diatoms groups, the sulphated polysaccharides are present in *Cylindrotheca closterium* and extracellular polysaccharides are present in *Phaedodactylum tricornutum, Nitzschia closterium, Skeletonema costatum* and *Chaetoceros* species (Penna, 1999, Guzmán-Murillo et al., 2007, Chen et al., 2011, Pletikapic et al., 2011).
In chlorophyta and rhodophyta microalgae groups, the sulphated exo-polysaccharides are present in *Chlorella* and *Porphyridium* species (Guzman et al., 2003, Geresh et al., 2002b). Among cyanophytes microalgae groups, the

extracellular polysaccharides are present in *Aphanothece halophytica* and the sulphated polysaccharides are present in *Arthrospira platensis, Anabaena, Aphanocapsa, Cyanothece, Gloethece, Phormidium* and *Nostoc* species (Li et al., 2001). The sulphated exopolysaccharides of microalgae species are composed of xylose, glucose, galactose and mannose as their major sugar residues while the major sugar residues present in extracellular polysaccharides comprise glucose, mannose, xylose and fucose (Raposo et al., 2013). Polysaccharides and especially the sulphated exo-polysaccharides are generally released into the surrounding medium by majority of the microalgae species (mentioned above) (Raposo et al., 2013).

The most common fresh-water microalgae used for human nutrition are *Spirulina* (blue green algae), *Chlorella* (Chlorophyta) and *Scenedesmus* species (Chlorophyta) (Misurcova et al., 2012). The storage polysaccharides of *Chlorella* and *Scenedesmus* species include starch, glucogen, hemicelluloses, glucuronorhamnans and extracellular polysaccharides while, in blue green microalgae such as *Spirulina* and *Cyanospora* species, polysaccharides include glycogen, starch, sulphated polysaccharides and exocellular polysaccharides (Misurcova et al., 2012). The structural rigid cell wall polysaccharides present in *Chlorella* and *Scenedesmus* species including cellulose, chitin and pectin comprise rhamnose, fucose, arabinose, xylose, mannose, galactose and glucose (Misurcova et al., 2012).

1.10.15 Cholesterol-lowering potential of microalgal polysaccharides

Soluble algal polysaccharides (37% of dry algae) from *Porphyridium* species (red microalgae) (14g/kg of diet) reduced the weight gain (16%) and exhibited cholesterol-lowering effects by increasing the faecal dry weight (66%) and bile acids excretion (68%) in control rats (Dvir et al., 2000). This study suggested that the formation of a viscous gel matrix by soluble algal polysaccharides treatment may have interfered with the lipid absorption and reduced the plasma cholesterol concentrations (29%) (Howarth et al., 2001). Additionally, compared with control rats, the increased viscosity of soluble polysaccharides lowered the gastrointestinal transit time (11%) and therefore may have delayed the nutrients absorption, resulting in reduced weight gain (Dvir et al., 2000, Howarth et al., 2001). White Leghorn chickens fed with either 5% or 10% *Porphyridium* algal biomass comprising 70% sulphated polysaccharides of algal dry weight showed reduced serum cholesterol concentrations by 11% and 28% compared with the control groups. Simultaneously, the egg yolk of chickens fed with algal biomass showed decreased cholesterol concentration by 10%. Reduced food intake with either 5% or 10% *Porphyridium* supplementation clearly shows the effect of increased viscosity with polysaccharides delayed nutrient absorption and therefore induced the satiety feeling (Ginzberg et al., 2000, Howarth et al., 2001).

1.10.16 Anti-oxidant and anti-inflammatory potential of microalgal polysaccharides

Crude extracellular polysaccharides (CEP) extracted from red algae *Rhodella reticulata* at a concentration of 6mg/mL showed efficient total anti-oxidant activity of 45.60% determined using ferrous oxidation assay (FOX) compared with vitamin E, a potent anti-oxidant compound (Chen et al., 2010).

Additionally, the superoxide anion (O_2^-) scavenging potential of CEP was comparatively high (340.65 U/mL) compared with vitamin E (170 U/mL) while vitamin E exhibited efficient hydroxyl radical scavenging activity (42%), compared with CEP (38%) (Chen et al., 2010). This study revealed the potent anti-oxidant potential of crude extracellular polysaccharides from *Rhodella reticulate*.

Similar *in vitro* study revealed that the water-soluble sulphated polysaccharides extracted from *Porphyridium* species at a concentration of 10mg/mL exhibited maximum anti-oxidant activity (87%) determined using FOX assay and inhibited the oxidative damage (79.7%) initiated by ferrous sulphate in 3T3-L1 adipocytes at a concentration of 37.5 µg/well (Tannin-Spitz et al., 2005).

Low molecular weight extracellular polysaccharide (EPS)-3 fragment (6.55kDa) extracted from *Porphyridium cruentum* showed stronger free radical scavenging activity (83.8%) using 1,1-diphenyl 2-picrylhydrazyl radical (DPPH) assay compared with high molecular weight extracellular polysaccharide fragments such as EPS-2 fragments (60.66 kDa) and EPS-1 fragments (256-kDa) (Sun et al., 2009). The EPS-3 fragments exhibited stronger hydroxyl radical scavenging activity of 69.3% at a concentration of 1mg/mL compared with vitamin C, a potent antioxidant that showed 50-60% hydroxyl scavenging activity at a higher concentration of 1.63 mg/mL and also stronger superoxide anion scavenging activity (9.3 fold) at a concentration of 2mg/mL compared with vitamin C (Xing et al., 2005, Sun et al., 2009). EPS-3 fragments via their potent anti-oxidant activity prevented the haemolysis of red blood cells induced by hydrogen peroxide (H_2O_2) at IC_{50} 0.81 mg/ml compared with high molecular weight polysaccharide fragments such as EPS-2 (IC_{50} 0.81 mg/ml) and EPS-3 (IC_{50} 1.09 mg/ml) (Sun et al., 2009).

The extracellular sulphated polysaccharides of microalgae, via their potent anti-oxidant activity could play an efficient therapeutic role in attenuating the oxidative stress-induced diabetic vascular complications such as diabetic retinopathy, neuropathy, vasculopathy and nephropathy (Giugliano et al., 1996).

Polymorphonuclear leukocyte migration and accumulation serve as the key steps for initiating the inflammatory response by inducing the secretion of pro-inflammatory cytokines that include interferon-α (IFN-α), TNF-α, IL-1β and interleukin-12 (IL-12); this chemotaxis process of inflammation contributes to inflammation-linked disorders such as inflammatory bowel disease, erythema and oedema (Matsui et al., 2003, Brazil et al., 2013). *In vitro* study using the polysaccharide fractions obtained from the red microalgae *Porphyridium* species at a concentration of 1% (weight (w)/volume (v)) showed 100% inhibition towards the migration of leucocytes and the chemotaxis-induced inflammatory response in human dermal microvascular endothelial cells (Matsui et al., 2003). This *in vivo* study using female subjects (aged 21-25) showed that pre-treatment (8% (w/v)) of sulphated polysaccharide extracts obtained from *Porphyridium* species prevented the occurrence of erythema showing 55% inhibition of redness on the inflammation-induced sites (volar forearm) of female subjects (Matsui et al., 2003).

1.11 Algae as a potential source of dietary minerals

Seaweeds generally contain a high percentage of mineral ions ranging between 7.5%-39% of dry algae (Rupérez, 2002, Bocanegra et al., 2009). Mineral

content in seaweeds is higher than fresh green leafy vegetables such as green onion (7.9%), lettuce (11.2%), radish (16.4%), coriander (21.6%) and parsley (13.5%) (Souzan and Abd el-aal, 2007).

All edible seaweeds including *Ulva* and *Caulerpa* species are rich in essential mineral ions including calcium (Ca^{++}), sodium (Na^{+}), potassium (K^{+}), magnesium (Mg^{++}), phosphorus (P^{-}), iron (Fe^{++}), iodine (I^{-}) and zinc (Zn^{++}) (Nisizawa et al., 1987). The Na^{+}/K^{+} ratio (0.1- 1.5) of seaweeds is comparatively similar to the green leafy vegetables Na^{+}/K^{+} ratio (0.04-0.3) (MacArtain et al., 2007, Souzan and Abd el-aal, 2007).

1.11.1 Mineral composition of green macroalgae (Chlorophyta)

Green macroalgal species such as *Caulerpa lentillifera* (46% of dry algae), *Codium reediae* (64% of dry algae), *Monostroma oxyspermum* (22% of dry algae), *Enteromorpha* species (23-29% of dry algae) and *Ulva fasciata* (23-29% of dry algae) contain high amounts of mineral ions (23-46% of dry algae) (McDermid and Stuercke, 2003). These green macroalgal species are also rich in essential mineral ions such as K^{+} (0.7-3.2 % of dry algae), Mg^{++} (1.2-2.9% of dry algae), P^{-} (0.1-0.4% of dry algae), Ca^{++} (0.39-0.95% of dry algae), Zn^{++} (3-32 µg/g of dry algae) and Fe^{++} (86-196 µg/g of dry algae) (McDermid and Stuercke, 2003). Compared with these macroalgal species, *Ulva fasciata* (2.9-3.2%) and *Monostroma oxyspermum* (3.14%) contain high potassium ion content while high Mg^{++} content was observed only in *Ulva fasciata* species (2.9-3.2% of dry algae) (McDermid and Stuercke, 2003). *Codium reediae* and *Caulerpa lentillifera* contain high Ca^{++} and Fe^{++} ion concentrations (Ca^{++}: 0.92-0.95% of dry algae; Fe^{++}: 196 µg/g and 167 µg/g of dry algae) compared with other macroalgal species whereas high Zn^{++} ion content was observed in *Caulerpa lentillifera* (17 µg/g of dry algae) and *Monostroma oxyspermum* (32 µg/g of dry algae) (McDermid and Stuercke, 2003). *Enteromorpha* species contain a sufficient amount of essential mineral ions that includes K^{+} (0.35% algal wet weight), Na^{+} (0.052% algal wet weight), Mg^{++} (0.46% algal wet weight), Ca^{++} (0.10% algal wet weight), Fe^{++} (220µg/g of algal wet weight) and Zn^{++} (12µg/g of algal wet weight) (MacArtain et al., 2007).

1.11.2 Mineral composition of brown macroalgae (Phaeophyta)

Brown macroalgae such as *Dictyota acutiloba, Dictyota sandvicensis, Sargassumechinocarpum* and *Sargassum obtusifolium* possess mineral ion contents ranging between 29-32% of dry algae (McDermid and Stuercke, 2003). *Sargassum* species including both *Sargassum echinocarpum* and *Sargassum obtusifolium* are rich in Mg^{++} (9.5% & 7.9% of dry algae) compared with the *Dictyota* species (5.0-7.5% of dry algae) while *Dictyota acutiloba* and *Sargassum echinocarpum* contain high Mg^{++} content (1.36% & 1.16% of dry algae) (McDermid and Stuercke, 2003). *Dictyota acutiloba* and *Sargassum obtusifolium* are rich in Zn^{++} (16 µg/g of dry algae), compared with *Dictyota sandvicensis* (13 µg/g of dry algae) and *Sargassum echinocarpum* (7 µg/g of dry algae) (McDermid and Stuercke, 2003). This study also showed that *Dictyota acutiloba* and *Dictyota sandvicensis* comprise high Fe^{++} (438 & 608 µg/g of dry algae) and, in addition, high calcium content (1.03-1.81% of dry algae) is present in both *Sargassum* or *Dictyota.*

Laminaria digita and *Himanthalia elongata* are rich in K^+ (2.01% & 1.35% of algal wet weight) while *Laminaria digita* is also rich in Fe^{++}(460 µg/g of dry algae) and Mg^{++} ion (0.40% of algal wet weight) (MacArtain et al., 2007). *Ascophyllum nodosum* possesses high Na^+ (1.17% of algal wet weight) and Ca^{++} (0.57% of algal wet weight) (MacArtain et al., 2007).

1.11.3 Mineral composition of red macroalgae (Rhodophyta)

Red macroalgae contain high mineral concentrations (23%-52% of dry algae) together with green (23-46% of dry algae) and brown macroalgal species (29-32% of dry algae) (McDermid and Stuercke, 2003). *Gracilaria* red algal species that include *Gracilaria coronopifolia, Gracilaria parvispora* and *Gracilaria salicornia* contain high mineral concentrations of about 48-53% of dry algae compared with other red algal seaweeds such as *Porphyra vietnamensis, Chondrus ocellatus* and *Ahnfeltiopsis* species with minerals content about 23-31% of dry algae (McDermid and Stuercke, 2003). In general, the red algal species discussed above are rich in K^+ (2-5% of dry algae). *Gracilaria* species such as *Gracilaria coronopifolia, Gracilaria parvispora* and *Gracilaria salicornia* species are rich in K^+ (16-22% of dry algae) and *Eucheuma denticulatum* also shows high K^+ (12% of dry algae) (McDermid and Stuercke, 2003). *Palmaria palmata* is rich in K^+ (1.2% of algal wet weight) (MacArtain et al., 2007, Bocanegra et al., 2009).

Chondrus ocellatus is rich in Zn^{++} (284 µg/g of dry algae) compared with *Gracilaria coronopifolia* (42 µg/g of dry algae), *Halymenia formosa* and *Ahnfeltiopsis concinna* (22µg/g of dry algae). Additionally, compared with these red macroalgal species d, *Halymenia formosa* contains high magnesium concentration (1.25% of dry algae) (McDermid and Stuercke, 2003). High Na^+ (1.6% of algal wet weight) was identified in *Chondrus crispus* species (MacArtain et al., 2007).

1.11.4 Mineral composition of microalgae

Marine microalgae such as *Tetraselmis suecica, Isochrysis galbana, Dunaliella tertiolecta* and *Chlorella stigmatophora* serve as rich sources of essential minerals (Ca^+, Na^+, K^+, Zn^{++} and Mg^{++}) and have been considered as the major source of mineral supplements in fish diets (Fabregas and Herrero, 1986). *Tetraselmis suecica* and *Dunaliella tertiolecta* comprise high Ca^{++} (2.08 and 2.09% of dry algae) compared to *Isochrysis galbana* (1.62% of dry algae) and *Chlorella stigmatophora* (1.51% of dry algae) (Fabregas and Herrero, 1986). *Tetraselmis suecica* and *Chlorella stigmatophora* contain high K^+ concentrations (1.20% and 1.12% of dry algae) while *Isochrysis galbana* contains high Mg^{++} (1.15% of dry algae) compared to these microalgal species; additionally, high Zn^{++} (1500 µg/g of dry algae) and Na^+ (1.04% of dry algae) content were observed in *Tetraselmis suecica* (Fabregas and Herrero, 1986).

1.12 Algae as a dietary source of n-3 PUFA

Macroalgae including green, red and brown macroalgal species are generally rich in n-3 PUFA (7.2-68% of total fatty acid) such as alpha-linolenic aid (ALA) (C18:3), EPA (C20:5) and DHA (22:6) compared with n-6 PUFA (2-45% of total fatty acid) (MacArtain et al., 2007, Bocanegra et al., 2009). The n-6/n-3 PUFA ratio in macroalgal groups ranges from about 0.50 to 1.80 (MacArtain et al., 2007, Bocanegra et al., 2009, Holdt and Kraan, 2011).

Microalgae such as diatoms, eustigmatophytes, chlorophytes, rhodophytes and cryptomonads are rich in EPA (3.2-34% of total fatty acid) while cryptomonads and pyrmnesiophytes groups are rich in DHA (0.2-11% of total fatty acid) (Volkman et al., 1989, Dunstan et al., 1992, Volkman et al., 1993, Dunstan et al., 1993). In microalgae, the n-6/n-3 PUFA ratio ranges from about 0.05 to 1.5 (Brown et al., 1997, Batista et al., 2013). Seaweeds being a rich source of n-3 PUFA are considered as functional foods (Lordan et al., 2011).

1.12.1 n-3 PUFA composition of green macroalgae (Chlorophyta)

Green macroalgal species contain sufficient amounts of n-3 PUFA that ranges from about 0.85% to 18% of dry algae (Holdt and Kraan, 2011, Pereira et al., 2012). *Enteromorpha* species and *Cladophora albida* contain high EPA (3.52 % & 2.02 % of dry algae) compared with other macroalgal species such as *Ulva* (1.50% of dry algae), *Codium* (1.40% of dry algae), *Codium fragile* (1.48% of dry algae) and *Chaetomorpha* (0.85% of dry algae) (Pereira et al., 2012). Among the green macroalgal species, the *Ulva* species is rich in ALA (4.4% of total fatty acid) while DHA is present in a minimal amount of about 0.8% in *Ulva* species and 0.86% in *Cladophora albida* compared with EPA content in green macroalgal species (Holdt and Kraan, 2011, Pereira et al., 2012). *Ulva* species serve as the richest dietary source of n-3 PUFA (6.6-18% of total fatty acid) and in addition the *Codium* species contain significant amounts of n-3 PUFA (7.40-9.52% of total fatty acid) compared with other macroalgae discussed above (Pereira et al., 2012).

1.12.2 n-3 PUFA composition of brown macroalgae (Phaeophyta)

The n-3 PUFA in brown algal species ranges about 6.6-25.0% of total fatty acid (Holdt and Kraan, 2011, Pereira et al., 2012). *Halopteris scoparia* and *Taonia atomaria* seaweeds consist of high percentage of EPA (14.39% and 13.55% of total fatty acid) compared with other brown seaweeds such as *Dictyota dichotoma* (6.57% of total fatty acid), *Sargassum vulgare* (8.60 % of total fatty acid) and *Cladostephus spongiosus* (11.46% of total fatty acid) (Pereira et al., 2012). The EPA content in *Laminaria* species, *Saccharina* species, *Undaria* species and *Fucus* species ranges about 3.6%-16% of total fatty acid and the ALA content ranges about 0.8%-3.9% of dry algae (Holdt and Kraan, 2011). *Sargassum vulgare* contains high amounts of DHA (1.5% of total fatty acid) compared with *Halopteris scoparia* (0.99% of total fatty acid) and *Taonia atomaria* (0.84% of total fatty acid) (Pereira et al., 2012). *Undaria pinnatifida* consists of about 11.2-12.0 % ALA of total fatty acid and 9.4-13.2% EPA of total fatty acid (Bocanegra et al., 2009).

1.12.3 n-3 PUFA composition of red macroalgae (Rhodophyta)

The red macroalgae species contains significant amounts of n-3 PUFA (2.90-68% of total fatty acid) (Holdt and Kraan, 2011, Pereira et al., 2012). The EPA content in *Porphyra* species varies between 6.0%-54% of total fatty acid while *Palmaria* species and *Chondrus* species comprise 35-47% EPA of total fatty acid (Holdt and Kraan, 2011). *Bornetia secundiflora* and *Jania* species consist of EPA about 25 g/100g fatty acid and 27 g/100 g fatty acid and the EPA content in *Pterocladiella capillacea* and *Peyssonnelia* species ranges about 15.26 g/100 g total fatty acid and 18.52 g/100g of fatty acid (Pereira et al.,

2012). The percentage EPA content in *Asparagopsis armata* and *Gracilaria* species varies between 1-3% of total fatty acid (Holdt and Kraan, 2011, Pereira et al., 2012). Among the red macroalgal species discussed above, the *Chondrus* and *Porphyra* species are rich in ALA content (0.2-4.3%) while the *Peyssonnelia* species contains significant amounts of DHA (4.86 g/100 g of total fatty acid) (Holdt and Kraan, 2011).

1.12.4 n-3 PUFA composition of microalgae

Microalgae including diatoms, chlorophyta and blue green algae are generally rich in n-3 PUFA (Volkman et al., 1991, Zhukova and Aizdaicher, 1995, Pratoomyot et al., 2005). Among the microalgal groups, diatoms such as *Thalassiosira* species and *Nitzschia cf. ovalis* comprise 16.65%-26.67% EPA of total fatty acid while the EPA content in the green microalgal species such as *Pavlova salina* and *Pavlova lutheri* ranges about 20-28% of total fatty acid (Volkman et al., 1991, Pratoomyot et al., 2005). *Nannochloropsis oculata* species (Eustigmatophyceae) and *Phaeodactylum trichornutum* contain significant amounts of EPA (25.9-39.8% and 29.7% of total fatty acid) (Zhukova and Aizdaicher, 1995, Holdt and Kraan, 2011).

Green microalgae including *Tetraselmis, Dictyosphaerium, Stichococcus, Chlorella* and *Scenedesmus* species are rich in ALA content ranging about 16.17%-26.47% of total fatty acid compared with the *Thalassiosira* species and *Nitzschia cf. ovalis* (diatoms) (0.37-1.10% of total fatty acid) (Volkman et al., 1991, Pratoomyot et al., 2005). Additionally, among the green microalgae discussed above, the *Dunaliella* species such as *Dunaliella tertiolecta, Dunaliella maritima* and *Dunaliella salina* contain high amounts of ALA (36.9%-42.6% of total fatty acid) (Zhukova and Aizdaicher, 1995). The *Anacystis* species of blue green algae comprise 23.18% ALA of total fatty acid (Jiang et al., 2004, Pratoomyot et al., 2005).

The percentage DHA content in *Schizochytrium mangrovei* varied from 32.29 to 39.14% of total fatty acid while *Schizochytrium mangrovei* contains low amounts of EPA (0.45 to 0.78 % of total fatty acid) and ALA (0.25 to 0.47% of total fatty acid) (Jiang et al., 2004).

Crypthecodinium cohnii contains 40-50% DHA of the total fatty acid (Jiang et al., 1999). The *Pavlova* species such as *Pavlova salina* and *Pavlova lutheri* contain high amounts of DHA (9.7-11% of total fatty acid) compared with the *Thalassiosira* species (1.33% of total fatty acid) and *Nitzschia cf. ovalis* (4.20% of total fatty acid) (Volkman et al., 1991, Pratoomyot et al., 2005).

1.13 Algae as a source of dietary amino acids

1.13.1 Amino acid composition of green macroalgae

Caulerpa lentillifera contains glutamic acid at 134.7 mg/g of protein and leucine content of about 77.9 mg/g of protein (Matanjun et al., 2009). The arginine and threonine contents in *Caulerpa lentillifera* are 57.1 and 58.4 mg/g of protein while the histidine and methionine contents are about 14.4-15.8 mg/g of dry algae (Matanjun et al., 2009). *Ulva pertusa* species contain higher amounts of arginine (149 mg/g of protein) compared to the glutamic acid and leucine content (69 mg/g of protein) (Bocanegra et al., 2009). Threonine and histidine content present in *Ulva pertusa* species varies between 31-40 mg/g of protein. In addition, methionine and tryptophan content in *Ulva pertusa* species ranges between 3-16mg/g of protein (Bocanegra et al., 2009). *Ulva lactuca*

possesses a high amount of glutamic acid and arginine (87.3 and 84.4 mg/g of protein) compared with the threonine (50.6 mg/g of protein) and leucine content (67.1 mg/g of protein). Cysteine and methionine content in *Ulva lactuca* varies between 13.3-15.7 mg/g of protein and histidine content is about 4.82 mg/g of protein (Wong and Cheung, 2000).

Ulva reticulata contains sufficient amounts of glutamic acid (129.8 mg/g of protein) and arginine (86.5 mg/g of protein) (Ratana-arporn and Chirapart, 2006). Threonine and leucine content in *Ulva reticulata* varies between 60.0-80.0 mg/g of protein (Ratana-arporn and Chirapart, 2006).

1.13.2 Amino acid composition of brown macroalgae

Laminaria species contain high amounts of glutamic acid (238-240 mg/g of protein) compared with *Undaria pinnatifida* (65-145 mg/g of protein) and *Hizikia fusiforme* (118-187 mg/g of protein) (Bocanegra et al., 2009). High arginine content was observed in *Hizikia fusiforme* (45-49 mg/g of protein) and *Undaria pinnatifida* (30-52 mg/g of protein) compared with the *Laminaria* species (33-36mg/g of protein) (Bocanegra et al., 2009). Threonine content in all the brown seaweeds such as *Laminaria* species, *Undaria pinnatifida* and *Hizikia fusiforme* varies between 10-54 mg/g of protein (Bocanegra et al., 2009). *Undaria pinnatifida* contain high amounts of leucine (74-84 mg/g of protein) compared with either *Laminaria* (49 mg/g of protein) or *Hizikia fusiforme* (5-7 mg/g of protein) (Bocanegra et al., 2009). The methionine and the histidine content in either *Undaria pinnatifida, Hizikia fusiforme* or *Laminaria* species ranges between 5-32 mg/g of protein (Bocanegra et al., 2009). Cysteine and tryptophan contents in all the brown algae seaweeds discussed above varies between 4-12 mg/g of protein (Bocanegra et al., 2009).

Sargassum polycystum contains glutamic acid at about 8 mg/g of protein and the leucine content at about 5 mg/g of protein and other amino acids including arginine, histidine and threonine present in the *Sargassum polycystum* at between 1-3 mg/g of protein (Matanjun et al., 2009).

1.13.3 Amino acid composition of red macro algae

Porphyra species contains significant amounts of glutamic acid (93-102 mg/g of protein) compared with the *Palmaria palmata* species (67-99 mg/g of protein) and *Eucheumia cottonii* (52 mg/g of protein) (Bocanegra et al., 2009, Matanjun et al., 2009). Glutamic acid content in *Hypnea charoides* and *Hypnea japonica* ranges from 98-110 mg/g of protein. *Hypnea* species that include either *Hypnea charoides* or *Hypnea japonica* contain high amounts of arginine (64-69 mg/ g of protein) compared with the *Palmaria palmata* (46-51 mg/g of protein), *Porphyra* species (59 mg/g of protein) and *Eucheumia cottonii* (3 mg/g of protein) (Wong and Cheung, 2000, Bocanegra et al., 2009, Matanjun et al., 2009). High leucine content in *Hypnea* species was observed (72-98 mg/g of protein) compared with either *Palmaria palmata* or *Porphyra* species (55-71 mg/g of protein) while the threonine content in either *Hypnea* species, *Palmaria palmata* or *Porphyra* species varies between 36-52 mg/g of protein (Bocanegra et al., 2009, Matanjun et al., 2009).

The histidine and methionine content in *Hypnea* species ranges between 7-19 mg/g of protein whereas in *Palmaria palmata* and *Porphyra* species, it ranges between 5-45 mg/g of protein (Bocanegra et al., 2009, Matanjun et al., 2009). *Palmaria palmata* contain high tryptophan content (30 mg/g of protein)

compared with *Porphyra* species (7 mg /g of protein) and among the red algal seaweeds discussed above, cysteine (12 mg/g of protein) was present only in *Porphyra* species (Bocanegra et al., 2009).

1.13.4 Amino acid composition of microalgae

Dunaliella bardawil and *Arthrospira maxima* contain high amounts of glutamate (126-127 mg/g of protein) compared with the other microalgae such as *Chlorella vulgaris* (116 mg/g of protein), *Scenedesmus obliquus* (107 mg/g of protein), *Spirulina platensis* (103 mg/g of protein) and *Aphanizomenon* species (103 mg/g of protein) while the arginine content in the microalgae discussed above ranges between 38-73 mg/g of protein (Christaki et al., 2011). *Dunaliella bardawil* and *Spirulina platensis* contain high amount of leucine (98-110mg/g of protein) (Christaki et al., 2011). Among the microalgae, *Chlorella vulgaris* and *Dunaliella bardawil* contain significant amounts of cysteine (12-14 mg/g of protein) (Christaki et al., 2011). The threonine content in *Spirulina platensis* and *Dunaliella bardawil* species ranged between 54-62 mg/g of protein and in addition high tryptophan content was observed in *Chlorella vulgaris* and *Arthrospira maxima* (14-21 mg/g of protein) (Christaki et al., 2011). Histidine and methionine content in the microalgae varies between 7-25mg/g of protein (Christaki et al., 2011).

1.14 Algae as a source of dietary carotenoids

1.14.1 Macroalgal carotenoids

Ulva fenestrare (chlorophyta) serves as a rich source of α-carotene, neoxanthin, violaxanthin and siphonoxanthin (Vershinin and Kamnev, 1996). *Laminaria* species (Phaeophyta) contain significant amount of fucoxanthin, β-carotene and violaxanthin (Haugan and Liaaen-Jensen, 1994, Terasaki et al., 2009, Rodríguez-Bernaldo de Quirós et al., 2010) while *Porphyra* species (Rhodophyta) are rich in β-carotene, lutein and zeaxanthin (Okai et al., 1996, Esteban et al., 2009).

1.14.2 Microalgal carotenoids

β-carotene, α-carotene and zeaxanthin are the major carotenoids present in *Dunaliella salina* (Yokthongwattana et al., 2005, Hu et al., 2008). *Hematococcus pluvialis* possessed high amounts of astaxanthin, canthaxanthin and lutein (Grewe and Griehl, 2008, Jaime et al., 2010) and *Chlorella* species are rich in astaxanthin, lutein, β-cryptoxantin and canthaxanthin (Inbaraj et al., 2006, Wu et al., 2007). *Scenedesmus* species contain significant amounts of lutein and β-carotene (Campo et al., 2007) whereas the *Spirulina* species are rich in β-carotene and zeaxanthin (Mohammed and Mohd, 2011, Christaki et al., 2012).

1.15 Magnesium and potassium ion role in attenuating the metabolic syndrome

The intracellular potassium ion (K_i^+) concentration was reduced in hypertensive (132mmol/L) and type diabetic subjects (121mmol/L), compared with control subjects (148 mmol/L) (Resnick et al., 2001). In addition, this study also suggested that the fasting K_i^+ depletion in the red blood cells of either type 2 diabetics or hypertensive subjects is closely associated with the intracellular calcium ion (Ca_i) and intracellular magnesium ion (Mg_i^{++})

homeostasis i.e. higher the K_i^+, lower the Ca_i^{++} and higher the Mg_i^{++} concentrations. Maintaining the steady-state K_i^+ concentrations via treatment could attenuate the pathogenesis of hypertension and type 2 diabetes (Resnick et al., 2001). The depletion of intracellular potassium ion (K_i^+) or magnesium ion (Mg_i^{++}) or increasing calcium ion (Ca_i^{++}) concentrations may lead to vascular smooth muscle contraction causing increased blood pressure (Yang et al., 2000), insulin resistance, impaired glucose and insulin metabolism (Altura and Altura, 1981, Kuriyama et al., 1982, Nadler et al., 1993, Barbagallo et al., 2001). Additionally, the depletion of K_i^+ and the corresponding Mg_i^{++} is also directly associated with increased blood lipid concentrations (Altura et al., 1990), decreased NO production and endothelial dysfunction (Yang et al., 2000). Improved insulin sensitivity by 1% dietary magnesium treatment may contribute to improved insulin resistance and glucose utilization (Volpe, 2008). Oral magnesium intake of 2.5g/day restored serum magnesium concentrations (0.74±0.10 vs 0.65±0.07 mmol/l) and improved the insulin sensitivity and metabolic control in subjects with type 2 diabetics (Rodriguez-Moran and Guerrero-Romero, 2003). Mg_i^{++} serves as the major cofactor for all enzymatic reactions specifically in the phosphorylation processes, playing an efficient role in inducing the phosphorylation of insulin receptor-tyrosine kinase that directly influences the insulin sensitivity and glucose metabolism (Barbagallo and Dominguez, 2007, Volpe, 2008). Dietary magnesium supplementation (10g/kg diet) lowered the blood pressure in DOCA-salt hypertensive rats by stimulating the vascular endothelial relaxant factors (Laurant et al., 1995).

In atherosclerotic rabbits, higher dietary magnesium intake (225-275 mg/kg/day) reduced serum triglyceride concentrations by 33% and serum cholesterol concentrations by 24% (Altura et al., 1990). Since magnesium is a divalent cation (Mg^{++}) like calcium ion (Ca^{++}), this lipid-lowering effect observed in atherosclerotic rabbits (Altura et al., 1990) was attributed to the ability of these cations to form insoluble salt complexes with either lipids or bile acid derivates to increase the excretion of lipids (Bhattacharyya et al., 1969, Renaud et al., 1983). Dietary Mg^{++} treatment (1%) in fructose-fed rats normalised the plasma concentrations of increased cholesterol from 3.10 mmol/L to 2.51 mmol/L, triglyceride concentrations from 1.13 mmol/L to 0.86 mmol/L, fasting insulin concentrations from 85.8 µIU/mL to 58.4 µIU/mL and fasting glucose concentrations from 5.8 mmol/L to 5.0 mmol/L (Olatunji and Soladoye, 2007). The hypolipidaemic effect of 1% dietary magnesium in fructose-fed rats has been attributed to the decreased activity of liver lipogenic enzymes (Olatunji and Soladoye, 2007) and increased lipoprotein lipase activity (Rayssiguier et al., 1991). In addition, potassium plays a crucial role in maintaining the vascular tone via activating the sodium (Na^+)-potassium (K^+) pump. In response to increased shear stress, K^+ is released from the endothelial cells and contributes to endothelial-dependent relaxation (Haddy et al., 2006). Spontaneously hypertensive rats (SHR) treated with K^+ (1 or 3.6%/ chow food) showed reductions in blood pressure by endothelial-dependent and -independent arterial relaxation (Wu et al., 1998). Based on this evidence and also since K_i^+ is directly associated with Mg_i^{++} concentrations in blood erythrocytes (Resnick et al., 2001), dietary supplementation of K^+ may attenuate the increased blood pressure in hypertensive patients (Haddy et al., 2006). In hypertensive rats fed with dietary K^+ (420 mg/kg body weight or 1.12

g/kg body weight), the damage caused by cerebral ischaemia was reduced (Rigsby et al., 2008).

1.16 Dietary fibres and their role in attenuating the metabolic syndrome

The mean daily intake of dietary fibre including both soluble and insoluble fibre among Japanese population was reported as 20.5g/day (Nakaji et al., 2002). Additionally, this report study also highlighted that a rapid decline (30%) in total dietary fibre intake between the years 1970 and 1998 may initiate major health problems in the future Japanese population (Nakaji et al., 2002). Soluble fibres such as guar gum and pectin are highly fermentable by gut microflora and also the gel-forming capacity of soluble fibre increases the viscosity of the gastrointestinal tract. This physicochemical property of soluble fibre plays a significant role in preventing cholesterol absorption and thereby lowering the serum total cholesterol and LDL (Hunt et al., 1993). The viscous nature of soluble fibre may also delay gastric emptying and therefore slow down glucose absorption and reduce post-prandial blood glucose concentrations and the corresponding increase in blood insulin concentrations (Jenkins et al., 1978, Edwards et al., 1987, Jenkins et al., 2000). Increased viscosity and gelling of dietary fibre would not necessarily limit the fat absorption and indeed it may delay the fat absorption and therefore increase the lipid excretion (Vahouny et al., 1988, Deuchi et al., 1995). SCFA such as acetate, propionate and butyrate released during the fermentation of soluble fibre in the colon inhibit food intake by increasing the expression of satiety hormones such as PYY and glucagon-like peptide-1 (GLP-1) to suppress weight gain (Scott et al., 2008). Insoluble fibre includes matrix fibres such as lignin, cellulose and hemi-cellulose. In general, insoluble fibre is not fermentable by gut microflora and their water retention capacity increases the faecal bulk without lowering serum total cholesterol concentrations (Hunt et al., 1993).

An observational study in healthy human subjects that included both young and old male adults with high carbohydrate intakes (304g/d and 218g/d) supplemented with high fibre diet rich in soluble fibre (23.6 g/d and 17.1 g/d) showed decreased serum cholesterol concentrations (3.80 vs 5.17 mmol/L and 4.99 vs 6.17 mmol/L), decreased fasting insulin concentrations (50.2 vs 67.4 pmol/L and 50.9 vs 64.6 pmol/L), decreased fasting glucose concentrations (5.05 vs 5.44 pmol/L and 5.05 vs 5.27 mmol/L), compared with subjects who had low soluble fibre intake (3.5g/d and 3.7 g/d) (Fukagawa et al., 1990). Diabetic subjects are recommended to consume food with moderate carbohydrates of $\geq$ 50% with dietary fibre intake of 25-50 g/d to lead to a healthy lifestyle (Anderson et al., 2004).

1.17 Inulin and oligofructose as soluble dietary fibres in attenuating the metabolic syndrome

1.17.1 Effect of inulin-type fructans on obesity

Inulin and oligofructose, the major storage carbohydrates present in vegetables, fruits and whole grain, improve health through their physiological effects as soluble dietary fibres (Cherbut, 2002, Kaur and Gupta, 2002). Inulin and oligofructose are considered to be soluble and undergo selective fermentation by *Bifidobacterium* species resulting in the release of SCFA such as acetate,

propionate and butyrate (Delzenne and Williams, 2002, Roberfroid, 2007). Obese rats fed with 10% or 20% prebiotic soluble fibres (inulin and oligofructose 1:1 ratio) showed reduced triglyceride accumulation in liver, and also in serum total cholesterol concentrations (Parnell and Reimer, 2010). It was suggested that inulin-type fructans can prevent the risk of obesity, type 2 diabetes, atherosclerotic coronary heart disease and insulin resistance by attenuating the lipid metabolism or hyperinsulinaemia (Roberfroid and Delzenne, 1998). High fat-fed mice treated with oligofructose-enriched inulin showed reduction in body weight gain, total body adiposity and liver fat content (Anastasovska et al., 2012). The anti-obesity effect of inulin and oligofructose has been attributed to the decreased energy intake regulated by increased circulating levels of satiety hormones such as GLP-1 and PYY (Cani et al., 2005, Delzenne et al., 2005). Treatment with inulin-type fructans increased the *Bifidobacterium* counts associated with increased production of GLP-1 and GLP-2 in the portal vein and prevented the fat mass development, glucose intolerance and metabolic endotoxaemia (Delzenne et al., 2011). Oligofructose treatment promotes satiety via up-regulation of GLP-1 to attenuate fat mass deposition and hepatic steatosis in obese rats; also oligofructose lowered plasma glucose concentrations in both streptozotocin-treated rats (STZ) and obese rats (Delzenne et al., 2007). Oligofructose supplementation reduced the body weight by decreasing the energy intake by stimulating the production of PYY and improving glucose utilisation in overweight adults (de Mesquita et al., 1991). Inulin-type fructans restored the insulin sensitivity in the subcutaneous adipose tissue by enhancing the phosphorylation of Akt and as well as inhibiting lipolysis by insulin in high fat mice (Dewulf et al., 2011). Also, treatment with the inulin-type fructans in high fat-fed mice normalised the subcutaneous adipose tissue mass and the adipocyte cells size by down-regulating the subcutaneous adipose tissue expression of G protein-coupled receptor 43 (GPR43) that primarily regulates the adipose tissue metabolism by inhibiting lipolysis and stimulating adipocyte differentiation (Hong et al., 2005, Ge et al., 2008, Dewulf et al., 2011).

1.17.2 Effect of inulin-type fructans on type 2 diabetes

Inulin treatment in healthy subjects increased plasma GLP-1 concentrations, postprandial serum concentrations of SCFA, and attenuated the increased serum concentrations of ghrelin and postprandial free fatty acids to reduce the risk factors of type 2 diabetes (Tarini and Wolever, 2010). Normal male Wistar rats treated with 10% oligofructose had reduced postprandial hyperglycaemia and hyperinsulinaemia (Kok et al., 1996). Oligofructose supplementation as 10% of the diet attenuated the postprandial serum concentrations of triglycerides by 40%, glucose by 12%, and insulin by 29% compared to rats fed with normal control diet (Kok et al., 1998). The improved glucose utilisation and hypolipidaemic effects observed in oligofructose-treated rats have been attributed to the increased serum concentrations of GIP and caecal concentrations of GLP-1 (D'Alessio et al., 1994, Knapper et al., 1995, Kok et al., 1998). Increased inulin (10g/day) in middle-aged men and women identified with moderately increased plasma concentrations of total cholesterol and triacylglycerol showed decreased fasting blood insulin concentrations and fasting plasma triacylglycerol concentrations (Jackson et al., 1999). Inulin and oligofructose treatment increased the portal vein and the distal colon

concentrations of GLP-1 and attenuated the increased serum concentrations of glucose, triacylglycerol and total cholesterol in C57B1/6J mice (Urias-Silvas et al., 2008). Inulin and oligofructose supplementation reduced food intake and therefore prevented the increase in body weight gain in mice fed with the standard control diet (Urias-Silvas et al., 2008). This study concluded that increased GLP-1 concentrations in the inulin and oligofructose-treated mice may play a crucial role in attenuating the body weight gain by inducing satiety and reducing the energy intake (Flint et al., 1998) and also reduced the blood glucose concentrations by stimulating insulin secretion from pancreatic ß-cells (Holz et al., 1993).

1.17.3 Effect of inulin-type fructans on hypertension and NAFLD

Synergy1 (1:1 inulin:oligofructose) mixture attenuated the elevated blood pressure by 9mmHg in rats fed with high fructose diet (Rault-Nania et al., 2008). Additionally, the antihypertensive activity of inulin-type fructans has been attributed to the reduction of oxidative stress induced by high fructose diet (Rault-Nania et al., 2008). The triglyceride-lowering effect of inulin and oligofructose in liver was exhibited via two ways, firstly by decreased *de novo* lipogenesis and secondly through increased production of propionate (Roberfroid and Delzenne, 1998). Young healthy male subjects fed 18% inulin-supplemented cereals showed decreased plasma concentrations of total cholesterol (18.3%) and triacylglycerol (27%) (Brighenti et al., 1999). Propionate selectively down-regulated hepatic fatty acid synthase (Delzenne and Williams, 2002). Hyperlipidaemic subjects (n = 54; both males and females) treated with inulin (8 g/d) for 3 weeks showed reduced blood concentrations of triglycerides and LDL cholesterol (Causey et al., 2000). Inulin-type fructans attenuated the increased plasma concentrations of triglycerides (36-50% reduction) and also the hepatic triglycerides concentration by 43-56% in fructose-fed rats (Rault-Nania et al., 2008). Dietary supplementation of either 10% inulin or oligofructose in rats fed with a high carbohydrate and high fat diet exhibited its triglyceride-lowering effect via inhibiting hepatic lipogenesis and preventing the increase in total serum cholesterol concentrations to play a potent role in preventing the NAFLD in humans (Delzenne et al., 2002).

1.17.4 Effect of inulin-type fructans on gut morphology

In obese mice, selective increases in *Bifidobacterium* counts following supplementation with prebiotics (oligofructose) prevented the increased intestinal permeability and improved the mucosal barrier function by a GLP-2 dependent mechanism attenuating the systemic and hepatic inflammation induced during obesity (Tsai et al., 1997, Cani et al., 2009). GLP-2 improved the mucosal barrier function via enhancing the rate of crypt cell proliferation, villus elongation and also by inhibiting apoptosis (Tsai et al., 1997). Inulin and oligofructose are resistant to digestion and absorption in the stomach and small intestine are highly susceptible to fermentation by gut microbes present in the large intestine to improve gut motility (Cherbut, 2002). Further, inulin and oligofructose by their faecal bulking effect increased the stool production to between 1.2 and 2.1 g per g of ingested substrate (Gibson et al., 1995, Cherbut, 2002) which resembles the faecal bulking effect of other fermentable soluble fibre such as pectins (1.3g/g) and guar gum (1.5g/g) (Cherbut, 2002). Increased

dosage of inulin from 20g/day to 40g/day enhanced the *Bifidobacterium* (beneficial gut microbe) counts by 16.5%/g of dry faeces in elderly constipated subjects (Kleessen et al., 1997). Inulin treatment at a higher dose of 40g/day increased the stool frequency to 7.5/week in constipated subjects (Kleessen et al., 1997). Similarly, chicory inulin treatment (4-15g/day) increased the faecal weight by 22g/day and also the stool frequency by 2.5 per week in healthy volunteers (Den Hond et al., 2000). Caecal and colonic mucosal contents of rats fed an inulin-supplemented diet (100g/kg diet) contained higher amounts of sulphomucins which are generally decreased in intestinal disease conditions such as inflammation and colon cancer and lower amounts of acidic mucins (Fontaine et al., 1996, Cherbut, 2002). Prebiotic supplementation by inulin and oligofructose (50g/kg diet) increased the height of villi, depth of crypt, number of goblet cells and the thickness of epithelial mucus layer in rat models fed with a commercial standard diet, playing a role in improving the gastrointestinal disorders and as well as in health maintenance (Kleessen et al., 2003).

1.17.5 Effect of inulin-type fructans on mineral ions absorption

Inulin and oligofructose resistant to digestion in the upper gastrointestinal tract are considered as prebiotics (O'Sullivan et al., 2010). Short chain carboxylic acids released during the fermentative process of inulin and oligofructose reduced the luminal pH and enhanced absorption of mineral ions such as magnesium and calcium
(Takahara et al., 2000, Scholz-Ahrens and Schrezenmeir, 2007, Lobo et al., 2009). Compared with control rats, rats fed with inulin-type fructans despite the increased faecal energy excretion (47-67%) showed maximum absorption of calcium, magnesium, iron, zinc and copper ions (Delzenne et al., 1995). Similarly, rats treated with oligofructose (50g/kg control diet) showed increased absorption of calcium and magnesium ions in the colon and rectum compared with the control rats (Ohta et al., 1995). Further study also determined that rats treated with inulin-type fructans increased intestinal absorption and the retention of calcium and magnesium ions compared with the control rats (Coudray et al., 2003). Rats treated with Synergy1 (inulin:oligofructose) mixture alone increased intestinal absorption (26%) and retention (25%) of calcium ions compared with the rats treated with either inulin or oligofructose (Coudray et al., 2003).

1.18 Focus and objectives of the study

Visceral fat depots enhanced during obesity serve as a major risk factor in the development of hypogonadism, cardiovascular disease, hypertension, type 2 diabetes, insulin resistance and NAFLD (Fox et al., 2007, Bjorndal et al., 2011). Also, testosterone deprivation in elderly men and prostate cancer patients undergoing androgen deprivation therapy is associated with increased visceral and subcutaneous fat mass, decreased lean mass, type 2 diabetes, cardiovascular complications, insulin resistance, dyslipidaemia and non-alcoholic fatty liver disease (Mudali and Dobs, 2004, Kawano, 2010, Hamilton et al., 2011). In modern societies, diets rich in high-carbohydrate and high-fat serve as the major causative factor for all metabolic disorders. In my study, I have induced obesity in rats using a high-carbohydrate, high-fat diet containing beef tallow 20%, condensed milk 39.5%, salt mixture 2.5% and fructose 17.5%

in the food with fructose 25% in the drinking water (Panchal et al., 2011b). These rats become hypertensive with increased cardiac stiffness, fatty liver, increased plasma liver enzymes and increased abdominal fat pads and so mimic the changes in chronic obesity in humans.

Objectives

The current non-drug and non-surgical treatments of obesity and its associated disorders are aimed at modifying dietary habits and increasing exercise to increase calorie expenditure and lowering calorie and fat intake (Anderson et al., 2011). This thesis will examine the role of testosterone deficiency in the development of the metabolic syndrome and then measure the responses to three possible interventions to reverse this syndrome. All studies will be undertaken in rats fed with high-carbohydrate, high-fat diet to mimic human metabolic syndrome.

1. To investigate whether testosterone deficiency in castrated rats enhances visceral obesity and its associated metabolic complications, and
2. To investigate the therapeutic effects of diet-based interventions with
    a) Seaweeds (*Ulva ohnoi* and *Derbesia tenuissima*)
    b) Microalgae mixture (*Scenedesmus dimorphus* and *Schroederiella apiculata*)
    c) Mineral ions (Mg^{++} and K^{+}) and
    d) Dietary soluble fibre (inulin and oligofructose mixture) in rats fed with high-carbohydrate, high-fat diet.

1.19 References

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CHAPTER 1.2

Seaweeds as potential therapeutic interventions for the metabolic syndrome

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Seaweeds as potential therapeutic interventions for the metabolic syndrome

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Abstract Seaweeds are a characteristic part of the traditional diet in countries such as Japan and Korea; these countries also have a lower prevalence of metabolic syndrome than countries such as the USA and Australia. This suggests that seaweeds may contain compounds that reduce the characteristic signs of obesity, diabetes, hypertension, fatty liver and inflammation in the metabolic syndrome. Potentially bioactive compounds from seaweeds include polysaccharides, peptides, pigments, minerals and omega-3 fatty acids. This review emphasises current research on these compounds in isolated cells, animal models and patients. Key problems for future research include chemical characterisation of the bioactive principles, defining pharmacological responses in all aspects of the metabolic syndrome, determining if a therapeutic dose has been administered, and defining oral bioavailability of the active ingredients.

Keywords Metabolic syndrome · Seaweeds · Polysaccharides · Peptides · Pigments · Minerals

1 Introduction

The metabolic syndrome, a cluster of metabolic disorders associated with obesity, insulin resistance, glucose intolerance, dyslipidaemia, hypertension and fatty liver, combines the major risk factors for cardiovascular disease, diabetes, kidney disease and blindness [1]. The prevalence of metabolic syndrome ranges from 1.7 to 56.7 % in both men and women in countries around the world [2, 3]. Dietary interventions such as the Mediterranean diet may improve the health of patients with metabolic syndrome [4].

The reported prevalence of metabolic syndrome in the Japanese population is 19.0 % in males aged 40–64 years, and 21.4 % in males aged 65–79 years [5]; considerably lower than the figures for the USA of 41.2 % for males aged 40–59 years and 49.9 % for males aged 60 years and above [6] and for Australia of 41.8 % in sedentary males aged 50–80 years [7]. The Japanese and Korean diets emphasise the use of soybean products, fish, seaweeds, vegetables, fruits and green tea suggesting that these products are associated with a decreased risk of cardiovascular mortality and metabolic syndrome [8, 9]. The average intake of seaweeds in Japan ranged from 3.0 to 6.9 g/day between 1950 and 1996 or around 1.6 kg dry weight/year [10]. Seaweeds are considered as functional foods, for example as the major source of the dietary intake of iodine in Japan estimated at 1–3 mg/day [11] and also producing bioactive peptides that may decrease blood pressure [12].

Seaweeds are aquatic photosynthetic plants separated into macroalgae or microalgae, with macroalgae classified into three types: brown algae (Phaeophyta), red algae (Rhodophyta) and green algae (Chlorophyta) [13]. Microalgae as unicellular organisms that are able to grow in both marine and fresh water [14] are classified into four major types: diatoms (bacillariophyta), yellow-brown flagellates (chlorophyta; prasinophyta; prymnesiophyta, cryptophyta, chrysophyta, rhodophyta and rhaphidiophyta), dinoflagellates (dinophyta) and blue-green algae (cyanophyta) [15].

Seaweeds contain polysaccharides (20–76 % of dry weight), proteins (15–40 % of dry weight), carotenoids, mineral ions (36 % of dry weight) and omega-3 fatty acids, usually eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA); all of the seaweed components may have the ability to improve health and prevent metabolic syndrome [15–17]. This review highlights recent studies on the potential bioactive components of seaweeds that may reverse the signs of metabolic syndrome.

2 Seaweed polysaccharides

Sulphated polysaccharides such as fucoidan from brown seaweeds [18], and carrageenans, alginates and porphyrans from red seaweeds have biological effects that could be useful

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in the prevention or reversal of metabolic syndrome (Fig. 1) [19, 20].

Treatment with 1 % fucoidan attenuated the body-weight gain, increased liver mass and especially the increased epididymal fat mass in high fat-fed mice; these changes involved attenuating the mRNA gene expression of major adipogenic markers such as aP2 and PPAR-γ and inhibiting acetyl carboxylase, an enzyme that plays a crucial role in the production of malonyl-CoA for fatty acid synthesis [21]. The plasma lipid profile was improved with decreased concentrations of triglycerides, cholesterol and low density lipoproteins [21]. Thus, fucoidan may prevent obesity by inhibiting adipogenesis and enhancing lipolysis.

Using 3T3-L1 adipocytes, fucoidan (200 μg/mL) decreased the lipid accumulation by 52 %, reduced triglyceride deposition by 15 % and increased hormone-sensitive lipase expression indicating stimulation of lipolysis [22]. Fucoidan (100 μg/mL) from *Undaria pinnatifida* suppressed adipogenesis in 3T3-L1 cells *via* down-regulating the mRNA gene expression of key adipogenic markers such as peroxisome proliferator activated receptor-γ (PPAR-γ), CCAAT/enhancer-binding protein α (C/EBPα) and adipocyte protein 2 (aP2) [23]. Fucoidan (100 μg/mL) also inhibited adipocyte differentiation and thereby prevented lipid accumulation in 3T3-L1 cells by suppressing mRNA gene expressions of key adipocyte differentiation markers such as tumor necrosis factor–α (TNFα), monocyte chemotactic protein-1 (MCP-1) and plasminogen activator inhibitor-1 (PAI-1) [23].

Crude fucoidan extracts obtained from *Undaria pinnatifida* (45 mg/kg bodyweight) prevented hyperglycaemia in genetically diabetic db/db female mice by improving the glucose utilisation and decreasing fasting blood glucose concentrations and serum insulin concentrations [24]. In alloxan-induced diabetic rats, fucoidan extracts obtained from *Saccharina japonica* species stimulated pancreatic release of insulin to lower plasma glucose concentrations [25]. The antidiabetic effects of fucoidan treatment improved diabetic retinopathy by attenuating the retinal expression of high glucose-induced vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1α (HIF-1α) in streptozotocin-induced diabetic mice [26].

In leptin receptor-deficient diabetic db/db mice, low molecular weight fucoidans (250 or 500 mg/kg) decreased white adipose tissue weight, serum concentrations of total cholesterol, low density lipoprotein cholesterol and triglycerides; further, fucoidan supplementation improved glucose tolerance by activating AMP-activated protein kinase and improved insulin sensitivity by increasing serum adiponectin concentrations [27]. In L6 myotubes, the low molecular weight fucoidans activated AMP–activated kinase to increase glucose uptake, improve endoplasmic stress-induced insulin resistance and decrease intracellular lipid contents by enhancing the fatty acid oxidation [27].

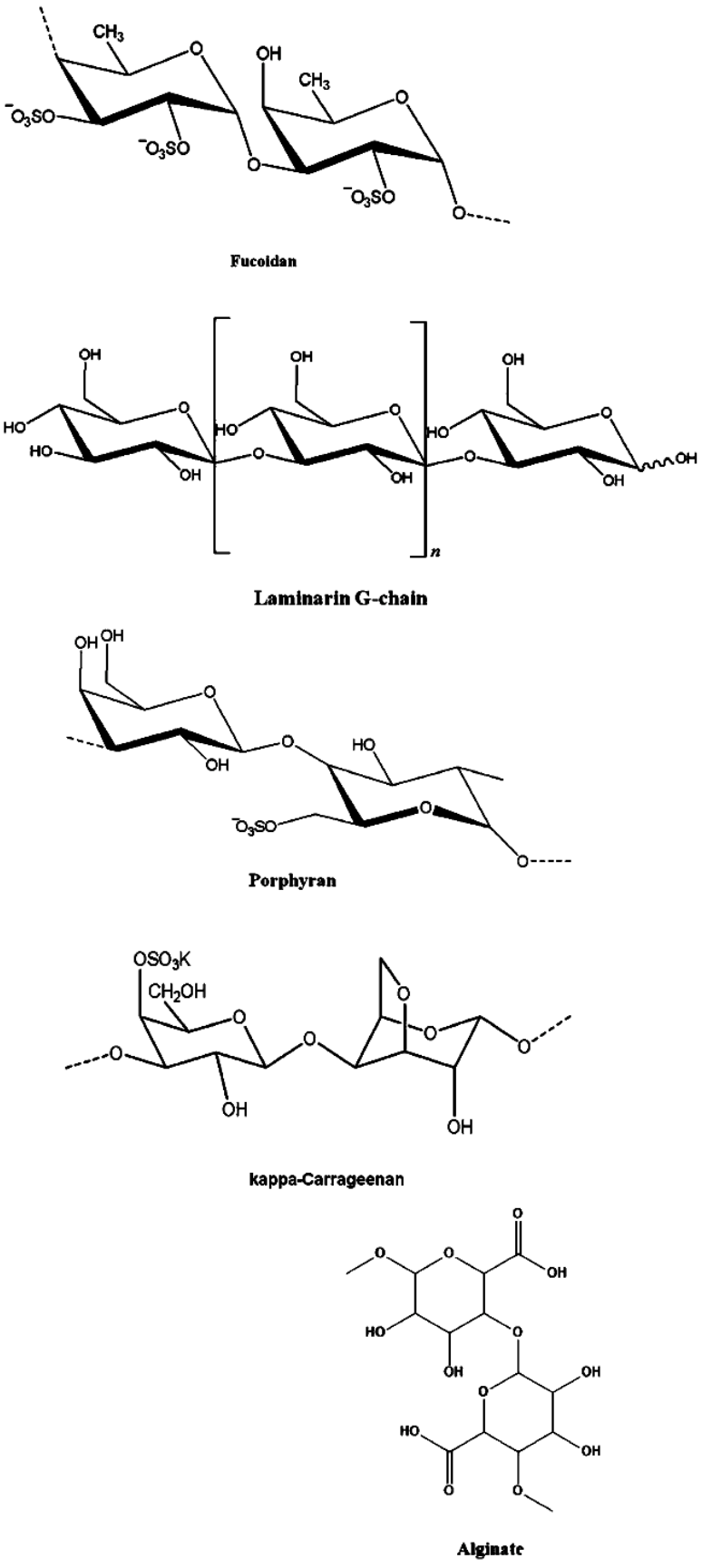
Possible anti-inflammatory actions of fucoidan fractions obtained from *Ecklonia cava* were shown as maximal inhibition of nitric oxide production at 100 μg/mL and the corresponding prostaglandin E2 (PGE_2) release at 200 μg/mL in lipopolysaccharide (LPS)-stimulated RAW macrophages, mediated *via* dose-dependent inhibition of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein expression [28]. Treatment with fucoidan attenuated the increased plasma concentrations of inflammatory cytokines including TNF-α and interleukin-6 (IL-6) in rats with ischaemia/reperfusion-induced myocardial damage [29].

Carrageenans are sulphated galactans from red seaweeds. Rats fed a diet including 10 % *Mastocarpus stellatus* improved antioxidant status in the caecum, increased coagulation times and reduced plasma triglycerides and cholesterol concentrations in healthy rats [30].

Alginates may decrease nutrient absorption in the small intestine to control the increased postprandial glucose concentrations and insulin secretion in type 2 diabetic patients, with long-term alginate treatment improving obesity management and attenuating the metabolic syndrome [31]. Relatively low concentrations of strongly gastric-gelling alginates dose-dependently reduced hunger responses by 20–30 % [32]. Sodium alginate (15 g in 500 mL) given to patients with body-mass index values of 23.5±1.7 kg/m^2 reduced the energy intake by 8.0 % by inducing satiety [33]. Treatment with 3 % sodium alginate from brown seaweeds such as *Laminaria hyperborea* and *Laminaria digitata* for 12 weeks increased the weight loss by 6.4 % in obese subjects [34]. Alginate supplementation to an energy-restricted diet of about 300 kcal/day for 12 weeks increased weight loss [34]. Thus, the increased viscosity from gel formation with some alginates may promote satiety with delayed gastric emptying, increased viscosity of digesta and slowed nutrient absorption in the small intestine [31]. Treatment with alginic acids (100 mg/kg body weight) from *Sargassum wightii* (brown macroalgae) prevented the inflammatory cell infiltration in arthritic rats by reducing the expression of C-reactive protein kinase and the corresponding increase in enzymes linked to inflammation such as cyclooxygenase, lipooxygenase and myeloperoxidase [35]. This mechanism could be useful in the treatment of obesity, recognised as a chronic low-grade inflammation [36]. Further, enzymatic cleavage of alginates produced oligosaccharides with 2–3 sugar units; these compounds almost abolished salt-induced hypertension in Dahl rats, possibly through direct vascular responses [37].

The adult intestine contains around 10^{14} microorganisms, mostly bacteria. Prebiotics are non-digestible carbohydrates that change this bacterial composition to modify gastrointestinal hormone secretion, glycaemic regulation and lipid metabolism, improving the risk factors for obesity [38]. These changes involve increased concentrations of short-chain carboxylic acids, including acetate, propionate and butyrate. The

Fig. 1 Seaweed polysaccharides



cell walls of seaweeds have a high fibre content of around 33–50 % dry weight; this fibre is a potential source of prebiotics. Porphyran, the major water-soluble sulphated polysaccharide isolated from the red algae *Porphyra yezoensis*, may act as a prebiotic to improve the gut microbiota in diabetic KK-ay mice, shown as increased caecum weight, increased propionate production and increased *Bacteroides* species counts, producing improved insulin resistance and fasting blood glucose concentrations with increased plasma adiponectin concentrations [39].

Animal studies conducted using seaweed polysaccharides such as laminarin, alginates and fucoidan showed that these brown algal polysaccharides are resistant to digestion in the upper gastrointestinal tract, but stimulate the population growth of beneficial microbes in the intestine such as *Bifidobacterium* species, *Bacteroidetes* and *Lactobacillus* species to improve the gut environment by enhancing the production of short chain carboxylic acids [40]. Additionally, propionate is directly associated with the expression of Glucose-dependent Insulinotropic Polypeptide (GIP), the key modulator of energy homeostasis, insulin secretion and glucose metabolism while butyrate is directly linked to the expression of satiety gut hormones such as Glucagon-Like Peptide (GLP-1) and peptide YY (PYY) that regulate weight management by inhibiting food intake [41].

The most common fresh-water microalgae used for human nutrition are *Spirulina* species (blue green algae), *Chlorella* species *(Chlorophyta)* and *Scenedesmus* species *(Chlorophyta)* [42]. The *in vitro* and *in vivo* animal studies highlight antioxidant, anti-hyperlipidaemic, anti-hyperglycaemic and anti-inflammatory activities of polysaccharides present in microalgal species [43]. These microalgae may improve health [17, 42]. As an example, 5 % *Spirulina* species improved vascular tone by stimulating the synthesis and release of nitric oxide by endothelial cells in either fructose-fed obese rats or lean rats [44]. Further, *Spirulina* intake at 4.5 g/day for 6 weeks by 36 hypertensive subjects aged 18–65 years reduced blood pressure [44]. Treatment of 19 type 2 diabetics with *Spirulina* species (8 g/d) for 12 weeks reduced plasma concentrations of triglycerides and inflammatory markers such as TNFα and IL-6 and increased plasma adiponectin concentrations showing the potential of *Spirulina* species as a functional food for diabetes management [45].

3 Seaweed peptides

The crude protein contents in red or green seaweeds are comparatively high, ranging between 15.2 and 26.6 % of dry algal weight, compared to the brown seaweeds (6.7 to 11.0 % of dry algal weight) [46]. Microalgae such as *Chlorella vulgaris, Dunaliella bardawil, Scenedesmus obliquus, Arthrospira maxima, Spirulina platensis* and *Aphanizomenon* species contain even higher protein contents that range from 39 to 71 % of dry algal weight, similar to high protein plant sources such as soy (65–70 %) and wheat (75–80 %) [47, 48]. Seaweeds serve as a good dietary source of essential amino acids such as leucine, isoleucine, valine, phenylalanine, threonine, tryptophan, methionine, arginine, histidine and lysine, similar to eggs and soy beans [19, 48, 49].

Seaweed peptides may improve aspects of the metabolic syndrome, especially hypertension. Short peptides from *Undaria pinnatifida* (Phaeophyta) inhibited angiotensin converting enzyme-1 (ACE-1), an enzyme that catalyses the conversion of inactive angiotensin I to active angiotensin II, a potent vasoconstrictor [50]. Peptides derived from *Porphyra yezoensis, Hizikia fusiformis, Ecklonia cava, Acanthophora delilei* have pronounced antihypertensive activity by inhibiting ACE combined with hypocholesterolaemic, anti-hyperglycaemic and antioxidant activities [19]. Phycoerythrin, the most abundant phycobiliprotein found in red seaweeds, attenuated the oxidative stress-induced secondary diabetic complications and reduced the increased plasma concentrations of glucose, triglycerides and cholesterol [12]. Additionally, macroalgal lectins showed possible anti-inflammatory activity in rodent models [12]. Phycocyanin proteins from the green microalgae *Spirulina platensis* possess antioxidant, anti-inflammatory and hepatoprotective potential [48]. Renin inhibitory pro-peptides such as isoleucine-arginine-leucine-isoleucine-isoleucine-valine-leucine-methionine-proline-isoleucine-leucine-methionine-alanine derived from *Palmaria palmata* showed effective antihypertensive activity *via* inhibition of the renin-angiotensin system [51].

4 Seaweed pigments

Seaweeds including the microalgae *Haematococcus pluvialis* are a major industrial source of carotenoids (Fig. 2) such as astaxanthin, for example using novel bio-reactors [52]. Low plasma carotenoid concentrations correlate with metabolic syndrome in both US adults and adolescents [53, 54]. Lower serum concentrations of lutein and zeaxanthin were reported in early atherosclerotic patients suggesting the possible beneficial effects of carotenoids on atherosclerosis [55]. Lutein treatment in pregnant women with gestational diabetes reduced the neonatal oxidative stress in the infants compared with infants born to untreated diabetic women [56]. One further step is the "carotenoid health index" to estimate the decreased risk of disease with increasing plasma concentrations of carotenoids [57].

Astaxanthin and fucoxanthin are present in high concentrations in some microalgae. Since astaxanthin has been reported as having hepatoprotective effects, antidiabetic activity, cardiovascular protective effects, antioxidant and anti-inflammatory activity, this carotenoid could attenuate the signs of the metabolic syndrome [58]. Chronic astaxanthin

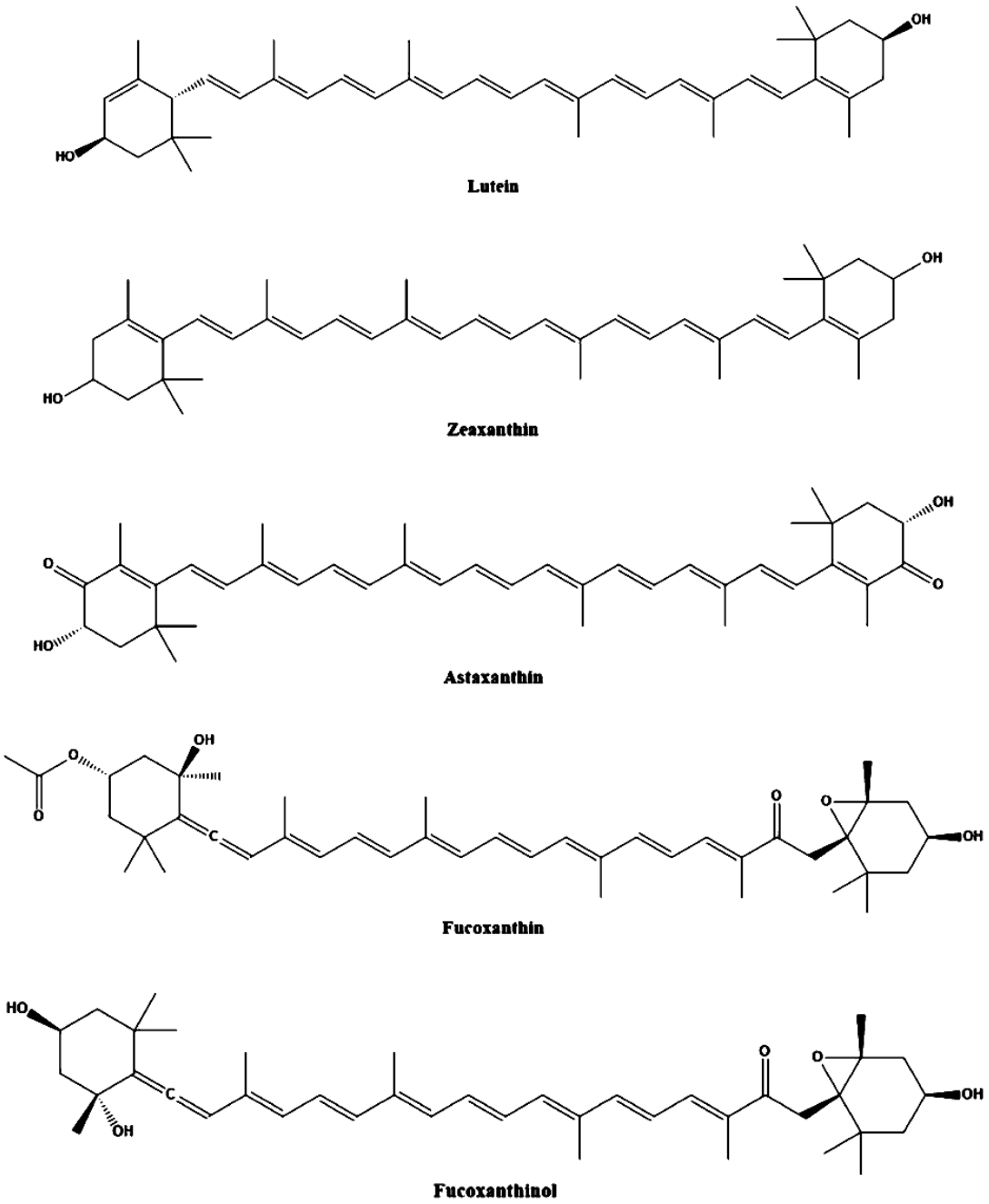


Fig. 2 Seaweed carotenoids

treatment of mice given a high fat, high fructose diet improved insulin sensitivity by activating post-receptor signalling and reducing oxidative stress, lipid accumulation and proinflammatory cytokines [59]. Astaxanthin functions as a modulator of PPARγ on adipocytes and macrophages, either as an agonist or antagonist, to initiate anti-diabetic and anti-inflammatory responses [60]. In cultured L6 myotubes, astaxanthin treatment enhanced glucose uptake by activating the insulin signalling pathway and restored the activation of insulin signalling pathway against TNF-α and palmitate-induced insulin resistance [61]. Astaxanthin treatment attenuated the production of reactive oxygen species and inflammatory cytokines such as IL-6, TNF-α and monocyte chemoattractant protein-1 (MCP-1) and decreased plasma C-reactive protein concentrations in diabetic rats [62]. In humans, astaxanthin consumption ranging from 1.8 mg/day to 22 mg/day may be cardioprotective by reducing the serum concentrations of triglycerides and total cholesterol with

corresponding increases in adiponectin and high density lipoprotein cholesterol (HDL-C) [63].

Fucoxanthin is present in edible brown seaweeds such as *Laminaria japonica, Hijikia fusiformis, Undaria pinnatifida* and *Sargassum fulvellum*. Fucoxanthin increased energy expenditure by stimulating uncoupling protein-1 expression in white adipose tissue [64]. In white adipose tissue, fucoxanthinol, a major metabolite of fucoxanthin, prevented inflammation and insulin resistance *via* down-regulating the mRNA expression of iNOS, COX-2 and the production of MCP-1, TNF-α and IL-6 [65].

Fucoxanthin treatment as 0.2 % of diet for 2 weeks in the diabetic/obese KK-A^y mouse model of hyperleptinaemia inhibited mRNA and protein expression of stearoyl-coenzyme A desaturase-1, the rate-limiting step in the synthesis of mono-unsaturated fatty acids from saturated fatty acids in the liver [66]. In these mice, fucoxanthin decreased serum leptin concentrations to improve leptin sensitivity to suppress white adipose tissue weight gain and improve glucose utilisation [66]. Treatment with fucoxanthin reduced white adipose tissue weight by 31–41 % while the combination of fucoxanthin and conjugated linolenic acid attenuated the body weight gain by 8 % and the white adipose tissue weight by 42 % in high fat-fed rats [67]. This anti-obesity effect of fucoxanthin was mediated by up-regulation of the expression of adiponectin, adipose triacylglycerol lipase as the rate-limiting enzyme in triacylglycerol hydrolysis and carnitine palmitoyl transferase 1A and also by the down-regulation of PPAR-γ expression as the master switch of adipogenesis in white adipose tissue. Other anti-obesity mechanisms could include regulating the fat deposition by increasing the energy expenditure and lipolysis by up-regulating uncoupling protein-1 and β_3-adrenoceptors in white adipose tissue, or by improving the plasma adipokine concentrations such as lowering plasma leptin concentrations or increasing plasma adiponectin concentrations [65, 68]. Fucoxanthin or its metabolite fucoxanthinol inhibited lipid accumulation in 3T3-L1 cells by downregulating the expression of key adipogenic markers including PPARγ, C/EBPα and sterol regulatory element-binding protein 1c and thereby suppressing the differentiation of murine 3T3-L1 preadipocytes to mature adipocytes [68].

Insulin resistance and type 2 diabetes may be controlled by inhibition of iNOS and COX-2 expression [64]. The potential antidiabetic effects of fucoxanthin using animal studies were highlighted [64, 65] showing that fucoxanthin prevented the development of diabetes and insulin resistance through the down-regulation of mRNA expression of pro-inflammatory mediators such as MCP-1, TNF-α, IL-6 and PAI-1 in white adipose tissue. In addition, fucoxanthin enhanced glucose uptake in skeletal muscle by up-regulation of the glucose transporter 4 (GLUT4).

5 Mineral composition of seaweeds

Seaweeds are one of many sources of important minerals such as zinc, magnesium, potassium and calcium [15, 69]. As examples, high K contents were reported in red macroalgal species such as *Gracilaria corticata* (1334 mg/9.5 g of dry algae), *Gracilaria pudumadensis* (1087 mg/9.5 g of dry algae) and the brown macroalgal species *Laminaria digitata* (1159 mg/9.5 g of dry algae) [69]. *Caulerpa veravelensis* (green macroalgae) and *Sargassum polycystum* (brown macroalgae) contained similar amounts of Ca (395 and 360 mg/9.5 g of dry algae) as standard dietary sources including low fat milk (305 mg/standard portion), low fat yoghurt with fruit (345 mg/standard portion) and yoghurt made from milk (275 mg/standard portion). *Ulva lactuca* (green macroalgae) contained high amounts of calcium (257 mg/9.5 g), comparable to the calcium content in cheese ranging between 204 and 224 mg/standard portion [69].

Zn supplementation may improve β-cell function, insulin resistance, glucose intolerance and hyperglycaemia in both type 1 and type 2 diabetics through its potent insulinomimetic action, and could prevent secondary complications such as diabetic retinopathy, renal failure and macrovascular complications [70, 71]. Zn supplementation as zinc sulphate (5 mg/kg of body weight) to OVE26 mice up-regulated the expression of nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a key regulator of antioxidant defence mechanisms, and metallotheonein, a potent antioxidant, with Zn treatment attenuating aortic inflammation, fibrosis and increased wall thickness [72].

Mg supplementation (200 mg/L drinking water) delayed the development of diabetes mellitus in Otsuka Long-Evans Tokushima Fatty (OLETF) rats by lowering plasma glucose concentrations in 38-week old OLETF rats and preventing the decrease in body weight and insulin concentrations in 60-week old OLETF rats [73]. Mg plays a crucial role in the regulation of glucose and insulin metabolism mainly through its impact on the tyrosine kinase receptor activity; further, Mg may directly increase GLUT4 activity [74]. In patients, decreased plasma Mg concentrations were directly related to the risk of development of type 2 diabetes and diabetic retinopathy [75]. Increased Mg intake reduced the incidence of type 2 diabetes among Japanese subjects by improving insulin resistance and reducing chronic low-grade inflammation [76]. Thus, Mg treatment of type 2 diabetics with low serum Mg concentrations (≤0.74 mmol/L) may improve insulin resistance and glucose utilisation [74].

Mg supplementation may have a direct influence on the improvement of vascular tone and prevent hypertension *via* stimulation of endothelium-dependent vasodilatation [74]. In humans, increased Mg intake by 100 mg/d provided cardioprotection by improving lipid metabolism, and glucose and insulin homoeostasis, and by lowering blood pressure

[74]. Dietary magnesium supplementation could markedly reduce the incidence of stroke, cardiovascular complications, hypertension and type 2 diabetes [74].

Modern Western diets are rich in Na with low K contents associated with increased peripheral vascular resistance and hypertension. Increased K intake *via* dietary supplements such as fruits and vegetables could prevent the development of hypertension and its associated cardiovascular complications [77]. In high Na-fed hypertensive rats, K supplementation attenuated the increased systolic blood pressure, reduced the incidence of stroke, and prevented cardiac hypertrophy and renal injury [77]. An optimal dietary intake of K of about 4.7 g/day and Na intake of about 1.5–2.3 g/day in humans may prevent hypertension primarily *via* stimulation of natriuresis to prevent the cardiovascular complications associated with increased Na intake [77].

An inverse association between dietary calcium intake and central obesity was shown in 506 adults aged between 18 and 60 years [78]. Three possible mechanisms for the anti-obesity responses to increased calcium intake include suppression of calciotropic hormones (calcitriol and parathyroid hormones) to decrease intracellular calcium concentrations in adipocytes and thereby enhancing lipolysis and thermogenesis, promoting the faecal excretion of fat by inhibiting the fat absorption, and stimulation of uncoupling protein-2 expression and thereby stimulating thermogenesis and fat oxidation [79].

6 Seaweed omega-3 fatty acids

The change from a hunter-gatherer diet to the modern diet has been associated with an increased ratio of omega-6 fatty acids to omega-3 fatty acids, with this change identified as the major risk factor for inflammatory-linked metabolic disorders such as cardiovascular disease, diabetes, obesity and rheumatoid arthritis [80]. The most common dietary omega-3 fatty acids are α-linolenic acid (ALA) from chia and linseeds, and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from fish oils. Fish concentrate these fatty acids from their diet, including seaweeds. Omega-3 fatty acids from either plant or fish sources are likely to affect all signs of the metabolic syndrome [81].

Few studies have concentrated on seaweeds to provide an adequate intake of omega-3 fatty acids to show responses in metabolic syndrome. In a standard dose of 9.5 g of dry seaweeds, *Euchema cottoni* species had the highest content of omega-3 fatty acids (256 mg) compared with the other seaweed species such as *Palmaria palmata* with 85.5 mg, *Sargassum polycystum species* with 77.5 mg and *Ulva labota* species with 95 mg; the content of omega-6 fatty acids in all the seaweeds ranged from 1.2 to 75.6 mg [69]. Microalgae such as *Chlorella minutissima* may produce EPA up to 45 % of the total fatty acid content [15]. These amounts of omega-3 fatty acids are much lower than the doses used in clinical trials of up to 4 g (EPA + DHA)/day [81]. While seaweeds may provide an industrial source of the omega-3 fatty acids, it seems unlikely that adding reasonable amounts of dried seaweeds to the diet would provide sufficient omega-3 fatty acids to show major health benefits.

7 Further studies

There are many areas of research in seaweeds that are being pursued around the world. Several research questions seem very important from our personal viewpoint as biomedical scientists with an interest in drug discovery and development and an emphasis on functional foods. The seaweeds are mixtures of partly defined complex chemicals such as polysaccharides and peptides and this complexity slows down the discovery of new therapeutic entities. The development of partial purification procedures could produce reproducible mixtures of bioactive ingredients from seaweeds, defined as nutraceuticals. These standardised products would then need to be tested for their biological activity in isolated cell systems, animal models of human disease and then in patients with disease states that may respond to these treatments. This is the outline of testing of new chemical entities as potential therapeutic interventions; a similar strong evidence basis is necessary for natural products such as seaweeds. If we wish to use seaweeds as a treatment of hypertension, obesity, diabetes, fatty liver and inflammation, as the key signs of the metabolic syndrome, then we need to have solid evidence that the seaweeds are effective in these conditions. These studies will need to define the effective non-toxic dose ranges for the seaweeds, as the presence of a bioactive compound is not sufficient evidence for therapeutic activity. One further complication is the oral bioavailability. These functional foods or nutraceuticals are likely to be given orally if intended for chronic treatment; access to the diseased tissues within the body requires absorption from the gut into the systemic circulation. This absorption may not be required in all cases; prebiotics are given for their role in modulating the gastrointestinal bacteria and so absorption into the blood stream may be counter-productive.

8 Conclusions

This review highlights the recent evidence that compounds derived from seaweeds such as polysaccharides (laminarin, fucoidan and alginates), peptides (phycoerythrin and phycocyanin), omega-3 fatty acids (especially EPA and DHA), carotenoids (astaxanthin, fucoxanthin and fucoxanthinol) and mineral ions (Mg, K, Ca and Zn) are effective functional foods and possible nutraceuticals. In particular, seaweeds may

be useful in controlling the signs of the metabolic syndrome such as obesity, hypertension, type 2 diabetes, cardiovascular complications and hyperlipidaemia. What we now know is only the beginning in defining the usefulness of dietary seaweeds and their components.

Conflict of Interest Both authors declare no conflict of interest related to the present work.

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CHAPTER 2

Materials and Methods

2. Materials and Methods

2.1 Materials

Freeze-dried seaweeds including two macroalgae species *Ulva ohnoi* and *Derbesia tenuissima* and micro-algae polyculture mixture comprising *Schroederiella apiculata* and *Scenedesmus dimorphus* were cultured and supplied by Dr Nicholas Paul, School of Marine & Tropical Biology, James Cook University, Townsville, Australia. Magnesium and potassium chloride (AR) grade were obtained from Chem-Supply Pty Ltd, Port Adelaide, Australia. Synergy1 mixture comprising 1/1 ratio of inulin and oligofructose was supplied by Invita Australia Pty Ltd.

2.2 Rats

Male Wistar rats (aged 8–9 weeks; weight 335-340 g) were obtained from The University of Queensland Biological Resources unit or the Animal Resource Centre, Perth and individually housed at the University of Southern Queensland's Animal House Facility, or bred at this facility. All experimental protocols were approved by the Animal Experimentation Ethics Committees of the University of Southern Queensland, The University of Queensland and the Queensland University of Technology, under the guidelines of the National Health and Medical Research Council of Australia.

2.3 Experimental diets and groups, induction of obesity and metabolic disorders

Rats were given high-carbohydrate, high-fat (H) diet for 16 weeks while the control group were given corn starch (C) diet. The composition of these diets is given in Table 1 (Panchal et al., 2011).

Treatment diets were prepared by adding either 5% or 2% of the respective supplements such as 5% seaweeds (*Ulva ohnoi* & *Derbesia tenuissima*), 5% microalgal mixture (*Schroederiella apiculata* & *Scenedesmus dimorphus*), 5% magnesium chloride, 2% potassium chloride and 5% Synergy1 (1/1 inulin: oligofructose) mixture with the C and H diet, replacing an equal amount of water in the diet. During each intervention, the rats were divided into 4 groups: (i) C (n=12), (ii) H (n=12), (iii) C+treatment (n=12), (iv) H+treatment (n=12). The control rats (C & H) were fed with C and H diet from 0 to 16 weeks and in the treatment protocol for first 8 weeks the rats were given normal C and H diet and the respective treatment diets were administered for the final 8 weeks of the 16 week protocol.

Table 1: Diet composition

Ingredient, g/kg	C	H
Corn starch	570	-
Powdered rat feed	155	155
Hubble, Mendel and Wakeman (HMW) Salt	25	25
Fructose	-	175
Beef tallow	-	200
Condensed milk	-	395

Water	250	50
Energy, kJ/g	11.23	17.93

Parameters were measured at the time points in Figure 1:

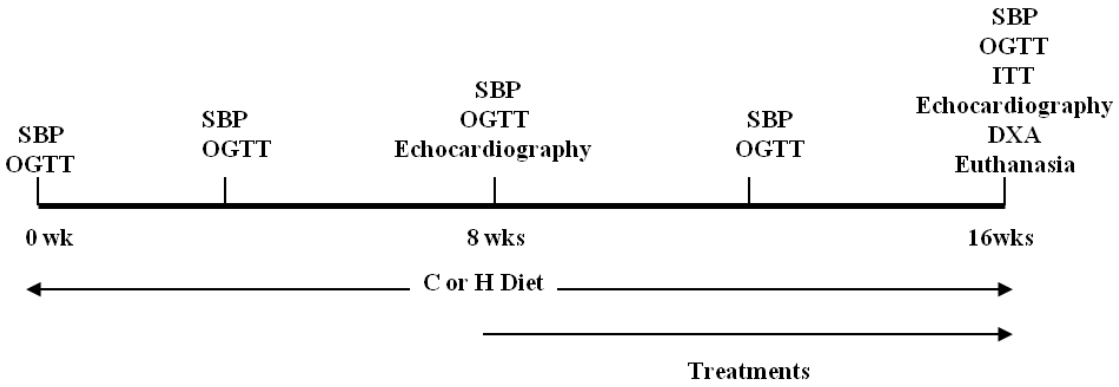

Figure 1.Schematic represenatation of the animal experimentation protocol. C, corn starch; H, high carbohydrate and high fat diet; SBP, systolic blood pressure; OGTT, oral glucose tolerance test; ITT, insulin tolerance test; DXA, dual-energy X-ray absorptiometry.

2.4 Assessment of physiological parameters

Body weight, food and water intakes were measured daily. Systolic blood pressure was measured after 0, 8 and 16 weeks under light sedation with i.p. injection of Zoletil (tiletamine 15 mg/kg, zolazepam 15 mg/kg), using an MLT1010 Piezo-Electric Pulse Transducer (AD Instruments) and inflatable tail-cuff connected to a MLT844 Physiological Pressure Transducer (AD Instruments) and PowerLab data acquisition unit (AD Instruments, Sydney, Australia). Waist circumference was measured with a standard tape measure. Rats were killed with an intraperitoneal (i.p.) injection of pentobarbitone sodium (100 mg/kg). Blood was taken from the abdominal aorta and centrifuged; the plasma was collected and frozen for further analyses. Feed conversion efficiency was calculated as previously described (Poudyal et al., 2012):

$$\text{Feed conversion efficiency (\%)} = \frac{\text{bodyweight gain (g)}}{\text{energy intake (kJ)}} * 100$$

2.5 Oral glucose tolerance test and insulin tolerance test

Oral glucose tolerance testing was performed after 0, 4, 8, 12 and 16 weeks of diet while insulin tolerance testing was performed after 16 weeks of diet. After 12 hours of fasting, blood glucose concentration was measured in blood samples taken from the tail vein. Subsequently, each rat was treated with glucose (2 g/kg body weight) via oral gavage. Tail vein blood samples were taken every 30 minutes up to 120 minutes following glucose administration. The blood glucose concentrations were analysed with a Medisense Precision Q.I.D glucose metre (Abbott Laboratories, Bedford, U.S.A.). For insulin tolerance testing, basal blood glucose concentrations were measured after 4–5 h of food deprivation as above. The rats were injected ip with 0.33 IU/kg body

weight insulin-R (Eli Lilly Australia, West Ryde, NSW, Australia) and tail vein blood samples were taken at 0, 15, 30, 45, 60, 90 and 120 min. Rats were withdrawn from the test if the blood glucose concentration dropped below 1.1 mmol/L and 4 g/kg body weight glucose was administered immediately by oral gavage to prevent hypoglycaemia.

2.6 Isolated heart preparation

The diastolic stiffness constant and the contractility of the hearts of the rats were assessed using the Langendorff heart preparation. Rats were euthanised with an intraperitoneal injection of pentobarbitone sodium (100 mg/kg). Once anaesthesised, heparin (1000 IU) was injected into the right femoral vein. The heart was stunned in ice-cold crystalloid perfusate (modified Krebs-Henseleit bicarbonate buffer (KHB) containing [in mM]: NaCl 119.1; KCl 4.75; $MgSO_4$ 1.19; KH_2PO_4 1.19; $NaHCO_3$ 25.0; glucose 11.0 and $CaCl_2$ 2.16) upon removal. The aorta was then isolated and cleared of extraneous fat and cannulated via the dorsal root (with the tip of the cannula positioned immediately above the coronary ostia of the aortic stump) and perfused in a non-recirculating Langendorff manner at 100cm of coronary perfusion pressure. The buffer was bubbled with carbogen (95% 0_2/5% CO_2), giving a pH of 7.4 and the temperature maintained at 36.9 ± 0.5°C. The hearts were punctured at the apex with a small piece of polyethylene tubing to facilitate the drainage of buffer. The isovolumetric ventricular function was measured by inserting a latex balloon into the left ventricle via the mitral orifice connected to a Capto SP844 MLT844 physiological pressure transducer and Chart software on a MacLab system. All left ventricular end-diastolic pressure values were measured by pacing the heart at 250 beats per minute using an electrical stimulator by touching two electrodes to the surface of the right atrium. End-diastolic pressure was measured using pressure volume relationship whereby increasing the volume load of water into the inserted latex balloon of left ventricle the amount of pressure developed on the latex balloon during the heart beats were recorded from 0 mmHg to 30 mmHg using the physiological pressure transducer. The right and left ventricles were then separated and weighed.

To assess myocardial stiffness, stress (δ, dyne/cm^2) and tangent elastic modulus (E, dyne/cm^2) for the mid wall at the equator of the left ventricle were calculated by assuming spherical geometry of the ventricle and considering the midwall equatorial region as representative of the remaining myocardium. Myocardial diastolic stiffness was calculated as the diastolic stiffness constant (κ, dimensionless), the slope of the linear relation between E and δ (Mirsky and Parmley, 1973).

2.7 Organ weights

Following euthanasia, the heart, liver, kidneys, visceral fat pads and spleen were removed and blotted dry for weighing. All organ weights were normalised relative to tibial length at the time of removal and values are presented in mg/mm.

2.8 Echocardiography

Echocardiography was performed by trained cardiac sonographers at the Medical Engineering Research Facility, The Prince Charles Hospital, Brisbane,

Australia. Rats were anaesthetised via intraperitoneal injection with Zoletil (tiletamine 15 mg/kg, zolazepam 15 mg/kg) and Ilium Xylazil (xylazine 10 mg/kg). Echocardiographic images were obtained using the Hewlett Packard Sonos 5500 (12MHz frequency fetal transducer) at an image depth of 3 cm using two focal zones. Measurements of left ventricular posterior wall thickness and internal diameter were made using two-dimensional M-mode taken at mid-papillary level. Left ventricular M-mode measurements at the level of the papillary muscles were used to define wall thicknesses and internal diameters at systole (s) and diastole (d). The measured wall thicknesses were the left ventricular posterior wall (LVPW) and the interventricular septum (IVS). The left ventricular internal diameters in diastole (LVIDd) and systole (LVIDs) were measured. Fractional shortening (FS;%) was defined as (LVIDd - LVIDs) / LVIDd x 100 (Brown et al., 2002). The left ventricular end-diastolic dimensions, ventricular wall thicknesses and fractional shortening were used as measurements of cardiac geometry and systolic function. Other measurements included early mitral flow diastolic velocity (E), peak velocity through the mitral valve at atrial contraction (A), deceleration time and the period between mitral valve closure and mitral valve opening (MC-MO) (Brown et al., 2002).

2.9 Body composition measurements

Dual-energy X-ray absorptiometric (DXA) measurements were performed on the rats after 16 weeks of feeding, 2 days before rats were killed for pathophysiological assessments, using a Norland XR36 DXA instrument (Norland Corp., Fort Atkinson, WI, 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)(Ward and Battersby, 2009). 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.

2.10 Organ bath studies

Contractility of the thoracic aorta was assessed using organ bath studies. Thoracic aortic rings (4 mm in length) were dissected out and suspended in an organ bath chamber with a resting tension of 10 mN. Tissues were bathed in a modified Tyrode solution containing [in mM]; NaCl 136.9; KCl 5.4; MgCl 1.05; $CaCl_2$ 1.8; $NaHCO_3$ 22.6; NaH_2P0_4 0.42; glucose 5.5; ascorbic acid 0.28 and sodium ethylene diamine tetra-acetic acid (EDTA) 0.1. The Tyrode solution was bubbled with carbogen (95 % O_2/ 5 % CO2) and the temperature maintained at 35 ± 0.5°C. Force of contraction was measured isometrically with Grass FT03C force transducers connected via amplifiers to a Macintosh computer via a MacLab system. The aortic rings were allowed to settle and equilibrate for approximately 60 min, with regular washing every 15 min. Cumulative concentration-response (contraction) curves were measured for noradrenaline (Sigma-Aldrich Australia, NA; from $1x10^{-8}$ to $lx10^{-4}$ M) to examine changes to the force of contraction; concentration-response (relaxation) curves were measured for acetylcholine (Sigma-Aldrich Australia, ACh; from $1x10^{-8}$ to $lx10^{-4}$ M) or sodium nitroprusside (Sigma-Aldrich Australia, SNP; from $1x10^{-8}$ to $lx10^{-4}$ M) in the presence of a submaximal contraction to noradrenaline (Panchal et al., 2011). Results were analysed as

the maximal increase or decrease in force of contraction, in mN, for each drug concentration.

2.11 Histology of heart and liver

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

Collagen distribution was observed in the left ventricle following picrosirius red staining. These sections followed the same deparaffinisation and hydration process as described above. Sections were then transferred to distilled water for a brief wash and then bathed in phosphomolybdic acid (0.2 % in distilled water) for 2 min to inhibit background autofluorescence and non-specific staining. Later, sections were placed in the collagen-selective stain picrosirius red (0.1 % Sirius Red F3BA in saturated picric acid) for 90 min. The sections were then washed in 0.1N HCl for 2 min, followed by dehydration with 95% and 100% (three times) ethanol (2 min each). Sections were then mounted as described above. Laser confocal microscopy (Zeiss LSM 510 upright Confocal Microscope) was used to determine the extent of collagen deposition in selected regions (Panchal et al., 2011).

2.12 Plasma analyses

Blood was collected from the abdominal aorta following euthanasia and centrifuged at 5000x *g* for 15 min within 30 min of collection into heparinised tubes. Plasma was separated and transferred to Eppendorf tubes for storage at −20°C before analysis. Plasma concentrations of total cholesterol, triglycerides, non-esterified fatty acids (NEFA), activities of plasma alanine transaminase (ALT) and aspartate transaminase (AST) were determined at The University of Queensland Veterinary Services, Gatton Campus, using kits and controls supplied by Olympus using an Olympus analyser (AU 400 Tokyo, Japan).

2.13 Statistical analyses

All data sets were represented as mean ± standard error of mean (SEM). Comparisons of findings between groups were made via statistical analysis of data sets using one-way (ANOVA). When interaction and/or the main effects

re significant, means were compared using Newman-Keuls multiple-comparison post hoc test. A p-value of <0.05 was considered as statistically significant. All statistical analyses were performed using Graph Pad Prism version 5.00 for Windows.

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CHAPTER 3

Effect of low testosterone in a high-carbohydrate, high-fat diet-induced rat model of metabolic syndrome

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Effect of low testosterone in a high-carbohydrate, high-fat diet induced rat model of metabolic syndrome

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Abstract

Consumption of high calorie diet contains higher proportions of fat as saturated and mono-unsaturated fatty acids, and refined carbohydrates serves as a major risk factor of visceral obesity associated metabolic syndrome. Testosterone deficiency in the androgen-deprived prostate cancer patients as well as in aged men showed fat mass development and increased risk factors of metabolic syndrome. In our study, we investigated the effects of testosterone deficiency on the pathogenesis of visceral adiposity induced metabolic syndrome in rats fed with high-carbohydrate, high-fat (H) diet. Castration in male Wistar rats (n=48) were performed in two methodologies: 1) surgical removal of testicles 2) leuprolide subcutaneous injection (2mg/kg bwt/month). In a 16 wk experimental protocol control male Wistar rats (n=24) and the castrated rats (n=48) were randomly placed into 6 groups (n=12 each): control rats on low fat-cornstarch (C) diet, leuprolide acetate injected rats on C diet (CLCAS), surgical castrated rats on C diet (CSCAS); control rats on high-carbohydrate (68% as fructose and sucrose), high-fat (24% as saturated and mono-unsaturated fatty acids) (H) diet, leuprolide acetate injected rats on H diet (HLCAS), and surgical castrated rats on H diet (HSCAS). Surgical castrated groups (CSCAS & HSCAS) showed diminished total testosterone concentrations (2.1-2.6 ng/mL) at 4 wk of experimental protocol while leuprolide acetate injected groups (CLCAS & HLCAS) showed lowered total testosterone concentrations after 8 wk (4.2-5.8 ng/mL). However, at the end of 16 wk compared to control diet fed groups (C&H), both the C diet fed castrated groups (CLCAS & CSCAS) and H diet fed castrated groups (HLCAS & HSCAS) showed increased abdominal circumference and visceral adiposity index (%) mainly as retroperitoneal fat depots. Additionally, a reduced lean mass with increased total body fat mass was observed only in HSCAS rats. Compared to control groups, the enhanced visceral adiposity in castrated groups were associated with increased risk factors of metabolic syndrome such as impaired oral glucose and insulin tolerance, increased cardiovascular stiffness and collagen deposition, inflammatory cells infiltration of hepatocytes and myocytes, non-alcoholic fatty liver disease and dyslipidemia. Further, among castrated groups, the CSCAS and HSCAS rats were hypertensive for 16 wk, whereas the CLCAS rats showed increased systolic blood pressure after 8 wk of experimental protocol. This hypertensive effect with diminished endothelial function has been attributed to the direct effect of reduced total testosterone concentrations. In CLCAS and HLCAS groups, systolic dysfunction and signs of eccentric hypertrophy were observed. This study demonstrates that testosterone deficiency may contribute to the development of visceral obesity linked metabolic syndrome.

Abbreviations used: ALT, alanine transaminase; AST, aspartate transaminase; C, corn starch diet-fed rats; CLCAS, corn starch diet-fed rats castrated with leuprolide acetate; CSCAS, corn starch diet-fed rats surgical castrated; H, high-carbohydrate, high-fat diet-fed rats; HLCAS, high-carbohydrate, high-fat diet-fed rats castrated with leuprolide acetate; HSCAS, high-carbohydrate, high-fat diet-fed rats surgical castrated; ESS, end-systolic wall stress; LVIDd, left ventricular internal diameter during diastole; LVIDs, left ventricular internal diameter during systole; LVPWd, left ventricular

posterior wall thickness during diastole; NEFA, non-esterified fatty acids; SBP, systolic blood pressure.

Introduction

Lifestyle changes including consumption of diet high in refined carbohydrates, saturated and mono-unsaturated fatty acids induce visceral obesity and its associated metabolic risk factors such as type 2 diabetes, insulin resistance, hypertension, endothelial dysfunction, cardiovascular disease, dyslipidemia, and non-alcoholic fatty liver disease (1-3). Androgen receptors regulate adipose tissue metabolism (4), and knock-out of androgen receptors in the adipocytes of high fat diet-fed mice showed hyperinsulinemia and hyperglycemia with increased risk to visceral obesity (5). Androgen-receptor knockout mice developed late-onset obesity with decreased lipolysis and energy expenditure (6, 7).

Testosterone treatment at higher concentration (600 mg/wk) attenuates visceral fat mass development in healthy young men (8). Similarly, testosterone supplementation (30mg/kg bwt) reduced the visceral adiposity with improved insulin sensitivity of visceral adipose tissue, and attenuated the metabolic symptoms in the high-fat diet fed rabbits (9). Testosterone influenced fat mass development by inhibiting the lipid uptake and lipoprotein-lipase activity in adipocytes, and also by stimulating lipolysis increasing the adipocytes β-adrenergic receptor density (10). Further, *in vitro* study in mouse C3H10T1/2 pluripotent cells showed testosterone treatment (30-300nM) promoted differentiation of mesenchymal pluripotent cells into myogenic lineage rather into adipogenic lineage with decreased mRNA expression of adipogenic transcription factors peroxisomal proliferator-activated receptor γ 2 (PPAR γ2) and CCAAT/enhancer binding protein α (C/EBP-α) (11). Similarly, testosterone supplementation inhibited the adipogenic differentiation in 3T3-L1 cells by suppressing the protein expression of PPAR-γ2 and C/EBP-α, which may be regulated by the activation of T-cell factor (TCF)/lymphoid-enhancer factor (LET) of Wnt signalling pathway (12).

Testosterone treatment at lower dose (2mg/kg bwt) increased the serum total testosterone concentrations from 0.2 to 10.6nmol/L, and improved the glucose utilization of muscle in castrated rats (13). Testosterone supplementation improved the insulin sensitivity with decreased plasma insulin concentration, and attenuated the impaired glucose tolerance in obese male Zucker rats (14). Long-term testosterone therapy in obese, diabetic men with low plasma total testosterone concentration (8.9nmol/L) improved the metabolic symptoms with reduced body weight and waist circumference, decreased fasting blood glucose concentration, reduced systolic and diastolic blood pressure, and improved lipid profile (15). Further, testosterone replacement increased the serum total testosterone concentrations by two-fold and attenuated the non-alcoholic fatty liver disease with reduced fat accumulation and apoptosis of hepatocytes in the high-fat diet fed castrated rats (16).

As discussed, testosterone plays a key role in attenuating metabolic risk factors such as fat mass development, type 2 diabetes, insulin resistance, hypertension and non-alcoholic fatty liver disease. Hence, testosterone deficiency may serve as a major risk factor of metabolic syndrome due to the increase in visceral obesity (17, 18).

Androgen deprivation therapy in prostate cancer men reduced the serum total testosterone concentration from 12.8nmol/L to 0.7nmol/L with increased total body fat mass and insulin resistance (19). Japanese-American men with lower baseline serum total testosterone concentrations (15.6-15.9nmol/L vs 16.8-17.7 nmol/L) showed increased intra-abdominal fat deposition and fasting plasma insulin concentration (20). Testosterone deprivation using gonadotrophin releasing hormone agonist leuprolide acetate increased the serum concentrations of total cholesterol and triglycerides in prostate cancer patients (21). Men diagnosed with lower serum total testosterone concentrations (6.8 nmol/L) showed increased body fat percentage with diminished insulin sensitivity and mitochondrial function (22). Further, hypogonadal prostate cancer patients with higher prevalence of central obesity, hyperglycemia, insulin resistance and dyslipidemia showed increases in the risk of cardiovascular disease (23).

Adiposity-induced testosterone deprivation is caused by high aromatase enzyme activity in adipocytes that converts testosterone into estradiol (24). Visceral obesity with increased serum 17β-estradiol concentrations (307.6pmol/L vs 146.4 pmol/L (baseline)), and decreased serum total testosterone concentrations (1.5nmol/L vs 8.7 nmol/L (baseline)) could play a significant role in inducing metabolic syndrome in the high-fat diet fed rabbits (9). Similarly, lower plasma testosterone concentrations (70%) measured in the obese male Zucker rats increased the visceral adiposity associated with increased arterial pressure, impaired glucose tolerance, and increased plasma insulin and total cholesterol concentration (14). Based on this evidence, this study hypothesizes that testosterone deficiency aggravates metabolic syndrome by enhancing the visceral adiposity. In this study, we have investigated the effect of testosterone deprivation achieved by surgical and chemical (leuprolide acetate) castration in a high-carbohydrate and high-fat diet induced rat model of metabolic syndrome.

MATERIALS AND METHODS

Castration

The experimental groups including surgically castrated male Wistar rats (n=48) and chemically castrated male Wistar rats (n=24) (9-10 wk old; 337.3±0.4 g) were supplied by The University of Southern Queensland and Animal Resource Centre, Perth, Australia and individually housed at The University of Southern Queensland animal house. Surgical castration conducted in this study was demonstrated by The University of Queensland School of Veterinary Sciences, Gatton, Australia. All experimentation was approved by the Animal Experimentation Ethics Committees of the University of Queensland and University of Southern Queensland under the guidelines of the National Health and Medical Research Council of Australia. Surgical castration was done under anesthesia with an intraperitoneal injection of Zoletil [tiletamine (15mg/kg) +zolazepam (15mg/kg), Virbac, Peakhurst, NSW, Australia] together with xylazine (10mg/kg, Rompun). Rats were surgically castrated by an incision made at the midpoint of the scrotum and the underlying tissue followed by removal of the testes part of the spermatic cord. The incision sites were sutured and rats were treated with carprofen 1mL/kg bwt for 2-3 days as an analgesic. Before starting the experimental protocol the castrated rats were fed with normal rat chow diet and monitored daily until they attained their

normal body weight (335-340 g). In a 16 wk protocol, chemical castration was carried out in male Wistar rats aged 8-9 wk (n=24) by a subcutaneous injection of gonadotrophin releasing hormone agonist (GnRH) leuprolide acetate (2mg/kg bwt/month) supplied by Abbott Pharmaceuticals, Australia. The leuprolide acetate injection solutions were prepared using 0.9% sterile saline and were administered at 0, 4, 8, &12 wk to suppress testosterone production.

Rats and diets

The experimental rats were individually housed in a temperature-controlled (20±2°C), 12-hour light/dark cycle environment with unrestricted access to water and the basal rat diet, for which the preparation and macronutrient composition of basal diets has been described in detail previously (2, 25). All experimentation was approved by the Animal Experimentation Ethics Committees of the University of Queensland and University of Southern Queensland under the guidelines of the National Health and Medical Research Council of Australia. The rats were randomly divided into 6 separate groups (n=12 each) and fed with cornstarch (C) diet, cornstarch + leuprolide acetate castration (CLCAS), cornstarch+ surgically castration (CSCAS), the high-carbohydrate, high-fat (H) diet, high-carbohydrate, high-fat + leuprolide acetate castration (HLCAS), high-carbohydrate, high-fat + surgical castration (HSCAS). The drinking water in all H groups included 25% fructose. Rats were monitored daily for body weight, food and water intakes. Feed efficiency (%) was calculated as previously described (2). Abdominal circumference was measured using a standard measuring tape during the period of anesthesia for systolic blood pressure measurements (2). Systolic blood pressure measurements and blood sampling were carried out at 0, 4, 8, 12, and 16 wk. Oral glucose tolerance test and insulin tolerance test were conducted at 0, 8, and 16 wk. All other measurements were made at wk 16.

Cardiovascular measurements and blood sampling

SBP was measured under light sedation following intraperitoneal injection of Zoletil (tiletamine 15 mg/kg, zolazepam 15 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 Power Lab data acquisition unit (AD Instruments, Sydney, Australia) (2). Simultaneously, 500µL blood was collected from tail vein of anesthetized rats and transferred immediately into the heparinized tubes. The tubes were kept on ice until centrifugation for 4^{o}C and 3500 rpm. The plasma samples were separated and stored at -20^{o}C until analysis of total testosterone concentrations. Echocardiographic examinations (Phillips iE33, 12MHz transducer) were performed to assess the cardiovascular structure and function in all rats at the end of protocol (2). Briefly, rats were anesthetized using Zoletil (tiletamine 25 mg/kg and zolazepam 25 mg/kg, i.p.; Virbac, Peakhurst, Australia) and Ilium Xylazil (xylazine 15 mg/kg, i.p.; Troy Laboratories, Smithfield, Australia) and positioned in dorsal recumbency. Electrodes attached to the skin overlying the elbows and right stifle facilitated the simultaneous recording of a lead II electrocardiogram. A short-axis view of the left ventricle at the level of the papillary muscles was obtained and used to direct acquisition of M mode images of the left ventricle for measurement of IVSd, LVPWd, LVIDs, and LVIDd. Measurements were taken in accordance with the guidelines of the

American Society of Echocardiography using the leading-edge method as described previously (2). The ventricular contractility indexes were calculated including ratio of SBP to LVIDs, ratio of SBP to systolic volume, and ratio of ESS to LVIDs (26, 27).

The left ventricular function of the rats in all treatment groups was assessed using the Langendorff heart preparation (2). Terminal anesthesia 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 (AD Instruments Australia and Pacific Islands, Bella Vista, NSW, Australia).

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 (2, 25).

Oral glucose tolerance and insulin tolerance tests

For oral glucose tolerance tests, basal blood glucose concentrations were measured in blood taken from the tail vein of overnight food-deprived rats using Medisense Precision Q.I.D glucose meter (Abbott Laboratories, Bedford, U.S.A). Fructose-supplemented drinking water in the H, HLCAS and HSCAS groups was replaced with normal water for the overnight food-deprivation period. The rats were given 2 g/kg body weight of glucose as a 40% aqueous solution via oral gavage. Tail vein blood samples were taken at 30, 60, 90, and 120 min following glucose administration. For insulin tolerance test, basal blood glucose concentrations were measured after 4-5 h of food deprivation as above. The rats were injected ip 0.33 IU/kg bwt insulin-R (Eli-Lilly Australia, West Ryde, NSW, Australia) and tail vein blood samples were collected at 0, 15, 30, 45, 60, 90, and 120 min. Rats were withdrawn from the test if the blood glucose concentration dropped below 1.1 mmol/L, and 4 g/kg glucose was administered immediately via oral gavage to prevent hypoglycemia.

Body composition measurements

Dual energy X-ray absorptiometric (DXA) measurements were performed on rats after 16 wk of feeding. This was done 2 days before rats were euthanized for pathophysiologic assessments, using a Norland XR36 DXA instrument (Norland Corp., Fort Atkinson, USA). DXA scans were analyzed using the manufacturer's recommended software for use in laboratory animals (Small Subject Analysis Software, version 2.5.3/1.3.1, Norland Corp., Fort Atkinson, USA) (2, 25). The precision error of lean mass for replicate measurements, with repositioning, was 3.2%. Visceral adiposity index (%) was calculated as follows: {[retroperitoneal fat (g)+omental fat (g)]/bodyweight (g)}*100 (2, 25).

Organ weights

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

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. 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 (2, 25). Thin sections (7 μm) of left ventricle and the liver were cut and stained with haematoxylin and eosin stain for determination of inflammatory cell infiltration with 20X and fat vacuole enlargement with 20X objectives using a Olympus BX51 microscope (Olympus, Melville, NY). Collagen distribution was measured in the left ventricle with picrosirius red stain. Laser confocal microscopy (Zeiss LSM 510 upright Confocal Microscope) was used to measure collagen deposition (2, 25).

Plasma biochemistry

Blood was centrifuged at 5,000 *g* for 15 min within 30 min of collection into heparinized tubes. Plasma was separated and transferred to Eppendorf tubes for storage at -20°C before analyzis. Activities of plasma enzymes and analyte concentrations were determined using kits and controls supplied by Olympus using an Olympus analyzer (AU 400, Tokyo, Japan) as previously described (2, 25). Plasma concentrations of total testosterone were measured using commercial ELISA (enzyme linked immune absorbent assay) testosterone kits (Catalog no: ADI-900-065; Sapphire Bioscience, Australia) according to manufacturer provided standards and protocols using a Titertek Multiskan MCC/340 spectrophotometer (Flow Laboratories) (2). In surgical castrated groups (CSCAS & HSCAS), total testosterone analyses were carried out at 0, 4, and 16 wk of experimental protocol while in control groups (C&H), and leuprolide acetate castrated groups (CLCAS & HLCAS), total testosterone analyses were conducted at 0, 4, 8, 12, and 16 wk.

Statistical analysis

Values are mean ± SE. Results were tested for variance using Bartlett's test, and variables that were not normally distributed were transformed (using $\log_{10}$ function) prior to statistical analyses. Data from C, CLCAS, CSCAS, H, HLCAS, and HSCAS groups were tested by two-way Anova. When interaction and/or the castration effects were significant, means were compared using Newman-Keuls multi-comparison post hoc test. Where transformation did not result in normality or constant variance, a Kruskal-Wallis nonparametric test was performed. $P<0.05$ was considered statistically significant. All statistical analyses were performed using Prism version 5.00 for Windows (Graph Pad, San Diego, CA).

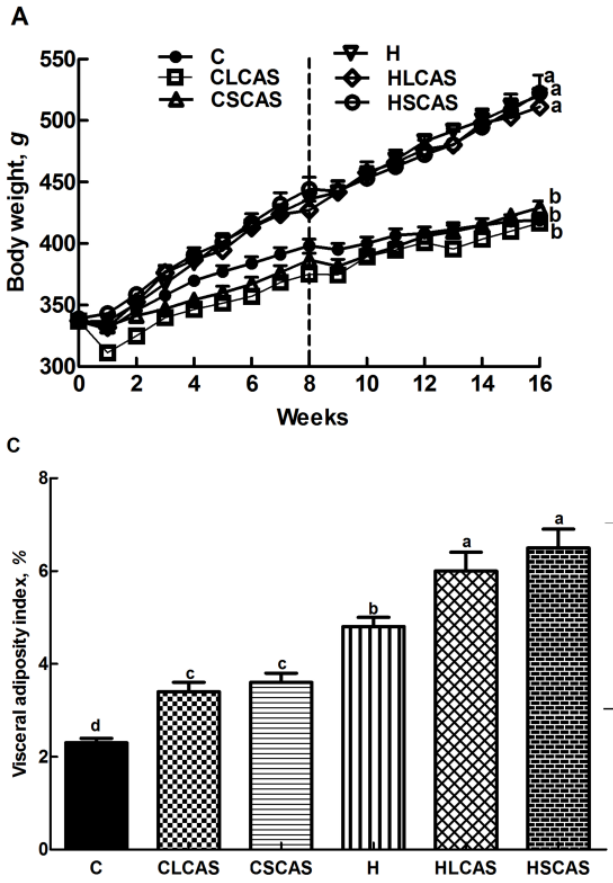
Results

Dietary intake, body parameters and metabolic variables

Food and water consumption were higher in C fed rats compared with H fed rats (Table 1). However, due to higher energy density of the H diet, an increased mean energy intake with increased feed efficiency (%) and body weight gain (%) was observed in H diet fed control rats (Table 1; Fig. 1A). In the cornstarch diet fed castrated groups, the CLCAS rats showed increased food intake compared to C and CSCAS rats (Table 1), while no changes in food and water intake were observed in HLCAS and HSCAS groups compared to the H diet fed control rats (Table 1). Compared to the control diets (C & H) fed groups, no changes in feed conversion efficiency (%) and body weight gain (%) were detected in either C or H diet fed castrated groups (CLCAS, CSCAS, HLCAS & HSCAS) (Table 1; Fig. 1*A*).

In the H diet fed control rats, higher total body fat mass (at 8 wk and 16 wk) with increased bone mineral content (at 16 wk) and abdominal fat (retroperitoneal + omental) mass was observed compared to the C fed rats (Table 2). Correspondingly, the H fed control rats also showed higher abdominal circumference and visceral adiposity index (%) (Fig. 1*B,C*). In the CLCAS and HLCAS castrated groups, reduced total body fat mass (at 8 wk) with decreased bone mineral content was observed (Table 2). Additionally, no changes in lean mass (at 8 wk) were observed in either CLCAS or HLCAS castrated rats (Table 2). The surgical castration groups (CSCAS and HSCAS rats) at 8 wk showed no changes in the total body fat mass and bone mineral content (Table 2), while a reduced lean mass (at 8 wk) was detected in both the CSCAS and HSCAS castrated groups (Table 2).

At 16 wk, no changes in either bone mineral content or total body fat mass were observed in the C fed groups (C, CLCAS and CSCAS) (Table 2). However, the lean mass was reduced in the CSCAS groups (Table 2). Compared to H and HLCAS groups, an increased total body fat mass with reduced lean mass was detected in the HSCAS rats (Table 2). The HLCAS rats showed decreased bone mineral content compared to H and HSCAS rats. This effect has been correlated to the moderate decrease in total body fat mass of HLCAS rats (Table 2). Compared to the control diets (C & H) fed groups, higher abdominal fat mass (Table 2) corresponded with increased abdominal circumference and visceral adiposity index (%) (Fig. 1*B,C*) was detected in both leuprolide acetate (CLCAS & HLCAS) and surgical castration (CSCAS & HSCAS) groups.



	P-Value		
Figure	**Diet**	**Castration**	**Interaction**
A	**<0.0001**	**0.41**	**0.88**
B	**<0.0001**	**<0.0001**	**0.54**
C	**<0.0001**	**<0.0001**	**0.74**

Fig. 1.Bodyweight (*A*), abdominal circumference (*B*), and visceral adiposity index percentage (*C*) measurements in C, CLCAS, CSCAS, H, HLCAS and HSCAS rats. Values are means ± SE; *n*=8-10 per group. Means within a row with unlike superscript letters (a-d) in each data set are significantly different (P<0.05).

Table 1. Dietary intake, feed conversion efficiency and body weight gain percentage measurements at 16 weeks in C, CLCAS, CSCAS, H, HLCAS, and HSCAS rats

Variable	C	CLCAS	CSCAS	H	HLCAS	HSCAS	*P*-Value		
							Diet	castration	Interaction
Food intake, *g/d*	31.6±2.0 [b]	36.1±2.2 [a]	31.9±2.3 [b]	20.8±1.3 [c]	24.0±1.8 [c]	21.1±1.8 [c]	<0.0001	0.003	0.82
Water intake, *ml/d*	31.0±2.0 [a]	26.8±2.5 [a]	32.0±2.4 [a]	19.9±1.5 [b]	17.8±1.5 [b]	19.8±1.6 [b]	<0.0001	0.15	0.71
Energy intake, *kJ/d*	360.7±2.4 [d]	412.9±3.4 [c]	364.5±3.1 [d]	447.9±3.5 [b]	490.3±8.3 [a]	454.5±3.9 [b]	<0.0001	<0.0001	0.36
Feed conversion efficiency (0-16 weeks), *%*	7.0±0.4 [b]	5.8±0.5 [b]	7.4±0.5 [b]	12.1±0.4 [a]	10.4±0.6 [a]	11.7±0.9 [a]	<0.0001	0.017	0.77
Body weight gain (0-16 weeks), *%*	25.5±1.4 [b]	23.6±2.4 [b]	26.8±1.6 [b]	54.4±1.6 [a]	51.5±3.4 [a]	54.1±4.3 [a]	<0.0001	0.50	0.95

Values are means ± SEM; *n*=9-10 per group. Means within a row with unlike superscript letter (a-d) in each data set are significantly different (P<0.05). C, cornstarch fed rats; CLCAS, corn starch+leuprolide acetate castrated rats; CSCAS, cornstarch+surgical castrated rats; H, high-carbohydrate, high-fat diet fed rats; HLCAS, high-carbohydrate, high-fat diet + leuprolide acetate castrated rats; HSCAS, high-carbohydrate, high-fat diet + surgical castrated rats.

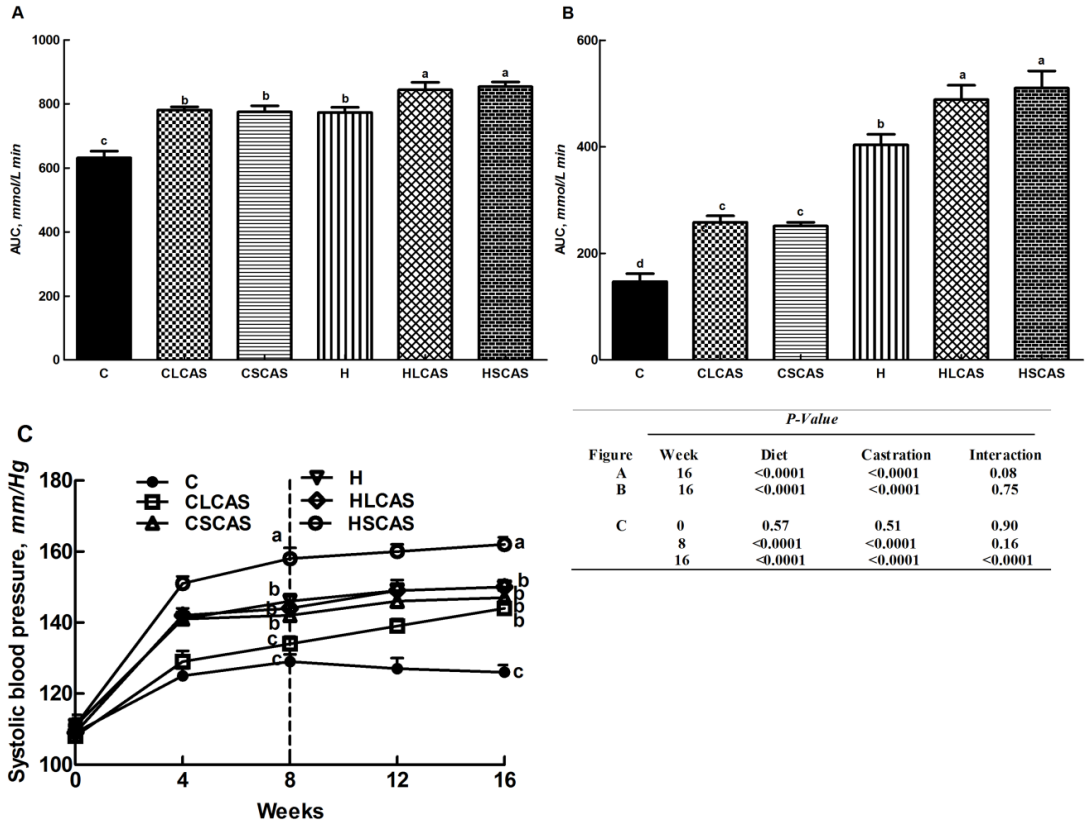
Table 2. Body parameters and organ wet weights in C, CLCAS, CSCAS, H, HLCAS, and HSCAS rats

Variable	C	CLCAS	CSCAS	H	HLCAS	HSCAS	*P*-Value		
							Diet	castration	Interaction
Bone mineral content, *g*									
1) 8 weeks	12.1±0.5 [ab]	10.5±0.2 [c]	11.3±0.3 [bc]	13.3±0.3 [a]	11.3±0.3 [bc]	13.0±0.5 [a]	0.0002	<0.0001	0.48
2) 16 weeks	13.3±0.5 [b]	12.0±0.2 [b]	12.4±0.4 [b]	15.9±0.4 [a]	13.3±0.5 [b]	16.2±0.6 [a]	<0.0001	<0.0001	0.024
Total fat mass, *g*									
1) 8 weeks	106.2±10.5 [b]	47.1±9.6 [c]	93.1±5.9 [b]	145.3±6.4 [a]	78.2±8.9 [b]	157.9±16.9 [a]	<0.0001	<0.0001	0.25
2) 16 weeks	114.2±10.9 [c]	112.8±8.7 [c]	121.5±10.4 [c]	190.7±7.9 [b]	161.2±14.9 [b]	259.0±23.1 [a]	<0.0001	0.007	0.05

Total lean mass, *g* 1) 8 weeks 2) 16 weeks	 296.3±4.4 [b] 312.4±11.4 [a]	 309.3±4.2 [ab] 275.6±5.1 [ab]	 271.4±6.4 [c] 270.8±11.5 [b]	 300.9±6.3 [ab] 313.5±7.7 [a]	 321.5±5.3 [a] 307.9±8.7 [a]	 267.4±9.5 [c] 228.7±14.1 [c]	 0.41 0.73	 <0.0001 <0.0001	 0.44 0.003
Abdominal circumference, *cm*	18.4±0.1 [d]	20.1±0.4 [c]	19.7±0.3 [c]	21.9±0.2 [b]	23.0±0.3 [a]	23.2±0.5 [a]	<0.0001	<0.0001	0.54
Retroperitoneal fat, *mg/mm*	122.3±7.9 [d]	220.5±18.3 [c]	231.3±17.6 [c]	365.7±17.4 [b]	479.2±54.4 [a]	536.6±26.4 [a]	<0.0001	<0.0001	0.52
Omental fat	65.5±5.9 [b]	82.6±6.2 [b]	90.0±5.8 [b]	131.9±9.2 [a]	147.4±12.6 [a]	136.9±13.2 [a]	<0.0001	0.16	0.51
Total abdominal fat, *mg/mm*	187.7±13.3 [d]	303.1±23.2 [c]	321.4±20.9 [c]	497.6±22.7 [b]	626.6±65.7 [a]	673.5±34.6 [a]	<0.0001	<0.0001	0.82
Liver weight, *mg/mm*	241.4±7.1 [b]	239.3±6.8 [b]	250.5±15.1 [b]	333.4±8.4 [a]	351.0±11.2 [a]	325.4±19.3 [a]	<0.0001	0.77	0.31

Values are means ± SEM; *n*=9-10 per group. Means within a row with unlike superscript letter (a-d) in each data set are significantly different (P<0.05).C, cornstarch fed rats; CLCAS, corn starch+leuprolide acetate castrated rats; CSCAS, cornstarch+surgical castrated rats; H, high-carbohydrate, high-fat diet fed rats; HLCAS, high-carbohydrate, high-fat diet + leuprolide acetate castrated rats; HSCAS, high-carbohydrate, high-fat diet + surgical castrated rats. Tissue wet weights were normalized against tibial length.

The H diet fed control rats showed impaired oral glucose and insulin tolerance compared to C fed rats (Fig. 2*A*,*B*). Castrated groups including CLCAS, HLCAS, CSCAS, and HSCAS rats showed diminished glucose utilization and insulin sensitivity compared to control diet (C & H) fed groups (Fig. 2*A*,*B*). Increased plasma concentrations of non-esterified fatty acids (NEFA), triglycerides, and total cholesterol were observed in H fed rats compared to the C fed control rats (Table 3). Further, no changes in plasma lipid profile were observed between the C diet fed control groups and castrated groups (C, CLCAS and CSCAS) (Table 3). In HLCAS rats, higher plasma concentrations of NEFA and triglycerides were detected compared to the H and HSCAS groups (Table 3). In C and H diet fed control rats, the plasma total testosterone concentrations was unchanged throughout 16 wk protocol (Table 3), while in CLCAS and HLCAS rats, diminished plasma total testosterone concentrations were observed at 12 wk and 16 wk (Table 4). In CSCAS and HSCAS groups, reduced total testosterone concentrations were observed at 4 wk and 16 wk of experimental protocol (Table 3).



		P-Value		
Figure	Week	Diet	Castration	Interaction
A	16	<0.0001	<0.0001	0.08
B	16	<0.0001	<0.0001	0.75
C	0	0.57	0.51	0.90
	8	<0.0001	<0.0001	0.16
	16	<0.0001	<0.0001	<0.0001

Fig. 2. Oral glucose tolerance (*A*), insulin tolerance (*B*), and systolic blood pressure (*C*) measurements in C, CLCAS, CCAS, H, HLCAS and HSCAS fed rats. Values are means ± SE; *n*=9-10 per group. Means with unlike superscript letters (a-d) in each data set are significantly different (P<0.05).

Table 3. Plasma lipids profile and total testosterone concentrations in C, CLCAS, CSCAS, H, HLCAS, and HSCAS rats

Variable	C	CLCAS	CSCAS	H	HLCAS	HSCAS	*P*-Value		
							Diet	Castration	Interaction
NEFA, *mmol/L (n=8)*	1.74±0.16 [d]	2.38±0.19 [cd]	2.47±0.17 [cd]	3.70±0.37 [b]	4.63±0.43 [a]	3.41±0.43 [bc]	<0.0001	0.05	0.11
Triglycerides, *mmol/L (n=8)*	0.60±0.03 [c]	0.60±0.06 [c]	0.56±0.05 [c]	1.43±0.1 [b]	1.98±0.31 [a]	1.06±0.22 [bc]	<0.0001	0.028	0.05
Total cholesterol, *mmol/L (n=8)*	1.37±0.06 [b]	1.65±0.05 [ab]	1.71±0.16 [ab]	1.88±0.06 [a]	1.89±0.11 [a]	1.84±0.17 [a]	0.0012	0.27	0.18
ALT activity, *U/L(n=8)*	25.9±1.6 [b]	27.6±1.9 [b]	29.0±2.6 [b]	41.8±2.0 [a]	40.4±5.9 [a]	39.6±1.6 [a]	<0.0001	0.95	0.60
AST activity, *U/L (n=8)*	64.3±2.9 [b]	64.1±3.5 [b]	78.3±5.5 [ab]	88.3±3.6 [a]	86.7±9.4 [a]	95.1±4.3 [a]	<0.0001	0.08	0.78
Total testosterone concentrations, *ng/mL (n=6-10)*									
Baseline	15.6±0.9	13.0±1.5	15.1±1.6	13.1±1.1	12.2±0.7	12.9±0.6	0.05	0.26	0.76
4 wk	14.2±1.0 [a]	15.3±0.9 [a]	2.1±0.2 [b]	15.1±0.4 [a]	15.9±1.1 [a]	2.6±0.3 [b]	0.33	<0.0001	0.97
8 wk	14.7±1.0	14.8±2.1	NM	14.7±0.9	15.1±0.6	NM	0.92	0.87	0.92
12 wk	15.7±1.4 [a]	7.2±1.3 [b]	NM	14.7±0.9 [a]	5.0±0.5 [b]	NM	0.21	<0.0001	0.63
16 wk	26.0±5.6 [a]	4.2±0.3 [b]	2.5±0.2 [b]	23.6±2.0 [a]	5.8±0.6 [b]	2.7±0.2 [b]	0.90	<0.0001	0.58

Values are means ± SE. Means within a row with unlike superscript letters (a-d) in each data set are significantly different (P<0.05). NEFA, non esterified fatty acids; ALT, alaninetransaminase; AST,aspartate transaminase; *n*,number of repetitive experiments; NM, not measured.

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Cardiovascular changes

The H diet fed rats showed higher SBP compared to C diet fed control rats (Fig. 2*C*). The CSCAS and HSCAS rats showed increased SBP throughout 16 wk of experimental protocol compared to the control (C & H) groups (Fig. 2*C*). The CLCAS rats showed higher SBP after 8 wk, but HLCAS rats showed no significant change in SBP compared to the H fed rats (Fig. 2*C*). Increased ESS:LVIDs and decreased SBP:LVIDs ratio as indexes of ventricular contractility were observed in CLCAS rats compared to C fed rats (Table 4). Alternatively, the HLCAS rats showed lower SBP:LVIDs ratio and SBP:systolic volume ratio with no noticeable change in ESS:LVIDs ratio (Table 4). Further, no changes in ventricular contractility indexes were measured in surgical castrated groups (CSCAS and HSCAS) (Table 4). Compared with control groups, increased systolic and diastolic volumes corresponding with increased LVIDd and LVIDs dimensions were observed in CLCAS and HLCAS rats (Table 4). Additionally, diminished fractional shortening (%) and ejection fraction (%) was observed in CLAS rats while the HLCAS rats showed lower fractional shortening percentage (Table 4). Furthermore, no detectable changes in cardiovascular structure and function were observed in the surgical castrated groups (CSCAS and HSCAS) (Table 4). The total heart wet weight (left ventricle + septum, right ventricle) was unchanged between the control groups and the castrated groups (Table 4). Compared with C fed rats, the left ventricle of H fed control rats and castrated groups (CLCAS, CSCAS, HLCAS, and HSCAS) showed increased infiltration of inflammatory cells (Fig. 3*A*-*F*). Also, in the high fat control rats and castrated groups, enhanced interstitial collagen deposition of myocytes (Fig. 3*G*-*L*) corresponding with increased diastolic stiffness constant (κ) was observed (Table 4).

Lower contractility responses to noradrenaline in isolated thoracic rings were measured in high fat control rats and castrated groups (Fig. 4*A*). Compared to C and CLCAS rats, lower smooth muscle-dependant relaxant responses to sodium nitroprusside were detected in CSCAS, H, HLCAS, and HSCAS groups (Fig. 4*B*) Compared to the C diet control groups, diminished endothelium-dependant relaxant responses to acetylcholine were measured in both the H fed control rats and castrated groups (Fig. 4*C*).

Table 4. Changes in cardiovascular structure and function in C, CLCAS, CSCAS, H, HLCAS, and HSCAS rats

Variable	C	CLCAS	CSCAS	H	HLCAS	HSCAS	*P*-Value		
							Diet	Castration	Interaction
Heart rate	253.4±22.9 [b]	237.6±11.0 [b]	227.4±17.4 [b]	350.1±10.3 [a]	290.1±14.9 [b]	281.8±15.0 [b]	<0.0001	0.01	0.30
LVIDd, *mm*	6.64±0.28 [c]	7.70±0.15 [ab]	6.63±0.30 [c]	7.09±0.22 [bc]	8.24±0.22 [a]	7.23±0.20 [bc]	0.008	<0.0001	0.95
LVIDs, *mm*	3.11±0.19 [c]	4.52±0.19 [a]	3.68±0.18 [b]	3.22±0.23 [c]	4.14±0.18 [ab]	3.48±0.25 [bc]	0.36	<0.0001	0.49
IVSd, *mm*	1.91±0.12	1.77±0.06	1.96±0.10	2.05±0.08	1.82±0.06	2.05±0.17	0.29	0.11	0.91
IVSs, *mm*	3.09±0.26 [ab]	2.80±0.08 [b]	3.13±0.20 [ab]	3.65±0.09 [a]	3.12±0.11 [ab]	3.43±0.20 [ab]	0.007	0.05	0.70
LVPWd, *mm*	1.70±0.09	1.79±0.03	1.91±0.12	2.04±0.06	1.75±0.05	2.08±0.15	0.05	0.06	0.14
LVPWs, *mm*	2.73±0.16 [b]	2.58±0.15 [b]	2.65±0.14 [b]	3.27±0.14 [a]	2.95±0.10 [ab]	3.04±0.13 [ab]	0.0004	0.24	0.80
Diastolic volume, *μL*	317.0±40.8 [c]	481.0±26.1 [ab]	318.0±38.2 [c]	427.0±39.8 [abc]	531.0±46.6 [a]	356.0±30.4 [bc]	0.037	0.0001	0.59
Systolic volume, *μL*	34.0±6.1 [c]	100.0±12.8 [a]	55.0±8.2 [bc]	38.0±6.9 [c]	77.0±8.6 [ab]	49.0±10.5 [bc]	0.27	<0.0001	0.34
SBP:LVIDs	42.4±2.9 [ab]	32.1±1.5 [c]	40.9±1.8 [abc]	48.3±3.8 [a]	36.9±2.4 [bc]	48.0±3.0 [a]	0.0009	0.0003	0.91
SBP:systolic volume	4826±956 [ab]	1599±198 [ab]	3016±418 [ab]	5562±1462 [a]	2291±479 [b]	4436±840 [ab]	0.17	0.0016	0.89

ESS:LVIDs	2.40±0.10[b]	2.84±0.16[a]	2.44±0.08[ab]	2.30±0.09[b]	2.56±0.11[ab]	2.69±0.09[ab]	0.63	0.009	0.06
Stroke volume, *μL*	283.0±37.5 [b]	381.0±22.1 [ab]	263.0±32.6 [b]	388.0±35.2 [ab]	454.0±43.3 [a]	306.0±24.4 [b]	0.009	0.0011	0.65
Cardiac output, *mL/min (n=8)*	70.1±9.2 [b]	90.6±7.3 [b]	56.3±7.9 [b]	135.2±10.9 [a]	130.9±12.9 [a]	84.8±5.6 [b]	<0.0001	0.0002	0.15
Relative wall thickness, *(n=8)*	0.56±0.05	0.47±0.02	0.58±0.05	0.56±0.02	0.45±0.02	0.60±0.05	1.00	0.004	0.87
Systolic wall stress, *(n=8)*	75.7±7.0 [b]	129.9±12.2 [a]	104.4±8.9 [ab]	74.8±7.1 [b]	106.4±7.3 [ab]	93.3±6.6 [b]	0.09	<0.0001	0.41
Fractional shortening, *% (n=8)*	53.1±2.3 [ab]	41.2±2.1 [c]	47.1±2.1 [b]	56.6±2.5 [a]	47.7±2.3 [b]	49.6±2.7 [abc]	0.04	0.0003	0.68
Ejection fraction, *%*	89.1±1.6 [a]	79.3±2.0 [b]	84.0±2.0 [ab]	91.2±1.5 [a]	85.2±1.7 [ab]	86.6±2.1 [a]	0.02	0.0004	0.53
Diastolic stiffness, *κ*	23.8±0.7 [b]	29.4±0.6 [a]	27.3±0.5 [a]	29.1±0.7 [a]	29.1±1.1 [a]	27.9±0.7 [a]	0.003	0.002	0.001
Estimated LV mass, *g*	0.81±0.03 [b]	1.01±0.04 [ab]	0.89±0.05 [ab]	1.16±0.07 [a]	1.07±0.06 [ab]	1.08±0.12 [ab]	0.001	0.65	0.12
LV+septum wet weight*, *mg/mm*	17.6±0.5	18.1±0.5	18.1±0.8	19.5±0.7	19.1±0.5	19.9±0.2	0.002	0.69	0.69
RV wet weight*, *mg/mm*,	2.4±0.2	2.8±0.2	3.4±0.6	4.0±1.2	3.2±0.2	2.8±0.1	0.33	0.94	0.17
Heart wet weight, *mg/mm*	20.0±0.6	20.9±0.7	21.5±1.4	23.7±1.6	22.3±0.5	22.7±0.3	0.01	0.88	0.37

Values are means ± SE; *n*=8-10 per group. Means within a row with unlike superscript letters (a-d) are significantly different (P<0.05). Left ventricular (LV)+ septum and right ventricular (RV) wet weights were normalized against tibial length. LVIDd, left ventricular internal diameter during diastole; LVIDs, left ventricular internal diameter during systole;IVSd, interventricular septum thickness during diastole; IVSs, interventricular septum thickness during systole; LVPWd, leftventricular posterior wall thickness during diastole; LVPWs, leftventricular posterior wall thickness during systole; SBP, systolic blood pressure; ESS, end systolic wall stress.

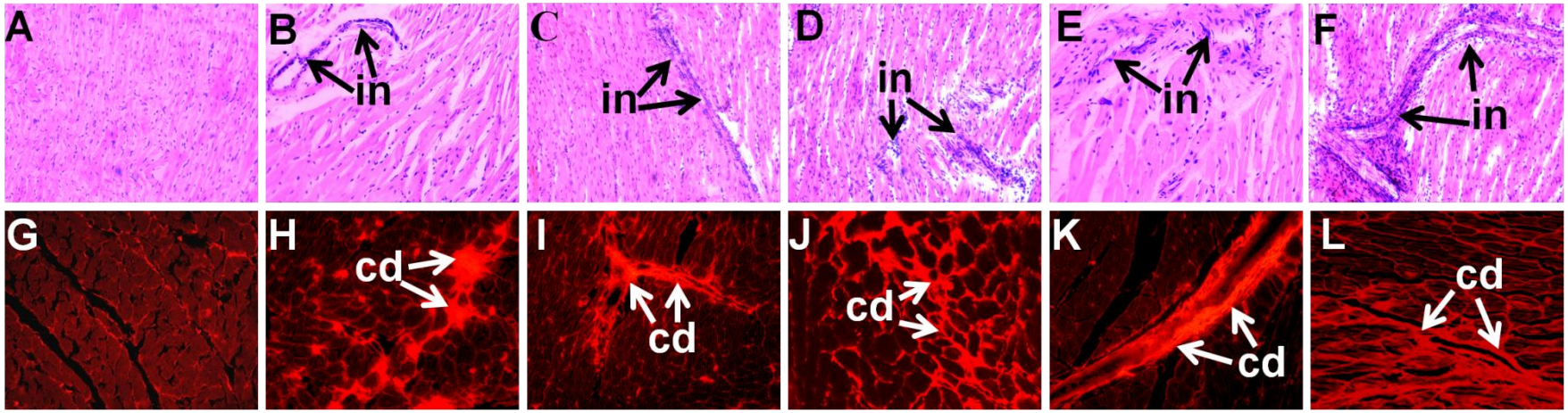

Fig. 3. *A-F*: haematoxylin and eosin stained pictures of left ventricle showing infiltration of inflammatory cells (in) in the myocytes of C (*A*), CLCAS (*B*), CSCAS (*C*), H (*D*), HLCAS (*E*) and HSCAS (*F*) rats at 20X magnification. *G-L*: picrosirius red-stained pictures of left ventricle showing interstitial collagen deposition (cd) in the myocytes of C(*G*), CLCAS(*H*), CSCAS(*I*), H(*J*), HLCAS(*K*) and HSCAS(*L*) rats at 20X magnification.

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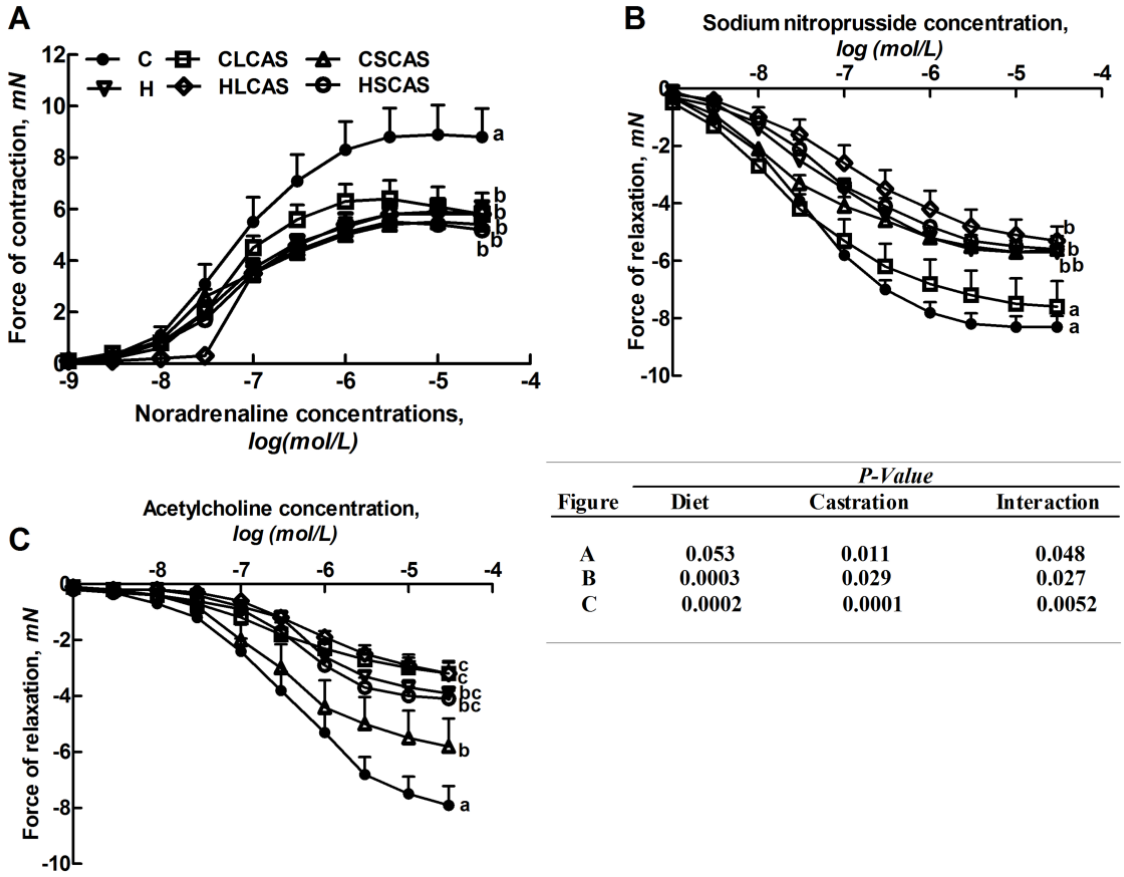

Figure	P-Value		
	Diet	Castration	Interaction
A	0.053	0.011	0.048
B	0.0003	0.029	0.027
C	0.0002	0.0001	0.0052

Fig. 4. Cumulative concentration response curves for noradrenaline-induced contraction (*A*), sodium nitroprusside-induced relaxation (*B*), and acetylcholine-induced relaxation (*C*) in thoracic aortic rings from C, CLCAS, CCAS, H, HLCAS and HSCAS fed groups. Values are means ± SE; *n*=9-10 per group. Means with unlike superscript letters (a-d) in each data set are significantly different (P<0.05).

Hepatic structure and function

Compared with C fed groups (C, CLCAS and CSCAS), the H fed groups including H, HLCAS, and HSCAS rats showed increased liver weight (Table 2) with higher plasma activities of ALT and AST (liver damage markers) (Table 3). Further, hepatocytes with enlarged fat vacuoles and higher infiltration of inflammatory cells in the portal region were observed in H, HLCAS, and HSCAS groups (Fig. 5*A*-*F* & 5*G*-*L*). In the hepatocytes of CLCAS and CSCAS rats, vacuolation with minimal enlarged fat vacuoles, and higher infiltration of inflammatory cells were detected in the portal region, compared to the C fed control rats (Fig. 5*A*-*C* & 5*G*-*I*).

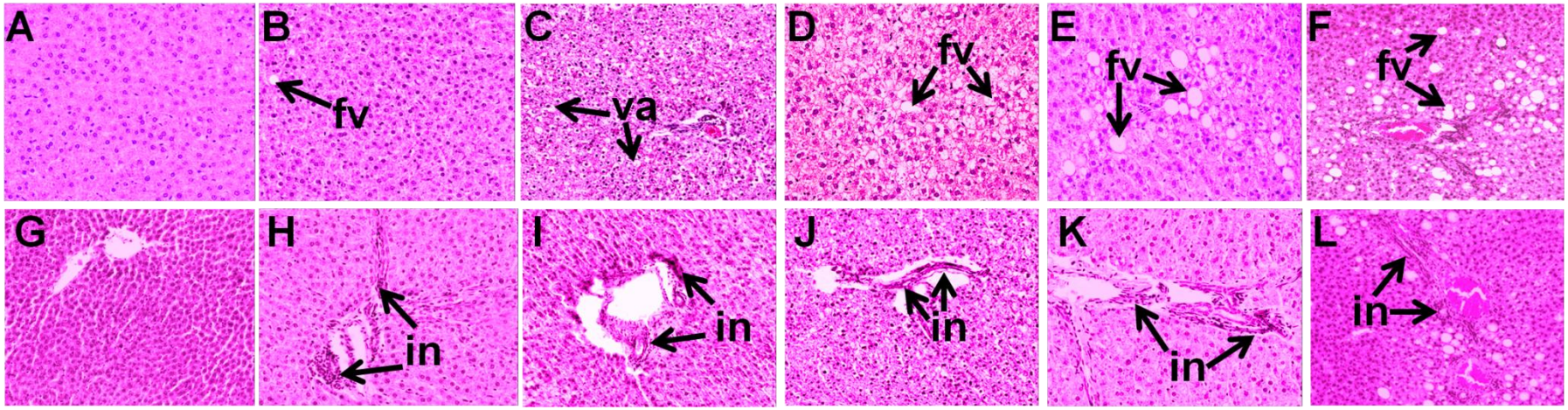

Fig. 5. Hematoxylin and eosin stained pictures of hepatocytes showing enlarged fat vacuoles (fv) and vacuolation (va) (Magnification x20) in rats fed with C (*A*), CLCAS (*B*), CSCAS (*C*), H (*D*), HLCAS (*E*) and HSCAS (*F*), and infiltration of inflammatory cells in rats fed with C (*G*), CLCAS (*H*), CSCAS (*I*), H (*J*), HLCAS (*K*) and HSCAS (*L*).

Discussion

As shown in our previous studies (2, 28), the H diet with higher amounts of carbohydrates (68% as fructose and sucrose) and fat (24% as saturated and mono-unsaturated fatty acids) increased the visceral obesity (Table 2; Fig 1*B,C*), and its associated metabolic risk factors including increased SBP (Fig. 2*C*), impaired glucose and insulin tolerance (Fig. 2*A,B*), collagen deposition (Fig. 3*J*) and cardiovascular stiffness (Table 4), non-alcoholic fatty liver disease (Table 3, Fig. 5*D&J*), infiltration of inflammatory cells in myocytes and hepatocytes (Fig. 3*D*&5*D*), endothelial dysfunction (Fig. 4) and dyslipidemia (Table 3). Testosterone regulates the development of skeletal muscle and adipose tissue (11, 29, 30), and its deficiency increases the metabolic risk factors including fat mass development, arterial stiffness and hypertension, type 2 diabetes, insulin resistance and cardiovascular disease in prostate cancer patients treated with androgen deprivation therapy and aged men (40-79 years) (24, 31-33). To explore the effects of low testosterone concentration on the pathogenesis of obesity and its associated metabolic complications (17, 19, 20), in this study we have investigated the effect of testosterone deprivation in the castrated rats fed with low-calorie (C) and high-calorie (H) diet.

As androgen receptors regulate adipose tissue metabolism (34), testosterone deficiency in either leuprolide acetate (CLCAS, HLCAS) or surgical (CSCAS, HSCAS) castrated groups (Table 3) showed increased visceral adiposity index (%) (Fig. 1*B,C*) with higher retroperitoneal fat deposition (Table 2). This effect may be due to the enhanced adipogenesis with increased lipoprotein lipase activity and/or decreased lipolysis in the castration groups (10, 11). Further, decreased lean mass in surgical castration groups (CSCAS and HSCAS) corresponding with increased total body fat mass in HSCAS rats has been correlated to the diminished mitochondrial function and energy expenditure (22, 35). The reduction in bone mineral content (at 8 wk) in the leuprolide acetate castration groups (Table 2) is positively associated with the reduced total body fat mass (46-56%) (36) (Table 2). At 16 wk, HLCAS groups showed reduced bone mineral content which may be due to the modest decrease in total body fat mass (15.7%) compared to H and HSCAS groups (Table 2).

Increased insulin resistance and glucose intolerance in the castrated groups (Fig. 2*A,B*) have been linked to the visceral adiposity mediated delivery of free fatty acids to the skeletal muscle and liver that diminishes the glucose disposal by inhibiting the insulin-stimulated glucose uptake and pancreatic β-cell function (37, 38).

Testosterone on its own could act as a potent vasodilator via an endothelium-independent mechanism (39, 40). Consequently, rats with decreased total testosterone concentration (83-85%) that appeared earlier (at 4 wk) in surgical castrated groups showed hypertension during 16 wk of experimental protocol (Fig. 2*C*) while CLCAS rats showed reduced total testosterone concentration (54%) only at 12 wk (Table 4) and became hypertensive after 8 wk of experimental protocol (Fig. 2*C*). This hypertensive effect of castrated groups clearly suggests that testosterone deficiency could have an independent effect in inducing the hypertension (41). Additionally, the increased SBP has been correlated with the diminished vascular function in either C or H fed castrated groups (Fig. 4*A-C*). Systolic dysfunction detected in the leuprolide acetate castrated groups (Table 4) has been directly associated with the enhanced

pressure volume overload showing signs of eccentric hypertrophy (Table 4) (42). Further, increased cardiac stiffness (Table 4) correlated with enhanced interstitial collagen deposition observed in either H control rats or castrated groups (Fig. 3*H-L*) is associated with the reduced glucose utilization (Fig. 2*A*) that may activate the protein kinase C (PKC) expression, and increase the transforming growth factor-1 (TGF-1) induced collagen deposition (43, 44). Also, enhanced visceral obesity in castrated groups may increase the infiltration of inflammatory cells in the heart (Fig. 3*A-F*) (45, 46).

Hepatic fat accumulation (Fig. 5*E&F*) identified with increased infiltration of inflammatory cells (Fig. 5*K&L*), and increased plasma ALT and AST activities (liver damage markers) in the H fed castrated groups are directly linked to the visceral adiposity-induced dyslipidemia (Table 3) (47, 48). Similarly, the testosterone deprived CSCAS and CLCAS groups showed enhanced visceral adiposity as vacuolated liver and minimal enlarged fat vacuoles (Fig. 5*B,C*) (16, 49).

In summary, testosterone deprivation increased the visceral obesity in castrated groups fed with either normal C diet or high-calorie H diet, and showed increased risk factors of metabolic syndrome such as insulin resistance, type 2 diabetes, cardiovascular stiffness and collagen deposition, inflammation, and non-alcoholic fatty liver disease. Additionally, testosterone deficiency independently increased the SBP in the castrated rats and showed endothelial dysfunction. These results support our hypothesis that visceral obesity enhanced by testosterone deprivation may play a key role in aggravating the obesity-linked metabolic syndrome.

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CHAPTER 4

Seaweed supplements normalize metabolic, cardiovascular and liver responses in high- carbohydrate, high-fat fed rats

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Article

Seaweed supplements normalise metabolic, cardiovascular and liver responses in high-carbohydrate, high-fat fed rats

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Abstract: For people in eastern Asia, increased seaweed consumption may be linked to lower incidence of metabolic syndrome. This study investigated the responses to two tropical green seaweeds, *Ulva ohnoi* (UO) and *Derbesia tenuissima* (DT), in a rat model of human metabolic syndrome. Male Wistar rats (330-340g) were fed either a corn starch-rich diet or a high-carbohydrate, high-fat diet with 25% fructose in drinking water, for 16 weeks; food was supplemented with dried seaweed for the final 8 weeks. High-carbohydrate, high-fat diet-fed rats showed the symptoms of metabolic syndrome leading to abdominal obesity, cardiovascular remodelling and non-alcoholic fatty liver disease. UO lowered total body fat mass by 24% (from 201.6±10.9 g to 153.1±14.6 g), systolic blood pressure by 29 mmHg (from 157±1 mmHg to 128±1 mmHg), and improved glucose utilisation and insulin sensitivity. In contrast, DT did not change total body fat mass but decreased plasma

triglycerides by 38% (from 1.29±0.19 mmol/L to 0.80±0.19 mmol/L) and total cholesterol by 17% (from 1.98±0.05 mmol/L to 1.65±0.09 mmol/L). UO and DT differed in composition of dietary fibre (UO: 18.1% dry weight soluble fibre as part of 40.9% total fibre; DT <0.1% dry weight soluble fibre as part of 23.4% total fibre). Increased magnesium intake from UO may improve glucose metabolism. Thus, UO was more effective in reducing metabolic syndrome than DT, possibly due to high soluble fibre and magnesium.

Keywords: Obesity; hypertension; fatty liver; tropical seaweeds; soluble fibre

1. Introduction

Obesity, defined as excess body fat, is a major health-care problem that increases the risk of cardiovascular and metabolic disorders such as hypertension, ischaemic stroke, insulin resistance, impaired glucose tolerance, hyperinsulinaemia and dyslipidaemia [1,2]. Current treatment of obesity is aimed at modifying dietary habits, increasing exercise to increase calorie expenditure and lowering calorie and fat intake [3,4], rather than drugs. Long-term drug treatment of obesity shows modest effects in many patients, and there are potential health hazards when combined drug therapies are used for obesity management [5].

Seaweeds are considered as part of a healthy diet, especially in Japan, Korea, China and the Philippines [6,7]. Seaweeds contain higher potassium, magnesium and calcium ion concentrations than other foods [7]. Seaweeds possess anti-diabetic, antioxidant, anti-obesity, anti-hyperlipidaemic and anti-inflammatory activities [8]. Seaweeds may prevent diet-induced cardiovascular disease as an alternative source of dietary fibre [9]. Fibre is the largest component of the seaweed biomass [10,11] and therefore may be present in sufficient amounts when included in the diet to prevent metabolic syndrome associated with obesity, type 2 diabetes and cardiovascular complications [12]. Seaweeds are typically low in fat (<5%), with a majority of this fat consisting of polyunsaturated fatty acids including the ω-3 fatty acids, such as α- and γ-linolenic acids, eicosapentaenoic acid and docosahexaenoic acid [13].

The combination of fibre as polysaccharides, with antioxidants, ω-3 fatty acids and minerals indicates that seaweeds could be targeted as functional foods for metabolic syndrome in westernized diets [6,8]. *Ulva* species showed cholesterol-lowering and cardioprotective properties, as well as anti-inflammatory potential [14,15]. Supplementation of a high-calorie diet with the edible green seaweed *Ulva linza* lowered intra-abdominal fat pads by 35%, blood pressure by 35%, blood glucose concentration by 31%, and serum cholesterol and triglycerides concentrations by 17 and 20% [16]. The fast growth rate and broad geographical distribution of tropical species of *Ulva*, such as *Ulva ohnoi* (UO), could provide a sustainable source of seaweed for new applications as functional foods as they are suitable for intensive aquaculture production [17]. Another tropical green seaweed with commercialisation potential is *Derbesia tenuissima* (DT), which has been targeted for its nutritional attributes, especially a higher content of polyunsaturated fatty acids [18]. In contrast to *Ulva*, there is little evidence for

Derbesia species as a functional food in metabolic syndrome, although methanolic extracts of *Derbesia* species showed a high inhibitory activity *in vitro* against protein tyrosine phosphatase 1B [19], a negative regulator of insulin receptors associated with signal transduction.

This study therefore measured the responses to two tropical green seaweeds, *Ulva ohnoi* and *Derbesia tenuissima*, on metabolic parameters and on the structure and function of the cardiovascular system and liver in rats fed a high-carbohydrate, high-fat diet. These rats showed symptoms of metabolic syndrome with metabolic abnormalities, cardiovascular remodelling, and non-alcoholic steatohepatitis [20]. Seaweed supplementation was given for the final 8 weeks of a 16 week protocol. The structure and function of the heart were characterised by echocardiography, isolated Langendorff heart preparation and histopathology, while the structure and function of the liver were characterised by histopathology and plasma biochemical analyses. In addition, metabolic function was characterised by fat measurements, glucose and insulin tolerance tests and plasma insulin concentrations.

2. Results

2.1 Rats

The experimental groups consisted of 72 male Wistar rats (9-10 weeks-old; 331.7±1.6 g) individually housed in a temperature-controlled (20±2°C), 12-hour light/dark cycle environment with unrestricted access to water and food, for which the preparation and macronutrient composition have been described previously [20,21]. The rats were randomly divided into 6 separate groups (n = 12 each) and fed with corn starch (C), corn starch + *Ulva ohnoi* 5% (CUO), corn starch + *Derbesia tenuissima* 5% (CDT), high-carbohydrate, high-fat (H), high-carbohydrate, high-fat + *Ulva ohnoi* 5% (HUO), or high-carbohydrate, high-fat + *Derbesia tenuissima* 5% (HDT). The seaweed-supplemented diets were prepared by adding 5% of seaweeds to replace an equivalent amount of water in the diet. The drinking water in all H groups included 25% fructose. The seaweed-supplemented diets were administered for 8 weeks starting 8 weeks after the initiation of the C or H diets.

2.2 Nutritional composition of seaweeds

The seaweed supplements differed in their concentrations of fibre, fatty acids and minerals. The total dietary fibre content of UO was 40.9% of dry algae with 18.1% as soluble fibre and 22.8% as insoluble fibre while DT contained 23.4% of dry algae, and this was exclusively insoluble fibre (Table 1). DT had higher fatty acid content (4.9% of dry algae) than UO (1.2% of dry algae), with higher omega-3 fatty acid content (38.8% of total fatty acids) and omega-6 fatty acid content (12.7% of total fatty acids) (Table 1). UO had higher proportion of mineral ions (13.5% of dry algae) than DT (11.5% of dry algae) (Table 1). Both seaweeds have higher magnesium, sodium and potassium ion concentrations than control diets (H and C) (Table S1), with HUO rats having higher magnesium intake (59.5 mg/day) than HDT (29.3 mg/day) and H (15.1 mg/day) rats (Table S2). Compared to C rats, the H rats showed increased fat intake including saturated fatty acids, mono-unsaturated fatty acids and poly-unsaturated fatty acids (Table S3). Neither UO nor DT altered the total fatty

acids intake in CUO, CDT, HUO, and HDT rats (Table S3). The highest intake of α-linoleic acid was measured in CDT (28.9 mg/day) and HDT (16.6 mg/day) treated rats. CDT rats had higher eicosapentaenoic acid intake (1.9 mg/day) than HDT rats (1.3 mg/day) (Table S3).

2.3 Metabolic variables

Consumption of food and water was higher in C rats than H rats (Table 2). Compared with C groups (C and CUO), increased energy intake occurred with no change in body weight gain and energy efficiency in H and HUO rats (Table 2). An increased body weight gain was observed in both CDT and HDT rats compared to C rats, while only HDT rats had an increased energy intake (Table 2).

Compared with C rats, lower lean mass (Table 3), higher total body fat mass (Figure 1*A*) and abdominal fat mass were measured in H rats (Table 3). H rats had higher abdominal circumference and visceral adiposity index (Table 3). HUO but not HDT lowered total body fat mass (Figure 1*A*). The glucose utilisation and insulin sensitivity were improved in HUO and HDT rats, compared with H rats (Figure 1*B* & 1*C*). Among C treated groups, CDT rats showed diminished glucose utilisation compared to C and CUO rats (Figure 1*B* & 1*C*). The bone mineral content was higher in H rats, compared with C rats with no effect of either seaweed treatment (Table 3). Increased plasma concentrations of NEFA, triglycerides and total cholesterol were observed in H rats, whereas no changes were observed in C groups (Table 4). Plasma NEFA concentrations were increased in HUO rats. DT treatment did not change the plasma NEFA concentration but normalised the plasma triglycerides and total cholesterol concentrations in HDT rats (Table 4). No changes were observed in plasma sodium, potassium and magnesium ion concentrations in the treatment groups (Table 4).

Table 1 Nutritional composition of seaweeds

Nutrients	***Ulva ohnoi (UO)***	***Derbesia tenuissima (DT)***
Total fibre (g/100g)	40.9	23.4
Insoluble fibre (g/100g)	22.8	23.4
Soluble fibre (g/100g)	18.1	<0.1
Total minerals (g/100g)	13.5	11.5
Essential minerals		
Magnesium (g/100g)	4.1	1.3
Potassium (g/100g)	2.9	1.3
Sodium (g/100g)	2.8	6.1
Calcium (g/100g)	0.3	0.5
Zinc (mg/100g)	0.6	6.2
Iron (mg/100g)	15.9	45.7
Total fatty acids (g/100g)	1.2	4.9
Saturated fatty acids (% of total fatty acids)	52.5	35.1
MUFA (% of total fatty acids)	28.6	13.4
PUFA (% of total fatty acids)	18.8	51.5

ω-3 PUFA (% of total fatty acids)	16.0	38.8
ω-6 PUFA (% of total fatty acids)	2.8	12.7

MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid

Table 2: Physiological variables in rats fed control diets C or H and with either *Ulva ohnoi* or *Derbesia tenuissima*

Variable	C	CUO	CDT	H	HUO	HDT	*P*-Value		
							Diet	Treatment	Interaction
Food intake, *g/d*	35.7±3.7[a]	33.6±3.6[a]	32.2±2.4[a]	22.0±2.0[b]	21.6±0.8[b]	21.9±1.3[b]	< 0.0001	0.78	0.81
Water intake, *ml/d*	32.0±3.4[a]	33.9±2.8[a]	33.6±2.4[a]	20.0±1.8[b]	24.8±0.8[b]	20.9±1.7[b]	< 0.0001	0.37	0.73
Energy intake, *kJ/d*	396.7±20.2[b]	398.9±11.1[b]	406.6±11.3[b]	469.9±9.3[a]	483.2±15.2[a]	478.2±4.1[a]	< 0.0001	0.75	0.86
Feed conversion efficiency, *%*	2.8±0.3[ab]	2.5±0.3[b]	4.1±0.4[a]	2.9±0.3[ab]	2.9±0.2[ab]	3.6±0.5[ab]	1.00	0.004	0.44
Body weight gain, *%*	11.1±1.1[b]	9.9±1.1[b]	16.7±1.5[a]	12.8±1.4[ab]	13.8±1.2[ab]	17.3±2.2[a]	0.10	0.001	0.54

[1]Values are mean ± SEM, *n*=8-10, Mean within a row with unlike superscript differ, P<0.05. C, corn starch fed rats; CUO, cornstarch rats treated with *Ulva ohnoi*; CDT, cornstarch rats treated with *Derbesia tenuissima*; H, high-carbohydrate, high-fat diet fed rats; HUO, high-carbohydrate, high-fat rats treated with *Ulva ohnoi*; HDT, high-carbohydrate, high-fat rats treated with *Derbesia tenuissima*.

Table 3 Metabolic variables in rats fed control diets C or H and with either *Ulva ohnoi* or *Derbesia tenuissima*

Variable	C	CUO	CDT	H	HUO	HDT	*P*-Value		
							Diet	Treatment	Interaction
Bone mineral content, *g*	12.4±0.3[c]	12.4±0.5[c]	13.5±0.4[bc]	15.1±0.5[a]	14.3±0.4[ab]	15.7±0.4[a]	< 0.0001	0.017	0.64
Total lean mass, *g*	319.1±10.3[ab]	302.4±2.5[bc]	333.2±6.6[a]	271.4±8.4[c]	292.3±11.8[bc]	284.4±3.9[c]	< 0.0001	0.23	0.04
Total body fat mass, *g*	85.5±7.4[c]	103.8±9.8[c]	100.1±7.9[c]	201.6±10.9[a]	153.1±14.6[b]	190.0±18.9[a]	< 0.0001	0.32	0.026
Abdominal circumference, *cm*	19.6±0.4[b]	18.8±0.2[b]	19.6±0.2[b]	22.0±0.4[a]	21.4±0.2[a]	22.2±0.6[a]	< 0.0001	0.08	0.95

Tissue wet weights, *mg/mm tibial length*									
Retroperitoneal fat	138.8±11.1[b]	127.8±10.6[b]	150.4±9.3[b]	331.5±20.9[a]	303.3±16.9[a]	322.0±18.8[a]	< 0.0001	0.32	0.76
Epididymal fat	105.3±5.7[b]	91.3±4.8[b]	115.3±8.4[b]	170.4±11.4[a]	200.3±15.4[a]	197.1±16.7[a]	< 0.0001	0.26	0.15
Omental fat	59.7±5.7[b]	63.1±4.6[b]	74.3±6.6[b]	131.6±10.6[a]	125.4±12.3[a]	115.3±14.4[a]	< 0.0001	0.99	0.27
Total abdominal fat	292.7±19.6[b]	268.9±26.0[b]	340.0±23.1[b]	670.1±46.9[a]	628.9±42.4[a]	634.4±40.2[a]	< 0.0001	0.49	0.45
Visceral adiposity index, *%*	3.7±0.3[b]	3.0±0.3[b]	3.9±0.3[b]	6.9±0.5[a]	7.0±0.4[a]	7.3±0.7[a]	< 0.0001	0.41	0.65
Liver weight	215.7±6.8[b]	236.6±13.9[b]	229.9±7.9[b]	277.5±13.6[a]	301.2±10.2[a]	269.3±18.0[a]	< 0.0001	0.17	0.55
LV+septum	17.1±0.3[b]	18.9±0.4[ab]	20.5±1.0[a]	17.2±0.5[b]	17.3±0.3[b]	18.1±1.0[b]	0.023	0.010	0.18
RV wet weight	2.2±0.2[ab]	2.7±0.2[ab]	2.9±0.4[a]	2.2±0.1[ab]	2.0±0.1[ab]	1.9±0.2[b]	0.004	0.67	0.10
Heart wet weight	19.3±0.4[b]	20.5±0.5[b]	23.3±1.4[a]	19.4±0.5[b]	19.2±0.5[b]	19.3±0.5[b]	0.006	0.030	0.025
Systolic blood pressure, *mmHg*	127±2[c]	126±3[c]	131±1[bc]	157±1[a]	128±1[bc]	135±3[b]	< 0.0001	< 0.0001	< 0.0001
Diastolic stiffness, *κ*	23.9±1.7[b]	23.3±0.4[b]	23.0±0.6[b]	29.8±2.2[a]	25.1±0.9[b]	23.4±1.0[b]	0.016	0.023	0.11

[1]Values are mean ± SEM, *n*=8-10, Mean within a row with the same superscript are not significantly different, P<0.05. C, corn starch fed rats; CUO, cornstarch rats treated with *Ulva ohnoi*; CDT, cornstarch rats treated with *Derbesia tenuissima*; H, high-carbohydrate, high-fat diet fed rats; HUO, high-carbohydrate, high-fat rats treated with *Ulva ohnoi*; HDT, high-carbohydrate, high-fat rats treated with *Derbesia tenuissima*.

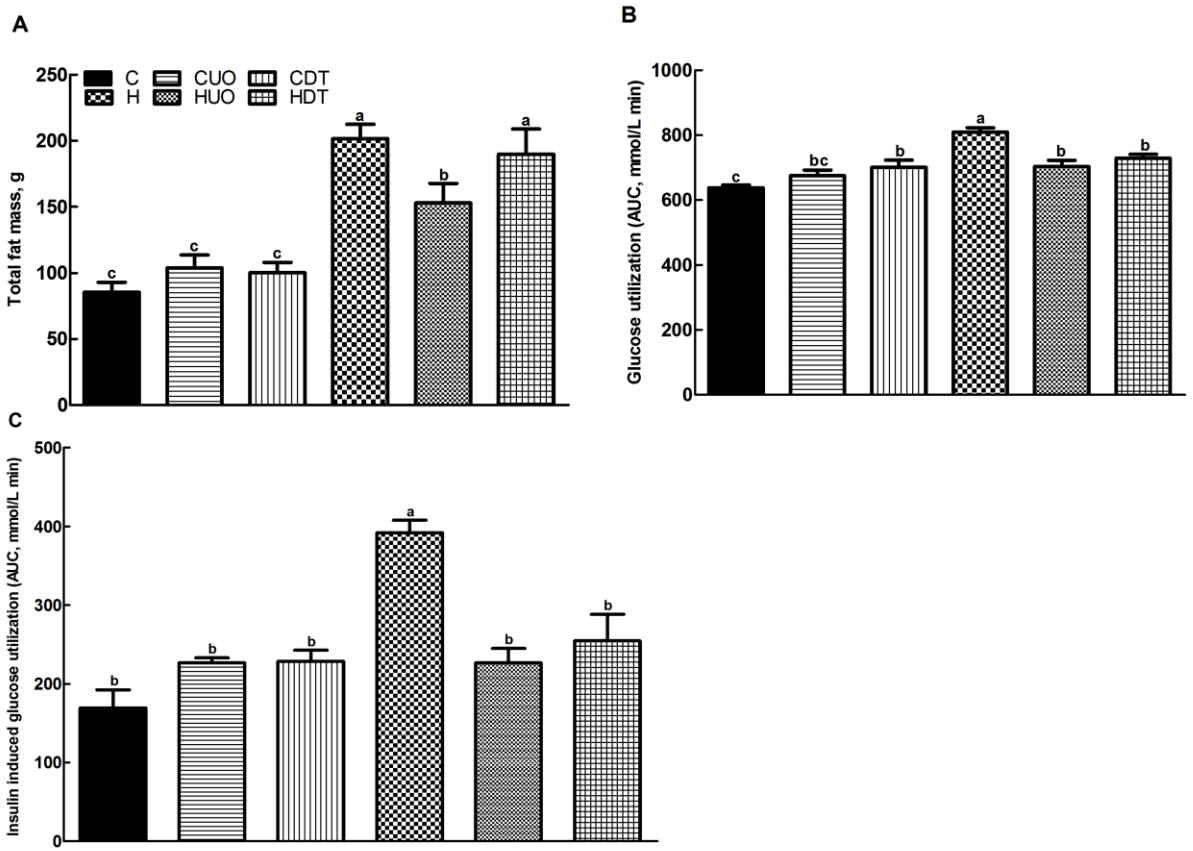
Table 4 Plasma biochemistry in rats fed control diets C or H and with either *Ulva ohnoi* or *Derbesia tenuissima*

Variable	C	CUO	CDT	H	HUO	HDT	*P*-Value		
							Diet	Treatment	Interaction
Plasma NEFA, *mmol/L*	1.47±0.18[c]	1.46±0.28[c]	1.55±0.09[c]	2.78±0.28[b]	3.73±0.29[a]	2.72±0.47[b]	< 0.0001	0.18	0.12

Plasma triglycerides, *mmol/L*	0.43±0.07[c]	0.41±0.07 [c]	0.54±0.07 [c]	1.29±0.19 [a]	1.17±0.14 [ab]	0.80±0.19 [bc]	< 0.0001	0.38	0.07
Plasma total cholesterol, *mmol/L*	1.52±0.09 [b]	1.51±0.04 [b]	1.53±0.05 [b]	1.98±0.05 [a]	1.87±0.19 [ab]	1.65±0.09[b]	0.0002	0.26	0.20
Plasma ALT activity, *U/L*	28.2±2.4 [b]	32.4±1.8 [b]	36.5±3.1 [b]	47.4±4.1 [a]	30.2±2.6 [b]	31.9±2.9 [b]	0.26	0.10	0.0002
Plasma AST activity, *U/L*	71.7±5.3 [b]	84.1±2.6 [b]	85.5±3.3 [b]	102.4±3.4 [a]	70.8±4.5 [b]	89.5±3.5 [b]	0.10	0.05	<0.0001
Plasma Na^+, *mmol/L*	143±1	143±1	141±1	143±0	140±1	140±1	0.07	0.03	0.24
Plasma K^+, *mmol/L*	5.55±0.38	5.94±0.33	6.43±0.31	6.03±0.64	6.75±1.04	5.20±0.48	0.97	0.57	0.19
Plasma Mg^{2+}, *mmol/L*	0.78±0.03	0.86±0.04	0.83±0.02	0.79±0.03	0.89±0.04	0.81±0.03	0.80	0.027	0.74

[1]Values are mean ± SEM, *n*=8-10, Mean within a row with the same superscript are not statistically different, P<0.05. C, corn starch fed rats; CUO, cornstarch rats treated with *Ulva ohnoi*; CDT, cornstarch rats treated with *Derbesia tenuissima*; H, high-carbohydrate, high-fat diet fed rats; HUO, high-carbohydrate, high-fat rats treated with *Ulva ohnoi*; HDT, high-carbohydrate, high-fat rats treated with *Derbesia tenuissima*.

FIGURE 1 Effects of seaweeds treatment on (*A)* total body fat mass, (*B)* oral glucose tolerance, and (*C)* insulin tolerance. Values are mean ± SEM, *n=8-10*. Endpoints means without a common letter differ, P<0.05. C, corn starch fed rats; CUO, cornstarch rats treated with *Ulva ohnoi*; CDT, cornstarch rats treated with *Derbesia tenuissima*; H, high carbohydrate, high fat diet fed rats; HUO, high carbohydrate, high fat rats treated with *Ulva ohnoi*; HDT, high carbohydrate, high fat rats treated with *Derbesia tenuissima*.



Cardiovascular structure and function

Systolic blood pressure was unchanged in C groups (Figure 2). The higher systolic blood pressure in H rats was lowered in HUO and HDT rats (Figure 2). The ratios of SBP:LVIDs and ESS:LVIDs were higher in H rats compared to C groups, while no change was observed in SBP:systolic volume ratio (Table S4). Ventricular contractility estimated as SBP:LVIDs and ESS:LVIDs was also normalised in HUO and HDT rats (Table S4). Further, no changes in echocardiographic parameters were observed in the seaweed treatment groups across both H and C groups (Table S4), except for an increase in left ventricular septal weight and heart weight in CDT but not in HDT rats (Table 3). Left ventricle showed increased infiltration of inflammatory cells (Fig. 3*D*) and collagen deposition (Figure 3*J*) in H rats, compared with C groups (Figures 3*A*,*B*,*C* & *G*,*H*,*I*). In HUO and HDT rats, the infiltration of inflammatory cells (Figure 3*E*,*F*) and the interstitial collagen deposition (Figure 3*K*,*L*) were normalised. The diastolic stiffness constant (κ) was normalised in HUO and HDT rats, compared to H rats (Table 3).

Lower contractile responses to noradrenaline in isolated thoracic rings were measured in H and HDT rats compared to C and HUO rats (Figure 4*A*). H rats showed lower smooth muscle-dependent and endothelium-dependent relaxant responses to sodium nitroprusside and acetylcholine (Figure 4*B*,*C*); both responses were higher in HUO rats and acetylcholine responses were higher in HDT rats (Figure 4*B*,*C*).

FIGURE 2 Effects of seaweeds treatment on systolic blood pressure. Values are mean ± SEM, *n=8-10*. Endpoints means without a common letter differ, P<0.05. C, corn starch fed rats; CUO, cornstarch rats treated with *Ulva ohnoi*; CDT, cornstarch rats treated with *Derbesia tenuissima;* H, high carbohydrate, high fat diet fed rats; HUO, high carbohydrate, high fat rats treated with *Ulva ohnoi*; HDT, high carbohydrate, high fat rats treated with *Derbesia tenuissima*.

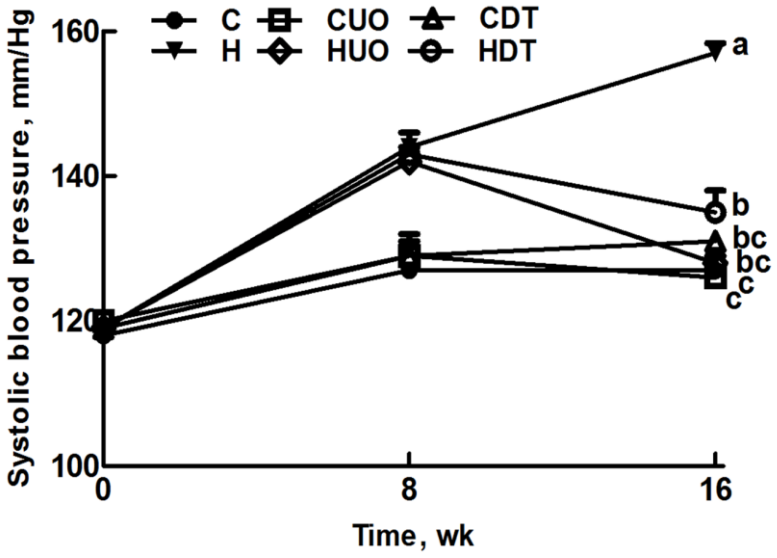


FIGURE 3 Effects of seaweeds treatment on inflammation and fibrosis in the heart. Haematoxylin and eosin staining of left ventricle showing infiltration of inflammatory cells *(A–F*, inflammatory cells marked as "in") (x20) from C *(A)*, CUO *(B)*, CDT *(C)*, H *(D)*, HUO *(E)*, and HDT *(F)* rats. Picrosirius red staining of left ventricle showing collagen deposition (G–M, fibrosis marked as "fi")(20x) from C *(A)*, CUO *(B)*, CDT *(C)*, H *(D)*, HUO *(E)*, and HDT *(F)* rats. C, corn starch fed rats; CUO, cornstarch rats treated with *Ulva ohnoi*; CDT, cornstarch rats treated with *Derbesia tenuissima*; H, high carbohydrate, high fat diet fed rats; HUO, high carbohydrate, high fat rats treated with *Ulva ohnoi*; HDT, high carbohydrate, high fat rats treated with *Derbesia tenuissima*.

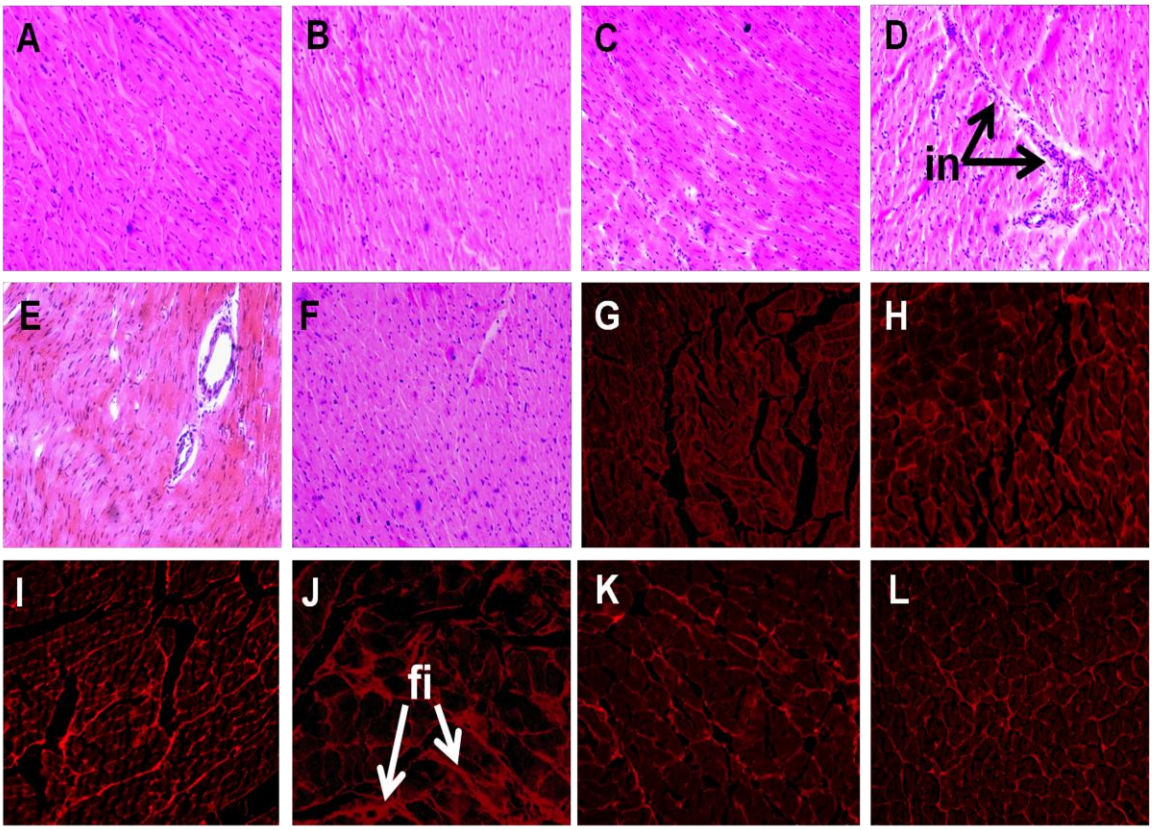
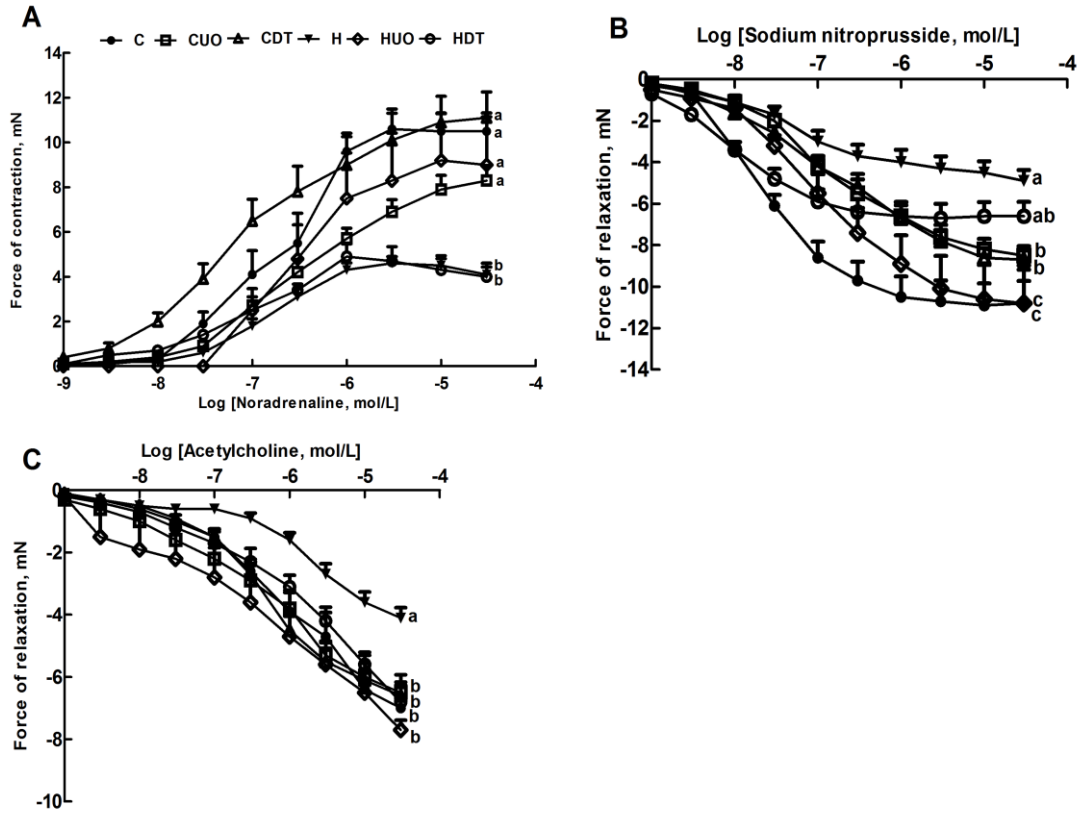


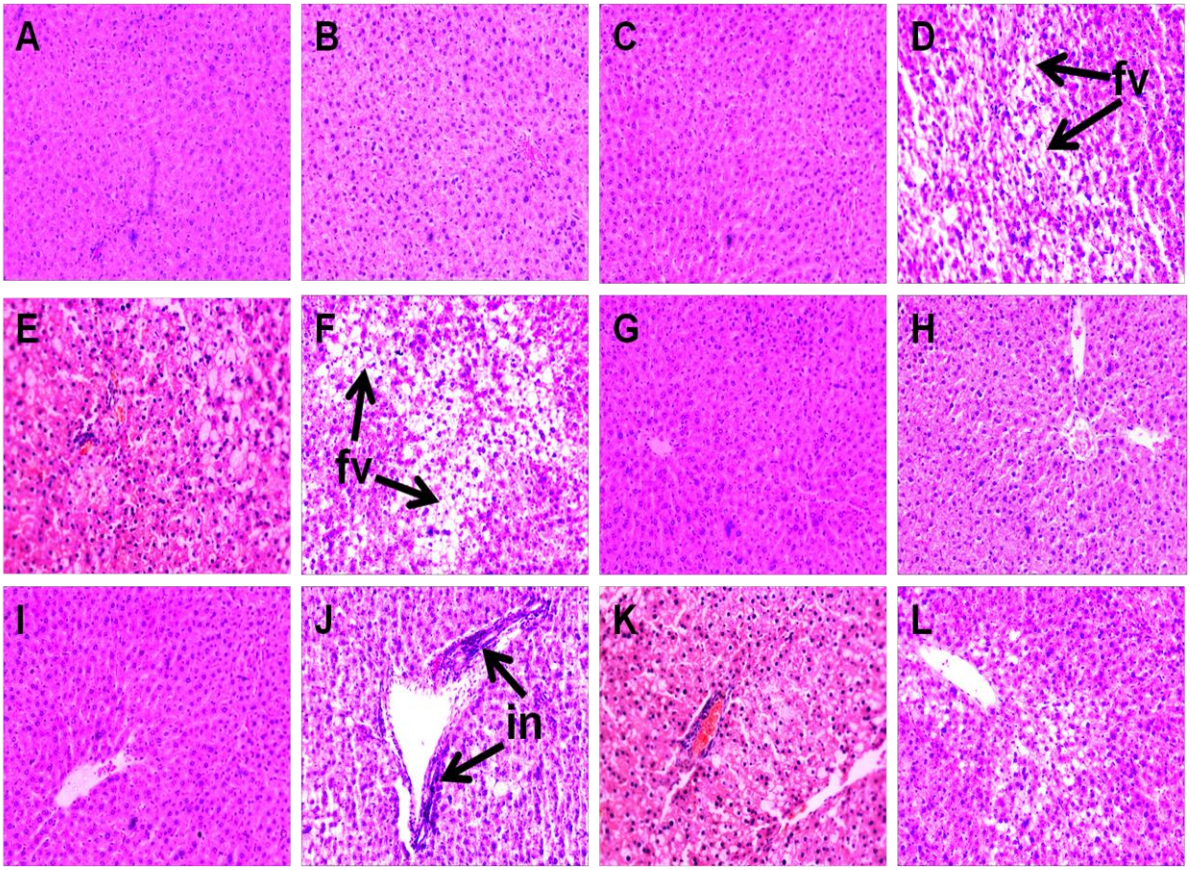
FIGURE 4 Effects of seaweeds treatment on noradrenaline-induced contraction *(A)*, sodium nitroprusside-induced relaxation *(B)*, and acetylcholine-induced relaxation *(C)* in thoracic aortic preparations from C, CUO, CDT, H, HUO, and HDT rats. Values are mean ± SEM, *n=8-10*. Endpoints means without a common letter differ, $P<0.05$. C, corn starch fed rats; CUO, cornstarch rats treated with *Ulva ohnoi*; CDT, cornstarch rats treated with *Derbesia tenuissima*; H, high carbohydrate, high fat diet fed rats; HUO, high carbohydrate, high fat rats treated with *Ulva ohnoi*; HDT, high carbohydrate, high fat rats treated with *Derbesia tenuissima*.



Liver structure and function

Compared to C groups, H rats showed increased liver weight (Table 3), higher infiltration of inflammatory cells and presence of enlarged fat vacuoles (Figures 5*A,B,C,D* & *G,H,I,J*). UO and DT treatment prevented the infiltration of inflammatory cells in HUO or HDT rats (Figure 5*K,L*). Liver weight was unchanged in HUO or HDT rats compared to H rats (Table 3). However, hepatocytes with enlarged fat vacuoles were observed in HDT rats but absent in HUO rats (Figure 5*E,F*). Plasma activities of liver enzymes ALT and AST were higher in H rats compared to C treatment groups whereas both the ALT and AST activities were normalised in HUO and HDT rats (Table 4).

FIGURE 5 Effects of seaweeds treatment on inflammation and fat deposition in the liver. Haematoxylin and eosin staining of liver showing enlarged fat vacuoles *(A-F*, marked as "fv") (x20) and inflammatory cells *(G–L*, marked as "in") (x20) from C *(A,G)*, CUO *(B,H)*, CDT *(C,I)*, H *(D,J)*, HUO *(E,K)*, and HDT *(F,L)* rats. C, corn starch fed rats; CUO, cornstarch rats treated with *Ulva ohnoi*; CDT, cornstarch rats treated with *Derbesia tenuissima*; H, high carbohydrate, high fat diet fed rats; HUO, high carbohydrate, high fat rats treated with *Ulva ohnoi*; HDT, high carbohydrate, high fat rats treated with *Derbesia tenuissima*.



3. Discussion

The prevalence of obesity is increasing in westernised populations, largely due to consumption of food that is rich in refined carbohydrates, omega-6 fatty acids, saturated and mono-unsaturated fatty acids [1,2]. Seaweeds are acknowledged as functional foods in eastern Asia, referring to the presence of nutrients that may reduce the potential damage from chronic diseases [6,8], but are not widely recognised as functional foods in westernised populations. In this study, we have demonstrated the ability of two tropical seaweeds that can be grown commercially, *Ulva ohnoi* (UO) and *Derbesia tenuissima* (DT), to attenuate or normalise a range of metabolic syndromes in rats that were induced using a diet with increased simple sugars and saturated fats [20-22]. *Ulva* represents a group of seaweeds present in all oceans, from tropical to temperate waters, that are highly suited to intensive aquaculture production [17]. *Derbesia* has been identified as a target group for functional food and intensive aquaculture production based on its unique nutritional and biochemical characteristics [18]. DT contains ω-3 polyunsaturated fatty acids including eicosapentaenoic acid and α-linolenic acid with a higher fat content than UO [13,18]. However, the intakes of α-linolenic acid (HUO:5.6 mg/day;HDT:16.6 mg/day) and eicosapentaenoic acid (HDT:1.3 mg/day) in this study are much lower than the intake of α-linolenic acid (350 mg/day) and eicosapentaenoic acid (550 mg/day) that attenuated diet-induced metabolic syndrome in previous studies using the same model [22]. Thus, the intakes of polyunsaturated fatty acids in this study are unlikely to explain the differences between seaweeds or be sufficient on their own to reverse metabolic changes in the H rats. Therefore, we now focus on three possible mechanisms whereby

increased intake of fibre or magnesium ions could improve metabolic syndrome by acting as prebiotics, improving cardiovascular function or decreasing inflammation.

Differing fibre contents with UO containing similar amounts of both soluble and insoluble fibre and DT containing only insoluble fibre could partially explain the different responses to these two seaweeds. Dietary fibres, including both soluble (ß-glucans, pectins, gums, mucilages and some hemicelluloses) and insoluble fibres (celluloses and hemicelluloses) from whole grains, vegetables and fruits, were directly associated with reducing the risk factors of cardiovascular disease, and the management of obesity, hypertension, hyperlipidemia and diabetes [23]. The American Dietetic Association recommends a daily dietary fibre intake of 25g for adult females and 38g for adult men [24]. Based on body surface area, conversion of the seaweed intake in the current study in rats to humans [25] would provide a fibre intake of between 5.8 and 15.6 g/day, lower than the recommendations, but similar to current intake in the USA. The physicochemical properties of seaweed fibre, such as the ability to absorb and hold water, ion exchange capacity and viscosity, will slow down absorption of compounds in the gut [26,27]. We suggest that the high soluble fibre content of UO could increase gastrointestinal viscosity and therefore inhibit intestinal absorption of fatty acids more effectively than DT, leading to the decreased total body fat mass with UO but not DT. Both UO and DT treatments improved glucose utilisation and insulin sensitivity, potentially through similar mechanisms. This has been shown with viscous soluble dietary fibre from linseed in young male Wistar rats [28]. Further, the fibre present in green seaweed is more similar to terrestrial crops than the fibre present in red and brown seaweed [29]. The fibre may work as a prebiotic, defined as polysaccharides that are not broken down in the stomach but are fermented in the intestine to improve gut bacteria [30]; these changes in intestinal bacteria could prevent obesity [31].

In addition to fibre, the mineral ion contents of the seaweed supplements were up to 3-fold higher than the control diets. The increased magnesium content in UO could delay the onset of diabetes as reported in OLETF rats where an increased magnesium intake of 16 mg/day [32] improved insulin sensitivity and glucose utilisation [33]. In contrast, magnesium deficiency aggravated the insulin resistance produced by high fat diets given to growing rats [34]. None of the other major minerals in the seaweeds such as potassium, calcium and zinc were present in sufficient quantities to have bioactive effects on the metabolic syndrome [35-37].

The potential influence of fibre and minerals on metabolic syndrome extended to other physiological changes in the high-carbohydrate, high-fat fed rats with attenuation or normalisation of the increases in blood pressure, ventricular diastolic stiffness, fibrosis and liver damage. High dietary fibre supplements in hypertensive subjects reduced systolic and diastolic blood pressures compared to subjects with low dietary fibre [38]. Increased dietary fibre may reduce blood pressure by reducing insulin concentrations and insulin resistance, by reducing body weight [6,39] or by increasing magnesium intake [33], or all three as in this study. The decreased infiltration of inflammatory cells probably precedes the decreased collagen synthesis and deposition in both the heart and liver. Both UO and DT supplementation improved liver structure and function, with multiple potential mechanisms such as improved insulin sensitivity and

glucose tolerance, decreased blood pressure, decreased body weight and decreased infiltration of inflammatory cells. Further, supplementation of DT to H rats reduced the plasma concentrations of triglycerides and total cholesterol. We suggest that the bioactive polysaccharides present in DT and UO may be responsible for these cardiovascular and liver responses [6,9] and deserve further study.

Inflammation is critically important in the development of obesity [40]; we showed increased infiltration of inflammatory cells in the heart and liver of H rats that was markedly attenuated by both UO and DT. In human adults with an increased fat intake of 78.8-84.2 g/day, increased dietary fibre intake of 14.3-16.6 g/day decreased serum C-reactive protein concentrations, a non-specific marker of inflammation [41]. Bioactive polysaccharides from many different seaweeds have demonstrated anti-inflammatory activity [42]. Furthermore, *in vivo* and *in vitro* studies observed that dietary fibre and short-chain carboxylic acids, such as propionate and butyrate, released during the bacterial fermentation of dietary fibre as prebiotics attenuated the production of pro-inflammatory cytokines including interleukin-8, interleukin-6 and tumour necrosis factor-α [43]. Similar modes of action are likely with green seaweed polysaccharides [29].

4. Experimental Section

4.1 Algal sources and nutritional components

Two species of green algae (Chlorophyta), *Ulva ohnoi* (UO) and *Derbesia tenuissima* (DT), were cultured in the aquaculture facilities of James Cook University, Townsville, Australia. All biomass was produced in large outdoor tanks with capacities of 2,500 L for *Derbesia* and >10,000 L for *Ulva*. Biomass was harvested on two occasions separated by ~12 months in 2011-2012. The biomass was rinsed in freshwater and freeze-dried, after which sub-samples were taken for analyses of fibre components (100 g dry-weight), and minerals and fatty acid concentrations (200 mg dry-weight).

In each sub-sample, 24 trace elements were quantified and mean values are reported (n=2 sub-samples per species). Al, Ca, K, Na, S, and P were analysed by Inductively Coupled Plasma Optical Emission Spectrometry, while metals and metalloids (As, B, Ba, Cd, Co, Cr, Cu, Fe, Hg, Mg, Mn, Mo, Ni, Pb, Se, Sr, V, and Zn) were analysed by Inductively Coupled Plasma Mass Spectrometry at the Advanced Analytical Centre, James Cook University, Townsville. Insoluble and soluble fibre was analysed on a combined sample of 100 g for each species containing 50 g from each harvest time. Fibre analyses were run using enzymatic-gravimetric methods by National Measurement Institute, Sydney, Australia (AOAC Official Method 985.29 for insoluble fibre; AOAC Official Method 993.19 for soluble fibre). Total crude lipids and fatty acids were extracted and analysed [13]. All remaining freeze-dried biomass was stored in vacuum-sealed bags under refrigeration until preparation of the food. All experimentation was approved by the Animal Ethics Committees of The University of Queensland and University of Southern Queensland under the guidelines of the National Health and Medical Research Council of Australia. Rats were monitored daily for body weight, and food and water intakes. Daily seaweed intake was calculated from the daily food intake. The

fatty acid concentrations of both C and H control diets used for the calculation of mean daily fatty acids intake were obtained from our previous study [22].
Abdominal circumference of rats was measured using a standard measuring tape during the period of anesthesia for systolic blood pressure (SBP) measurements and energy efficiency was calculated [20-22]. SBP measurements, and oral glucose and insulin tolerance tests were conducted at 0, 8 and 16 weeks. Oral glucose and insulin tolerance tests were performed as described [20-22]. Rats were withdrawn from the test if the blood glucose concentration dropped below 1.1 mmol/L, and 4 g/kg glucose was administered immediately via oral gavage to prevent hypoglycemia. Dual energy X-ray absorptiometric (DXA) measurements were performed on rats after 16 weeks of feeding, 2 days before euthanasia [20-22]. Visceral adiposity index (%) was calculated [20-22].

4.2 Cardiovascular measurements

Systolic blood pressure was measured under light sedation following intraperitoneal injection of Zoletil (tiletamine 15 mg/kg, zolazepam 15 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 (AD Instruments, Sydney, Australia) [20-22].
Echocardiographic examinations (Phillips iE33, 12MHz transducer) were performed to assess the cardiovascular structure and function in all rats at 16 weeks [20-21]. Rats were anaesthetised using Zoletil (tiletamine 25 mg/kg and zolazepam 25 mg/kg, intraperitoneally (i.p).; Virbac, Peakhurst, Australia) and Ilium Xylazil (xylazine 15 mg/kg, i.p.; Troy Laboratories, Smithfield, Australia). The ventricular contractility indexes were calculated including ratio of SBP to LVIDs, ratio of SBP to systolic volume, and ratio of ESS to LVIDs [21,44].
The left ventricular function of the rats was assessed using the Langendorff heart preparation [20-22]. Terminal anaesthesia was induced via i.p. injection of pentobarbitone sodium (Lethabarb®, 100 mg/kg). Blood (~5 mL) was taken from the abdominal aorta after heparin (200 IU; Sigma-Aldrich Australia, Sydney, Australia) administration through the right femoral vein. 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 (AD Instruments Australia and Pacific Islands, Bella Vista, NSW, Australia).
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 [20-22].
4.3 Organ weights

The right and left ventricles were separated after Langendorff experiments and weighed. Liver, and retroperitoneal, epididymal, and omental fat pads were removed following heart removal and blotted dry for weighing. Organ weights were normalized relative to the tibial length at the time of their removal (in mg/mm) [20-22].

4.4 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 photographed. 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 [20-22]. Thin sections (7 μm) of left ventricle and the liver were cut and stained with haematoxylin and eosin stain for determination of inflammatory cell infiltration with 20X and fat vacuole enlargement with 40X objectives using a Olympus BX51 microscope (Olympus, Melville, NY). Collagen distribution was measured in the left ventricle with picrosirius red stain. Laser confocal microscopy (Zeiss LSM 510 upright Confocal Microscope) was used to visualize collagen deposition [20-22].

4.5 Plasma biochemistry

Blood was centrifuged within 30 min of collection into heparinised tubes at 5,000 *g* for 15 minutes. Plasma was separated and transferred to Eppendorf tubes for storage at -20°C before analysis. Activities of plasma enzymes and analyte concentrations were determined using kits and controls supplied by Olympus using an Olympus analyzer (AU 400, Tokyo, Japan) [20-22].

4.6 Statistical analysis

All data are presented as mean ± SEM. Data from C, CUO, CDT, H, HUO and HDT groups were compared in a series of two-way ANOVAs (Analysis of Variance) with two types of "Diet", high carbohydrate and high fat diet or control cornstarch diet, and three types of "Treatment", a control and treatments supplemented with each seaweed, as the two fixed factors in the analyses. Homogeneity of variance for ANOVA was assessed using Bartlett's test and variables that were not normally distributed were log-transformed prior to analysis. Where the main effects were significant ($P<0.05$), means were compared using Newman-Keuls multiple comparisons. Where transformations did not result in normality or homogeneity of variance, a Kruskal-Wallis non-parametric test was performed. All statistical analyses were run using GraphPad Prism version 5.00 for Windows (San Diego, California, USA).

5. Conclusions

Our findings suggest that soluble dietary fibre as a major component in UO could play a key role in attenuating the signs of the metabolic syndrome such as hypertension, endothelial dysfunction, diminished insulin sensitivity and glucose utilisation, increased cardiac stiffness, increased collagen deposition,

increased liver damage and increased fat mass in diet-induced obese rats. Further, we would suggest that the insoluble fibre of both DT and UO may improve glucose metabolism and that the increased magnesium intake in UO could delay the onset of diabetes. Thus, these tropical seaweeds serve as a commercially viable source of dietary fibre, as they can be produced in large quantities by aquaculture, to attenuate the signs of metabolic syndrome.

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Author Contributions

SAK, MM, LCW and NAP performed the experiments; NAP and LB designed the experiments; all co-authors contributed to the manuscript. This manuscript forms part of the PhD thesis of SAK under the supervision of LB.

Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary tables

Table S1. Micronutrient profile of diets

Mineral ions	Corn starch (mg/kg)	High-carbohydrate, high-fat diet (mg/kg)	*Ulva ohnoi* (mg/kg)	*Derbesia tenuissima* (mg/kg)
K	4760	4450	29350	13400
Na	1475	1335	27650	61100
Mg	914	689	41300	13000
Ca	7190	7790	2985	4870
Fe	256.5	185	58.5	360
P	2910	2500	742	3620
Mn	23.5	18.2	13.0	29.9
B	3.4	2.9	85.9	78.0
Al	17.6	20.9	16.3	13.7
Sr	14.0	12.4	36.3	77.2
Zn	22.1	18.3	12.0	36.3
Cu	8.6	7.1	11.7	16.9
Ni	0.4	0.3	2.8	2.0
Mo	0.3	0.1	0.3	0.4
V	3.3	5.3	1.0	1.4
As	1.4	1.2	0.0	4.3
Cr	10.0	16.0	1.8	3.2
Pb	<= 0.05	<= 0.05	0.3	0.9
Se	<= 1	<= 1	1.3	3.1
Co	<= 0.1	<= 0.1	0.3	0.5

Hg	<= 0.5	<= 0.5	1.3	4.4
Cd	<= 0.05	<= 0.05	0.2	0.3
Sum total	**18291.0**	**18049.2**	**134477.1**	**114723.4**

Mineral ions profile (ppm, mean of samples, n=2 ± SD<15040).

Table S2. Essential mineral ion intake in rats fed control diets C or H and with either *Ulva ohnoi* or *Derbesia tenuissima*

Variable	C	CUO	CDT	H	HUO	HDT	*P*-Value		
							Diet	Treatment	Interaction
Magnesium intake, *mg/d*	32.6±3.3 [c]	97.8±7.8 [a]	50.4±3.8 [b]	15.1±1.3 [d]	59.5±5.1 [b]	29.3±1.8 [c]	< 0.0001	< 0.0001	0.05
Potassium intake, *mg/d*	169.9±17.4 [a]	204.5±16.3 [a]	174.9±13.0 [a]	97.2±8.2 [b]	127.9±10.9 [b]	112.0±6.7 [b]	< 0.0001	0.05	0.87
Sodium intake, *mg/d*	52.7±5.4 [c]	93.9±7.5 [b]	146.0±10.9 [a]	29.2±2.5 [d]	58.7±5.0 [c]	96.0±5.8 [b]	< 0.0001	< 0.0001	0.17
Calcium intake, *mg/d*	256.7±26.3 [a]	241.1±19.3 [ab]	239.5±17.8 [ab]	170.1±14.4 [b]	171.6±14.6 [b]	175.7±10.5 [b]	< 0.0001	0.92	0.81
Zinc intake, *mg/d*	0.79±0.08 [a]	0.75±0.06 [a]	0.77±0.06 [a]	0.40±0.03 [b]	0.41±0.03 [b]	0.44±0.03 [b]	< 0.0001	0.90	0.84

[1]Values are mean ± SEM, *n*=8-10, Mean within a row with the same superscript are not statistically different, P<0.05. C, corn starch fed rats; CUO, cornstarch rats treated with *Ulva ohnoi*; CDT, cornstarch rats treated with *Derbesia tenuissima*; H, high-carbohydrate, high-fat diet fed rats; HUO, high-carbohydrate, high-fat rats treated with *Ulva ohnoi*; HDT, high-carbohydrate, high-fat rats treated with *Derbesia tenuissima*.

Table S3. Total fat, SF, MUFA and PUFA intake in rats fed control diets C or H and with either *Ulva ohnoi* or *Derbesia tenuissima*

Variable	C	CUO	CDT	H	HUO	HDT	*P*-Value		
							Diet	Treatment	Interaction
Total fat intake, *g/d (n=8-10)*	0.29±0.02 [b]	0.28±0.02 [b]	0.34±0.03 [b]	5.23±0.44 [a]	5.19±0.44 [a]	5.29±0.32 [a]	< 0.0001	0.96	0.99
Saturated fatty acid, *g/d (n=8-10)*	0.08±0.01 [b]	0.09.±0.01 [b]	0.10±0.00 [b]	2.77±0.23 [a]	2.75±0.23 [a]	2.80±0.17 [a]	< 0.0001	0.98	0.99
MUFA, *g/d (n=8-10)*	0.10±0.01 [b]	0.09±0.01 [b]	0.10±0.01 [b]	2.28±0.19 [a]	2.29±0.19 [a]	2.31±0.14 [a]	< 0.0001	0.99	0.99
PUFA, *g/d (n=8-10)*	0.10±0.01 [b]	0.09±0.01 [b]	0.10±0.01 [b]	0.15±0.01 [a]	0.15±0.01 [a]	0.17±0.01 [a]	< 0.0001	0.32	0.61
ALA, m*g/d (n=8-10)*	13.2±1.4 [c]	21.4±1.7 [b]	28.9±2.2 [a]	5.0±0.0 [d]	5.6±0.5 [d]	16.6±0.9 [c]	< 0.0001	<0.0001	0.03
EPA, m*g/d (n=8-10)*	0.0±0.0 [c]	0.0±0.0 [c]	1.9±0.1 [a]	0.0±0.0 [c]	0.0±0.0 [c]	1.3±0.1 [b]	0.0002	<0.0001	<0.0001

[1]Values are mean ± SEM, *n*=8-10, Mean within a row with the same superscript are not statistically different, P<0.05. C, corn starch fed rats; CUO, cornstarch rats treated with *Ulva ohnoi*; CDT, cornstarch rats treated with *Derbesia tenuissima*; H, high-carbohydrate, high-fat diet fed rats; HUO, high-carbohydrate, high-fat rats treated with *Ulva ohnoi*; HDT, high-carbohydrate, high-fat rats treated with *Derbesia tenuissima*.
[2] MUFA, mono-unsaturated fatty acid; PUFA, polyunsaturated fatty acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid .

Table S4 Cardiovascular structure and function in rats fed control diets C or H and with either *Ulva ohnoi* or *Derbesia tenuissima*

Variable	C	CUO	CDT	H	HUO	HDT	*P*-Value		
							Diet	Treatment	Interaction
LVIDd, *mm*	7.3±0.2	7.7±0.2	7.8±0.2	7.7±0.2	7.9±0.1	7.7±0.4	0.38	0.39	0.56
LVIDs, *mm*	3.1±0.3 [b]	4.3±0.3 [a]	4.4±0.3 [a]	3.5±0.3 [b]	4.4±0.1[a]	3.3±0.1 [b]	0.36	0.001	0.016
Heart rate	332.7±33.1 [a]	252.3±12.5 [b]	245.0±6.8 [b]	373.0±15.5 [a]	217.6±5.1 [b]	393.6±33.5 [a]	0.007	< 0.0001	0.001
IVSd, *mm*	1.8±0.0	1.9±0.0	2.0±0.1	1.9±0.0	1.9±0.0	1.9±0.1	1.00	0.18	0.18
IVSs, *mm*	3.30±0.09	3.00±0.13	3.35±0.10	3.39±0.10	3.20±0.09	3.44±0.12	0.15	0.017	0.84
LVPWs, *mm*	3.00±0.07	2.93±0.09	3.15±0.12	3.26±0.13	2.95±0.09	3.28±0.11	0.12	0.036	0.52
LVPWd, *mm*	1.83±0.06 [b]	1.76±0.04 [b]	1.99±0.04 [a]	1.85±0.03 [ab]	1.85±0.04 [ab]	1.83±0.07 [ab]	0.68	0.104	0.040
Diastolic volume, μL	377.8±35.7	463.0±35.9	497.0±31.5	482.0±35.4	516.0±21.6	485.0±36.3	0.09	0.13	0.24
Systolic volume, μL	48.0±6.3[b]	88.0±15.0 [a]	95.0±15.4 [a]	53.0±11.8 [b]	90.0±7.4 [a]	40.0±5.0 [b]	0.08	0.004	0.012
SBP:LVIDs	35.2±1.8[b]	29.4±2.0[c]	30.4±1.9[c]	47.3±3.9 [a]	29.2±0.9 [b]	40.1±1.6[b]	0.0006	<0.0001	0.033
SBP:systolic volume	3096±525[ab]	1731±363[b]	1716±340[b]	4818±1129[a]	1497±137 [b]	3690±458[ab]	0.035	0.003	0.18
ESS:LVIDs	2.02±0.07[b]	2.10±0.06[b]	2.09±0.08[b]	2.44±0.09[a]	2.17±0.07[b]	2.02±0.08[b]	0.032	0.09	0.008
Stroke volume, μL	330.0±33.9	375.0±32.5	456.0±44.7	429.0±29.5	426.0±16.4	445.0±33.8	0.09	0.12	0.26
Cardiac output, *mL/min*	112.9±20.1[bc]	93.6±7.4[c]	128.7±20.6 [abc]	159.5±11.9 [ab]	92.6±4.1[c]	172.2±16.7 [a]	0.022	0.001	0.23
Relative wall thickness	0.52±0.02	0.49±0.02	0.49±0.03	0.47±0.03	0.47±0.01	0.49±0.02	0.23	0.81	0.57

Systolic wall stress	71.4±4.5	90.7±6.8	92.1±6.4	87.5±9.4	95.9±4.9	68.1±5.4	0.48	0.12	0.035
Fractional shortening, %	50.1±2.4 [ab]	49.8±1.3[ab]	47.1±2.0 [ab]	51.3±3.5 [ab]	45.0±1.1 [b]	56.7±1.4 [a]	0.30	0.14	0.012
Ejection fraction, %	86.9±1.6[ab]	81.2±1.3 [b]	84.5±1.8 [b]	87.8±2.6 [ab]	83.5±0.9 [b]	91.8±0.8 [a]	0.018	0.003	0.17
Estimated LV mass, *g*	0.90±0.10 [b]	1.04±0.04 [b]	1.36±0.12 [a]	1.03±0.08 [b]	1.17±0.03 [ab]	1.09±0.06 [ab]	0.96	0.010	0.025

[1]Values are mean ± SEM, *n*=8-10, Mean within a row with the same superscript are not statistically different, P<0.05. C, corn starch fed rats; CUO, cornstarch rats treated with *Ulva ohnoi*; CDT, cornstarch rats treated with *Derbesia tenuissima*; H, high-carbohydrate, high-fat diet fed rats; HUO, high-carbohydrate, high-fat rats treated with *Ulva ohnoi*; HDT, high-carbohydrate, high-fat rats treated with *Derbesia tenuissima*.

[2] LVIDd, left ventricular internal diameter during diastole; LVIDs, left ventricular internal diameter during systole; IVSd, interventricular septum during diastole; IVSs, interventricular septum thickness during systole; LVPWd, left ventricular posterior wall thickness during diastole; LVPWs, left ventricular posterior wall thickness during systole; SBP, systolic blood pressure; ESS, end systolic wall stress.

CHAPTER 5

Microalgae attenuates obesity linked cardiac, hepatic and metabolic changes in high-carbohydrate, high-fat diet fed rats

The Journal of Nutrition

Microalgae attenuates obesity linked cardiac, hepatic and metabolic changes in high-carbohydrate, high-fat diet fed rats[1-3]

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Abstract

Healthy diet includes consumption of low-calorie diet, high in protein, fiber, minerals and omega-3 fatty acids and is considered as an effective long-term lifestyle intervention for obesity management. As a dietary intervention, this study investigated the responses to microalgae mixture, *Scenedesmus dimorphus* and *Schroederiella apiculata* (SC), containing all essential dietary supplements including fiber (19.6% of dry algae), proteins (46.1% of dry algae), omega-3 fatty acids (2.8% of dry algae) and minerals (3.7% of dry algae), in a high-carbohydrate, high-fat diet induced metabolic syndrome rat model. Male Wistar rats were divided into 4 groups. Two groups were fed with corn starch diet containing 68% carbohydrates as polysaccharides, while the other two groups were fed a high-carbohydrate (68%), high-fat (24%) diet supplemented with 25% fructose drinking water. This high-calorie diet contained fructose and sucrose as the main carbohydrates and saturated and mono-unsaturated fatty acids as the major dietary fatty acids from beef tallow. The high-carbohydrate, high-fat diet fed rats showed increased visceral obesity and its associated metabolic symptoms such as hypertension, insulin resistance, cardiovascular remodelling, and nonalcoholic fatty liver disease. SC supplementation (5% of diet) lowered the total body and abdominal fat mass with increased lean mass, and attenuated the visceral obesity linked metabolic symptoms such as hypertension, impaired glucose and insulin tolerance, endothelial dysfunction, cardiac stiffness and collagen deposition, inflammatory cells infiltration of heart and liver, and non-alcoholic fatty liver disease in the high-carbohydrate and high-fat diet fed rats. This study suggests that insoluble fiber (19.6% of dry algae) supplementation from SC may contribute to the reversal of diet-induced metabolic syndrome.

Key words: Healthy eating**;** obesity management; visceral obesity; metabolic symptoms; microalgae; insoluble fiber.

Introduction

High calorie diet including Western diet high in fat and refined carbohydrates elevates the development of intra-abdominal obesity, and its associated metabolic risk factors such as impaired glucose tolerance, insulin resistance, increased systolic blood pressure, dyslipidemia, endothelial dysfunction, and cardiovascular complications (1-4). A healthy dietary pattern including the intake of vegetables, alginate from brown seaweed, fruits, and whole grain fiber in overweight subjects has been shown to promote weight loss, prevent obesity and its associated metabolic risk factors (5-8). In addition to physical activity, healthy eating is considered as a long-term lifestyle intervention for obesity management which includes food consumption low in total dietary fats especially saturated and trans fats, low in refined carbohydrates, and serves as the high dietary source of proteins and fiber (9, 10).

As a healthy diet containing omega-3 fatty acids (eicosapentaenoic acid and docosahexaenoic acid), proteins, aminoacids, pigments, vitamins and minerals, microalgae has been considered as a functional food for humans and animals (11, 12). Further, bioactive ingredients such as ß-carotene, astaxanthin, lutein, eicosapentaenoic acid, docosahexaenoic acid, phycobiliproteins, and insoluble fiber extracted from *Chlorella*, *Spirulina*, *Dunaliella*, *Porphyridium*, and *Scenedesmus* species were reported for their antioxidant, cardio-protective, hepatoprotective, anti-inflammatory, and anti-hyperlipidemic effects (13-15).

Chlorella pyrenoidosa treatment (100mg/kg bwt) in streptozotocin (STZ) induced diabetic rats lowered the plasma glucose concentrations from 356 mg/dL to 158 mg/dl, and improved the lipid profiles of plasma, liver, and kidney with decreased concentrations of total cholesterol, triglycerides, and non-esterifed fatty acids (NEFA) (16). *Parachlorella beijerinckii* supplementation (5%/ diet) inhibited visceral fat accumulation, and improved glucose metabolism with reduced serum concentrations of glucose, insulin, and triglycerides in high-fat diet fed C57BL/6J mice (17). *In vitro* study using *Chlorella* extracts reduced the lipid accumulation in 3T3-L1 cells which may be regulated by the inhibition of adipogenesis in 3T3-L1 adipocytes (18).
Chlorella vulgaris improved the glucose and insulin tolerance by stimulating the insulin signalling pathway, and attenuated dyslipidemia in the high-fat diet fed Balb/C mice (19). A similar study showed *Chlorella vulgaris* supplementation (10% of the diet) improved the glucose metabolism which is influenced by the increased protein expression of glucose transporter type 4 (GLUT4) in the high-fat diet fed rats (20). Oral treatment of *Spirulina maxima* either in 5% or 10% aqueous suspension lowered the blood glucose concentration and reduced the plasma concentrations of triglycerides, total cholesterol, and low density lipoprotein cholesterol (LDL) in fructose-fed rats (21).
Spirulina treatment (8g/day) attenuated the increased plasma concentrations of triglycerides, total cholesterol, and LDL cholesterol in type 2 diabetic patients with dyslipidemia (22). Further, *Spirulina* supplementation decreased the serum and hepatic concentrations of total cholesterol, triglycerides, and non-esterified fatty acids with reduced macrophage infiltration of visceral adipose tissue in a mouse model of metabolic syndrome (23). *Scenedesmus quadricauda* crude extracts inhibited lipid peroxidation of linoleic acid (24).
Dietary supplementation of *Chlorella pyrenoidosa* (4% & 8% of the diet) reduced the systolic and diastolic blood pressure by down-regulating the angiotensin-converting enzyme (ACE) activity in rats treated with N_{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME)-induced endothelial dysfunction (25). Similarly, *Chlorella regularis* fractional supplements prevented the elevation of blood pressure in the stroke-prone spontaneously hypertensive rats which may be due to the higher concentrations of arginine (6.4% /dry weight), a potent endothelium-dependant vasorelaxant (26, 27). *Spirulina* improved the vascular tone by stimulating the endothelium-mediated synthesis and release of nitric oxide, cyclooxygenase-dependent metabolite of arachidonic acid, and by decreasing the endothelium-mediated synthesis and release of eicosanoids (28). *Spirulina* supplementation (5% of diet) attenuated hypercholesterolemia-induced atherosclerosis by reducing the serum concentrations of triglycerides, total cholesterol, and LDL in high-cholesterol diet fed rabbits (29). The anti-hypertensive and anti-hyperlipidemic effect of *Spirulina* exhibited in humans may prevent the risk factors of cardiovascular diseases (28).
Spirulina attenuated the hepatic changes with increased reactive oxygen species (ROS) from liver mitochondria, increased plasma liver enzyme activities such as alanine transaminase (ALT) and aspartate transaminase (AST), liver fibrosis and hepatic nuclear-factor kappa B (NF-κB) activation in choline-deficient high fat diet fed rats (CDHF) (30). *Chlorella* crude extracts improved the gut morphology by enhancing the villi length, crypt depth, and

intestinal proliferation in a rat model of experimental short bowel syndrome (31).

As discussed above, both *Chlorella* and *Spirulina* have been examined for their biological effects in attenuating metabolic risk factors such as obesity, hypertension, type 2 diabetes, insulin resistance, dyslipidemia and non-alcoholic fatty liver disease. Microalgae serve as a reservoir of biologically active compounds, presenting unique structures and functions, the biological effects of which remain unexplored (32). *Scenedesmus* species, common freshwater green microalgae, are well-known for their nutritional value and have been considered as one among the novel food products of microalgae (33). This study has investigated the therapeutic effects of microalgae mixture of *Scenedesmus dimorphus* and *Schroederiella apiculata* in the high-carbohydrate and high-fat diet induced metabolic syndrome rat model.

Materials and Methods

Algal sources and nutritional components

Scenedesmus dimorphus [8] and *Schroederiella apiculata* (SC) microalgal mixture were cultured in the aquaculture facilities of James Cook University (JCU), Townsville, Australia. All biomass was produced in large outdoor tanks with capacities of >10,000L for microalgae culture. Biomass was harvested on two occasions separated by ~12 months from 2011-2012. At each time, the biomass was rinsed in freshwater and freeze-dried, after which sub-samples (~200mg dry weight) were taken for analyses of fiber components, minerals and fatty acid concentrations.

[8] Abbreviations used: ALT, alanine transaminase; AST, aspartate transaminase; SC, *Scenedesmus dimorphus*+*Scenedesmus apiculata*; C, corn starch diet-fed rats; CSC, corn starch diet-fed rats treated with *Scenedesmus dimorphus*+*Scenedesmus apiculata*; H, high-carbohydrate, high-fat diet-fed rats; HSC, high-carbohydrate, high-fat diet-fed rats treated with *Scenedesmus dimorphus*+*Scenedesmus apiculata*; ESS, end-systolic wall stress; IVSd, interventricular septum thickness during diastole; LVIDd, left ventricular internal diameter during diastole; LVIDs, left ventricular internal diameter during systole; LVPWd, left ventricular posterior wall thickness during diastole; NEFA, non-esterified fatty acids; SBP, systolic blood pressure.

In each sub-sample, 24 trace elements were quantified and mean values are reported (n = 2 sub-samples per species). Al^{3+}, Ca^{2+}, K^{+}, Na^{+}, S^{2-}, and P^{3-} were analysed by Inductively Coupled Plasma Optical Emission Spectrometry, while metals and metalloids (As^{2-}, B^{+}, Ba^{2+}, Cd^{2+}, Co^{+}, Cr^{3+}, Cu^{2+}, Fe^{3+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Mo^{+}, Ni^{2+}, Pb^{2+}, Se^{2+}, Sr^{2+}, V^{2+}, and Zn^{2+}) were analyzed by Inductively Coupled Plasma Mass Spectrometry at the Advanced Analytical Centre, JCU, Townsville. Insoluble and soluble fibers were analyzed on a combined sample of 100g for each species containing 50g from each harvest time. Fiber analyses were run using enzymatic-gravimetric methods by Grain Growers, Sydney, Australia (NATA Accred. 66; AOAC Official Method 985.29 for insoluble fibre; AOAC Official Method 993.19 for soluble fibre). All remaining freeze-dried biomass was stored in vacuum-sealed bags under refrigeration until use in the diet formulation.

Rats and diets

The experimental groups consisted of 48 male Wistar rats (9-10 weeks old; 337.3 ± 0.3 g) supplied by The University of Queensland Biological Resources

unit and individually housed in a temperature-controlled (20±2°C), 12-hour light/dark cycle environment with unrestricted access to water and the group-specific rat diet at the University of Southern Queensland Animal House. The preparation and macronutrient composition of basal diets, including the dietary fatty acid profiles, have been described in detail previously (34, 35). All experimentation was approved by the Animal Experimentation Ethics Committees of the University of Queensland and University of Southern Queensland under the guidelines of the National Health and Medical Research Council of Australia.

The rats were randomly divided into 4 separate groups (n = 12 each) and fed with corn starch (C), corn starch + *Scenedesmus dimorphus* and *Scenedesmus apiculata* mixture 5% (CSC), high-fat (H), high-carbohydrate, high-fat + *Scenedesmus dimorphus* and *Scenedesmus apiculata* mixture 5% (HSC). The microalgae mixture-supplemented diets were prepared by adding 5% of microalgal mixture to replace an equivalent amount of water in the diet. The drinking water in all H groups included 25% fructose. The microalgae mixture-supplemented diets were administered for 8 wk starting 8 wk after the initiation of the C or H diets. Rats were monitored daily for body weight, food and water intakes. Daily seaweed intake was calculated from the daily food intake. The fatty acid concentrations of both C and H control diets used for the calculation of mean daily fatty acids intake in the control rats, and as well as in the microalgae treatment groups were obtained from our previously published study (36).

Abdominal circumference and body length of rats were measured using a standard measuring tape during the anesthesia for SBP measurements (35). BMI and energy efficiency were calculated (35). Systolic blood pressure measurements, oral glucose tolerance tests, and insulin tolerance tests were conducted at 0, 8, and 16 wk. All other measurements were made at wk 16.

Cardiovascular measurements

Systolic blood pressure was measured under light sedation following intraperitoneal injection of Zoletil (tiletamine 15 mg/kg, zolazepam 15 mg/kg; Virbac, Peakhurst, NSW, Australia) using a MLT1010 Piezo-Electric Pulse Transducer and inflatable tail-cuff connected to a MLT844 Physiologic Pressure Transducer using PowerLab data acquisition unit (AD Instruments, Sydney, Australia) (35).

Echocardiographic examinations (Phillips iE33, 12MHz transducer) were performed to assess the cardiovascular structure and function in all rats at the end of protocol (35). Briefly, rats were anesthetized using Zoletil (tiletamine 25 mg/kg and zolazepam 25 mg/kg, i.p.; Virbac, Peakhurst, Australia) and Ilium Xylazil (xylazine 15 mg/kg, i.p.; Troy Laboratories, Smithfield, Australia) and positioned in dorsal recumbency. Electrodes attached to the skin overlying the elbows and right stifle facilitated the simultaneous recording of a lead II electrocardiogram. A short-axis view of the left ventricle at the level of the papillary muscles was obtained and used to direct acquisition of M mode images of the left ventricle for measurement of IVSd, LVPWd, LVIDs, and LVIDd. Measurements were taken in accordance with the guidelines of the American Society of Echocardiography using the leading-edge method as described previously (35). The ventricular contractility indexes were calculated

including ratio of SBP to LVIDs, ratio of SBP to systolic volume, and ratio of ESS to LVIDs (37, 38).

The left ventricular function of the rats in all treatment groups was assessed using the Langendorff heart preparation (35). Terminal anesthesia 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 physiologic pressure transducer and Chart software on a MacLab system (AD Instruments Australia and Pacific Islands, Bella Vista, NSW, Australia).

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 (35, 38).

Oral glucose tolerance and insulin tolerance tests

For oral glucose tolerance test, basal blood glucose concentrations were measured in blood taken from the tail vein of overnight food-deprived rats using Medisense Precision Q.I.D glucose meter (Abbott Laboratories, Bedford, U.S.A). Fructose-supplemented drinking water in the H, HUO and HDT groups was replaced with normal water for the overnight food-deprivation period. The rats were given 2 g/kg body weight of glucose as a 40% aqueous solution via oral gavage. Tail vein blood samples were taken at 30, 60, 90, and 120 min following glucose administration. For insulin tolerance test, basal blood glucose concentrations were measured after 4-5 h of food deprivation as above. The rats were injected ip 0.33 IU/kg insulin-R (Eli-Lilly Australia, West Ryde, NSW, Australia) and tail vein blood samples were collected at 0, 15, 30, 45, 60, 90, and 120 min. Rats were withdrawn from the test if the blood glucose concentration dropped below 1.1 mmol/L, and 4 g/kg glucose was administered immediately via oral gavage to prevent hypoglycemia.

Body composition measurements

Dual energy X-ray absorptiometric (DXA) measurements were performed on rats after 16 weeks of feeding. This was done 2 days before rats were euthanised for pathophysiological assessments, using a Norland XR36 DXA instrument (Norland Corp., Fort Atkinson, USA). DXA scans were analyzed using the manufacturer's recommended software for use in laboratory animals (Small Subject Analysis Software, version 2.5.3/1.3.1, Norland Corp., Fort Atkinson, USA) (34, 35). The precision error of lean mass for replicate measurements, with repositioning, was 3.2%. Visceral adiposity index (%) was calculated (34, 35).

Organ weights

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

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. 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 (34, 35). Thin sections (7 μm) of left ventricle and the liver were cut and stained with haematoxylin and eosin stain for determination of inflammatory cell infiltration with 20X and fat vacuole enlargement with 20X objectives using a Olympus BX51 microscope (Olympus, Melville, NY). Collagen distribution was measured in the left ventricle with picrosirius red stain. Laser confocal microscopy (Zeiss LSM 510 upright Confocal Microscope) was used to measure collagen deposition (35, 38).

Plasma biochemistry

Blood was centrifuged at 5,000 *g* for 15 min within 30 min of collection into heparinized tubes. Plasma was separated and transferred to Eppendorf tubes for storage at -20°C before analysis. Activities of plasma enzymes and analyte concentrations were determined using kits and controls supplied by Olympus using an Olympus analyzer (AU 400, Tokyo, Japan) as previously described (35, 38).

Statistical analysis

All data are presented as mean ± SEM. Data from C, CSC, H, and HSC groups were compared in a series of two-way ANOVAs with "Diet", high carbohydrate and high fat diet or control cornstarch diet, and "Treatment" diet supplemented with seaweed as the fixed factors. Homogeneity of variance for ANOVA was assessed using Bartlett's test and variables that were not normally distributed were log-transformed prior to analysis. Where the main effects were significant ($P<0.05$), means were compared using Newman-Keuls multiple comparisons. Where transformations did not result in normality or homogeneity of variance, a Kruskal-Wallis non-parametric test was performed. All statistical analyses were run using GraphPad Prism version 5.00 for Windows (San Diego, California, USA).

Results

Nutritional composition of microalgal mixture and essential mineral ions and fatty acids intake

The total dietary fiber content of SC was 19.6% of dry algae with <0.1% as soluble fiber and 19.6% as insoluble fiber (Table 1). The total fatty acid content of SC was 4.84% of dry algae of which the omega-3 fatty acid represents 86% of total poly-unsaturated fatty acids compared to the omega-6 fatty acid content, which represents 14% of total poly-unsaturated fatty acids (Table 1). Mineral ions content of SC ranges about 3.7% of dry algae (Table

1). SC contains high magnesium, sodium, potassium, and zinc ion concentrations, compared to control diets (H and C) (Table S1). However, only CSC rats showed high magnesium (38.2 mg/day), sodium (55.6 mg/day), potassium (181.1 mg/day) and zinc (0.96 mg/day) intake compared to C control rats, while the HSC rats had higher zinc intake (0.52 mg/day) compared to the H control rats (0.38 mg/day) (Table S2). SC supplementation increased the total dietary fiber intake in CSC (584.5 mg/day) and HSC rats (369.5 mg/day), compared to C and H control rats (Table S2), while increased protein intake was also measured in CSC (1.86 mg/day) and HSC (1.75 mg/day) rats (Table S2). Compared to C rats, the H diet fed control rats showed increased fat intake including saturated fatty acids, mono-unsaturated fatty acids and poly-unsaturated fatty acids (Table S3). Microalgal mixture (SC) supplementation did not change the total fatty acids intake in either CSC or HSC rats (Table S3), while increased intake of α-linolenic acid (ALA) was measured in CSC (42.1 mg/day) and HSC (23.6 mg/day) treated rats. CSC rats showed increased eicosapentaenoic acid (EPA) (0.28 mg/day) and docosahexaenoic acid (DHA) (0.36mg/day) intake compared to HSC rats with EPA intake 0.18mg/day and DHA intake 0.23mg/day (Table S3).

Physiology and metabolic variables

Food and water consumption were higher in C rats compared with H rats (Table 2). Compared with C fed groups (C & CSC), increased energy intake with significant increase in body weight gain and energy efficiency was observed in H and HSC fed groups (Table 2). Compared with C fed rats, higher total body fat mass (Fig. 1*A*) and abdominal fat mass was observed in H fed rats (Table 3). H fed control rats had higher abdominal circumference and visceral adiposity index (Table 3). HSC lowered total body fat mass (Fig. 1*A*) and total abdominal fat mass with decreased abdominal circumference and visceral adiposity index (Table 3). The glucose utilization and insulin sensitivity were improved in HSC treated rats, compared with H fed rats (Fig. 1*B* & 1*C*). There was no change among C fed groups (Fig. 1*B* & 1*C*). The bone mineral content was higher in H fed control rats, compared with C fed rats (Table 3), while in HSC treated rats, the bone mineral content was reduced (Table 3). Increased plasma concentrations of NEFA, triglycerides and total cholesterol were observed in H fed rats, whereas no changes were observed in C fed groups (Table 4). SC treatment normalized the increase plasma concentrations of NEFA, triglycerides and total cholesterol in HSC treated rats (Table 4). No changes in plasma potassium and magnesium ion concentrations were observed in the treatment groups (Table 4), while increased plasma sodium concentrations were observed in C and H control rats compared to CSC and HSC treated rats (Table 4).

Table 1 Nutritional composition of microalgae poly-culture

Nutrients	Units	*Scenedesmus dimorphus & Schroederiella apiculata*
Total fibre	g/100g DW	19.6
Insoluble Fibre	g/100g DW	19.6
Soluble Fibre	g/100g DW	<0.1
Total protein	g/100g DW	46
Total minerals	g/100g DW	3.7
Essential minerals		
Magnesium	g/100g DW	0.41
Potassium	g/100g DW	1.14
Sodium	g/100g DW	0.32
Calcium	g/100g DW	0.27
Zinc	mg/100g DW	0.01
Iron	mg/100g DW	0.27
Total fatty acids	g/100g DW	4.84
Saturated fatty acid	% / total fatty acid	19
MUFA	%/ total fatty acid	14
PUFA	%/ total fatty acid	67
n-3 PUFA	%/ total PUFA	86
n-6 PUFA	%/ total PUFA	14

MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid. DW, dry weight.

Table S1. Mineral ions profile of microalgae and control diets

Mineral ions	Corn starch mg/kg	High carbohydrate and high fat diet (mg/kg)	*Scenedesmus species* (mg/kg)
K	4760.0	4450.0	9010.0
Na	1475.0	1335.0	5325.0
Mg	913.5	689.0	4415.0
Ca	7190.0	7790.0	2670.0
Fe	256.5	185.0	2650.0
P	2910.0	2500.0	11800.0
Mn	23.5	18.2	631.0
B	3.4	2.9	29.3
Ba	23.8	16.1	23.5
Al	17.6	20.9	105.0
Sr	14.0	12.4	35.2
Zn	22.1	18.3	110.0
Cu	8.6	7.1	404.0
Ni	0.4	0.3	2.7
Mo	0.3	0.1	1.1
V	3.3	5.3	0.6
As	1.4	1.2	<=1
Cr	10.0	16.0	3.7
Pb	<= 0.05	<= 0.05	0.6
Se	<= 1	<= 1	<= 1
Co	<= 1	<= 1	5.7
Hg	<= 0.5	<= 0.5	<= 0.5
Cd	<= 0.05	<= 0.05	<= 0.05
Sum total	**18291.0**	**18049.0**	**37202.3**

Mineral ions profile (ppm, mean of samples, n=2 ± SD<15040)

Table S2. Essential mineral ion, total protein and total fiber intake in rats fed control diets C or H and microalgae treated diets CSC and HSC.

Variable	C	CSC	H	HSC	*P*-Value		
					Diet	Treatment	Interaction
Magnesium intake, *mg/d*	28.6±1.5 [b]	38.2±2.8 [a]	14.4±0.9 [c]	19.3±1.1 [c]	< 0.0001	0.0002	0.19
Potassium intake, *mg/d*	148.7±7.8 [b]	181.1±13.1 [a]	93.1±5.8 [c]	105.3±5.8 [c]	< 0.0001	0.016	0.26
Sodium intake, *mg/d*	46.1±2.4 [b]	55.6±4.0 [a]	27.9±1.7 [c]	32.1±1.8 [c]	< 0.0001	<0.015	0.33
Calcium intake, *mg/d*	224.7±11.8 [a]	248.9±18.1 [a]	163.0±10.1 [b]	170.2±9.4 [b]	< 0.0001	0.24	0.52
Zinc intake, *mg/d*	0.79±0.01 [b]	0.96±0.03 [a]	0.38±0.03 [b]	0.52±0.01 [a]	< 0.0001	< 0.0001	0.52
Total fiber, *mg/d*	231.3±12.2 [c]	584.5±42.4 [a]	154.8±9.6 [d]	369.5±20.4 [b]	< 0.0001	< 0.0001	0.009
Total protein, *g/d*	0.99±0.05 [b]	1.86±0.14 [a]	1.22±0.08 [b]	1.75±0.09 [a]	0.54	< 0.0001	0.08

[1]Values are mean ± SEM, *n*=9-10, Mean within a row with unlike superscript differ, P<0.05. C, corn starch fed rats; CSC, cornstarch rats treated with *Schroederiella apiculata* &*Scenedesmus dimorphus*; H, high carbohydrate, high fat diet fed rats; HSC, high carbohydrate, high fat rats treated with *Schroederiella apiculata* &*Scenedesmus dimorphus*.

Table S3. Total fat, SF, MUFA and PUFA intake in rats fed control diets C or H and microalgae treated diets CSC and HSC

Variable	C	CSC	H	HSC	*P*-Value		
					Diet	Treatment	Interaction
Total fat intake, *g/d*	0.25±0.01 [b]	0.36±0.03 [b]	5.01±0.31 [a]	5.19±0.29 [a]	< 0.0001	0.49	0.87
Saturated fatty acid, *g/d*	0.07±0.00 [b]	0.09.±0.01 [b]	2.66±0.16 [a]	2.74±0.15 [a]	< 0.0001	0.65	0.78
MUFA, *g/d*	0.09±0.01 [b]	0.11±0.01 [b]	2.21±0.14 [a]	2.27±0.13 [a]	< 0.0001	0.67	0.83
PUFA, *g/d*	0.09±0.01 [c]	0.15±0.01 [b]	0.14±0.01 [b]	0.18±0.01 [a]	0.0003	<0.0001	0.33
ALA, m*g/d*	11.6±0.6 [d]	42.1±3.1 [a]	4.8±0.3 [c]	23.6±1.3 [b]	< 0.0001	<0.0001	0.002
EPA, m*g/d*	0.00±0.00 [c]	0.28±0.02 [a]	0.00±0.00 [c]	0.18±0.01 [b]	<0.0001	<0.0001	<0.0001
DHA, m*g/d*	0.00±0.00 [c]	0.36±0.03 [a]	0.00±0.00 [c]	0.23±0.01 [b]	<0.0001	<0.0001	<0.0001

[1]Values are mean ± SEM, *n*=9-10, Mean within a row with unlike superscript differ, P<0.05. C, corn starch fed rats; CSC, cornstarch rats treated with *Schroederiella apiculata* &*Scenedesmus dimorphus*; H, high carbohydrate, high fat diet fed rats; HSC, high carbohydrate, high fat rats treated with *Schroederiella apiculata* and *Scenedesmus dimorphus.*

Table 2 Physiological variables in rats fed control diets C or H and in microalgae treated diets CSC or HSC.

Variable	C	CSC	H	HSC	*P*-Value		
					Diet	Treatment	Interaction
Food intake, *g/d*	31.3±1.6 [a]	34.2±2.5 [a]	20.9±1.3 [b]	21.5±1.2 [b]	< 0.0001	0.32	0.51

Water intake, *ml/d*	31.3±1.8[a]	32.6±2.3[a]	19.0±1.4[b]	20.9±1.5[b]	< 0.0001	0.38	0.87
Energy intake, *kJ/d*	356.3±2.4[d]	417.9±10.8[c]	440.0±5.1[b]	475.4±5.1[a]	< 0.0001	<0.0001	0.06
Feed conversion efficiency, *%*	2.0±0.2[c]	2.0±0.2 [c]	4.5±0.3 [a]	3.3±0.3 [b]	<0.0001	0.024	0.024
Body weight gain (8-16 weeks), *%*	7.2±0.9 [c]	8.6±1.0 [c]	19.7±1.2 [a]	15.5±1.5 [b]	0.002	0.001	0.144

[1]Values are mean ± SEM, *n*=9-10, Mean within a row with unlike superscript differ, P<0.05.C, corn starch fed rats; CSC, cornstarch rats treated with *Schroederiella apiculata &Scenedesmus dimorphus*; H, high carbohydrate,high fat diet fed rats; HSC, high carbohydrate, high fat rats treated with *Schroederiella apiculata* & *Scenedesmus dimorphus.*

Table 3 Metabolic variables in rats fed control diets C or H and in microalgae treated diets CSC or HSC.

Variable	**C**	**CSC**	**H**	**HSC**	***P*-Value**		
					Diet	**Treatment**	**Interaction**
Bone mineral content, *g*	12.4±0.4 [c]	11.8±0.2 [c]	16.2±0.5 [a]	13.9±0.4 [b]	< 0.0001	0.0009	0.038
Total fat mass, *g*	97.4±6.7 [c]	63.4±6.9 [d]	197.8±14.1[a]	129.1±10.4[b]	< 0.0001	<0.0001	0.094
Total lean mass, *g*	287.0±3.9 [b]	320.7±6.6 [a]	279.1±10.9 [b]	317.3±7.1[a]	0.621	0.059	0.360
Abdominal circumference, *cm*	18.2±0.1[c]	18.7±0.2[c]	22.2±0.3[a]	20.5±0.1[b]	< 0.0001	0.0038	<0.0001
Tissue wet weights, *mg/mm tibial length*							
Retroperitoneal fat	119.0±9.0[c]	121.8±12.7[c]	365.3±26.3[a]	235.6±13.1[b]	< 0.0001	<0.0001	0.0020
Epididymal fat	92.8±7.5[c]	100.9±7.8[c]	208.2±14.3[a]	133.7±11.9[b]	< 0.0001	0.0039	0.0005
Omental fat	60.0±5.0[b]	75.3±8.1[b]	125.3±10.6[a]	70.9±5.9[b]	0.0003	0.016	<0.0001
Total abdominal fat	271.8±19.4[c]	298.1±27.8 [c]	698.8±47.2[a]	440.2±28.4 [b]	< 0.0001	0.0010	<0.0001
Visceral adiposity index, *%*	3.3±0.2[c]	3.5±0.3[c]	6.6±0.5[a]	4.7±0.4[b]	< 0.0001	0.027	0.007
Liver weight	239.3±4.4 [b]	221.8±4.3 [b]	337.5±9.8 [a]	226.1±6.1 [b]	< 0.0001	<0.0001	<0.0001
LV+septum wet weight*, *mg/mm*	17.3±0.4 [b]	18.1±0.6 [b]	20.0±0.6 [a]	17.2±0.5 [b]	0.10	0.07	0.002

RV wet weight*, *mg/mm*	2.3±0.1 [bc]	3.0±0.2 [a]	4.0±1.2 [bc]	2.0±0.1 [bc]	0.57	0.30	0.036
Heart wet weight, *mg/mm*	19.6±0.4 [b]	21.1±0.6 [b]	24.0±1.6 [a]	19.2±0.5 [b]	0.18	0.08	0.0014
LV+septum wet weight*, *mg/mm*	17.3±0.4 [b]	18.1±0.6 [b]	20.0±0.6 [a]	17.2±0.5 [b]	0.10	0.07	0.002
Diastolic stiffness, κ	23.8±0.7[b]	22.8±0.5[b]	29.8±2.2[a]	24.8±1.1[b]	0.005	0.029	0.14

[1]Values are mean ± SEM, *n*=8-10, Mean within a row with unlike superscript differ, P<0.05. C, corn starch fed rats; CSC, cornstarch rats treated with *Schroederiella apiculata &Scenedesmus dimorphus*; H, high carbohydrate,high fat diet fed rats; HSC, high carbohydrate, high fat rats treated with *Schroederiella apiculata* & *Scenedesmus dimorphus.*

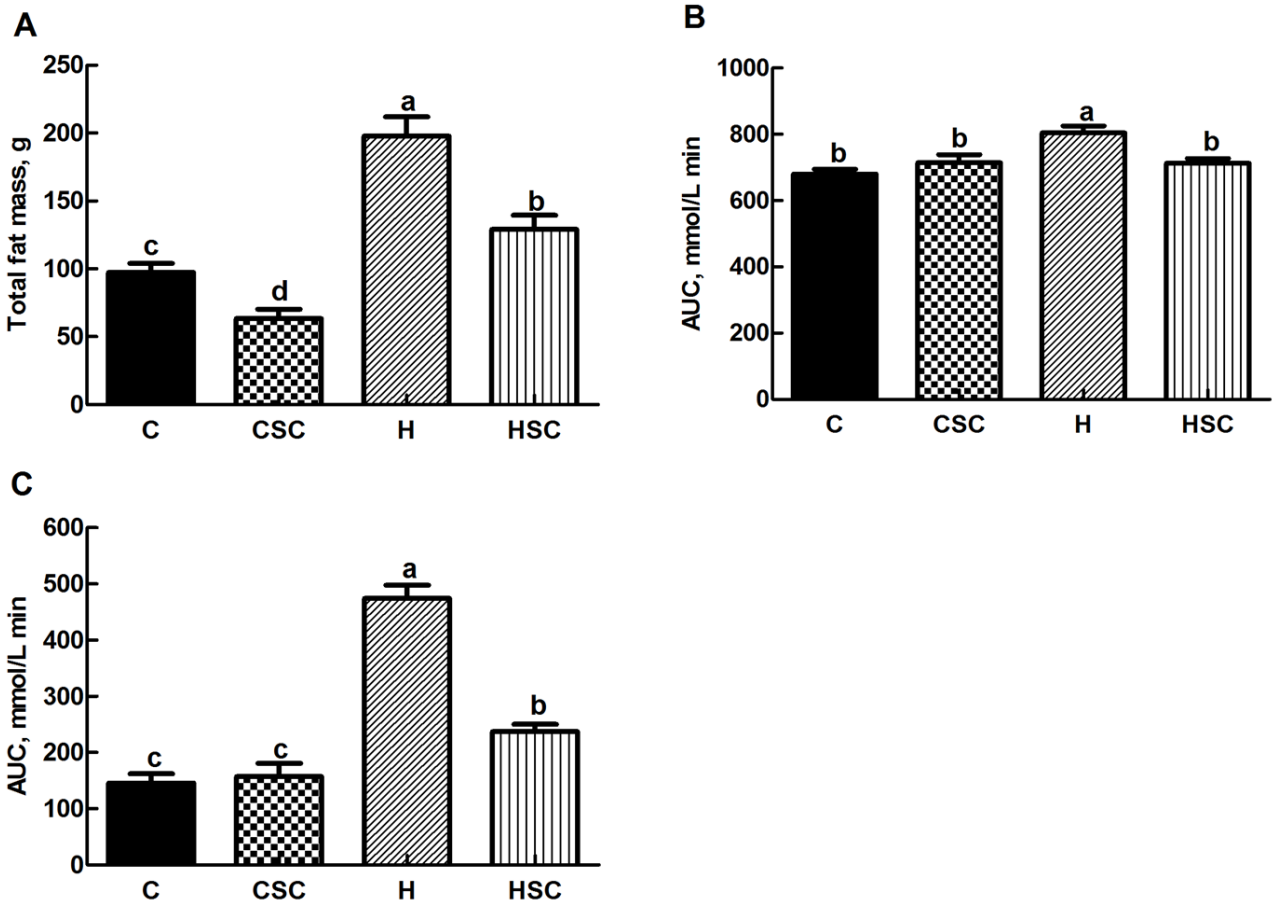

FIGURE 1 Seaweeds treatment on (*A)* total body fat mass, (*B)* oral glucose tolerance, and (*C)* insulin tolerance. Values are mean ± SEM, *n=8-10*. Endpoints means without a common letter differ, P<0.05. C, corn starch fed rats; CSC, cornstarch rats treated with *Scenedesmus dimorphus+Schroederiella apiculata*; H, high carbohydrate, high fat diet fed rats; HSC, high carbohydrate, high fat rats treated with *Scenedesmus dimorphus+Schroederiella apiculata*.

Table 4 Plasma biochemistry in rats fed control diets C or H and in microalgae treated diets CSC and HSC.

Variable	C	CSC	H	HSC	*P*-Value		
					Diet	Treatment	Interaction
Plasma NEFA, mmol/L	1.50±0.08 [b]	1.89±0.08 [b]	4.16±0.12 [a]	1.62±0.18 [b]	< 0.0001	<0.0001	<0.0001
Plasma triglycerides, mmol/L	0.51±0.07[b]	0.56±0.03[b]	2.69±0.04 [a]	0.39±0.05 [b]	< 0.0001	<0.0001	<0.0001
Plasma total cholesterol, mmol/L	1.48±0.06 [b]	1.64±0.03 [b]	2.01±0.05 [a]	1.64±0.04 [b]	<0.0001	0.032	<0.0001
Plasma ALT activity, U/L	27.5±2.5[b]	26.6±2.9[b]	44.4±2.4 [a]	22.7±1.8[b]	0.012	<0.0001	0.0002
Plasma AST activity, U/L	70.8±3.5 [b]	70.5±9.3 [b]	103.3±5.6 [a]	78.4±2.9 [b]	0.002	0.041	0.046
Plasma Na+, mmol/L	142.3±0.4 [a]	140.0±0.5 [b]	142.8±0.4 [a]	140.0±0.2 [b]	0.53	<0.0001	0.53
Plasma K+, mmol/L	5.43±0.39	5.70±0.26	6.28±0.78	6.95±0.58	0.06	0.39	0.71
Plasma Mg++, mmol/L	0.75±0.01	0.81±0.04	0.75±0.03	0.80±0.02	0.86	0.05	0.86

[1]Values are mean ± SEM, *n*=8, Mean within a row with unlike superscript differ, P<0.05. C, corn starch fed rats; CSC, cornstarch rats treated with *Schroederiella apiculata* &*Scenedesmus dimorphus*; H, high carbohydrate, high fat diet fed rats; HSC, high carbohydrate, high fat rats treated with *Schroederiella apiculata* &*Scenedesmus dimorphus*

Cardiovascular changes

Systolic blood pressure was unchanged in C fed groups (125-130 mmHg) (Fig. 2). The increased systolic blood pressure (152 mmHg) in H fed rats was lowered in HSC treated rats (140 mmHg) (Fig. 2). The ratio of ESS:LVIDs was higher in H fed control rats compared to C fed groups, while no change was observed in SBP:LVIDs and SBP:systolic volume ratios (Table S4). The ESS:LVIDs ratio index of ventricular contractility were normalized in HSC rats (Table S4). Further, no change in cardiac structure and function was observed in the microalgae treatment groups compared to H or C fed group (Table S4). The left ventricular septum weights and heart weights were increased in the H fed rats compared to the C fed rats (Table 3). SC mixture supplementation normalized the left ventricle+septum weight and total heart wet weight in the HSC rats (Table 3). Left ventricle showed increased inflammatory cells infiltration (Fig. 3*C*) and interstitial collagen deposition (Fig. 3*G*) in H fed rats, compared with C fed groups (Fig. 3*A*,*B* & *E*,*F*). In HSC treated rats, the inflammatory cells infiltration (Fig. 3*D*) and the interstitial collagen deposition (Fig. 3*H*) were normalized. The diastolic stiffness constant (κ) was normalized in HSC treated rats, compared with H fed rats (Table 3).

Lower contractile responses to noradrenaline in isolated thoracic rings were measured in H and HSC fed rats compared to C and CSC fed groups (Fig. 4*A*). Lower smooth muscle-dependent and endothelium-dependent relaxant responses to sodium nitroprusside and acetylcholine were measured in H fed control rats (Fig. 4*B*,*C*); both responses were higher in HSC fed rats (Fig. 4*B*,*C*).

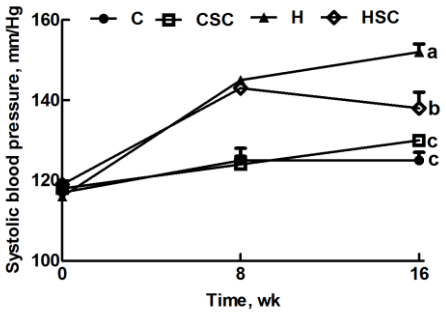

FIGURE 2 Seaweeds treatment on systolic blood pressure. Values are mean ± SEM, *n=8-10*. Endpoints means without a common letter differ, P<0.05. C, corn starch fed rats; CSC, cornstarch rats treated with *Scenedesmus dimorphus+Schroederiella apiculata*; H, high carbohydrate, high fat diet fed rats; HSC, high carbohydrate, high fat rats treated with *Scenedesmus dimorphus+Schroederiella apiculata*.

Table S4. Cardiovascular structure and function in rats fed control diets C or H and microalgae treated diets CSC and HSC.

Variable	C	CSC	H	HSC	*P*-Value		
					Diet	Treatment	Interaction
Heart rate	304.5±30.5 [ab]	241.4±20.6 [b]	332.5±14.1 [a]	372.0±20.0 [a]	0.0013	0.60	0.028
LVIDd, *mm*	7.1±0.3	7.6±0.1	7.8±0.2	7.2±0.1	0.45	0.80	0.008
LVIDs, *mm*	3.7±0.4	4.1±0.2	3.7±0.2	3.4±0.2	0.20	0.85	0.20
LVPWd, *mm*	1.72±0.04 [b]	1.81±0.04 [b]	2.03±0.04 [a]	1.83±0.04 [b]	0.0006	0.27	0.0006
LVPWs, *mm*	2.70±0.10	2.99±0.08	3.10±0.08	3.06±0.12	0.187	0.087	0.614
IVSd, *mm*	1.87±0.09	1.86±0.07	2.04±0.03	1.91±0.03	0.08	0.26	0.33
IVSs, *mm*	2.97±0.15	3.09±0.08	3.47±0.16	3.13±0.13	0.05	0.42	0.09
Diastolic volume, *µL*	378.0±43.7	467.0±24.8	493.0±42.5	385.0±17.9	0.63	0.78	0.007
Systolic volume, *µL*	64.0±15.9	76.0±8.9	57.0±7.7	44.0±6.7	0.07	0.96	0.24
SBP:LVIDs	36.6±3.8	32.5±1.7	40.9±1.8	37.9±1.9	0.06	0.16	0.82
SBP:systolic volume*(n=8-10)*	3482±983	1982±314	3016±418	3328±451	0.47	0.33	0.14
ESS:LVIDs	2.02±0.07[b]	2.21±0.06[b]	2.44±0.08[a]	2.11±0.09[b]	0.044	0.36	0.002
Stroke volume, *µL*	315.0±30.5 [b]	392.0±21.1 [ab]	436.0±37.8 [a]	340.0±13.5 [b]	0.22	0.73	0.004
Cardiac output, *mL/min*	95.2±10.8 [b]	94.8±9.9 [b]	142.6±9.6 [a]	125.9±6.7 [ab]	0.0003	0.37	0.39

Relative wall thickness	0.51±0.03	0.48±0.02	0.53±0.02	0.52±0.01	0.17	0.35	0.64
Systolic wall stress	88.6±8.4	91.5±5.7	91.4±5.7	72.8±5.8	0.23	0.24	0.11
Fractional shortening, *%*	47.8±3.5	48.2±1.2	52.0±1.6	52.4±1.8	0.07	0.86	1.00
Ejection fraction	84.5±2.7	85.4±1.3	88.5±1.1	88.7±1.3	0.043	0.75	0.84
Estimated LV mass, *g*	0.90±0.07 [b]	1.09±0.06 [ab]	1.24±0.06 [a]	0.97±0.04 [b]	0.07	0.50	0.0005

[1]Values are mean ± SEM, *n*=8, Mean within a row with unlike superscript differ, P<0.05. C, corn starch fed rats; CSC, cornstarch rats treated with *Schroederiella apiculata & Scenedesmus dimorphus*; H, high carbohydrate, high fat diet fed rats; HSC, high carbohydrate, high fat rats treated with *Schroederiella apiculata & Scenedesmus dimorphus.*

[2] LVIDd, left ventricular internal diameter during diastole; LVIDs, left ventricular internal diameter during systole;IVSd, interventricular septum thickness during diastole; IVSs, interventricular septum thickness during systole; LVPWd, leftventricular posterior wall thickness during diastole; LVPWs, leftventricular posterior wall thickness during systole; SBP, systolic blood pressure; ESS, end systolic wall stress.

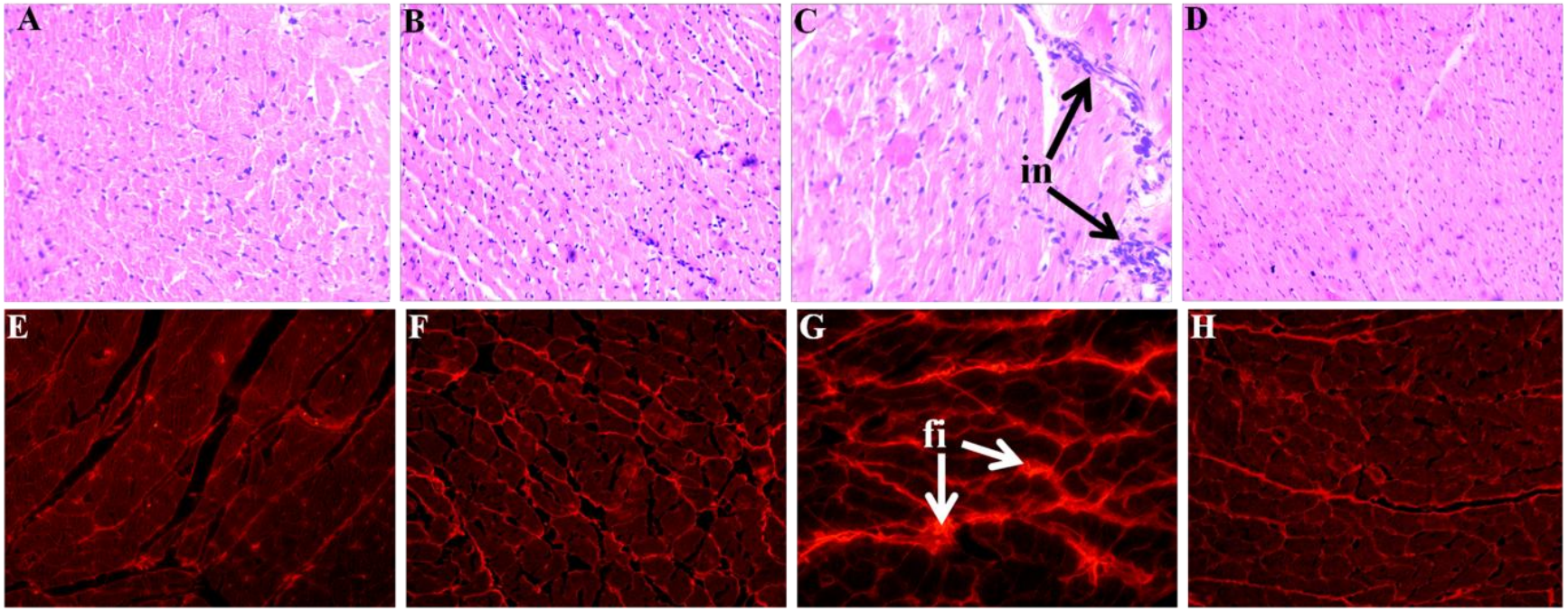

FIGURE 3 Seaweeds treatment on inflammation and fibrosis in the heart. Haematoxylin and eosin staining of left ventricle showing infiltration of inflammatory cells *(A–D*, inflammatory cells marked as "in") (x20) from C *(A)*, CSC *(B)*, H *(C)* and HSC *(D)*. Picrosirius red staining of left ventricle showing collagen deposition (*E–H*, fibrosis marked as "fi")(20x) from C *(E)*, CSC *(F)*, H *(G)* and HSC *(H)* rats. C, corn starch fed rats; CSC, cornstarch rats treated with *Scenedesmus dimorphus+Schroederiella apiculata*; H, high carbohydrate, high fat diet fed rats; HSC, high carbohydrate, high fat rats treated with *Scenedesmus dimorphus+Schroederiella apiculata*.

Table 3 Metabolic variables in rats fed control diets C or H and in microalgae treated diets CSC or HSC.

Variable	C	CSC	H	HSC	*P*-Value		
					Diet	Treatment	Interaction
Bone mineral content, *g*	12.4±0.4 [c]	11.8±0.2 [c]	16.2±0.5 [a]	13.9±0.4 [b]	< 0.0001	0.0009	0.038
Total fat mass, *g*	97.4±6.7 [c]	63.4±6.9 [d]	197.8±14.1 [a]	129.1±10.4 [b]	< 0.0001	<0.0001	0.094
Total lean mass, *g*	287.0±3.9 [b]	320.7±6.6 [a]	279.1±10.9 [b]	317.3±7.1 [a]	0.621	0.059	0.360

Abdominal circumference, *cm*	18.2±0.1[c]	18.7±0.2[c]	22.2±0.3[a]	20.5±0.1[b]	< 0.0001	0.0038	<0.0001
Tissue wet weights, *mg/mm tibial length*							
Retroperitoneal fat	119.0±9.0[c]	121.8±12.7[c]	365.3±26.3[a]	235.6±13.1[b]	< 0.0001	<0.0001	0.0020
Epididymal fat	92.8±7.5[c]	100.9±7.8[c]	208.2±14.3[a]	133.7±11.9[b]	< 0.0001	0.0039	0.0005
Omental fat	60.0±5.0[b]	75.3±8.1[b]	125.3±10.6[a]	70.9±5.9[b]	0.0003	0.016	<0.0001
Total abdominal fat	271.8±19.4[c]	298.1±27.8 [c]	698.8±47.2[a]	440.2±28.4 [b]	< 0.0001	0.0010	<0.0001
Visceral adiposity index, *%*	3.3±0.2[c]	3.5±0.3[c]	6.6±0.5[a]	4.7±0.4[b]	< 0.0001	0.027	0.007
Liver weight	239.3±4.4 [b]	221.8±4.3 [b]	337.5±9.8 [a]	226.1±6.1 [b]	< 0.0001	<0.0001	<0.0001
LV+septum wet weight*, *mg/mm*	17.3±0.4 [b]	18.1±0.6 [b]	20.0±0.6 [a]	17.2±0.5 [b]	0.10	0.07	0.002
RV wet weight*, *mg/mm*	2.3±0.1 [bc]	3.0±0.2 [a]	4.0±1.2 [bc]	2.0±0.1 [bc]	0.57	0.30	0.036
Heart wet weight, *mg/mm*	19.6±0.4 [b]	21.1±0.6 [b]	24.0±1.6 [a]	19.2±0.5 [b]	0.18	0.08	0.0014
LV+septum wet weight*, *mg/mm*	17.3±0.4 [b]	18.1±0.6 [b]	20.0±0.6 [a]	17.2±0.5 [b]	0.10	0.07	0.002
Diastolic stiffness, *κ*	23.8±0.7[b]	22.8±0.5[b]	29.8±2.2[a]	24.8±1.1[b]	0.005	0.029	0.14

[1]Values are mean ± SEM, *n*=8-10, Mean within a row with unlike superscript differ, P<0.05. C, corn starch fed rats; CSC, cornstarch rats treated with *Schroederiella apiculata &Scenedesmus dimorphus*; H, high carbohydrate, high fat diet fed rats; HSC, high carbohydrate, high fat rats treated with *Schroederiella apiculata &Scenedesmus dimorphus.*

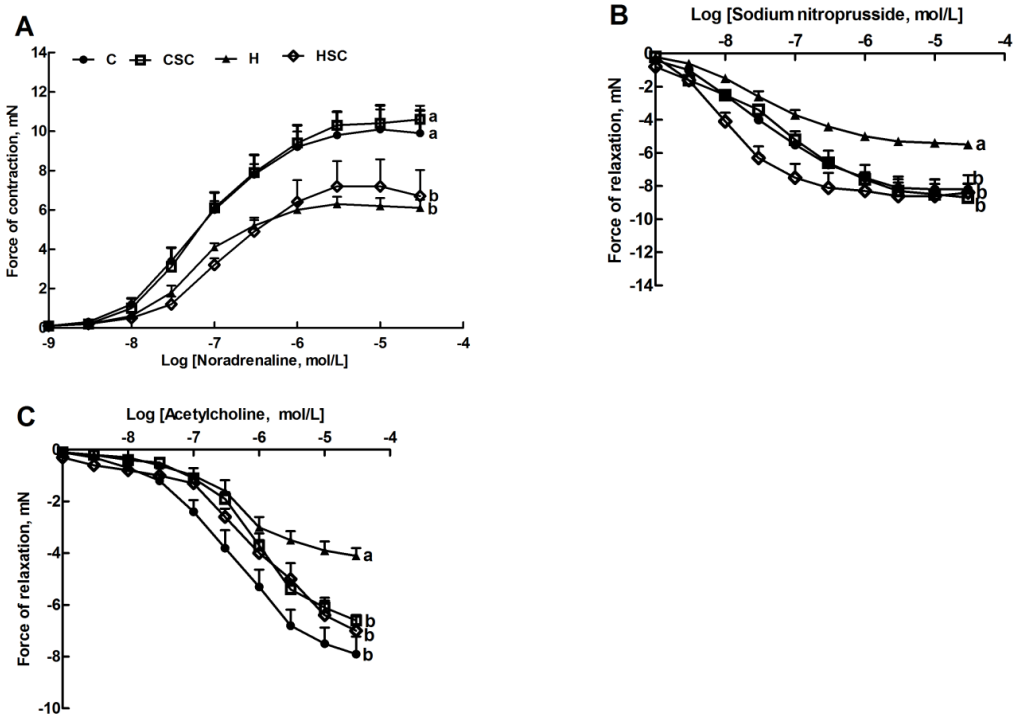

FIGURE 4 Microalgae mixture treatment on noradrenaline-induced contraction *(A)*, sodium nitroprusside-induced relaxation *(B)*, and acetylcholine-induced relaxation *(C)* in thoracic aortic preparations from C, CSC, H and HSC rats. Values are mean ± SEM, *n=9-10*. Endpoints means without a common letter differ, P<0.05. C, corn starch fed rats; CSC, cornstarch rats treated with *Scenedesmus dimorphus+Schroederiella apiculata*; H, high carbohydrate, high fat diet fed rats; HSC, high carbohydrate, high fat rats treated with *Scenedesmus dimorphus+Schroederiella apiculata.*

Hepatic structure and function

Compared to C fed groups, the H fed rats showed increased liver weight (Table 3), higher infiltration of inflammatory cells and presence of enlarged fat vacuoles (Fig. 5*A,B,C* & *E,F,G*). SC treatment prevented the infiltration of inflammatory cells in HSC treated rats (Fig. 5*H*). Liver weight was normalized in HSC fed rats compared to H fed control rats (Table 3). Further, hepatocyte with enlarged fat vacuoles wasn't observed in HSC treated rats (Fig. 5*D*). Plasma activities of liver enzymes ALT and AST were higher in H fed rats compared to C treatment groups whereas both the ALT and AST activities were normalized in HSC treated rats (Table 4).

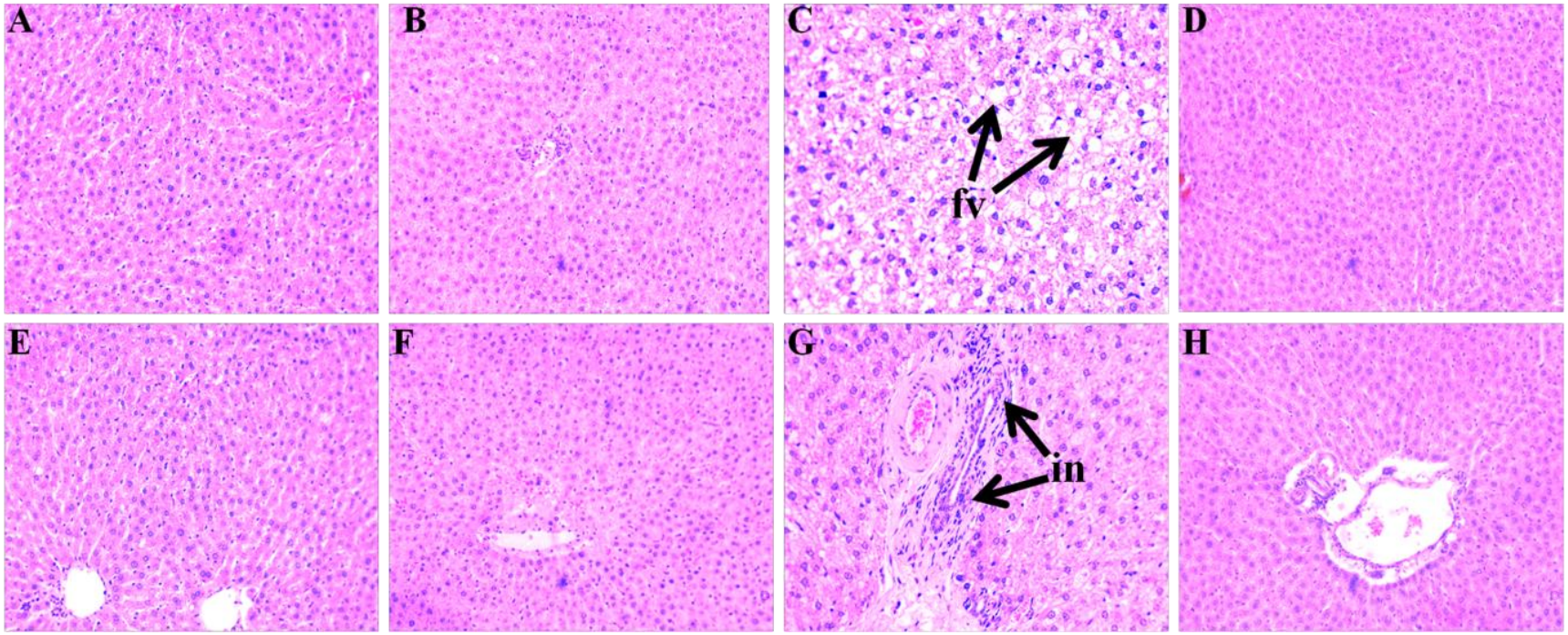

FIGURE 5 Microalgae mixture treatment on inflammation and fat deposition in the liver. Haematoxylin and eosin staining of liver showing enlarged fat vacuoles *(A-D*, marked as "fv") (x20) and inflammatory cells *(E–H*, marked as "in") (x20) from C *(A,E)*, CSC *(B,F)*, H *(C,G)*, HSC *(D,H)*. C, corn starch fed rats; CSC, cornstarch rats treated with *Scenedesmus dimorphus+Schroederiella apiculata*; H, high carbohydrate, high fat diet fed rats; HSC, high carbohydrate, high fat rats treated with *Scenedesmus dimorphus+Schroederiella apiculata*

Table 4 Plasma biochemistry in rats fed control diets C or H and in microalgae treated diets CSC and HSC.

Variable	C	CSC	H	HSC	*P*-Value		
					Diet	Treatment	Interaction
Plasma NEFA, mmol/L	1.50±0.08 [b]	1.89±0.08 [b]	4.16±0.12 [a]	1.62±0.18 [b]	< 0.0001	<0.0001	<0.0001
Plasma triglycerides, mmol/L	0.51±0.07[b]	0.56±0.03[b]	2.69±0.04 [a]	0.39±0.05 [b]	< 0.0001	<0.0001	<0.0001

Plasma total cholesterol, mmol/L	1.48±0.06 [b]	1.64±0.03 [b]	2.01±0.05 [a]	1.64±0.04 [b]	<0.0001	0.032	<0.0001
Plasma ALT activity, U/L	27.5±2.5[b]	26.6±2.9[b]	44.4±2.4 [a]	22.7±1.8[b]	0.012	<0.0001	0.0002
Plasma AST activity, U/L	70.8±3.5 [b]	70.5±9.3 [b]	103.3±5.6 [a]	78.4±2.9 [b]	0.002	0.041	0.046
Plasma Na+, mmol/L	142.3±0.4 [a]	140.0±0.5 [b]	142.8±0.4 [a]	140.0±0.2 [b]	0.53	<0.0001	0.53
Plasma K+, mmol/L	5.43±0.39	5.70±0.26	6.28±0.78	6.95±0.58	0.06	0.39	0.71
Plasma Mg++, mmol/L	0.75±0.01	0.81±0.04	0.75±0.03	0.80±0.02	0.86	0.05	0.86

[1]Values are mean ± SEM, *n*=8, Mean within a row with unlike superscript differ, P<0.05. C, corn starch fed rats; CSC, cornstarch rats treated with *Schroederiella apiculata &Scenedesmus dimorphus*; H, high carbohydrate, high fat diet fed rats; HSC, high carbohydrate, high fat rats treated with *Schroederiella apiculata &Scenedesmus dimorphus*.

Discussion

Consumption of high calorie Western diet with higher amount of saturated and mono-unsaturated fatty acids, and refined carbohydrates, serves as the major risk factor of body weight gain and visceral obesity (39-41). In our study compared to low calorie-C (11.4 kJ/g) diet fed rats, rats fed a high calorie-H (21.4 kJ/g) diet with an increased energy intake developed increased body weight gain and increased feed conversion efficiency percentage (Table 2). Since visceral obesity is considered as the main driving factor to metabolic syndrome (35, 42), increased intra-abdominal fat mass in H fed rats (Table 3) was associated with impaired glucose and insulin tolerance (Fig. 1B&C), increased systolic blood pressure (Fig. 2), increased cardiac stiffness (Table 3) and collagen deposition (Fig. 3G), inflammation of heart (Fig. 3C), endothelial dysfunction (Fig. 4A-C), impaired plasma lipid profile (Table 4), and hepatocytes with enlarged fat vacuoles and inflammatory cells infiltration (Fig. 5C&G). Dietary intake of SC supplements including zinc, total protein, α-linolenic acid, eicosapentanoic acid and docosahexaenoic acid in HSC treated rats (Table S2&S3) are inadequate to reverse the H diet induced metabolic changes (36, 43, 44), while the high dietary insoluble fiber intake (369.5-584.5 mg/day) (Table S2) measured with SC supplementation could prevent the metabolic syndrome in H diet fed rats.

Based on the body surface area, the translated human dose of daily dietary fiber intake from CSC and HSC treated rats varies between 8.3-13.1 g/day (45), which is equivalent or higher than the dietary fiber intake (7.2-9.3g/day) of known weight loss diets such as the South Beach diet and Atkins diet (46). Dietary fiber intake (7g/day) reduced body weight by 5kg in overweight patients (47). In our study, increased total dietary fiber intake in HSC rats (Table S2) may reduce the body weight gain percentage (Table 1) (48). Further, SC mixture supplementation reduced total body fat mass by 35% (Fig. 1A) and abdominal fat mass by 37% (Table 3) in HSC rats to contribute to the attenuation of body weight (%) in HSC rats. Similar to Kristensen et al (48), insoluble fiber (19.6% of dry algae) supplementation from 5% SC treatment may enhance the fecal energy loss by fecal bulking effect (49, 50), and therefore possibly decrease the fat digestibility (49, 51) in the HSC rats (Table 3). Also, increased lean mass by 14% and normalized liver weight in HSC treated rats (Table 3) determines the hepatic and skeletal muscle β-oxidation of fatty acids by the insoluble fiber supplementation from SC (50, 52).

In HSC rats, improved glucose and insulin tolerance (Fig. 1B&1C) is regulated by the decrease in visceral obesity associated plasma NEFA concentrations (Table 4) that diminish the insulin sensitivity of skeletal muscle (42, 53, 54). Additionally, increased lean mass detected with SC supplementation (Table 3) may contribute to the improved glucose utilization and insulin sensitivity in HSC treated rats.

Further, improvement in insulin sensitivity with SC supplementation reduced the systolic blood pressure (Fig. 2) associated with decreased ventricular contractility in HSC rats (Table S4). This anti-hypertensive effect of SC treatment may be caused by the decrease in the activity of sympathetic nervous system that stimulates vasoconstriction during hyperinsulinemia and insulin resistance (42, 55). Visceral obesity associated hypertension, hyperglycemia, insulin resistance, and dyslipidemia increase the arterial stiffness leading to endothelial dysfunction (42, 56). Similarly, H fed rats with enhanced visceral

obesity showed diminished vasoconstriction, and decreased endothelial and vascular smooth muscle functions (Fig. 4A,B,C). Improved vascular function detected with SC mixture supplementation (Fig. 4B,C) has been directly associated with improved glucose utilization, insulin resistance and lipid profile (Table 4).

SC supplementation did not change the cardiovascular structure from echocardiographic examination (Table S4) but attenuated the interstitial collagen deposition and cardiac stiffness (Table 3) in HSC rats (Fig. 3G). These changes in the cardiovascular structure may occur due to the hypoglycemic effect of SC mixture which possibly decreases the protein kinase C expression and inhibits the collagen deposition induced by transforming growth factor (TGF-1) (57, 58). Insoluble fiber treatment from 5% SC attenuates the visceral adiposity induced inflammatory cells infiltration in the heart and liver tissue of HSC rats (59) (Fig. 3C&5G). SC mixture treatment improved the liver morphology (Fig. 5D,H) and function by the attenuation of impaired lipid profile (60) (Table 3).

In conclusion, insoluble fiber supplementation from SC increases the lean mass, prevents fat deposition especially visceral fat and improves the metabolic risk factors such as impaired glucose and insulin tolerance, hypertension, endothelial dysfunction, collagen deposition and cardiac stiffness, inflammatory cells infiltration of heart and liver, and non-alcoholic fatty liver disease in the diet induced obese rats. Further characterization of insoluble polysaccharides present in SC will be carried out using standard techniques.

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CHAPTER 6

Responses to increased magnesium and potassium intake in a high-carbohydrate, high-fat diet-induced metabolic syndrome model in rats

The Journal of Nutrition

Responses to increased magnesium and potassium intake in a high-carbohydrate, high-fat diet-induced metabolic syndrome model in rats

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Abstract

Consumption of Western diets associated with the reduced magnesium intake showed higher prevalence of metabolic syndrome among U.S adults. The high-carbohydrate (68% as sucrose and fructose), high-fat (24% as saturated and mono-unsaturated fat) (H) diet mimicking "Western diets" in our diet-induced rat model of metabolic syndrome contained lower magnesium content (689 mg/kg diet vs 914 mg/kg diet) compared to the normal low fat (8%) cornstarch diet (C). Henceforth, the H fed rats were detected with higher incidence of metabolic syndrome which includes increased fat mass development (total body fat mass and abdominal fat mass), insulin resistance, impaired glucose tolerance, dyslipidemia, non-alcoholic fatty liver disease, endothelial dysfunction and cardiovascular complications detected with increased systolic blood pressure, stroke volume, cardiac output, left ventricular septum weight, total heart weight, collagen deposition, inflammatory cells infiltration of myocytes and cardiac stiffness. High magnesium (Mg^{++}) supplementation (as 5% (w/w) magnesium chloride of H diet) attenuated the metabolic syndrome in the H diet-induced obese rats. In addition, Mg supplementation as a divalent cation (Mg^{++}) increased fecal lipid excretion in the high fat rats that may prevent the fat mass development. Increased plasma magnesium (Mg^{++}) concentration (25-27%) identified in rats fed with potassium (K^{+}) (as 2% potassium chloride (w/w) of the diet) supplemented C or H diets, improved insulin resistance, impaired glucose tolerance, impaired lipid profile, inflammation of heart, collagen deposition and cardiac stiffness in the high fat rats. However, no changes in fat mass development were observed with K^{+} supplementation. Furthermore, increased circulating concentrations of both magnesium and potassium observed in either Mg^{++} or K^{+} treated high fat rats improved the cardiovascular complications in the diet-induced obese rats showing reduced systolic blood pressure, improved endothelial-function, reduced cardiac output and left ventricle + septum and total heart wet weight. In summary, Mg^{++} supplementation performed better in the prevention of H-diet induced metabolic syndrome than K^{+} supplementation. Also, this study highlights the interdependent therapeutic effects of both magnesium and potassium in the improvement of insulin resistance, type 2 diabetes, impaired lipid profile, and cardiovascular complications.

Key words: Western diets; magnesium intake; magnesium and potassium; metabolic syndrome.

Introduction

Western lifestyle includes consumption of "Western diets" containing higher proportion of fat (as saturated and mono-unsaturated), refined carbohydrates (as sucrose and fructose) and minerals (as sodium chloride) (1). About half of the US population accustomed to the "Western dietary pattern" failed to meet the US-1989 dietary recommendations for intake of essential minerals such as calcium, magnesium and zinc (1). These dietary lifestyle changes may increase the prevalence of metabolic syndrome which includes abdominal obesity, insulin resistance, type 2 diabetes, hypertension, and hypertriglyceridemia in US adults (2-4). An observational study on Korean adults identified lower intake of magnesium and copper ions in the metabolic syndrome groups, supporting an adequate mineral intake in the control of metabolic syndrome. Song et al (5) showed an inverse association between dietary magnesium

intake and metabolic syndrome prevalence in middle aged and older U.S. women. "Western dietary pattern" with reduced magnesium intake (349 mg/day) was associated with a higher prevalence of metabolic symptoms in an urban Mexican population (2). Furthermore, magnesium deficiency aggravates metabolic complications (6, 7).

Rats fed with high carbohydrate (as sucrose) magnesium-deficient diet showed increased plasma concentrations of triglycerides and total cholesterol (8). Also, increased insulin resistance was observed in rats fed the magnesium-deficient high-fat diet (9). In U.S young adults aged 18 to 30 years, high magnesium intake may prevent the risk factors of metabolic syndrome (10). Oral supplementation of 5% magnesium chloride solution improved the insulin sensitivity and metabolic complications in type 2 diabetics (11). Similarly, high dietary magnesium intake (3.2-44.2 mg/kg body weight) improved the insulin sensitivity in overweight and obese individuals in the Newfoundland population (12). Oral magnesium treatment for 4 wk in Dahl rats fed with high salt (8% sodium chloride) prevented the blood pressure elevation and improved hypercontractility to potassium chloride in isolated superior mesenteric arteries (13).

Potassium ion homeostasis is directly linked to intracellular magnesium metabolism (14). The intracellular potassium ion concentration in red blood cells is directly proportional to the intracellular magnesium ion concentration (15). These results emphasized the interaction and interdependency of intracellular potassium (K_i^+) concentration with intracellular magnesium (Mg_i^{++}) and calcium (Ca_i^{++}) towards the development of hypertension and diabetes. However, increased potassium intake (from 1% to 3.6%) in the spontaneously hypertensive rats decreased the systolic blood pressure and improved the endothelial-dependent and independent vasorelaxation (16). Although previous studies have explored the independent effects of magnesium and potassium in regulating the blood pressure and glucose utilisation (16, 17), the interdependent therapeutic effects in the prevention of metabolic syndrome are poorly understood. Further, there is a need for well-designed therapeutic trials with oral magnesium supplements against the metabolic syndrome (18). Therefore, in this study we have investigated the therapeutic responses to high potassium (as potassium chloride 2% of control diet) and high magnesium (as magnesium chloride 5% of control diet) supplementation in the high-carbohydrate, high-fat diet induced rat model of metabolic syndrome (19). The high-carbohydrate (68% as sucrose and fructose), high-fat (24% as saturated and mono-unsaturated fat) control diet used in this study ccontains low magnesium content (689mg/kg diet) compared to the normal low fat (8%) cornstarch diet (914 mg/kg diet), while the potassium content in both the control diets were similar (4.5-4.8g/kg diet).

Materials and Methods

Rats and diets

The experimental groups consisted of 72 male Wistar rats (9-10 weeks-old; weighing 335.0 ± 1.1 g) supplied by The University of Queensland Biological Resources unit and individually housed in a temperature-controlled (20±2°C), 12-hour light/dark cycle environment with access *ad libitum* to water and the group-specific rat diet at the University of Southern Queensland Animal House. All experimentation was approved by the Animal Experimentation

Ethics Committees of the University of Queensland and University of Southern Queensland under the guidelines of the Australian code of practice for the care and use of animals for the scientific purposes (7th edition 2004) and the National Health and Medical Research Council of Australia.

The rats were randomly divided into 6 groups (n = 12 each) and fed the following diets: basal corn starch (C); basal corn starch + potassium chloride 2% (CK); basal corn starch + magnesium chloride 5% (CM); basal high-carbohydrate, high-fat (H); basal high-carbohydrate, high-fat + potassium chloride 2% (HK); basal high-carbohydrate, high-fat + magnesium chloride 5% (HM).

The preparation and macronutrient composition of basal diets, including the dietary fatty acid profiles, have been described in detail previously (19, 20). Treatment diets were prepared by adding either 5% (w/w) magnesium chloride hexahydrate or 2% (w/w) potassium chloride in the basal C and H diets that replaces an equivalent amount of water added in the basal diets, without affecting its basal nutrients composition. Rats were fed on the basal diets for 8 wk followed by a further 8 wk of feeding the mineral salt-supplemented diets. The drinking water in all high-carbohydrate, high-fat-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 as described previously (19, 21).

Abdominal circumference and body length of rats were measured using a standard measuring tape during the period of anesthesia for systolic blood pressure measurements (19, 21). Body weight gain and energy efficiency were calculated as described (19, 21). Systolic blood pressure measurements, and oral glucose and insulin tolerance tests were conducted at 0, 8, and 16 wk. Oral glucose and insulin tolerance tests were performed as described (19, 21). Rats were withdrawn from the test if the blood glucose concentration dropped below 1.1 mmol/L, and 4 g/kg glucose was administered immediately via oral gavage to prevent hypoglycemia. Dual energy X-ray absorptiometric (DXA) measurements were performed on rats after 16 wk of feeding, 2 days before euthanasia (19, 21). Visceral adiposity index (%) was calculated (19, 21).

Cardiovascular measurements

Systolic blood pressure was measured as previously described (19) under light sedation following intraperitoneal injection of Zoletil (tiletamine 15 mg/kg, zolazepam 15 mg/kg; Virbac, Peakhurst, NSW, Australia) using a MLT1010 Piezo-Electric Pulse Transducer and inflatable tail-cuff connected to a MLT844 Physiologic Pressure Transducer using PowerLab data acquisition unit (ADInstruments, Sydney, Australia).Anesthesia using intraperitoneal Zoletil (tiletamine 15 mg/kg and zolazepam 15 mg/kg i.p.; Virbac, Peakhurst, NSW, Australia) was used for echocardiographic examination (Hewlett Packard Sonos 5500, 12 MHz transducer) performed at 16 wk as previously described (19). The left ventricular (LV) function of the rats in all treatment groups was assessed using the Langendorff heart preparation as previously described (19). Terminal anesthesia 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 physiologic pressure transducer and Chart software on a MacLab system (ADInstruments Australia and Pacific Islands, Bella Vista, NSW, Australia). 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 (19, 21).

Lipid excretion analysis

After completion of 16 wk experimental protocol, rats (n=6/per group) from both control (C & H) and treatment groups (CK, CM, HK & HM) were maintained in metabolism cages and fed with their respective diets over a period of 12-h (light/dark cycle). The collected feces were then weighes and ground using mortar and pestle. The extraction of dietary lipids was undertaken in 1 g of powdered feces by manual solvent extraction using a 2:1 chloroform/methanol mixture with 0.1% vitamin E as an antioxidant. The solvent mixture contents were mixed on a rotating device for 40 min with 20 mL of chloroform/methanol solvent and then centrifuged at 2500 rpm for 5 min. The extraction procedure was repeated twice and subsequently washing with double distilled water to remove all polar material. Extracts were pooled and chloroform was evaporated under a steam of nitrogen on a hot plate at 60^{o}C until the beakers reached constant weight for the calculation of gravimetric extractable lipid content (22). The percentage lipid excretion was calculated based on the formula [(Amount of lipid excreted in g/Total amount of lipid consumed in g)*100)]. The amount of extractable lipids present in the basal diets (C: 6.2 g/ diet and H: 187g/kg diet) was obtained from our previous study (21).

Organ weights

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

Histology

Two rats per group were used 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 as previously described (19, 21). Thin sections (7 μm) of left ventricle and the liver were cut and stained with haematoxylin and eosin stain for determination of inflammatory cell infiltration with 20X and fat vacuole enlargement with 40X objectives using a Olympus BX51 microscope (Olympus, Melville, NY). Collagen distribution was measured in the left ventricle with picrosirius red stain. Laser confocal microscopy (Zeiss

LSM 510 upright Confocal Microscope) with color intensity quantitated using NIH-imageJ software (National Institute of Health, USA) was used to determine the extent of collagen deposition in selected tissue sections (19, 21).

Plasma biochemistry

Blood was centrifuged at 5,000 x*g* for 15 min within 30 min of collection into heparinized tubes. Plasma was separated and transferred to Eppendorf tubes for storage at -20°C before analysis. Activities of plasma enzymes (alanine transaminase (ALT) and aspartate transaminase (AST)) and plasma analyte concentrations which includes non-esterified fatty acids (NEFA), triglycerides, creatinine, total cholesterol, magnesium (Mg), potassium (K), and sodium (Na) were determined using kits and controls supplied by Olympus using an Olympus analyser (AU 400, Tokyo, Japan) as previously described (19, 21).

Statistical analysis

All data are presented as mean ± SEM. Results were tested for variance using Bartlett's test and variables that were not normally distributed were transformed (using log 10 function) prior to statistical analyses. Data from C, CK, CM, H, HK, and HM groups were tested by two-way ANOVA. When interaction and/or the main effects were significant, means were compared using Newman-Keuls multiple comparison *post hoc* test. Where transformations did not result in normality or constant variance, a Kruskal-Wallis 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 5.00 for Windows (San Diego, California, USA).

Results

Dietary intake, body parameters, and glucose utilization

Food and water consumption were increased in both CK and CM-treated rats, compared with C-fed rats (Table1). Similarly, in H-fed groups, water intake was increased by mineral supplementation (Table1) while an increased food intake was detected only in HM rats (Table 1). Compared with basal-diet fed groups (C and H), 2% K supplementation did not affect the energy intake corresponding with no changes in body weight gain and feed conversion efficiency (Table 1). In contrast, rats fed on Mg supplemented diets showed increased energy intake but reduced body weight gain and feed conversion efficiency (Table 1). Compared to C and H basal diet-fed rats, no changes in lean mass were observed in either 2% K or 5% Mg treatment groups (Table1). 2% K treatment of corn starch-fed rats elicited no changes in total body fat mass, abdominal circumference, visceral adiposity index, and abdominal fat mass (including retroperitoneal fat, epidydmal fat, and omental fat) (Table 1). In contrast, 5% Mg supplementation in corn starch-fed rats showed no changes in total body fat mass but attenuated the abdominal circumference and abdominal fat mass in CM rats (Table1). In H-fed rats, 5% Mg supplementation reduced total body fat mass, abdominal circumference, and abdominal fat mass (Table 1). The glucose utilization and insulin sensitivity were improved in rats fed with either 2% K or 5% Mg supplemented H diet, compared with H fed rats. In addition, CM treated rats improved glucose utilization compared to C and CK fed groups, while no changes were observed in insulin sensitivity in between the C fed groups (Table 1).

Table 1 Physiological and metabolic variables in rats fed control diets C or H and with either potassium chloride or magnesium chloride

Variables	C	CK	CM	H	HK	HM	Diet	Treatment	Interaction
Food intake, g/d *(n=10)*	30.1±2.4[b]	36.1±2.4[a]	35.6±1.9[a]	22.0±2.0[c]	22.3±1.9[c]	30.6±2.1[b]	<0.0001	0.007	0.121
Water intake, mL/d *(n=10)*	29.2±1.9[c]	35.8±1.8[a]	37.8±2.4[a]	19.8±1.8[e]	22.1±1.7[d]	33.1±2.3[b]	<0.0001	<0.0001	0.089
Body weight gain, %*(n=10)*	11.3±1.5[ab]	12.9±0.6[a]	3.9±0.7[c]	12.8±1.4[a]	11.5±1.1[a]	2.5±0.5[c]	0.613	<0.0001	0.284
Feed conversion efficiency, %	3.3±0.4 [a]	3.0±0.1 [a]	1.0±0.2 [b]	2.7±0.3 [a]	2.5±0.2 [a]	0.4±0.1 [b]	0.006	<0.0001	0.972
Energy intake, kJ/d *(n=10)*	339.6±5.1[c]	427.2±17.9[b]	405.9±10.3[b]	467.6±11.3[b]	465.2±4.4[b]	662.6±10.6[a]	<0.0001	<0.0001	<0.0001
Bone mineral content, g *(n=8-10)*	12.9±0.2[bc]	12.5±0.2[b]	11.2±0.3[cd]	14.8±0.4[a]	13.3±0.3[b]	13.0±0.5[b]	<0.0001	<0.0001	0.227
Total fat mass, g*(n=8-10)*	90.4±5.5[b]	80.5±6.6[b]	71.4±5.5[b]	182.3±8.1[a]	159.7±16.8[a]	101.8±15.4[b]	<0.0001	<0.0001	0.011
Total lean mass, g *(n=8-10)*	302.2±4.4	311.7±7.4	280.6±4.2	305.8±11.2	287.8±2.9	284.9±7.6	0.373	0.13	0.097
Abdominal circumference, cm *(n=10)*	19.6±0.4[c]	19.9±0.1[c]	18.1±0.3[d]	22.1±0.4[a]	22.0±0.2[a]	19.3±0.4[c]	<0.0001	<0.0001	0.159
Visceral adiposity index, %*(n=8-10)*	3.3±0.1[b]	2.6±0.2[b]	1.3±0.1[c]	6.1±0.4[a]	5.7±0.8[a]	3.1±0.4[b]	<0.0001	<0.0001	0.265
Tissue wet weight*, mg/mm*(n=8-10)*									
Retroperitoneal fat	121.7±5.9[b]	117.1±5.6[b]	33.1±5.3[c]	293.1±18.9[a]	269.1±32.8[a]	131.2±25.0[b]	<0.0001	<0.0001	0.144
Epididymal fat	96.7±4.6[b]	91.7±6.3[b]	46.5±3.2[c]	170.4±11.4[a]	161.6±22.2[a]	92.1±10.1[b]	<0.0001	<0.0001	0.422
Omental fat	52.6±3.8[c]	55.8±3.8[c]	19.5±2.3[d]	131.6±10.6[a]	99.5±8.5[b]	55.6±7.6[c]	<0.0001	<0.0001	0.006
Total abdominal fat mass	270.9±11.6 [b]	249.3±17.3 [b]	99.7±9.9 [c]	595.2±35.7 [a]	530.2±62.9 [a]	278.8±41.1 [b]	<0.0001	<0.0001	0.117
OGTT, mmol/L x min *(n=8-10)*									
Week 0	669.5±17.0	667.7±16.2	712.1±8.9	662.7±12.5	714.5±18.4	717.4±24.9	0.280	0.022	0.262

Week 8	624.6±10.7[a]	661.4±29.1[b]	656.4±9.5[b]	801.0±14.9[a]	813.8±9.6[a]	804.2±12.8[a]	<0.0001	0.333	0.667
Week 16	607.8±12.1[c]	603.3±13.0[c]	555.8±10.5[d]	797.3±16.9[a]	684.3±18.9[b]	697.9±16.7[b]	<0.0001	<0.0001	0.003
ITT, mmol/L x min*(n=10)*									
Week 16	112.0±3.9 [c]	92.8±4.1 [c]	83.4±4.9 [c]	392.5±19.5 [a]	332.7±17.9 [b]	112.1±18.2 [c]	<0.0001	<0.0001	<0.0001
Lipid excreted, %	2.2±0.3 [d]	3.8±0.4 [d]	5.4±0.8 [cd]	12.0±1.0 [b]	8.6±1.1 [bc]	16.5±2.5 [a]	<0.0001	0.0015	0.041

Each value is a mean±S.E.M. Means with superscript letters a,b,c,d without a common letter differ (P <0.05)*. Tissue wet weights were normalized against tibial length. C, corn starch fed rats; CK, corn starch rats treated with 2% potassium chloride; CM, corn starch rats treated with 5% magnesium chloride; H, high-carbohydrate ,high-fat fed rats; HK, high-carbohydrate, high-fat rats treated with 2% potassium chloride; HM, high-carbohydrate, high-fat rats treated with 5% magnesium chloride.

Systolic blood pressure and cardiovascular changes

Systolic blood pressure was unchanged in all C treatment groups. Compared with H fed rats, an increased systolic blood pressure was normalized in either HK or HM rats (Table 2). Compared to C fed rats, rats fed with CM diet showed increased left ventricular posterior wall thickness in diastole (LVPWd) (Table 2), while rats fed the HM diet reduced left ventricular posterior wall thickness in systole (LVPWs) and interventricular septum thickness in systole (IVSs) (Table 2). In H fed groups, the HK rats showed increased interventricular septum thickness during diastole (IVSd) (Table 2).

The H fed control rats showed increased stroke volume and cardiac output compared to C fed groups (table 2). K treatment in H rats normalized the stroke volume and cardiac output, while with the Mg supplementation the cardiac output alone was normalized but showed a reduction in fractional shortening and ejection fraction percentage in the HM treated rats compared to H rats (Table 2). Additionally, the estimated left ventricular mass was normalized in either HK or HM treated rats (Table 2). Further, no changes in cardiac structure from echocardiographic examination were observed in either 2% K or 5% Mg treatment groups compared to C or H fed groups (Table 2).

Compared with C and H fed groups, the left ventricular septum wet weight and total heart wet weight were reduced in either CM or HM rats. Similarly, with the K supplementation both the left ventricular septum and total heart wet weight were normalized in the HK treated rats compared with the control groups (C and H) (Table 2). The diastolic stiffness constant (κ) was reduced in either HK or HM fed rats compared to H fed rats.

Table 2 Cardiovascular structure and function in rats fed control diets C or H and with either potassium chloride or magnesium chloride

Variables	C	CK	CM	H	HK	HM	Diet	Treatment	Interaction
LVIDd, *mm(n=6-10)*	7.23±0.22	6.83±0.41	7.03±0.26	7.85±0.18	6.80±0.39	7.60±0.21	0.096	0.038	0.435
LVIDs, *mm(n=6-10)*	3.23±0.16 ^{b}	3.23±0.53^{ab}	3.98±0.18^{ab}	3.52±0.23 ^{ab}	3.25±0.49^{ab}	4.53±0.22^{a}	0.263	0.004	0.695
LVPWd, *mm(n=6-10)*	1.72±0.03^{b}	1.90±0.08^{ab}	2.10±0.09 ^{a}	1.95±0.05 ^{ab}	1.95±0.12 ^{ab}	1.77±0.02 ^{b}	0.763	0.272	0.001
LVPWs, *mm(n=6-10)*	2.89±0.11^{ab}	3.25±0.14^{a}	2.80±0.06 ^{ab}	3.22±0.11 ^{a}	2.98±0.29 ^{ab}	2.50±0.12 ^{b}	0.514	0.007	0.070
IVSd, *mm(n=6-10)*	1.86±0.07^{b}	1.98±0.09^{ab}	1.98±0.07^{ab}	1.95±0.05^{b}	2.23±0.10^{a}	1.77±0.02^{b}	0.466	0.005	0.01
IVSs, *mm(n=6-10)*	3.13±0.11^{ab}	3.33±0.28^{ab}	3.10±0.04^{ab}	3.60±0.08^{a}	3.57±0.23^{a}	2.80±0.05^{b}	0.264	0.004	0.037
Diastolic volume, *µL(n=6-10)*	347±37	352±61	372±44	514±35	346±67	465±39	0.034	0.188	0.197
Systolic volume, *µL(n=6-10)*	38.0±5.3^{b}	51.0±25.3^{ab}	68.0±10.0^{ab}	51.0±10.5^{ab}	50.0±25.4^{ab}	101.0±14.9^{a}	0.251	0.033	0.560
Stroke volume, *µL(n=6-10)*	309.0±36.1^{b}	301.0±44.8^{b}	304.0±34.9^{b}	463.0±28.2^{a}	296.0±44.8^{b}	364.0±27.4^{ab}	0.028	0.075	0.115
Cardiac output, *mL/min(n=6-10)*	107.5±20.3^{b}	105.1±20.1^{b}	84.5±13.6^{b}	168.4±9.8^{a}	95.2±21.4^{b}	96.3±9.2^{b}	0.146	0.022	0.124
Relative wall thickness*(n=6-10)*	0.52±0.02 ^{ab}	0.58±0.04 ^{ab}	0.59±0.04 ^{ab}	0.50±0.02 ^{b}	0.63±0.06 ^{a}	0.47±0.01 ^{b}	0.266	0.015	0.042

Variables	C	CK	CM	H	HK	HM	Diet	Treatment	Interaction
Systolic wall stress*(n=6-10)*	68.9±4.9^{b}	69.1±13.9^{b}	91.9±5.4^{ab}	89.1±7.5^{ab}	75.0±14.5^{b}	113.2±7.7^{a}	0.037	0.004	0.611
Fractional shortening, *%(n=6-10)*	52.5±2.5^{a}	54.7±4.7^{a}	44.6±1.2^{ab}	55.5±2.2^{a}	54.0±4.7^{a}	40.6±1.7^{b}	0.925	0.001	0.300
Ejection fraction, *%(n=6-10)*	86.9±1.6^{ab}	87.9±4.2^{ab}	81.8±0.9^{ab}	90.6±1.4^{a}	87.7±3.6^{ab}	78.6±1.8^{b}	0.959	0.001	0.347
Diastolic stiffness, *κ(n=8-10)*	21.0±1.5^{b}	24.3±0.6^{b}	22.6±0.8^{b}	29.1±2.0^{a}	24.1±0.5^{b}	23.2±0.6^{b}	0.009	0.253	0.004
Estimated LV mass, *g (n=6-10)*	0.92±0.05^{b}	0.90±0.06^{b}	0.99±0.03^{b}	1.19±0.05^{a}	0.98±0.05^{b}	0.93±0.04^{b}	0.028	0.073	0.011
LV+septum wet weight*,*mg/mm(n=9-10)*	17.1±0.6^{b}	16.7±0.6^{bc}	14.1±0.6^{c}	20.8±0.3^{a}	17.2±0.4^{b}	15.7±0.6^{b}	<0.0001	<0.0001	0.013
RV wet weight*, *mg/mm (n=9-10)*	2.3±0.5	2.3±0.1	2.0±0.2	2.8±0.3	2.1±0.2	1.9±0.1	0.770	0.105	0.402
Heart wet weight, *mg/mm (n=9-10)*	20.5±0.7^{b}	18.6±0.6^{bc}	16.1±0.5^{d}	23.1±0.3^{a}	19.3±0.4^{bc}	17.7±0.7^{c}	0.001	<0.0001	0.241
Liver weight, *mg/mm*	240.6±6.2^{ab}	213.9±11.9^{b}	214.4±14.7^{b}	274.4±12.7^{a}	296.3±15.6^{a}	253.9±11.4^{ab}	<0.0001	0.129	0.113
Systolic blood pressure, *mmHg(n=10)*	127±2^{b}	134±1^{b}	130±1^{b}	157±1^{a}	130±3^{b}	128±3^{b}	<0.0001	<0.0001	<0.0001

Each value is a mean±S.E.M. Means with superscript letters a,b,c,d without a common letter differ (P <0.05)*. Tissue wet weights were normalized against tibial length. C, corn starch fed rats; CK, corn starch rats treated with 2% potassium chloride; CM, corn starch rats treated with 5% magnesium chloride; H, high-carbohydrate, high-fat fed rats; HK, high-carbohydrate, high-fat rats treated with 2% potassium chloride; HM, high-carbohydrate, high-fat rats treated with 5% magnesium chloride.

Vascular responses in thoracic aortic rings

Compared to H fed rats, no changes in contractile responses to noradrenaline in isolated thoracic rings were observed in C control rats (Fig. 1A) while higher contractile responses to noradrenaline in isolated thoracic rings were measured in both K (CK & HK) and Mg treatment groups (CM & HM) (Fig. 1A). The C treatment groups showed higher smooth-muscle dependent and endothelium-dependent relaxant responses to sodium nitroprusside and acetylcholine, compared to H control rats (Fig. 1B,C). In addition, the CM rats showed higher smooth-muscle dependent relaxant response to sodium nitroprusside compared to all the treatment groups (Fig. 1B). Both K and Mg supplementation improved the smooth-muscle dependent and endothelium-dependent relaxant responses to sodium nitroprusside and acetylcholine in HK and HM rats (Fig. 1B,C).

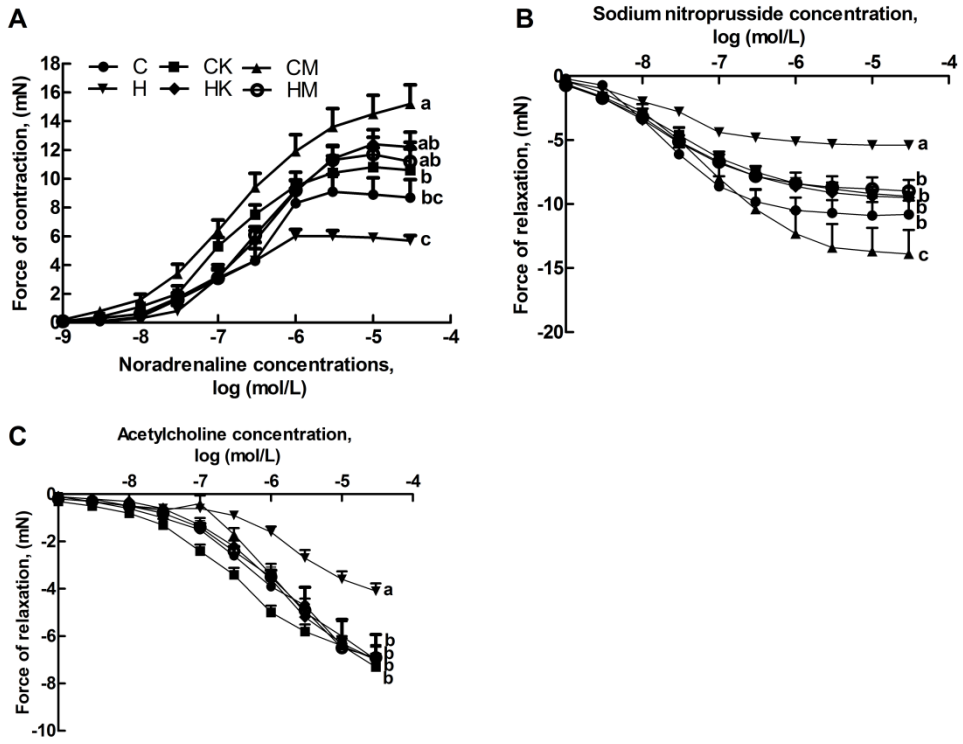

FIGURE 1 Effect of magnesium and potassium treatment on noradrenaline-induced contraction *(A)*, sodium nitroprusside-induced relaxation *(B)*, and acetylcholine-induced relaxation *(C)* in thoracic aortic preparations from C, CK, H, and HM rats. Values are mean ± SEM, *n=10*. Endpoints means without a common letter differ, P<0.05. C, corn starch fed rats; CK, corn starch rats treated with 2% potassium chloride; CM, corn starch rats treated with 5% magnesium chloride; H, high-carbohydrate, high-fat fed rats; HK, high-carbohydrate, high-fat rats treated with 2% potassium chloride; HM, high-carbohydrate, high-fat rats treated with 5% magnesium chloride.

Plasma biochemistry and lipid excretion analysis

Decreased plasma concentrations of non-esterified fatty acids and triglycerides were observed in the corn starch-fed animals compared with high fat–fed rats (Table 3). Within the H-fed rats, decreased plasma concentrations of non-esterified fatty acids and triglycerides were observed in HK and HM rats (Table 3). Additionally, no changes in plasma concentrations of total cholesterol and alanine transaminase (ALT) activity were observed among either C or H treatment groups while decreased plasma aspartate transaminase (AST) activity was observed in C rats (Table 3). Compared with control treatment groups, increased plasma potassium and magnesium ion concentrations with no changes in plasma sodium ion concentration were observed in both 5% Mg or 2% K treatment groups (Table 2). Among all the treatment groups, the percentage lipid excreted was significantly increased in HM rats (Table 1).

Table 3 Plasma biochemistry in rats fed control diets C or H and with either potassium chloride or magnesium chloride

Variable	C	CK	CM	H	HK	HM	Interaction		
							Diet	Treatment	Interaction
Plasma NEFA, mmol/L *(n=10)*	1.50±0.11 ^{c}	1.43±0.19 ^{c}	1.28±0.17 ^{c}	3.61±0.34 ^{a}	2.35±0.37 ^{b}	1.16±0.07 ^{c}	<0.0001	<0.0001	0.0001
Plasma triglycerides, mmol/L *(n=10)*	0.56±0.07 ^{b}	0.40±0.04 ^{b}	0.39±0.03 ^{b}	2.37±0.30 ^{a}	0.72±0.14 ^{b}	0.28±0.02 ^{b}	<0.0001	<0.0001	<0.0001
Plasma total cholesterol, mmol/L *(n=10)*	1.62±0.06 ^{b}	1.59±0.08 ^{b}	1.69±0.13 ^{b}	1.70±0.07 ^{b}	1.69±0.07 ^{b}	2.16±0.05 ^{a}	0.002	0.001	0.032
Plasma creatinine, µmol/L *(n=10)*	44.9±0.9	46.6±2.2	48.9±1.4	46.6±1.6	48.9±1.3	50.6±1.6	0.133	0.039	0.975
Liver enzymes & mineral ions									
Plasma ALT activity, U/L *(n=10)*	27.0±2.1	36.2±5.3	43.7±5.8	37.2±2.9	36.8±5.4	36.6±4.9	0.743	0.224	0.179
Plasma AST activity, U/L *(n=10)*	68.9±4.1 ^{b}	80.3±5.8 ^{ab}	87.3±2.0 ^{ab}	100.9±7.9 ^{a}	104.5±6.8 ^{a}	104.6±14.7 ^{a}	0.001	0.379	0.659
Plasma Na+, mmol/L *(n=10)*	143±1	140±1	142±1	141±1	139±1	141±1	0.109	0.038	0.847
Plasma K+, mmol/L *(n=10)*	5.19±0.33 ^{b}	7.23±0.48 ^{a}	7.11±0.36 ^{a}	4.74±0.24 ^{b}	6.70±0.25 ^{a}	6.53±0.44 ^{a}	0.001	<0.0001	0.002
Plasma Mg+, mmol/L *(n=10)*	0.73±0.01 ^{c}	0.93±0.05 ^{b}	1.25±0.05 ^{a}	0.71±0.02 ^{c}	0.89±0.04 ^{b}	0.98±0.04 ^{b}	0.001	<0.0001	0.002

Each value is a mean±S.E.M. Means with superscript letters a,b,c,d without a common letter differ (P <0.05)*. C, corn starch fed rats; CK, corn starch rats treated with 2% potassium chloride; CM, corn starch rats treated with 5% magnesium chloride; H, high-carbohydrate, high-fat fed rats; HK, high-carbohydrate, high-fat rats treated with 2% potassium chloride; HM, high-carbohydrate, high-fat rats treated with 5% magnesium chloride.

Histology staining

Compared with C treatment groups, increased infiltration of inflammatory cells and collagen deposition were observed in myocytes of H fed rats (Fig. 2A,B,C&D; Fig. 5A,B,C&D). The infiltration of inflammatory cells and collagen deposition in myocytes were normalized in either HK or HM rats (Fig. 2E,F; Fig. 5E,F). Compared with C and HM treatment groups, liver sections showed inflammatory cells infiltration and enlarged fat vacuoles in both H and HK rats (Fig. 3A-F; Fig. 4A-F).

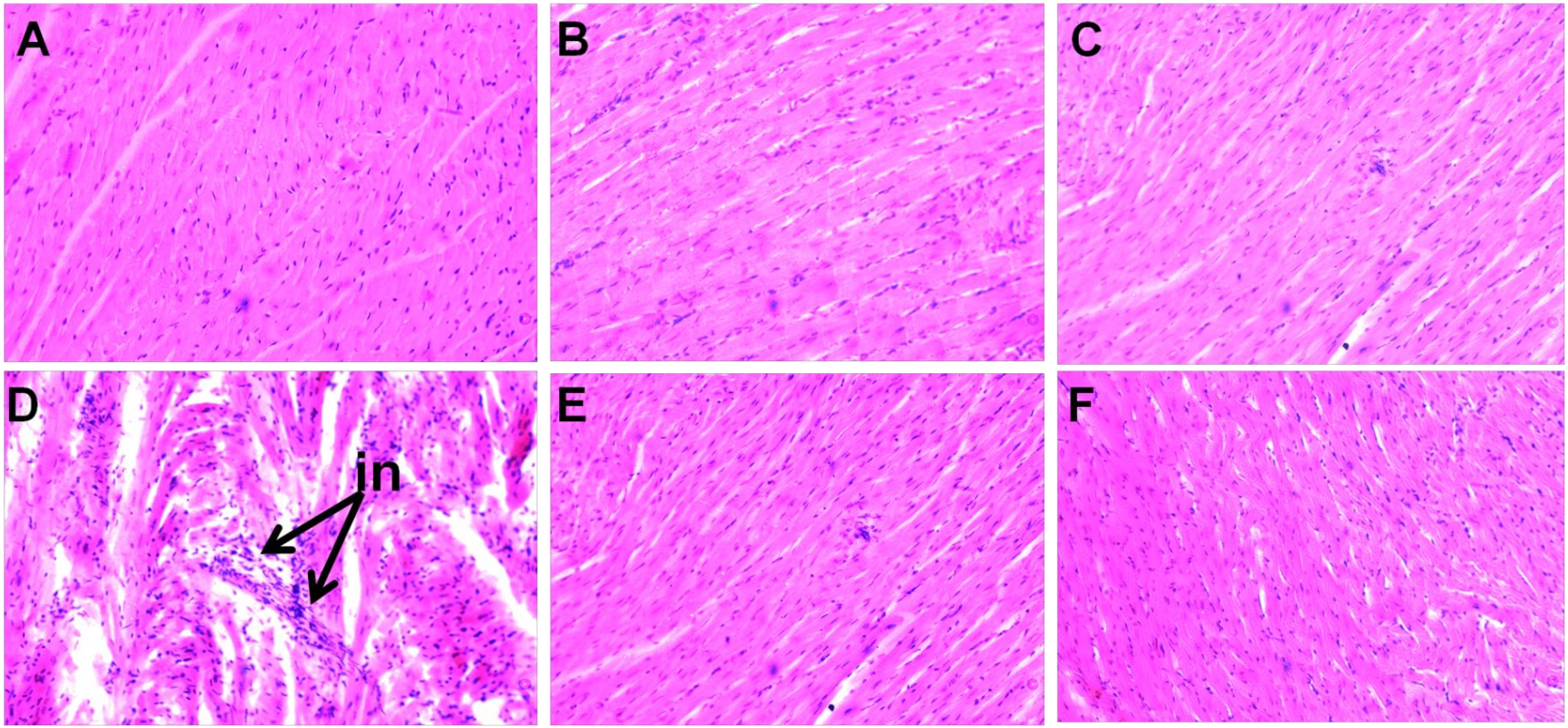

FIGURE 2 Effect of magnesium and potassium treatment on inflammation in the heart. Haematoxylin and eosin staining of left ventricle showing infiltration of inflammatory cells *(A–F*, inflammatory cells marked as “in”) (x20) from C *(A)*, CK *(B)*, CM *(C)*, H *(D)*, HK *(E)*, and HM *(F)*. C, corn starch fed rats; CK, corn starch rats treated with 2% potassium chloride; CM, corn starch rats treated with 5% magnesium chloride; H, high-carbohydrate, high-fat fed rats; HK, high-carbohydrate, high-fat rats treated with 2% potassium chloride; HM, high-carbohydrate, high-fat rats treated with 5% magnesium chloride.

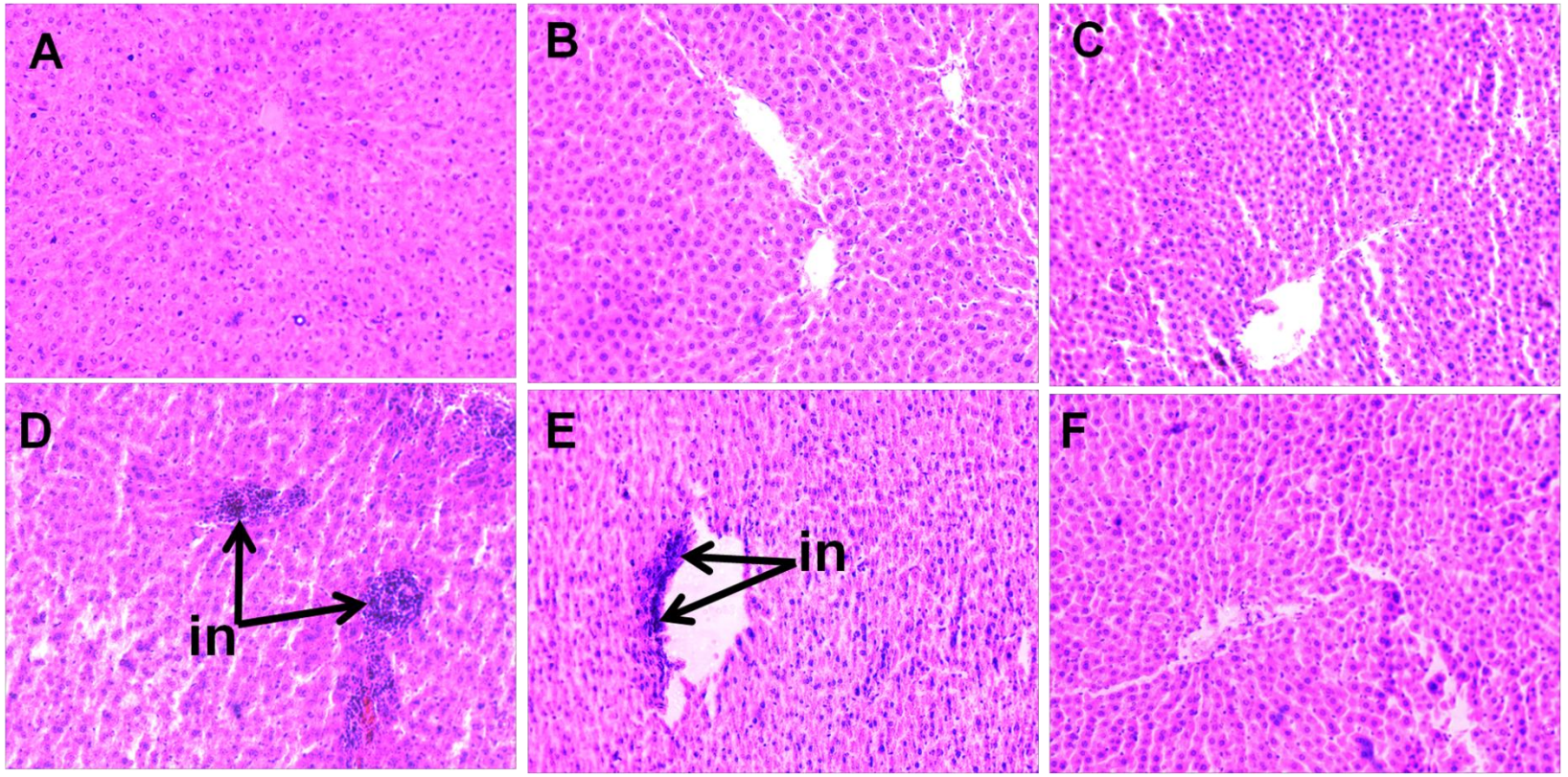

FIGURE 3 Effect of magnesium and potassium treatment on inflammation in the liver. Haematoxylin and eosin staining of hepatocytes showing infiltration of inflammatory cells *(A–F*, inflammatory cells marked as "in") (x20) from C *(A)*, CK *(B)*, CM *(C)*, H *(D)*, HK *(E)*, and HM *(F)*. C, corn starch fed rats; CK, corn starch rats treated with 2% potassium chloride; CM, corn starch rats treated with 5% magnesium chloride; H, high-carbohydrate ,high-fat fed rats; HK, high-carbohydrate, high-fat rats treated with 2% potassium chloride; HM, high-carbohydrate, high-fat rats treated with 5% magnesium chloride.

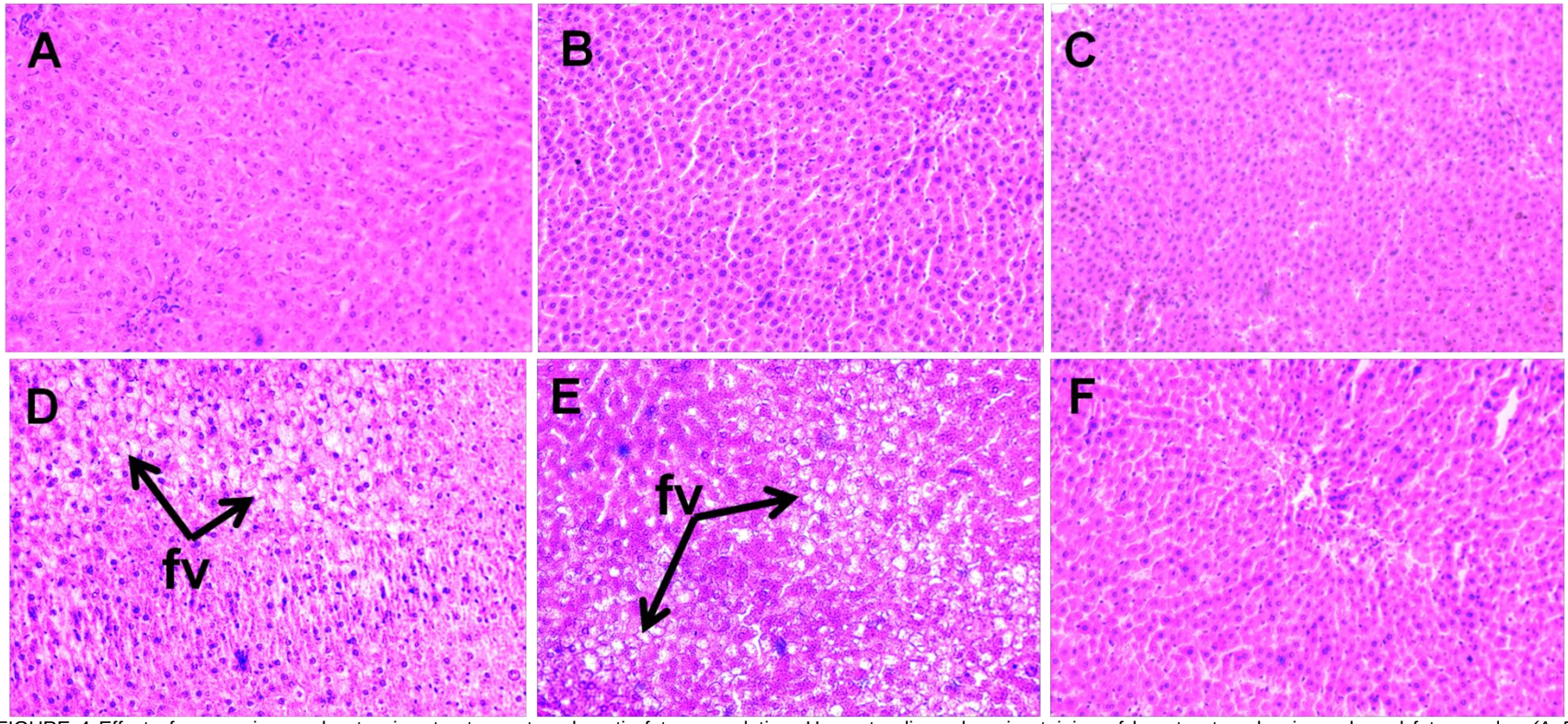

FIGURE 4 Effect of magnesium and potassium treatmment on hepatic fat accumulation. Haematoxylin and eosin staining of hepatocytes showing enlarged fat vacuoles *(A–F*, fat vacuoles are marked as "fv") (x20) from C *(A)*, CK *(B)*, CM *(C)*, H *(D)*, HK *(E)*, and HM *(F)*. C, corn starch fed rats; CK, corn starch rats treated with 2% potassium chloride; CM, corn starch rats treated with 5% magnesium chloride; H, high-carbohydrate ,high-fat fed rats; HK, high-carbohydrate, high-fat rats treated with 2% potassium chloride; HM, high-carbohydrate, high-fat rats treated with 5% magnesium chloride.

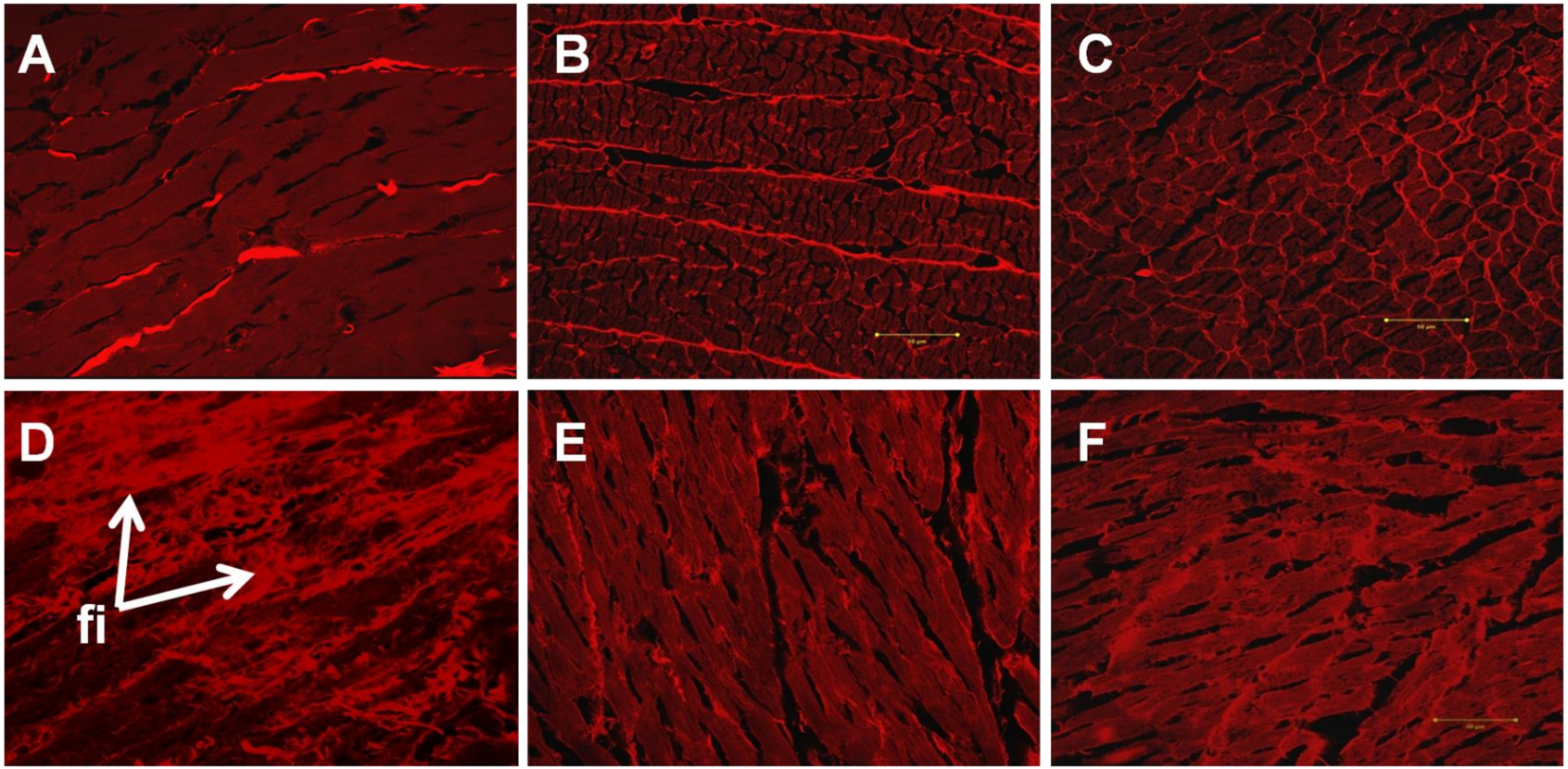

FIGURE 5 Effect of magnesium and potassium treatmment on fibrosis of heart. Picrosirius red staining of heart showing collaged deposition *(A–F*, fibrosis is marked as "fi") (x20) from C *(A)*, CK *(B)*, CM *(C)*, H *(D)*, HK *(E)*, and HM *(F)*. C, corn starch fed rats; CK, corn starch rats treated with 2% potassium chloride; CM, corn starch rats treated with 5% magnesium chloride; H, high-carbohydrate ,high-fat fed rats; HK, high-carbohydrate, high-fat rats treated with 2% potassium chloride; HM, high-carbohydrate, high-fat rats treated with 5% magnesium chloride.

Discussion

In this study, we investigated the effects of either 5% Mg or 2% K dietary supplementation in a high-fat diet model of the metabolic syndrome (19). Notably, only Mg treatment decreased body weight gain compared to the rats fed the control diet. This effect could be attributed to reduction in total body fat mass rather than fat-free mass; fat mass decreased from 36.2% body weight to 25.4% while fat-free mass increased from 60.8% to 71.2%. Since lipid excretion was increased in these rats, it is tempting to speculate that decreased fat mass is due to decreased fat absorption rather than decreased lipogenesis and fat deposition. In support of this view is the observation that Mg treatment had no effect upon body fat mass in the C-fed animals where dietary fat availability was lower and consequently effects of Mg on absorption of fatty acids were likely to be less; although increased compared to the basal diet fed animals, this increase in lipid excretion was not significant. This effect of Mg administration has been attributed to the ability of divalent cations such as calcium (Ca^{2+}) and magnesium (Mg^{2+}) to form insoluble salt complexes with either fatty acids or bile acids and thereby enhance their faecal excretion (23, 24). In contrast, supplementation of the diets with the monovalent cation, K+, had no effects.

Mg supplementation improved the insulin sensitivity (Table 1) possibly by enhancing the insulin receptor-tyrosine kinase activity (18, 25), and therefore showed improved glucose utilization in the HM rats (Table 1). Further, increased plasma magnesium concentrations (25-27%) with K treatment suggested a possible increase in intracellular magnesium concentrations (15) that attenuates the insulin resistance and impaired glucose tolerance (18, 25) in the HK rats (Table 1).

Normalized systolic blood pressure in either K or Mg treated high fat rats (Table 2) is mediated by the improvement in endothelial-dependent and -independent vaso-relaxant responses (16, 26), improving vascular function in both HK and HM rats (Fig. 1B,C). Also, reduction in volume overload augmented by diminished cardiac output and reduced estimated left ventricular mass in HK and HM treated rats (Table 2) may contribute in the prevention of hypertension (27). In response to these cardiovascular changes, both the left ventricular +septum and total heart wet weight were normalized in HK and HM rats (Table 2). Systolic dysfunction was detected with high magnesium supplementation in HM rats (Table 2). Further, no changes in cardiovascular modelling were observed in either K or Mg supplementation (Table 2). The hypoglycemic effect of K and Mg treatment has been associated with the improved cardiovascular structure showing reduced collagen deposition of heart (Fig. 5E&F) (28, 29), and normalized cardiac stiffness in the high fat-treated rats (Table 2). Increased circulating concentrations of magnesium (25%-38%) observed in either K or Mg treated rats (Table 3) may prevent the infiltration of inflammatory cells in the heart (Fig. 2E,F) (5, 30). Similarly, the hypolipidemic effect of HK rats showing reduced plasma NEFA (35%) and triglycerides (70%) concentrations correlated with the increased circulating concentrations of magnesium by 25% (31, 32), while, in HM rats, higher plasma magnesium concentrations (38%) reduced visceral fat accumulation (53%) and exhibited stronger hypolipidemic effect with 68% reduction in NEFA concentrations and 88% reduction in plasma triglycerides

concentrations (32, 33). Hypercholesterolemia was detected in HM rats (Table 3) in contradiction to previous studies (31, 32).
Magnesium supplementation improved the liver morphology by attenuating the visceral adiposity induced infiltration of inflammatory cells (Fig. 3F) (34) and hepatic fat accumulation (Fig. 4F) (35), while, HK rats detected with visceral obesity showed no changes in liver morphology compared to high-fat fed rats (Fig. 3D,E; Fig. 4D,E). However, the increased liver weight (Table 2) and plasma liver damage enzyme markers (ALT and AST activities) remained unchanged in both HK and HM rats (Table 3).
In summary, compared with 2% K treatment, 5% Mg treatment performed well in preventing the metabolic syndrome by attenuating the increased abdominal and total body fat mass, improving insulin sensitivity and glucose utilization, collagen deposition, cardiac stiffness, increased plasma nonesterified fatty acids and triglycerides concentration, increased systolic blood pressure and endothelial dysfunction observed in H rats. This study also postulated that increased plasma concentrations of magnesium observed with 2% K supplementation may contribute to the attenuation of diminished insulin sensitivity and glucose utilization, collagen deposition, cardiac stiffness, inflammation, increased plasma nonesterified fatty acids and triglycerides concentration in H fed rats. Increased plasma concentrations of potassium and magnesium ions observed in either 2% K or 5% Mg supplementation prevented hypertension and endothelial dysfunction in the high-calorie diet induced obese rats.

Conclusion

This was our initial study to examine the therapeutic effect of 2% K/5% Mg treatment in the H diet induced metabolic syndrome rat model. Further, this study also highlights the interaction and interdependency of both magnesium and potassium ions in attenuating the metabolic syndrome induced by H diet. Future studies will be conducted using low doses of magnesium chloride and potassium chloride in H diet induced metabolic syndrome rat model.

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CHAPTER 7

Effects of inulin and oligofructose in high-carbohydrate, high-fat diet-induced metabolic syndrome in rats

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Effects of inulin and oligofructose in high-carbohydrate, high-fat diet-induced metabolic syndrome in rats

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Abstract

Inulin and oligofructose, well known for their prebiotic effects to attenuate weight gain and promote weight loss via inhibiting energy intake, have been suggested for obesity management. The present study investigated the physiological effects of inulin and oligofructose mixture (Synergy1) as soluble dietary fibre in rats fed with a high-carbohydrate, high-fat (H) diet as a model of diet-induced metabolic syndrome. Cardiovascular structure and function were determined using echocardiography and histology, body composition parameters (lean and total body fat mass) using DXA, liver structure and function using plasma markers and histology and gut morphology using histology were also measured. Dietary supplementation with Synergy1 mixture prevented body weight gain and fat mass accumulation (abdominal and total body fat mass) via enhancing the energy excretion by increasing the faecal load. Increased faecal lipid excretion suggested the inhibition of intestinal fatty acid absorption and may contribute to the decreased visceral adiposity observed in Synergy1 treated H fed rats. Decreased adiposity was associated with attenuation of symptoms characteristic of the metabolic syndrome including hypertension, cardiac fibrosis, increased cardiac stiffness, endothelial dysfunction, impaired hepatic structure and function, impaired glucose and insulin tolerance, impaired plasma lipid profile, inflammatory cells infiltration in myocytes, hepatocytes and ileum, and impaired ileum morphology.

Key words: Inulin and oligofructose: Energy intake: Synergy1: Prebiotic: Soluble dietary fibre: Metabolic syndrome: High-carbohydrate, high-fat diet-fed rats.

Introduction

Obesity and its associated metabolic syndrome are increasing globally due to lack of physical activity and consumption of "Western diet" rich in fat and refined carbohydrates [1; 2]. Consumption of foods rich in dietary fibre has been suggested as a potential dietary preventative measure for obesity-linked metabolic syndrome arising from a high calorie diet, rich in saturated fats and refined sugars [1; 3; 4]. Individuals with high dietary fibre intake that includes cereal fibre, fruit fibre, barley ß-glucans, guar gum, oat ß-glucans, pectin and psyllium have shown decreased occurrence of metabolic symptoms such as coronary heart disease, stroke, hypertension, type 2 diabetes, obesity and gastrointestinal diseases [3; 4]. Also, dietary fibre supplementation promotes weight loss in obese individuals by energy intake reduction [3; 4; 5]. The average dietary fibre intake among US children and adults is less than half of the recommended dietary fibre intake (14g/1000 kcal) and is associated with increased risk for development of obesity and its associated metabolic disorders [3; 6]. The mean daily dietary fibre intake among the Japanese populations in 1952 of 20.5g/day declined by 27% between by 1998 to make this change the major health problem among the Japanese population for the future generations [7].

Inulin and oligofructose, containing β (2→1) glucosidic linkages, are the major non-digestible/fermentable soluble fibres of Jerusalem artichoke, chicory root, garlic and dahlia tubers [8; 9]. Inulin-type fructans resistant to human digestive enzymes (α-glucosidase, maltase, isomaltase and sucrase) are fermented by the colonic microbes. They promote the growth of *Bifidobacterium* and *Lactobacillus* species that have been suggested to exhibit a beneficial role in

human health by reducing the risk factors of diseases such as osteoporosis, atherosclerosis, type 2 diabetes and colon cancer [8; 10; 11]. Inulin and oligofructose treatment in either lean or obese mice decreased the energy intake by 6% and 11% via up-regulation of satiety hormones such as glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) and has been suggested to have a potential therapeutic effect in treatment of obesity [12]. High fat-fed rats treated with oligofructose showed increased colonic and portal plasma expression of glucagon like peptide-1 (GLP-1), reduced energy intake oncomitant with body weight gain and fat mass accumulation [13; 14]. In humans, oligofructose consumption (16 g/day) attenuated the energy intake via up-regulation of satiety hormones such as GLP-1 and PYY [15]. Furthermore, it has been suggested that reduced adipocyte differentiation and the adipocyte cell size in high fat-fed mice receiving inulin-type fructan dietary supplements is mediated by diminishing the over-expression of G-protein coupled receptor 43 (GPR43) in subcutaneous adipose tissue [16]. Additionally, oligofructose supplementation promotes weight loss via reducing the energy intake and improving glucose utilisation in overweight adults [17]. Inulin-type fructans improve glucose tolerance by increasing the GLP-1 expression which promotes the insulin secretion by enhancing the proliferation of pancreatic beta-cells [18; 19]. Daily intake of inulin (10 g/day) in healthy middle aged men and women lowered the fasting insulin concentrations after 4 weeks of treatment [20]. Similarly, oligofructose supplementation (10% of the diet) reduced the serum concentrations of both insulin and glucose in rats [21]. Furthermore, inulin and oligofructose (10% of the diet) treatment also increased the erythrocyte glucose uptake in rats [22]. Rodent studies suggested the hypotriglyceridaemic effect of inulin and oligofructose may be attributed to the inhibition of hepatic *de novo* lipogenesis via down-regulating the lipogenic enzymes [23; 24]. *In vivo* and *in vitro* studies using rat models showed that propionate, a short chain carboxylic acid released during the colonic fermentation of inulin-type fructans, inhibits fatty acid synthesis by reducing the mRNA expression of fatty acid synthase [23; 25]. This hypotriglyceridaemic effect of inulin-type fructans has been suggested as the mechanism for their beneficial actions in preventing obesity-linked non-alcoholic fatty liver disease [23; 25]. Stimulating the growth of *Bifidobacterium* species [26; 27; 28] can reverse the metabolic endotoxaemia associated with the obesity, inflammation and insulin resistance of the metabolic syndrome. Stimulating the proliferation of *Bifidobacteria* improves the gut barrier function via attenuating the bacterial/endotoxin translocation and also enhances colonic epithelial mucosal layer thickness, villi height and crypt depth [29; 30].

In summary, although the biological effects of inulin-type fructans have been evaluated using different models in experimental animals and humans, the physiological effects of inulin and oligofructose as dietary fibre in obesity-linked metabolic syndrome remains relatively unexplored. In this study, we investigated the effect of an inulin and oligofructose mixture (Synergy1) on cardiovascular, gut morphology, hepatic and metabolic parameters in a well-characterised [31] diet-induced model of human metabolic syndrome.

Materials and methods

Synergy1 mixture

Orafti(R)Synergy1 is a oligofructose-enriched inulin mixture [32] supplied by Invita Australia Pty Ltd, NSW 2093, containing 92±2 g of oligofructose+inulin, 8±2 g of sugars (glucose+fructose+sucrose) with ash (sulphated) content <0.2g in each 100g. The caloric value of the Synergy1 mixture is 693 kJ/100 g of powdered mixture.

Rats and diets

The experimental groups consisted of 48 male Wistar rats (7-8 weeks old; starting body weight 336.3 ± 0.5 g) supplied by The University of Queensland Biological Resources unit and individually housed in a temperature-controlled (20±2°C), 12-hour light/dark cycle environment with *ad libitum* access to water and the group-specific rat diet at the University of Southern Queensland Animal House. All experimentation was approved by the Animal Experimentation Ethics Committees of the University of Queensland and University of Southern Queensland under the guidelines of the National Health and Medical Research Council of Australia. Rats were randomly divided into 4 separate groups (n = 12 each) and fed with corn starch (C), corn starch + 5% Synergy1 (CSY1), high-carbohydrate, high-fat (H), high-carbohydrate, high-fat + Synergy1 (HSY1). Body weight, food and water intakes were measured daily and feed efficiency (%) was calculated as described in our previous study [31; 33]. The preparation and macronutrient composition of basal diets, including the dietary fatty acid profiles, have been described in detail previously [31; 34]. The Synergy1 mixture-supplemented diets were prepared by adding 5% of Synergy1 mixture replacing an equivalent amount of water in the diet. The Synergy1-supplemented diets were administered for 8 weeks starting 8 weeks after the initiation of the corn starch or high-carbohydrate, high-fat diet. The drinking water in all high-carbohydrate, high-fat-fed groups was augmented with 25% fructose for the duration of the study.

Cardiovascular measurements

Systolic blood pressure was measured as previously described [31; 33], under light sedation (Zoletil (tiletamine 15 mg/kg, zolazepam 15 mg/kg by intraperitoneal injection; 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).Echocardiographic examination (Hewlett Packard Sonos 5500, 12 MHz transducer) was performed at 16 weeks as previously described [31; 33]. Left ventricular (LV) function of rats in all treatment groups was assessed using the Langendorff heart preparation as previously described [31; 33]. Terminal anaesthesia was induced via i.p. injection of pentobarbitone sodium (Lethabarb®, 100 mg/kg). Following 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). Aortic contraction was determined using thoracic aortic rings (~4 mm in length) 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 [31; 33].

Oral glucose and insulin tolerance tests

For oral glucose tolerance test, basal blood glucose concentrations were measured in blood taken from the tail vein of overnight food-deprived rats using Medisense Precision Q.I.D glucose metre (Abbott Laboratories, Bedford, U.S.A). Fructose-supplemented drinking water in the H and HSY1 groups was replaced with normal water for the overnight food-deprivation period [31; 33]. 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 min following glucose administration. Insulin tolerance testing was performed after 5 h of food deprivation in rats given an intraperitoneal injection of 0.33 IU insulin/kg body-weight. Tail vein blood samples were taken at 15, 30, 45, 60, 90 and 120 min for blood glucose measurements after intraperitoneal insulin administration [31; 33].

Body composition measurements

Dual energy X-ray absorptiometric (DXA) measurements were performed on rats after 16 weeks of feeding, 2 days before rats were euthanised for pathophysiological assessments, using a Norland XR36 DXA instrument (Norland Corp., Fort Atkinson, USA). DXA scans were analysed using the manufacturer's recommended software for use in laboratory animals (Small Subject Analysis Software, version 2.5.3/1.3.1, Norland Corp., Fort Atkinson, USA) as previously described [31]. The precision error of lean mass for replicate measurements, with repositioning, was 3.2%. Visceral adiposity index (%) was calculated as in our previous study [31; 33].

Organ weights

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

Gut characteristics

The stomach, small intestine, caecum and colon were weighed and measured. Weights were normalised relative to the tibial length at the time of their removal (in mg/mm).

Lipid excretion and faecal output analysis

Rats from all 4 groups (n=6 each) including C, CSY1, H and HSY1 were individually housed in metabolic cages and fed with their respective diets over a period of 12 hours, while in H and HSY1 rats alone the drinking water was supplemented with 25% fructose. After 12 hours, faecal samples were collected from these groups (C, CSY1, H and HSY1) and measured for their dry weight. The collected faecal samples from each treatment groups were stored separately in an air tight sealed container at -20 degrees until further

determinations were performed. Freeze dried faecal samples were weighed and grounded using mortar and pestle. Dietary lipids were extracted from 1 g of powdered faeces by manual solvent extraction using a 2:1 chloroform/methanol mixture with 0.1% vitamin E as an antioxidant. The solvent and faeces were mixed on a rotating device for 40 min with 20 mL of chloroform/methanol solvent and then centrifuged at 2500 rpm for 5 min. The extraction procedure was repeated twice and subsequently washing with double distilled water to remove all polar material. Extracts were pooled and chloroform evaporated under a steam of nitrogen on a hot plate at 60^{o}C until the beakers reached constant weight allowing calculation of gravimetric extractable lipid content [35]. The percentage lipid excretion was calculated based on the formula [(Amount of lipid excreted in g/Total amount of lipid consumed in g)*100)]. The amount of extractable lipids present in either C or H diets, (6.2 and 187g/kg diet respectively), [36] was used for calculations.

Histology

Two rats per group were allocated 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 as previously described [36; 37]. 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 20X and fat vacuole enlargement with 20X objectives using a Olympus BX51 microscope (Olympus, Melville, NY). Collagen distribution was measured in the left ventricle with picrosirius red stain. Laser confocal microscopy (Zeiss LSM 510 upright Confocal Microscope) with colour intensity quantitated using NIH-imageJ software (National Institute of Health, USA) was used to determine the extent of collagen deposition in selected tissue sections [36; 37]. Faecal matter-free ileal tissue segments were removed from the mesenteric border with rinsing and fixed in a 10% neutral buffered formalin for 3 days and then dehydrated and embedded in paraffin wax as previously described [36; 37]. The ileal segments were cut at 5-μm-thickness and stained with haematoxylin-eosin for determination of infiltration of inflammatory cells, mucosal thickening, glands (crypts) cell proliferation and morphology with 20X objectives using a Olympus BX51 microscope (Olympus, Melville, NY) [38; 39].

Plasma analyses

Briefly, blood was centrifuged at 5,000x*g* for 15 min within 30 min of collection into heparinised tubes. Plasma was separated and transferred to Eppendorf tubes for storage at -20°C before analysis. Activities of plasma enzymes such as alanine transaminase (ALT) and aspartate transaminase (AST), and plasma analytes concentration including non-esterified fatty acids (NEFA), triglycerides and total cholesterol were determined using kits and controls supplied by Olympus using an Olympus analyser (AU 400, Tokyo, Japan) as previously described [36; 37].

Statistical analysis

All data are presented as mean ± SEM. Results were tested for variance using Bartlett's test and variables that were not normally distributed were transformed (using log 10 function) prior to statistical analyses. Data from C, CSY1, H and HSY1 groups were tested by two-way ANOVA. When interaction and/or the main effects were significant, means were compared using Newman-Keuls multiple comparison *post hoc* test. Where transformations did not result in normality or constant variance, a Kruskal-Wallis non-parametric test was performed. A *P*-value of <0.05 was considered as statistically significant. All statistical analyses were performed using Graph Pad Prism version 5.00 for Windows (San Diego, California, USA).

Results

Dietary intake, body weight and metabolic parameters

Food and water consumption was increased in corn starch treatment groups (C & CSY1), compared with H treatment groups (H & HSY1); supplementation with Synergy1 had no effect (Table1). Owing to the different energy densities of the diets, both diet and Synergy1 supplementation changed energy intake; in cornstarch-fed rats, Synergy1 mixture supplementation increased energy intake which, coupled with decreased body weight gain, decreased markedly feed conversion efficiency (Table 1). Compared to C fed rats, the high calorie diet fed H rats had higher energy intake with an increased body weight gain by 2.3 fold coupled with increased feed conversion efficiency percentage (Table 1). Synergy1 supplementation did not change the energy intake in HSY1 treated rats but reduced the body weight gain percentage by 2.6 fold with decreased feed conversion efficiency (Table 1).

H fed rats showed increased total body fat mass with no change in lean mass compared to C fed rats (Table 2). CSY1 treated rats did not show a change in total body fat mass while in HSY1 rats a reduced total body fat mass was observed compared to H fed control rats (Table 1). In addition to this, in the HSY1 treated rats reduced abdominal circumference (11%) corresponded with decreased total abdominal fat mass of 39% (as retroperitoneal fat, epididymal fat and omental fat mass) and reduced visceral adiposity index % (28%) (Table 2). No changes were observed in CSY1 treated rats compared to C rats (Table 2). The H diet fed rats showed impaired glucose and insulin tolerance compared with C fed groups (Table 2). Further, Synergy1 supplementation improved the impaired oral glucose tolerance and as well as the insulin tolerance in HSY1 treated rats (Table 2).

Gut weight, stool output and lipid excretion analysis

The H treatment groups showed increased total gut weight that includes stomach, small intestine, caecum and colon weight, compared with the C treatment groups. No change was observed in the total colon length measurements including proximal colon and distal colon length in either C or H treatment groups (Table 3). Synergy1 supplementation with either C or H diet increased the caecum weight with contents by 24-31% as well as the colon weight with contents by 43-76% in CSY1 and HSY1 treated rats (Table 3). Synergy1 supplementation with either C or H diet increased the stool weight by 1.9g in CSY1 treated rats and 1.7g in HSY1 treated rats (Table 3).

Compared with C treatment groups, increased faecal lipid excretion (%) was observed in H fed rats (Table 3). Synergy1 treatment further increased the

faecal lipid excretion (%) by 38% in HSY1 treated rats, compared with H fed rats (Table 3).

Table 1 Changes in dietary intakes, feed conversion efficiency and body weight gain %

Variable	C	CSY1	H	HSY1	*P*-Value		
					Diet	Treatment	Interaction
Food intake (g/d)	31.8±1.7[a]	33.0±2.2[a]	20.9±1.4[b]	20.8±1.2[b]	<0.0001	0.73	0.68
Water intake (ml/d)	31.2±1.7[a]	32.7±2.1[a]	18.7±1.5[b]	19.3±1.5[b]	<0.0001	0.54	0.78
Energy intake (kJ/d)	362.4±3.6[c]	389.3±5.5[b]	438.6±5.7[a]	446.6±5.5[a]	<0.0001	0.0017	0.07
Feed conversion efficiency (%)	2.4±0.1[b]	0.9±0.5[c]	4.6±0.3[a]	1.8±0.1[b]	<0.0001	<0.0001	0.09
Body weight gain %	8.7±0.3[b]	3.4±2.1[c]	20.2±1.3[a]	7.9±0.6[b]	<0.0001	<0.0001	0.0155

C, corn starch; CSY1, corn starch + Synergy1; H, high carbohydrate, high fat; HSY1, high carbohydrate, high fat + Synergy1**.** Each value is a mean ± SEM. Mean within a row with unlike superscript differ, P<0.05.Body weight gain percentage calculated as percentage of body weight increases from 8 weeks to 16 weeks for all groups.Ten animals per group.

Table 2 Body parameters, organ wet weights and visceral adipose index % measurements

Variable	C	CSY1	H	HSY1	*P*-Value		
					Diet	Treatment	Interaction
Bone mineral content (g)	12.3±0.4^{c}	11.9±0.3^{c}	16.4±0.5^{a}	13.8±0.6^{b}	<0.0001	0.0026	0.0231
Total fat mass (g)	95.2±4.9b^{c}	69.6±6.7^{c}	210.1±14.9^{a}	121.5±7.3^{b}	<0.0001	<0.0001	0.0021
Total lean mass (g)	279.0±2.7	299.9±7.9	279.1±10.9	308.5±7.4	0.581	0.0032	0.590
Abdominal circumference (cm)	18.2±0.2^{c}	17.9±0.2^{c}	21.9±0.3^{a}	19.4±0.2^{b}	<0.0001	<0.0001	<0.0001
Retroperitoneal fat (mg/mm)	122.6±9.8^{c}	117.5±20.8^{c}	359.5±33.9^{a}	195.4±16.4^{b}	<0.0001	0.0005	0.0009
Epididymal fat (mg/mm)	95.8±6.0^{c}	93.4±13.8^{c}	212.1±15.4^{a}	139.2±11.6^{b}	<0.0001	0.0040	0.0066
Omental fat (mg/mm)	56.8±4.5^{c}	53.4±6.2^{c}	116.5±7.7^{a}	84.3±6.9^{b}	<0.0001	0.0089	0.0315
Total Abdominal fat (mg/mm)	275.1±18.9^{c}	264.3±40.1^{c}	688.0±54.5^{a}	418.9±32.3^{b}	<0.0001	0.0009	0.0020
Visceral adiposity index (%)	3.3±0.2^{c}	3.2±0.4^{c}	6.5±0.5^{a}	4.7±0.4^{b}	<0.0001	0.0201	0.0361
Liverweight (mg/mm)	243.5±4.9^{c}	260.7±11.2b^{c}	342.6±11.7^{a}	285.3±10.3^{b}	<0.0001	0.050	0.0006
OGTT (AUC) (mmol/L min)							
Week 0	640.5±20.6	633.5±15.6	610.5±25.1	629.6±17.1	0.40	0.76	0.52
Week 8	654.8±9.6^{b}	660.0±14.5^{b}	826.1±6.9^{a}	845.1±11.4^{a}	<0.0001	0.277	0.533
Week 16	663.8±14.9^{c}	653.4±10.6^{c}	838.7±20.3^{a}	728.7±13.8^{b}	<0.0001	0.0004	0.0025
ITT (AUC) (mmol/L min)	187.4±24.4^{c}	155.2±11.4^{c}	531.0±18.5^{a}	323.9±11.0^{b}	<0.0001	<0.0001	<0.0001

C, corn starch; CSY1, corn starch + Synergy1; H, high carbohydrate, high fat; HSY1, high carbohydrate, high fat + Synergy1; OGTT, oral glucose tolerance; ITT, insulin tolerance; AUC, Area under the curve. Each value is a mean ± SEM. Mean within a row with unlike superscript differ, P<0.05, eight to ten animals per group.

Table 3 Gut weight, stool weight and lipid excretion measurements

Variable	C	CSY1	H	HSY1	*P*-Value		
					Diet	Treatment	Interaction
Stomach (mg/mm)	80.4±9.5^{b}	89.0±8.6^{b}	133.2±12.9^{a}	118.9±13.7^{ab}	0.052	0.566	0.132
Small intestine (cm)	143.5±2.2	139.7±4.4	148.8±1.8	142.2±1.7	0.165	0.067	0.614
Proximal colon (cm)	4.7±0.1	5.7±0.3	4.7±0.3	5.8±0.4	0.867	0.0011	0.867
Distal colon (cm)	3.7±0.1^{a}	3.5±0.3^{ab}	3.0±0.1^{b}	3.3±0.1^{ab}	0.0135	0.775	0.158
Total colon (cm)	8.3±0.2	9.2±0.5	7.8±0.3	9.1±0.4	0.420	0.005	0.590
Small intestine (mg/mm)	216.7±9.8^{b}	209.9±18.5^{b}	260.3±5.3^{a}	231.2±8.3^{ab}	0.0081	0.130	0.342
Caecum (mg/mm)	80.9±5.5^{c}	105.6±4.0^{b}	101.1±5.5^{b}	124.8±5.0^{a}	0.0032	0.0003	0.497
Colon (mg/mm)	30.3±1.9^{c}	53.3±3.2^{a}	40.2±3.5^{b}	56.9±2.9^{a}	0.0275	<0.0001	0.2907
Total gut (mg/mm)	408.3±21.7^{b}	457.8±24.3^{b}	534.9±18.2^{a}	525.6±18.9^{a}	<0.0001	0.343	0.168
Fecal weight (g/12h)	1.08±0.14^{d}	2.95±0.15^{b}	1.77±0.11^{c}	3.42±0.19^{a}	0.001	<0.0001	0.47
Lipid excretion (%)	7.37±0.97^{c}	8.92±0.45^{c}	14.37±0.61^{b}	19.81±1.52^{a}	<0.0001	0.002	0.06

C, corn starch; CSY1, corn starch + Synergy1; H, high carbohydrate, high fat; HSY1, high carbohydrate, high fat + Synergy1; Each value is mean ± SEM. Means within a row with unlike superscript differ, P<0.05. *Eight to ten animals per group. Tissue wet weights were normalized with the tibial length in mg/mm.

Systolic blood pressure and cardiovascular changes

Systolic blood pressure was unchanged in the C treatment groups. Compared to H fed rats, an increased systolic blood pressure was normalised in HSY1 treated rats (Table 4). Further, no change in cardiac structure and function from echocardiographic examination was observed in either CSY1 or HSY1 treatment groups, compared with the control groups (Table 4). Compared to C fed groups, an increase in both the left ventricular septum weight and the total heart weight was observed in H fed rats whereas no change was observed in HSY1 treated rats (Table 4). The diastolic stiffness constant (κ) value that determines the cardiac stiffness was normalised in HSY1 treated rats, compared with H fed rats (Table 4).

Table 4 Changes in cardiovascular structure and function

Variable	C	CSY1	H	HSY1	*P*-Value		
					Diet	Treatment	Interaction
LVIDd (mm)	7.73±0.22	7.71±0.12	7.87±0.18	7.95±0.18	0.295	0.868	0.781
Heart rate	308.0±24.1[a]	237.0±9.0[b]	301.3±17.2[a]	259.9±12.3[ab]	0.617	0.0014	0.363
IVSd (mm)	1.92±0.07	1.88±0.03	2.03±0.04	1.90±0.01	0.145	0.060	0.308
IVSs (mm)	3.07±0.13	3.17±0.10	3.19±0.16	2.89±0.10	0.527	0.430	0.121
LVIDs (mm)	4.28±0.31	4.10±0.22	4.17±0.21	4.17±0.41	0.947	0.765	0.765
LVPWs (mm)	2.79±0.07	2.99±0.28	2.96±0.11	2.95±0.11	0.695	0.567	0.527
Diastolic volume (μL)	493.0±39.2	481.0±22.4	517.0±34.7	532.0±35.9	0.275	0.965	0.691
Systolic volume (μL)	90.00±15.5	77.0±13.3	80.0±12.4	90.0±18.7	0.922	0.922	0.455
Stroke volume (μL)	402.0±28.6	404.0±17.7	437.0±29.3	442.0±30.3	0.187	0.898	0.956
Cardiac output, (mL/min)	119.8±4.9	96.2±6.4	130.4±9.8	116.6±13.6	0.107	0.054	0.602
LVPWd (mm)	1.79±0.11	1.88±0.03	1.99±0.04	1.83±0.03	0.238	0.578	0.054
Relative wall thickness	0.48±0.02	0.49±0.01	0.51±0.01	0.47±0.01	0.708	0.267	0.069
Systolic wall stress	94.2±7.2	85.2±9.4	107.2±8.0	85.9±8.6	0.418	0.080	0.467
Fractional shortening (%)	45.0±3.0	49.3±1.1	47.2±2.1	46.5±1.7	0.887	0.397	0.242
Ejection fraction (%)	82.4±2.5	84.3±2.2	84.8±1.8	83.4±3.0	0.758	0.918	0.500
Diastolic stiffness (κ)	22.9±0.6[b]	22.9±0.5[b]	27.8±0.5[a]	22.6±1.2[b]	0.0045	0.0015	0.0015
Estimated LV mass (g)	1.09±0.09	1.09±0.04	1.25±0.06	1.14±0.03	0.089	0.364	0.364

LV+septum wet weight* (mg/mm)	16.79 ± 0.36^{b}	16.21 ± 1.06^{b}	19.63 ± 0.57^{a}	17.82 ± 0.60^{ab}	0.0012	0.219	0.665
RV wet weight* (mg/mm)	2.24 ± 0.10	2.54 ± 0.18	2.76 ± 0.16	2.39 ± 0.23	0.343	0.689	0.030
Heart wet weight (mg/mm)	19.04 ± 0.35^{b}	18.75 ± 1.20^{b}	22.39 ± 0.67^{a}	20.27 ± 0.71^{ab}	0.0041	0.1374	0.2562
Systolic blood pressure (mmHg)	124 ± 2^{b}	118 ± 2^{b}	152 ± 2^{a}	123 ± 3^{b}	<0.0001	<0.0001	<0.0001

C, corn starch; CSY1, corn starch + Synergy1; H, high carbohydrate, high fat; HSY1, high carbohydrate, high fat + Synergy1; LV, left ventricle; LVIDd, left ventricular internal diameter thickness in diastole; LVIDs, left ventricular internal diameter thicknessin systole; IVSd, inter ventricular septum thickness in diastole; IVSs, inter ventricular septum thickness in systole; LVPWd, leftventricular posterior wall thickness in systole; LVPWs, left ventricular posterior wall thickness in systole. Each value is mean ± SEM. Means within a row with unlike superscript differ, P<0.05. Tissue wet weights were normalised with the tibial length in mg/mm. *Eight to ten animals per group.

Vascular responses in thoracic aortic rings

Among all the treatment groups, no change in contractility in isolated thoracic rings to noradrenaline was observed (Figure 1A). Compared to C treatment groups, the H fed rats showed a diminished endothelium-dependent vascular response in thoracic aortic rings to acetylcholine and also a diminished endothelium-independent vascular response in thoracic aortic rings to sodium nitroprusside (Figure 1B,C). Synergy1 supplementation normalised the vascular response in thoracic aortic rings to acetylcholine as well as to sodium nitroprusside in HSY1 treated rats, compared with H diet fed rats (Figure 1B,C).

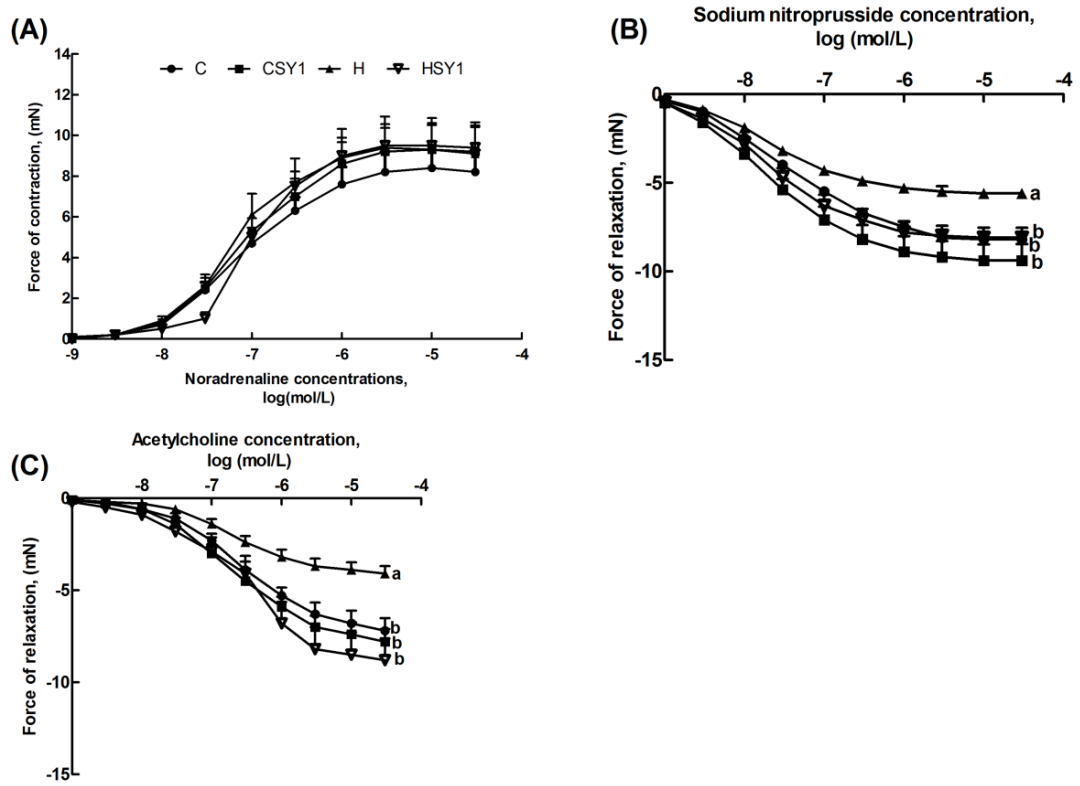

Fig. 1. Cumulative concentration-response curves for noradrenaline (A), sodium nitroprusside (B) and acetylcholine (C) in thoracic aortic rings from rats fed with corn starch (C), corn starch + synergy1 (CSY1), high carbohydrate, high fat (H), high carbohydrate, high fat + synergy1 (HSY1) fed rats. Values are means for ten rats per group, with standard errors represented with vertical bars. Mean values with an unlike letter were significantly different (P<0.05).

Plasma biochemistry

In C treatment groups, increased plasma concentrations of non esterified fatty acids (NEFA) by 77.5% were observed in CSY1 treated rats compared to C fed rats. H fed rats showed increased plasma NEFA and triglycerides concentration compared to C and CSY1 groups (Table 5). Compared with H fed rats, plasma concentrations of NEFA and triglycerides were normalised in HSY1 treated rats (Table 5). Additionally, increased plasma total cholesterol concentration was observed in either H or HSY1 rats, compared to the C treatment groups (Table 5). The H diet fed rats showed increased plasma activity of alanine transaminase (ALT) and aspartate transaminase (AST) compared to C treatment groups (Table 5). Synergy1 supplementation normalised the plasma ALT and AST activity in HSY1 treated rats (Table 5).

Table 5 Plasma biochemistry

Variable	C	CSY1	H	HSY1	*P*-Value		
					Diet	Treatment	Interaction
NEFA (mmol/L)	1.65±0.27[a]	2.93±0.47[b]	5.12±0.33[a]	3.78±0.51[b]	0.0018	0.192	0.096
Triglycerides (mmol/L)	0.49±0.07[b]	0.54±0.09[b]	2.69±0.43[a]	0.65±0.11[b]	0.0054	0.0071	0.0238
Total cholesterol (mmol/L)	1.54±0.06[b]	1.58±0.09[b]	1.99±0.04[a]	1.88±0.08[a]	0.0011	1.000	0.822
ALT activity (U/L)	29.63±2.81[b]	32.14±2.93[b]	43.93±2.55[a]	33.59±1.96[b]	0.672	0.322	0.951
AST activity (U/L)	74.43±3.21[b]	74.89±4.23[b]	96.81±7.23[a]	70.60±3.16[b]	0.0129	0.0151	0.0017

C, corn starch; CSY1, corn starch + Synergy1; H, high carbohydrate, high fat; HSY1, high carbohydrate, high fat + Synergy1; Each value is mean ± SEM. Means within a row with unlike superscript differ, P<0.05. *Eight to ten animals per group.

Histology

Compared with C treatment groups, increased collagen deposition and inflammatory cells infiltration was observed in the myocytes of H fed rats (Fig. 2(E),2(F)&2(G); Fig. 2(A),2(B)&2(C)). Increased inflammatory cells infiltration and enlarged fat vacuoles of hepatocytes was also observed in the H diet fed rats, compared to C treatment groups (Fig. 3(E),3(F)&3(G); Fig. 3(A),3(B)&3(C)). Synergy1 treatment in H diet fed rats showed no signs of collagen deposition in left ventricle as well as enlarged fat vacuoles in hepatocytes (Fig. 2(H); Fig. 3(D)). The inflammatory cells infiltration of both heart and liver was attenuated in the HSY1 treated rats (Fig. 2(D); Fig 3(H)). The ileum of H diet fed rats showed increased infiltration of inflammatory cells, elongated crypt cells and diminished epithelial mucosal thickening, compared to C treatment groups (Fig. 4(A), 4(B)&4(C)). SY1 supplementation attenuated the intestinal inflammation in high-fat fed rats showing no noticeable signs of ileal infiltration of inflammatory cells (Fig. 4(D)). Improved gut morphology showing enhanced crypt cells proliferation with developed villi and mucosal thickening was detected in HSY1 treated rats (Fig. 4(D)).

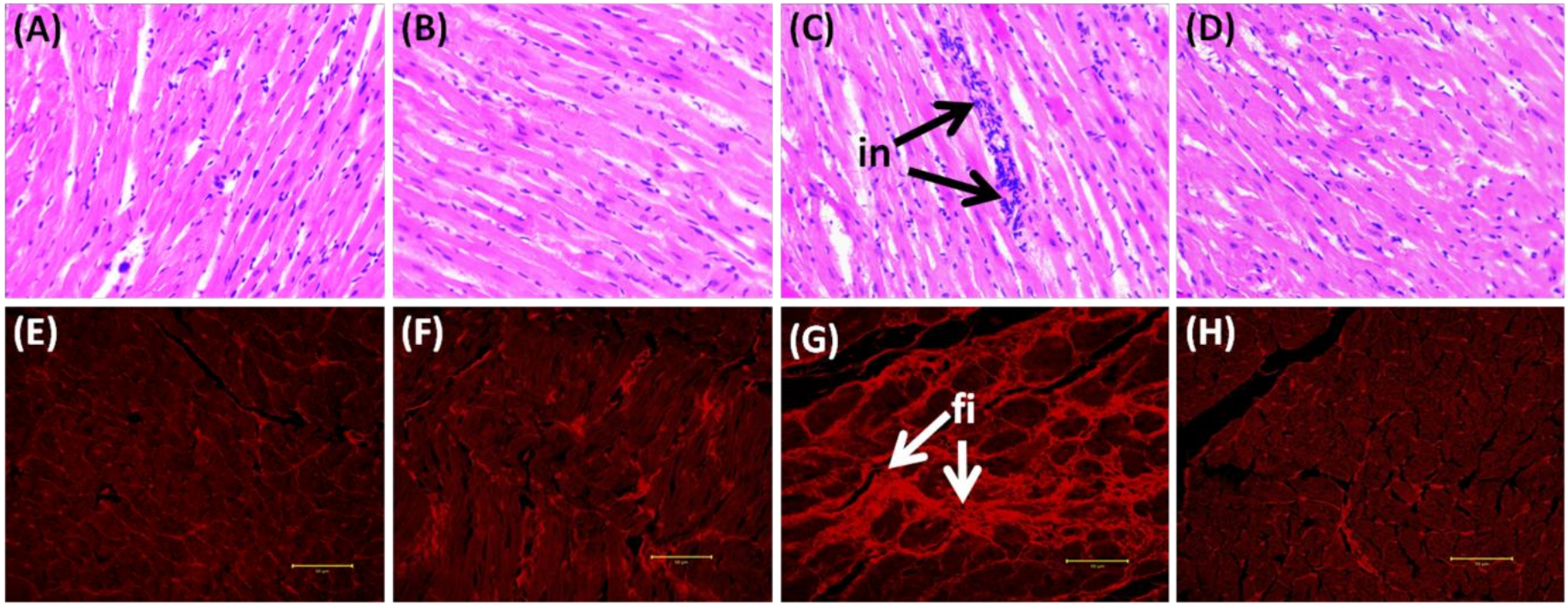

Fig. 2. Haematoxylin and eosin staining of left ventricle (x20) showing infiltration of inflammatory cells (A–D, inflammatory cells marked as "in") in rats fed with corn starch diet (A), cornstarch diet + Synergy1 (B), high carbohydrate, high fat diet (C), high carbohydrate, high fat diet+ Synergy1 (D). Picrosirius red staining of left ventricular interstitial collagen deposition (E–H, fibrosis marked as "fi")(20x) in corn starch diet (E), cornstarch diet + Synergy1 (F), high carbohydrate, high fat diet (G), high carbohydrate, high fat diet + Synergy1 (H).

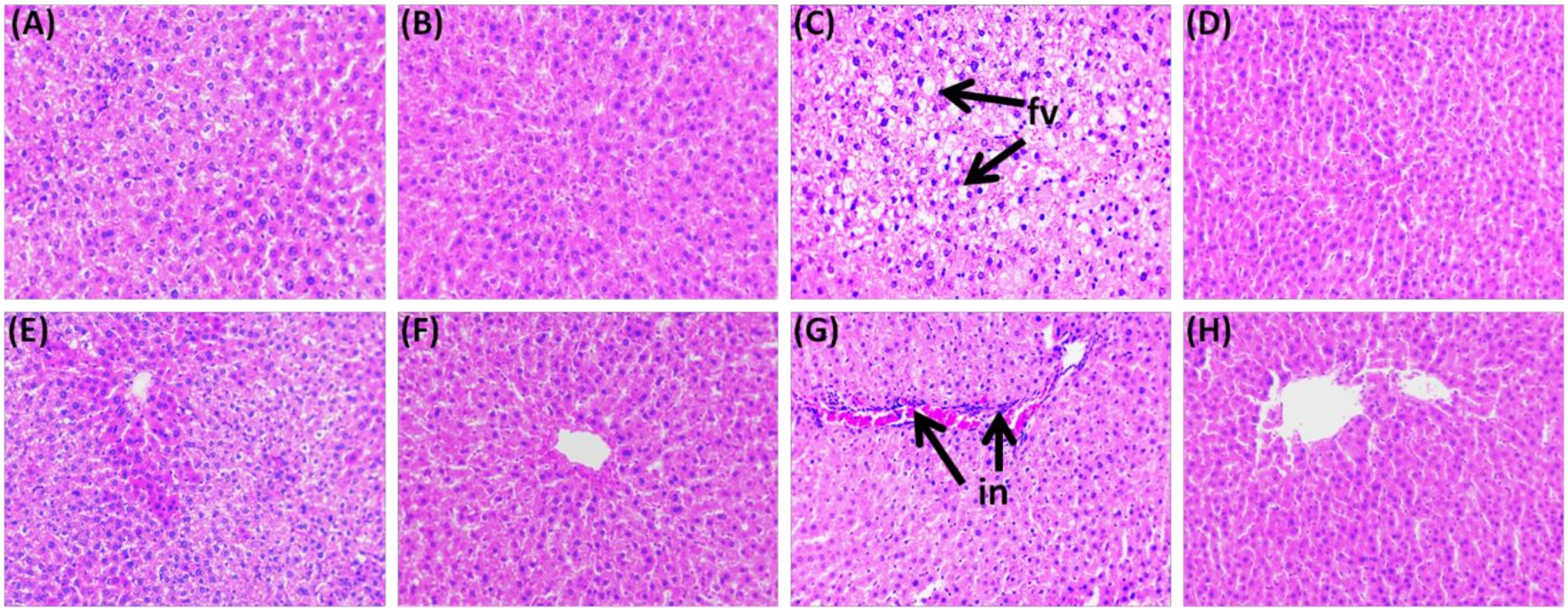

Fig. 3. Haematoxylin and eosin staining of hepatocytes (x20) showing hepatocytes with enlarged fat vacuoles (A–D, fat vacuoles marked as “fv”) and inflammatory cells infiltration (E-H, inflammatory cells marked as “in”) (20x) in rats fed with corn starch diet (A, E), cornstarch diet + Synergy1 (B,F), high carbohydrate, high fat diet (C,G), high carbohydrate, high fat diet + Synergy1 (D,H).

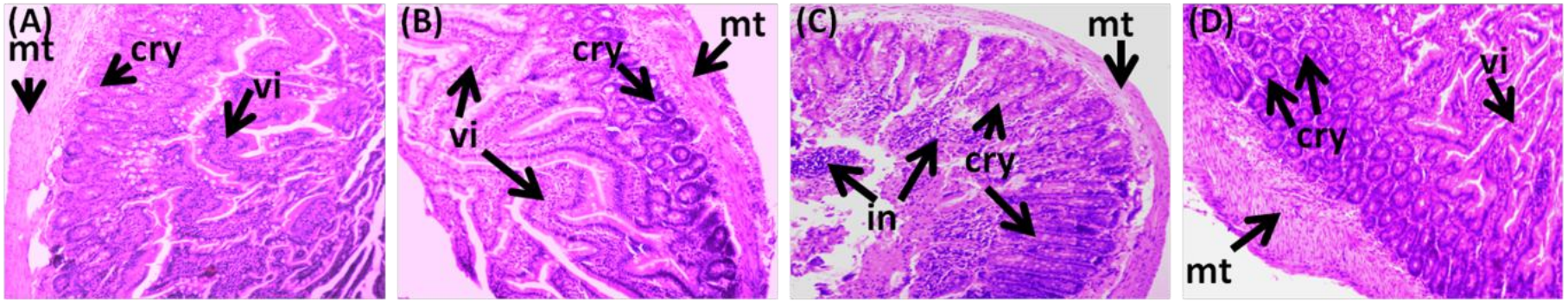

Fig. 4. Haematoxylin and eosin staining of ileum (x20) showing ileum with inflammatory cells infiltration (A-D, inflammatory cells marked as “in”), crypt cells (A-D) marked as “cry”, villi (A-D) marked as “vi” and mucosal thickening (A-D) marked as “mt” in rats fed with corn starch diet (A, E), cornstarch diet + Synergy1 (B,F), high carbohydrate, high fat diet (C,G), high carbohydrate, high fat diet + Synergy1 (D,H).

Discussion

Previous studies using rodents and human subjects have shown that prebiotic fibre (inulin and oligofructose) supplementation prevented the weight gain and the fat mass development by reducing the food intake via up-regulating the expression of satiety hormones such as glucagon-like peptide (GLP-1) and peptide YY (PYY) [15; 19; 40]. The present study found that dietary supplementation with 5% inulin:oligofructose did not affect the food intake in either corn starch or high carbohydrate-high fat-fed rats, but nevertheless attenuated weight gain. This appears to be due to increased faecal loss (1.7-1.9g/12h) possibly due to an increase in colonic motility [41] enhancing energy excretion [42]. Further, increased faecal lipid excretion in high fat rats treated with Synergy1 mixture is indirect evidence of inhibition of intestinal fatty acid absorption [41]. To compensate this faecal energy loss accompanied by increased faecal lipid excretion, the total energy expenditure via increased oxidation of endogenous fat may be triggered [43; 44] with the Synergy1 mixture supplementation showing reduced total body fat and abdominal fat mass development in HSY1 rats [45] (Table 2). Tissue partioning was also improved marginally by Synergy1 supplementation with fat mass not only being decreased but lean mass increased, 7% and 10% for cornstarch-fed and fat-fed rats respectively. Rodent studies showed that oligofructose treatment (10% or 20% of diet) reduced the postprandial glycaemia and insulinaemia [8]. Inulin supplementation (10% of diet) lowered the blood glycaemic response in healthy human volunteers [46]. In our study, SY1 supplementation improved the glucose utilisation and as well as the insulin-stimulated glucose utilisation in H rats (Table 2). Though the anti-hyperglycaemic mechanism of inulin and oligofructose is not clearly understood [8], this study suggests that SY1 mixture supplementation may slow down the digestion and the absorption of digestible carbohydrates by shortening the small intestinal transit time [8; 41]. Also possibly SY1 mixture may inhibit the hepatic glucose production via enhancing the production of short chain carboxylic acids especially propionic acid [47; 48]. Propionic acid indirectly inhibits the hepatic glucose production by decreasing the plasma free fatty acid concentrations [8; 49]. In our study compared with H control rats, a 26% decrease in plasma free fatty acids concentration was observed in the HSY1 rats (Table 5).

The anti-hypertensive effect (Table 4) of Synergy1 treatment coupled with improved vascular function (Fig. 1(B), (C)) has been associated with the decreased total abdominal fat mass (39%) which includes retroperitoneal, epididymal and omental fat (Table 2) [50], where visceral depots serves as the major site of secreting angiotensinogen and angiotensin-converting enzymes (ACEs) [51; 52]. Synergy1 treatment with its hypoglycaemic effect prevented cardiac stiffness (Table 4) and its associated collagen deposition in the HSY1 treated myocytes (Fig. 2G), which may be due to decrease in protein kinase C (PKC) expression and inhibition of collagen deposition induced by the transforming growth factor-1 (TGF-1) [53; 54].

Consumption of high-fat diet gradually slows down the gastric emptying due to the suppression of gastrointestinal motility [55; 56]. In our study, the high-fat (as 24%) [34] diet fed control rats showed increased total gut weight by 31% compared to the low-fat (as 0.8%) [34] cornstarch diet fed rats [46] (Table 3). However, Synergy1 mixture treatment showed no significant changes in the total gut weight but increased the caecal weight with contents in CSY1

(23%/normalized wet weight) and HSY1 (19%/normalized wet weight) rats (Table 3). This increase in caecal wet weight may be due to an increase in caecal pool size which is mainly accelerated by the colonic fermentation of Synergy1 (inulin and oligofructose) mixture that generates the short chain carboxylic acids (acetate, butyrate and propionate) [57] and decreases the caecal pH [8; 58]. This increased fermentation action of oligofructose-enriched inulin (Synergy1) mixture could increase the colon weight in CSY1 (43%) or HSY1 (29%) rats by enhancing the intestinal colonic motility [59]. As a result, increased stool production by 1.7-1.9g/12h was observed in the CSY1 and HSY1 rats (Table 3) [8; 41].

Though inulin and oligofructose on its own is well known for its hypolipidaemic effect [20; 57], in our study we suggest Synergy1 mixture supplementation attenuated the visceral obesity induced dyslipidaemia[60] in the HSY1 rats. However, no changes in plasma total cholesterol concentrations were detected in the HSY1 treated rats (Table 5). Synergy1 mixture treatment prevented the abdominal obesity associated hepatic fat accumulation [61] (Fig. 3(H)) and improved the liver function (Table 5).

Increased visceral adiposity index percentage by 3.2-3.3% in the H fed control rats (Table 2) may infiltrate more macrophages associated with stimulation of adipocyte-derived inflammatory cytokines such as interleukin-6 (IL-6), tumour necrosis factor- alpha (TNF-α), plasminogen activator inhibitor-1 (PAI-1) and nuclear transcription factor κ-B (NF-κ B) [62]. Therefore, H fed control rats showed enhanced visceral adiposity with increased infiltration of inflammatory cells in both heart (Fig. 2(C)) and liver (Fig. 3(G)). Synergy1 supplementation attenuated the visceral adiposity induced infiltration of inflammatory cells in both heart (Fig. 2(D)) and liver (Fig. 3(H)) of HSY1 rats [62].

A diminished mucosal wall thickness and higher inflammation of intestinal ileum (Fig. 4(C)) detected in H fed control rats may be due to the high-fat diet induced intestinal permeability of the lipopolysaccharide (LPS)-containing gut microbes [63]. Recruitment of LPS-containing gut microbes increases the circulating concentrations of LPS (metabolic endotoxaemia) [28; 65] that inhibits the expression of mucosal tight junction proteins ZO-1 and occludin [63; 64]. Synergy1 supplementation being bifidogenic in action [57] may enhance the growth of *Bifidobacteria* by inhibiting the H diet-induced obesity [66; 67], and therefore prevent the LPS-induced inflammation [64] of intestinal ileum in the HSY1 rats (Fig. 4(D)). *Bifidobacteria* that provides the healthier microvillus environment prevents the bacterial translocation of the (LPS)-containing gut microbes [63; 68; 69] and improves the intestinal barrier function [70]. Henceforth, an improved ileal wall thickness was detected in the HSY1 rats (Fig. 4(D)). The fermentable Synergy1 treatment may enhance the secretion of gut hormone glucagon like peptide-2 (GLP-2) and improve the ileal crypt cells proliferation (Fig. 4(D)) and villi morphology (Fig. 4(D)) in the HSY1 rats [67; 71]. As a future outcome, this study will examine the *Bifidobacteria* counts in the collected faecal samples of control groups (C and H) and Synergy1 treatment groups.

In conclusion, as an effective prebiotic inducing satiety, inulin and oligofructose has been suggested for the treatment of obesity and its associated metabolic disorders [12; 14]. The present study highlights for the first time the physiological effects of Synergy1 (inulin:oligofructose) mixture supplementation as dietary fibre [4; 9] in attenuation of diet-induced metabolic

syndrome. This study suggested that Synergy1 treatment by increasing the faecal load and faecal lipid excretion prevents the body weight gain and fat mass development specifically visceral obesity, and showed improved oral glucose and insulin tolerance, lowered systolic blood pressure, improved vascular function, improved lipid profiles and gut morphology.

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CHAPTER 8

Conclusion and future outcomes

Conclusions and future outcomes

Obesity associated with increased visceral adiposity serves as a key risk factor for metabolic complications such as insulin resistance, type 2 diabetes, hypertension, cardiovascular complications, dyslipidaemia and non-alcoholic fatty liver disease (Després and Lemieux, 2006, Lavie et al., 2009, Mathieu et al., 2009). Adaptation to "Western diet" among world populations (O'Dea, 1991, Ravussin et al., 1994, Novotny et al., 2009), which includes consumption of high calorie diet containing higher amounts of saturated and mono-unsaturated fats and refined carbohydrates plays a leading role in inducing obesity associated metabolic complications such as insulin resistance, type 2 diabetes, hypertension and cardiovascular disease (Cordain et al., 2005, Manzel et al., 2014). The high-carbohydrate (68% as fructose and sucrose), high-fat (24% as saturated and mono-unsaturated fat) diet mimicking "Western diet" in an established rat model of diet-induced metabolic syndrome (Panchal et al., 2011 b), showed increased fat mass development (both total body and visceral fat), insulin resistance, impaired glucose utilisation, hypertension, endothelial dysfunction, cardiovascular stiffness, collagen deposition, inflammation of heart and liver, dyslipidaemia and non-alcoholic fatty liver disease.

In **Chapter 1,** I discussed the effect of testosterone deprivation on the total body fat mass development and metabolic risk factors in prostate cancer patients who underwent androgen-deprivation therapy and aged men. Further, I highlighted the interdependency of visceral adiposity and testosterone deprivation in the pathogenesis of metabolic syndrome (Zitzmann, 2009) that led me to investigate the hypothesis that testosterone deficiency on its own could aggravate the visceral adiposity-induced metabolic syndrome in a diet-induced rat model mimicking human metabolic syndrome. Seaweed consumption, well accepted among Japanese, Chinese and Korean populations, showed lesser risk factors of metabolic syndrome (Brown et al., 2014). As a long-term lifestyle intervention for the obesity-linked metabolic syndrome management, I emphasised all possible bioactive nutrients such as dietary polysaccharides (as soluble and insoluble fibre), ω-3 fatty acids (eicosapentaenoic acid and docosahexaenoic acid), proteins, mineral ions (Mg, K, Ca, Fe and Zn) and bioactive carotenoids present in seaweeds and microalgal groups.

A recent population cohort study (1999-2010) on US adults (n=30,433) who had higher mean dietary fibre intake (16.3-17.0 g) showed a lower incidence of metabolic syndrome compared to the individuals with lower mean dietary fibre intake (14.5-15.5g) (Grooms et al., 2013). Additionally, as dietary fibre intake is reported for its cardiovascular benefits (Satija and Hu, 2012), Grooms et al (2013) suggested the need to increase the dietary fibre intake as a preventive measure of metabolic syndrome among US adults through various dietary strategies. Further, by highlighting the low magnesium status that influences the development of metabolic syndrome in US population (Rosanoff et al., 2012), there is a clear need to increase magnesium intake to meet an adequate intake of 200-420 mg/day for the prevention of metabolic syndrome in all age groups of the USA population (Rosanoff et al., 2012) .

Based on the literature evidence in **Chapter 1**, I found that both seaweeds and microalgae provide sufficient amounts of dietary fibre ranging from 16.4 to 74.6% of dry algae (Lahaye, 1991, Dvir et al., 2000) and magnesium content from 1.2 to 9.5% of dry algae (Fabregas and Herrero, 1986, Mcdermid and

Stuercke, 2003). Therefore, I suggested in **Chapter 1.1** (Kumar and Brown, 2013) that bioactive nutrients present in seaweeds and microalgae could provide an effective therapeutic effect in the attenuation of metabolic syndrome. To test this hypothesis, I investigated the effects of two-tropical marine seaweeds (*Ulva ohnoi* and *Derbesia tenuissima*) and a microalgal co-culture (*Scenedesmus dimorphus* and *Schroederiella apiculata*) in the high-carbohydrate, high-fat diet induced rat model of metabolic syndrome. Further, in understanding the therapeutic effects of magnesium in the prevention of metabolic syndrome, in **Chapter 1** I discussed all possible biological effects of magnesium treatment in the reversal of metabolic symptoms such as insulin resistance, type 2 diabetes and hypertension. I identified that magnesium supplementation in the treatment of obesity-linked metabolic syndrome remains unexplored. Therefore, I investigated the effect of high magnesium supplementation in the treatment of metabolic syndrome in the diet-induced obese rats. Since magnesium and potassium ion homeostasis are interlinked (Altura and Altura, 1984), I observed that no research study has targeted the interdependent mechanisms of magnesium and potassium in the attenuation of metabolic complications (Resnick et al., 2001). This observation convinced me to extent my investigation study further on high potassium intake and the prevalence of metabolic syndrome in the diet-induced obese rats.

Although inulin and oligofructose are well accepted as prebiotics (Kaur and Gupta, 2002), still their physiological effects as a soluble dietary fibre supplementation (Cherbut, 2002) in prevention of obesity related metabolic complications remains poorly understood. So, I investigated the effect of Synergy1 (inulin:oligofructose) mixture as a soluble dietary fibre in the high-carbohydrate, high-fat diet induced rat model of metabolic syndrome.

Chapter 3 in this thesis showed testosterone deprivation induced by either surgical castration or chemical castration with leuprolide acetate (a gonadotrophin-releasing-hormone (GnRH)-receptor agonist) increased the visceral obesity and its associated risk factors such as hypertension, insulin resistance, impaired glucose utilisation, cardiovascular stiffness with collagen deposition, inflammation, non-alcoholic fatty liver disease and dyslipidaemia in the diet-induced obese rats. Thus, my research findings emphasised the need of targeting the visceral adiposity development as a preventive measure of metabolic syndrome in the testosterone-deprived prostate cancer patients. Also, as a long-term therapeutic intervention, I would strongly recommend consuming "food as medicine" for the better control of metabolic symptoms in prostate cancer patients following androgen deprivation therapy.

In **Chapter 4** in this thesis, I explored the therapeutic effect of two Australian-grown tropical marine seaweeds *Ulva ohnoi* and *Derbesia tenuissima* in the high-carbohydrate, high-fat diet induced rat model of metabolic syndrome. *Ulva ohnoi* as an effective dietary supplement of soluble fibre and magnesium attenuated the total body fat mass development and prevented the signs of metabolic syndrome such as hypertension, endothelial dysfunction, diminished insulin sensitivity and glucose utilization, increased cardiac stiffness, increased collagen deposition and increased liver damage in the diet-induced obese rats. The insoluble fibre present in *Derbesia tenuissima* is suggested to improve the diminished glucose utilisation and insulin sensitivity, and prevent dyslipidaemia, and infiltration of inflammatory cells in the heart and liver of obese rats. In **Chapter 5,** the insoluble fibre supplementation of microalgae

polyculture mixture (*Scenedesmus dimorphus* and *Schroederillea apiculata*) increased the lean mass and attenuated the visceral fat induced metabolic syndrome in diet-induced obese rats.

My seaweed and microalgae study results highlight the possible biological effects of mineral ions, particularly magnesium, and dietary polysaccharides (fibre) in attenuating the obesity-linked metabolic syndrome. The therapeutic responses to seaweeds and microalgae are suggested to be unique because they rely on a high fibre content, compared to our previous studies on natural polyphenolic compounds such as rutin and caffeine in the attenuation of diet-induced metabolic syndrome (Panchal et al., 2011, Panchal et al., 2012b).

As a strategy to increase the dietary fibre intake in the prevention of metabolic syndrome (Grooms et al., 2013), dietary supplementation of algal fibre (16.4-74.6g/100 g of wet algae) (Lahaye, 1991, Dvir et al., 2000) with the regular fibre diets (0.8-8.9 g/100 g (w/w)) which includes brown rice, prunes, porridge, lentils green/brown, cabbage, carrots, apples and bananas (MacArtain et al., 2007) could supply the recommended dietary fibre intake of 25-50g/day for metabolically sick patients (Anderson et al., 2004). Seaweeds contribute approximately 21% of Japanese meals (Joshinaga et al., 2001, Zava and Zava, 2011). I would suggest that increasing dietary fibre supplementation of seaweeds to about 25-40% (w/w) with the regular fibre (8.9 g/100 g (w/w)) diets would serve the recommended daily dietary fibre intake of 25-35g in the metabolically sick patients. Similarly, the dietary magnesium supplementation (98-574 mg/100 g of wet algae) from seaweeds together with the magnesium consumption from regular diets (11-520mg/100 g (w/w)) which includes brown rice, whole milk, cheddar cheese, lentils green/brown, spinach, bananas, brazil nuts and peanuts (MacArtain et al., 2007) could meet the recommended magnesium intake of 240-420 mg/day (Food and Nutrition Board, 1997) suggested for the better control of metabolic syndrome in the US population (Rosanoff et al., 2012). Further, seaweeds supplementation (21% (w/w)) with the regular magnesium (520mg/100 g (w/w)) diets could meet the highest percentile of recommended dietary magnesium intake of 431-531.3 mg/day to lead a healthy lifestyle. In addition to Japanese, Chinese and Korean populations (Jensen, 1993, Matsumura, 2001), consumption of seaweeds (20-40% (w/w)) supplemented diet is recommended to all populations as seaweeds are usually served in cooked form that flavour the dishes (Mabeau and Fleurence, 1993, Zava and Zava, 2011). Additionally, with my research findings, I would strongly recommend that consumption of seaweeds and microalgae could meet the emerging demands of all essential dietary supplements which include fibre (soluble and insoluble fibre), magnesium, potassium, calcium, omega-3 polyunsaturated fatty acids, bioactive carotenoids such as β-carotene, astaxanthin, fucoidan, fucoxanthin and lutein in the treatment of metabolically sick patients (MacArtain et al., 2007, Teas et al., 2009). Further, large scale aquaculture production of edible seaweeds and microalgae containing all essential nutrients could be achieved efficiently using standard culture methods (Borowitzka, 1992, Lawton et al., 2013b, Magnusson et al., 2014). Apart from waste water treatment (Abdel-Raouf et al., 2012), I strongly support our collaborators from the James Cook University for their initiatives to increase the algal biomass production commercially (Lawton et al., 2013a, Lawton et al., 2014, Carl et al., 2014) that could be further investigated for its biological significance in the attenuation of metabolic

complications such as type 2 diabetes, obesity, insulin resistance, hypertension and cardiovascular disease (Gomez-Gutierrez et al., 2011, Lordan et al., 2011). With an increase in algal biomass production, the awareness to know the benefits and consumption of seaweed intake to lead a healthy lifestyle would become more popular in both developed and developing countries.

In **Chapter 6,** I investigated the therapeutic effect of high magnesium (as magnesium chloride) and potassium (as potassium chloride) supplementation in the diet-induced obese rats. High magnesium treatment attenuated obesity and its associated metabolic symptoms such as hypertension, endothelial dysfunction, diminished insulin sensitivity and glucose utilisation, increased cardiac stiffness, increased collagen deposition, dyslipidaemia and increased liver damage. While the high potassium supplementation showed no change in fat mass development but prevented the diminished insulin sensitivity with impaired glucose tolerance, hypertension, collagen deposition and increased cardiac stiffness, and dyslipidaemia in the high fat rats. In addition to these physiological effects, increased plasma magnesium and potassium ion concentrations observed in either magnesium chloride or potassium chloride treated obese rats, suggested possible interdependent therapeutic effects in reversing the diet-induced metabolic syndrome.

In **Chapter 7,** I showed the possible physiological effects of Synergy1 (inulin and oligofructose) mixture supplementation as soluble dietary fibre in attenuating the diet-induced metabolic syndrome in obese rats. Synergy1 treatment by increasing the fecal load and fecal lipid excretion, and also possibly by increasing the *Bifidobacteria* counts, prevented the obesity and its associated metabolic risk factors such as impaired glucose and insulin tolerance, hypertension, endothelial dysfunction, cardiovascular stiffness and collagen deposition, dyslipidaemia, non-alcoholic fatty liver disease, intestinal ileal inflammation and impaired ileum morphology in high carbohydrate, high fat fed rats. As a lifestyle intervention, seaweeds (*Ulva ohnoi*, *Derbesia tenuissima*) and microalgae (*Scenedesmus dimorphus* and *Schroederiella apiculata*) including nutrient supplements magnesium, potassium and dietary fibre, could play an efficient therapeutic role in attenuating the obesity-linked metabolic syndrome.

In summary, testosterone deficiency aggravates the diet-induced metabolic syndrome via promoting visceral fat deposition. This research finding persuaded me to control the visceral adiposity development as an effective therapeutic treatment of metabolic syndrome. As a long-term treatment of obesity, lifestyle modifications are considered as essential compared to either pharmacological or surgical treatments (Glandt and Raz, 2011, Ritter et al., 2012). Further, as a lifestyle intervention (Ritter et al., 2012), healthy eating, so "Foods as medicine", has been emphasised by various research studies for the better management of obesity-related metabolic complications (Panchal et al., 2012a, Poudyal et al., 2013, Panchal et al., 2011a). In my research findings, dietary supplementation with microalgae mixture (*Scenedesmus dimorphus* and *Schroederiella apiculata*), magnesium and Synergy1 mixture (inulin:oligofructose) to prevent visceral obesity showed effective control of metabolic syndrome in the diet-induced obese rats, when compared to the other dietary interventions including potassium and seaweeds (*Ulva ohnoi* and *Derbesia tenuissima*) supplementation identified with no changes in visceral obesity. Thus, my research findings persuaded me to focus on novel

therapeutic targets for the prevention of visceral obesity-associated metabolic symptoms.

8.1 Future targets and outcomes

Algal polysaccharides were reported for their prebiotic effects showing resistance to digestion in the upper gastrointestinal tract and stimulation of the population growth of beneficial gut microbes such as *Bifidobacterium* species, *Bacteroidetes* and *Lactobacillus* species (Kuda et al., 2005, Wang et al., 2006, Deville et al., 2007, Lynch et al., 2010). Further, stimulation of beneficial gut microbes is considered as a novel therapeutic target in the treatment of obesity-linked metabolic syndrome (Tuohy et al., 2009, Cani and Delzenne, 2011). Colonisation of microbes from obese animals densely populated with *Firmicutes* in germ-free mice showed higher total body fat compared to the colonisation of microbes from lean animals (Turnbaugh et al., 2006). Similarly, genetically modified obese mice (*ob/ob*) with increased *Firmicutes* to *Bacteroidetes* ratio promoted adiposity (Ley et al., 2005, Ley et al., 2006). Research studies also confirmed that obesity-related changes in gut microbiota promote fat storage as triglycerides by suppressing the fasting induced adipocyte factor (fiaf) and enhancing the visceral adiposity (Fig. 1) (Backhed et al., 2004, Backhed et al., 2007).

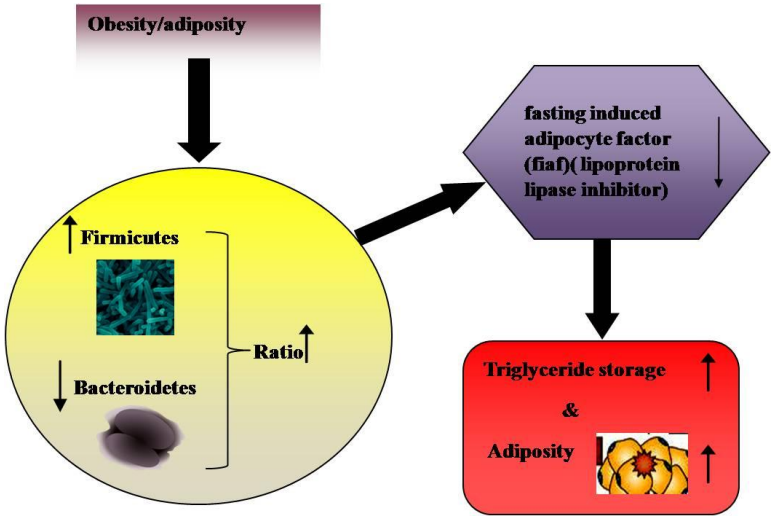

Fig. 1 Role of gut microbes in enhancing adiposity

So, my future work will be on the characterisation of polysaccharides from seaweeds (*Ulva ohnoi*, *Derbesia tenuissima*) and microalgal culture (*Scenedesmus dimorphus* and *Schroederiella apiculata*) which will be carried out using standard methods. The polysaccharide extracts isolated from seaweeds and microalgal culture will then be tested for their possible biological effects as prebiotics (O'Sullivan et al., 2010, Conterno et al., 2011) in the high-carbohydrate, high-fat diet induced rat model of metabolic syndrome. This will require characterising and comparing the beneficial gut microbes present in the faecal samples of high fat control groups and Synergy1 (a known prebiotic)-treated high fat groups. Further, investigation will be carried out using beneficial gut microbe supplementation, in a high-carbohydrate, high-fat diet induced rat model of metabolic syndrome.

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Appendix

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Responses to oleic, linoleic and α-linolenic acids in high-carbohydrate, high-fat diet-induced metabolic syndrome in rats

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Abstract

We investigated the changes in adiposity, cardiovascular and liver structure and function, and tissue fatty acid compositions in response to oleic acid-rich macadamia oil, linoleic acid-rich safflower oil and α-linolenic acid-rich flaxseed oil (C18 unsaturated fatty acids) in rats fed either a diet high in simple sugars and mainly saturated fats or a diet high in polysaccharides (cornstarch) and low in fat. The fatty acids induced lipid redistribution away from the abdomen, more pronounced with increasing unsaturation; only oleic acid increased whole-body adiposity. Oleic acid decreased plasma total cholesterol without changing triglycerides and nonesterified fatty acids, whereas linoleic and α-linolenic acids decreased plasma triglycerides and nonesterified fatty acids but not cholesterol. α-Linolenic acid improved left ventricular structure and function, diastolic stiffness and systolic blood pressure. Neither oleic nor linoleic acid changed the left ventricular remodeling induced by high-carbohydrate, high-fat diet, but both induced dilation of the left ventricle and functional deterioration in low fat-diet-fed rats. α-Linolenic acid improved glucose tolerance, while oleic and linoleic acids increased basal plasma glucose concentrations. Oleic and α-linolenic acids, but not linoleic acid, normalized systolic blood pressure. Only oleic acid reduced plasma markers of liver damage. The C18 unsaturated fatty acids reduced *trans* fatty acids in the heart, liver and skeletal muscle with lowered stearoyl-CoA desaturase-1 activity index; linoleic and α-linolenic acids increased accumulation of their C22 elongated products. These results demonstrate different physiological and biochemical responses to primary C18 unsaturated fatty acids in a rat model of human metabolic syndrome.

Keywords: Metabolic syndrome; Omega-3; Omega-6; Omega-9; Flax; Macadamia; Safflower

1. Introduction

The quality of dietary fat plays a significant role in the development and progression of chronic conditions such as obesity [1]. It is generally accepted that dietary saturated fatty acids (SFAs) and *trans* fatty acids are detrimental and unsaturated fatty acids are beneficial to cardiovascular health [2]. Unsaturated fatty acids are traditionally classified by chemical structure into monounsaturated fatty acids (MUFAs) with a single double bond or polyunsaturated fatty acids (PUFAs) with more than one double bond rather than by their biological activity [3,4]. PUFAs are further classified into n-3 and n-6 series based on position of the first double bond from the methyl end of the fatty acids [3]. Most dietary fat sources contain some or all of the 18-carbon fatty acid series including both saturated and unsaturated fatty acids such as stearic acid (C18:0; SFA), oleic acid (C18:1n-9; MUFA), linoleic acid (C18:2n-6; n-6 PUFA) and α-linolenic acid (C18:3n-3; n-3 PUFA). Oleic acid has a single double bond at the n-9 position; linoleic acid has two double bounds at the n-6 and n-9 positions, while α-linolenic acid has three double bonds at the n-3, n-6 and n-9 positions; these double bonds are *cis* configuration [5]. Oleic acid is the predominant dietary MUFA generally derived from animal fats such as tallow and lard as well as from plant-based oils such as olive oil, high-oleic sunflower oil and canola oil [6]. Linoleic and α-linolenic acids are mainly derived from plant-based oils such as safflower and flax oils, respectively [5].

However, there is considerable controversy as to the biological responses to dietary fatty acids in disease states such as metabolic syndrome. Despite being structurally similar, different dietary fatty acids, even within the same class, elicit different physiological responses [7–11]. The inconsistencies in the data reporting the biological responses to dietary fatty acids reflect both the small number of comparative studies on the individual fatty acids and the assumption that similar chemical structures will have similar biological actions [9]. We have recently demonstrated the nonhomogeneous responses to the major n-3 PUFAs, α-linolenic acid, eicosapentaenoic acid and docosahexaenoic acid, on the components of metabolic syndrome in diet-induced obese rats, the same model used in the current study [12].

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The dietary pool of 18-carbon unsaturated fatty acids is characterized by varying amounts of oleic, linoleic and α-linolenic acids, although typical modern human diets contain much more linoleic acid than α-linolenic acid [4,13,14]. The biological actions of these C18 unsaturated fatty acids on one or more components of metabolic syndrome remain poorly studied or controversial. Most studies with oleic acid suggest that MUFAs are a useful dietary replacement for lipogenic carbohydrates and saturated fats [6,15]. However, the role of chronic MUFA supplementation in reversing the risk factors of metabolic syndrome remains largely unclear. Linoleic and α-linolenic acids are often grouped together but produce markedly different responses on risk factors of metabolic syndrome [9,12]. Both linoleic and α-linolenic acids are eicosanoid precursors, but the linoleic acid-derived eicosanoids are usually proinflammatory, while the α-linolenic acid-derived eicosanoids are usually anti-inflammatory [7]. Additionally, α-linolenic acid is the least studied of the n-3 series PUFAs [7].

The differences in physiological responses to dietary oleic, linoleic and α-linolenic acids have not been evaluated in the same disease model. This is necessary, as shown in our recent study with dietary n-3 PUFA in diet-induced obesity in rats [12], to determine whether different classes of unsaturated fatty acids (n-9, n-6 and n-3 series) produce different responses. In this study, we compared the effects of MUFA-rich macadamia oil (63% oleic, 17% palmitoleic acids), n-6 PUFA-rich safflower oil (75% linoleic acid) and n-3 PUFA-rich flax oil (58% α-linolenic acid) on cardiovascular, hepatic and metabolic parameters in a diet-induced rat model of the human metabolic syndrome. Additionally, we investigated the changes in fatty acid composition of plasma, adipose tissue, liver, heart and skeletal muscle after dietary oil supplementation. In addition to serving as a control to the high-carbohydrate, high-fat diet (24% fat, mostly *trans* and SFA without n-3 PUFA), a corn starch-rich diet (0.8% fat, with comparable proportions of SFA, MUFA and PUFA without *trans* and little n-3 PUFA) also served as a model of a low-fat diet with the same carbohydrate intake for selective evaluation of the responses to the individual unsaturated fatty acids.

2. Materials and methods

2.1. Rats and diets

The experimental groups consisted of 96 male Wistar rats (9–10 weeks old; 336±2g) supplied by The University of Queensland Biological Resources unit and individually housed in a temperature-controlled (20°C±2°C), 12-h light/dark cycle environment with *ad libitum* access to water and the group-specific rat diet at the University of Southern Queensland Animal House. All experimentation was approved by the Animal Experimentation Ethics Committees of The University of Queensland and the University of Southern Queensland under the guidelines of the National Health and Medical Research Council of Australia. The rats were randomly divided into eight separate groups (n=12 each) and fed with corn starch (C); corn starch+oleic acid-rich macadamia oil (COA); corn starch+linoleic acid-rich safflower oil (CLA); corn starch +α-linolenic acid-rich flaxseed oil (CALA); high carbohydrate, high fat (H); high carbohydrate, high fat+oleic acid-rich macadamia oil (HOA); high carbohydrate, high fat+linoleic acid-rich safflower oil (HLA); and high carbohydrate, high fat+α-linolenic acid-rich flaxseed oil (HALA). This study was done together with our previous study with major n-3 PUFAs to allow future interstudy comparisons and therefore shares the same C and H groups [12].

Body weight and food and water intakes were measured daily, and feed efficiency (%) was calculated as described in our previous study [16]. The preparation and macronutrient composition of basal diets, including the dietary fatty acid profiles, have been described in detail previously [16–18]. The macronutrient composition of the

Table 1
Composition and fatty acid profiles of the diet and the oil supplements (macadamia oil, safflower oil, and flax oil)

Composition	Basal diets		Oil supplements[a]		
	C	H	Macadamia oil	Safflower oil	Flaxseed oil
Ingredients					
Corn starch, g/kg	570.0	–	–	–	–
Powdered rat feed[b], g/kg	155.0	155.0	–	–	–
HMW salt mixture, g/kg	25.0	25.0	–	–	–
Fructose, g/kg	–	175.0	–	–	–
Beef tallow, g/kg	–	200.0	–	–	–
Condensed milk, g/kg	–	395.0	–	–	–
Water, ml/kg	250.0	50.0	–	–	–
Total energy density, kJ/g	11.2	17.9	34.4	34.0	33.5
Micronutrient composition, g/kg					
Total carbohydrate	600.2	515.7	–	–	–
Total fat[c]	8.1	239.0	–	–	–
Total protein	31.8	58.1	–	–	–
Total fiber	7.4	7.4	–	–	–
Total vitamins	0.3	0.3	–	–	–
Total minerals	0.1	0.4	–	–	–
Ash	0.6	0.0	–	–	–
Total moisture	296.3	124.0	–	–	–
Fatty acid (g/100g of total recoverd fatty acid) (n=3/group)					
C14:0	10.0±0.9	3.7±0.1	0.0±0.0	0.0±0.0	0.0±0.0
C16:0	17.5±0.4	24.6±0.5	7.9±0.1	7.0±0.01	5.8±0.0
C16:1n-7	0.0±0.0	0.0±0.0	17.3±0.1	0.1±0.0	0.0±0.0
C18:0	0.6±0.0	24.2±0.2	3.1±0.02	2.6±0.0	5.0±0.0
C18:1n-9	34.5±2.3	0.9±0.5	62.8±0.3	13.6±0.01	16.0±0.0
C18:1*trans*-7	0.0±0.0	40.8±0.8	0.0±0.0	0.0±0.0	0.0±0.0
C18:2n-6	30.5±0.8	2.7±0.1	2.1±0.02	75.1±0.02	14.6±0.0
C18:3n-3	4.7±0.1	0.1±0.1	0.0±0.0	0.9±0.0	58.2±0.0
C20:0	0.0±0.0	0.2±0.1	2.6±0.02	0.3±0.0	0.2±0.0
C20:1n-9	0.0±0.0	0.0±0.0	2.7±0.02	0.2±0.0	0.0±0.0
Total SFA	28.3±1.4	53.1±0.7	14.2±0.1	9.9±0.01	11.0±0.01
Total MUFA	35.7±2.3	44.1±0.6	82.8±0.1	13.9±0.01	16.0±0.0
Total PUFA	36.0±0.9	2.8±0.0	2.9±0.02	76.2±0.01	73.0±0.01

HMW=Hubble, Mendel and Wakeman salt mixture (Hubbell R.B., Mendel L.B., Wakeman A.J. New salt mixture for use in experimental diets. *J Nutr* 1937;14:273–85) (MP Biochemicals, Seven Hills, New South Wales, Australia).

[a] Oil-supplemented diets prepared by adding 30 ml of respective oil in the basal diets, replacing equivalent amounts of water.

[b] Meat-free rat and mouse feed (Speciality Feeds, Glen Forrest, Western Australia, Australia) contains (g/kg of feed) the following: carbohydrate, 707.1; proteins, 194.0; fat, 48.0; fiber, 48.0; total vitamins, 2.1; and total minerals, 0.85.

[c] Primarily derived from powdered rat feed in the C diet.

basal diets and the fatty acid composition of macadamia, safflower and flaxseed oil supplements (Proteco Gold Pty. Ltd., Kingaroy, Queensland, Australia) are described in Table 1. The oil-supplemented diets were prepared by adding 3% of the respective oil to replace an equivalent amount of water in the diet. The oil-supplemented diets were administered for 8 weeks starting 8 weeks after the initiation of the corn starch or high-carbohydrate, high-fat diet. The drinking water in all high-carbohydrate, high-fat diet-fed groups was augmented with 25% fructose for the duration of the study.

2.2. Cardiovascular measurements

Systolic blood pressure was measured as previously described [16] under light sedation following intraperitoneal injection of Zoletil (tiletamine 15 mg/kg, zolazepam 15 mg/kg; Virbac, Peakhurst, New South Wales, Australia) using an MLT1010 Piezo-Electric Pulse Transducer and inflatable tail-cuff connected to an MLT844 Physiological Pressure Transducer using PowerLab data acquisition unit (ADInstruments, Sydney, Australia).

Anesthesia using intraperitoneal injection of Zoletil (tiletamine 15 mg/kg and zolazepam 15 mg/kg; Virbaca) and Ileum Xylazil (xylazine 10 mg/kg; Troy Laboratories, Smithfiled, New South Wales, Australia) was used for echocardiographic examination (Hewlett Packard Sonos 5500, 12-MHz transducer) performed at 16 weeks as previously described [16–18], in accordance with the guidelines of the American Society of Echocardiography using the leading-edge method [19].

The left ventricular function of the rats in all groups was assessed using the Langendorff heart preparation as previously described [16–18]. Terminal anesthesia was induced via intraperitoneal 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, New South Wales, Australia).

Thoracic aortic rings (~4 mm in length) were suspended in an organ bath chamber with a resting tension of approximately 10 mN. Cumulative concentration–response (contraction) curves were measured for noradrenaline (Sigma-Aldrich Australia); concentration–response (relaxation) curves were measured for acetylcholine (Sigma-Aldrich Australia) and sodium nitroprusside (Sigma-Aldrich Australia) in the presence of a submaximal (70%) contraction to noradrenaline [17].

2.3. Oral glucose tolerance

For oral glucose tolerance test, basal blood glucose concentrations were measured in blood taken from the tail vein of overnight food-deprived rats using Medisense Precision Q.I.D glucose meter (Abbott Laboratories, Bedford, MA, USA). Fructose-supplemented drinking water in the H, HOA, HLA and HALA groups was replaced with normal water for the overnight food-deprivation period. The rats were given 2 g/kg body weight of glucose as a 40% aqueous solution via oral gavage. Tail vein blood samples were taken at 30, 60, 90 and 120 min following glucose administration.

2.4. Body composition measurements

Dual-energy X-ray absorptiometric (DXA) measurements were performed on rats after 16 weeks of feeding, 2 days before rats were euthanized for pathophysiological assessments, using a Norland XR36 DXA instrument (Norland Corp., Fort Atkinson, WI, USA). DXA scans were analyzed using the manufacturer's recommended software for use in laboratory animals (Small Subject Analysis Software, version 2.5.3/1.3.1; Norland Corp.) as previously described [18]. The precision error of lean mass for replicate measurements, with repositioning, was 3.2%. Visceral adiposity index (%) was calculated as in our previous study [16].

2.5. Organ weights

The right and left ventricles were separated after perfusion experiments and weighed. Liver and retroperitoneal, epididymal and omental fat pads were removed following heart removal and blotted dry for weighing. Organ weights were normalized relative to the tibial length at the time of their removal (in mg/mm). Immediately after weighing, the left ventricle, liver and retroperitoneal fat were stored at −20°C in 50-ml polypropylene centrifuge tubes for fatty acid analysis.

2.6. Fatty acid analysis

The extraction of tissue and dietary lipids was undertaken by manual solvent extraction using a 2:1 chloroform/methanol mixture with 0.1% butylated hydroxy-toluene as an antioxidant as described in previous studies [16]. Fatty acid methyl esters were analyzed on an Agilent J&W DB-23 column (60 m × 0.25 mm × 0.25 μm) (Agilent Technologies, Santa Clara, CA, USA) by a Shimadzu GC-17A equipped with an FID detector as previously described [16]. A multi-fatty acid standard mixture was used for checking the performance of the GC and as a recovery test for the sample preparation procedure. Quantitation of fatty acids in all samples was based on a linear calibration equation obtained from the C17 standard. For identification purposes, a 28-fatty-acid methyl ester mixture standard (Nu-Check Prep. Inc., Elysian, MN, USA) was used for retention time (RT) calibration. A plot of carbon number versus log RT for the saturated series, one degree of unsaturation or two degrees of unsaturation allowed a relationship to be developed for identification purposes. All fatty acids were expressed as g/100 g of total recovered fatty acids.

2.7. Histology

Two rats per group were taken exclusively for histological analysis. Two slides were prepared per tissue specimen, and two random, nonoverlapping fields per slide were taken to avoid biased analysis. Organs were also collected from rats used for perfusion studies. Immediately after removal, heart and liver tissues were fixed in 10% neutral buffered formalin for 3 days and then dehydrated and embedded in paraffin wax as previously described [16–18]. Thin sections (7 μm) of the left ventricle and the liver were cut and stained with hematoxylin and eosin stain for determination of inflammatory cell infiltration with 20× and fat vacuole enlargement in liver with 40× objectives using a Olympus BX51 microscope (Olympus, Melville, NY, USA). Collagen distribution was measured in the left ventricle with picrosirius red stain. Laser confocal microscopy (Zeiss LSM 510 upright confocal microscope) with color intensity quantitated using National Institutes of Health (Bethesda, MD, USA) ImageJ software was used to determine the extent of collagen deposition in selected tissue sections [17].

2.8. Plasma biochemistry

Briefly, blood was centrifuged at 5000*g* for 15 min within 30 min of collection into heparinized tubes. Plasma was separated and transferred to 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 analyzer (AU 400, Tokyo, Japan) as previously described [16–18]. Plasma insulin concentrations (ALPCO, Salem, NH, USA) were estimated using a commercial enzyme-linked immunosorbent assay (ELISA) kit according to manufacturer-provided standards and protocols.

2.9. Statistical analysis

All data are presented as mean±S.E.M. Results were tested for variance using Bartlett's test, and variables that were not normally distributed were transformed (using log 10 function) prior to statistical analyses. Data from C, COA, CLA, CALA, H, HOA, HLA and HALA groups were tested by two-way analysis of variance. When interaction and/or the main effects were significant, means were compared using Newman–Keuls multiple-comparison *post hoc* test. Where transformations did not result in normality or constant variance, a Kruskal–Wallis nonparametric test was performed. A P value of $<.05$ was considered as statistically significant. All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (San Diego, CA, USA).

3. Results

3.1. Dietary intake, body composition and plasma biochemistry

α-Linolenic acid- and oleic acid- but not linoleic acid-supplemented groups showed reduced body weight gain, in this order, compared to their respective controls. HOA, HLA and HALA groups showed decreased visceral adiposity and body mass index, with decreases in abdominal circumference only in the latter two groups compared to H rats (Table 2). Abdominal circumference and body mass index were not affected in CLA and COA rats, respectively, compared to C rats (Table 2). However, total body fat increased in HOA rats and remained unaffected in all other oil-supplemented groups compared to their respective controls (Table 2). Total body lean mass decreased in all oil-supplemented groups (Table 2). Bone mineral content decreased in CALA group but increased in HOA and HALA groups. These changes in body composition were associated with loss of retroperitoneal fat in HOA, HLA and HALA (HALA<HLA<HOA); epididymal fat in HALA; and omental fat in HLA and HALA groups.

Food and water intakes were decreased in H, HOA, HLA and HALA rats compared to C, COA, CLA and CALA rats, respectively (Table 2). Linoleic acid supplementation decreased food intake in CLA but not in HLA groups compared to C and H rats, respectively, and oleic acid and α-linolenic acid supplementation increased dietary intakes in HOA and HALA groups, respectively (Table 2). Water intake was unchanged by dietary oils (Table 2). Consequently, oleic acid and α-linolenic acid but not linoleic acid supplementation increased energy intakes compared to C and H rats (Table 2). α-Linolenic acid

Table 2
Dietary intakes, body composition and anthropometrics, organ wet weights, plasma fatty acid composition and biochemistry in groups fed the C, COA, CLA, CALA, H, HOA, HLA and HALA diet

Variable	C	COA	CLA	CALA	H	HOA	HLA	HALA	*P* value		
									Diet	Treatment	Interaction
Food intake, g/d (n=10)	33.5±1.1^{a}	34.0±0.8^{a}	28.9±0.3^{b}	35.1±0.6^{a}	22.2±0.5^{e}	25.9±0.7^{cd}	21.1±0.3^{e}	25.8±1.0^{d}	<.0001	<.0001	.07
Water intake, ml/d (n=10)	28.7±2.5^{ab}	31.6±2.2^{a}	28.0±0.3^{ab}	28.2±2.1^{ab}	20.0±1.0^{c}	24.4±1.4^{bc}	19.3±0.2^{c}	21.7±1.3^{c}	<.0001	.0408	.87
Supplemented oil intake, ml/d (n=10)	0.0±0.0^{c}	1.0±0.0^{a}	0.9±0.0^{a}	1.0±0.0^{a}	0.0±0.0^{c}	0.8±0.0^{b}	0.6±0.0^{b}	0.7±0.0^{b}	<.0001	<.0001	<.0001
Energy intake, kJ/d (n=10)	375.6±12.2^{d}	417.7±9.9^{c}	354.3±4.3^{d}	431.6±6.7^{bc}	466.8±8.8^{b}	589.3±19.9^{a}	466.4±7.9^{b}	562.0±18.3^{a}	<.0001	<.0001	.0114
Feed conversion efficiency, % (n=10)	3.1±0.3^{bc}	2.3±0.4^{cd}	2.1±0.3^{cd}	1.3±0.2^{d}	3.6±0.3^{bc}	6.6±1.0^{a}	4.5±0.3^{b}	3.9±0.3^{bc}	<.0001	.0015	.0012
Body weight gain (week 8–16), % (n=10)	10.8±1.3^{bc}	5.6±1.0^{d}	9.6±0.8^{c}	4.1±0.8^{d}	15.3±1.4^{a}	10.6±1.4^{bc}	13.8±1.0^{ab}	9.1±0.8^{c}	<.0001	<.0001	.98
Visceral adiposity index, % (n=10)	4.3±0.3^{cd}	3.4±0.3^{d}	3.0±0.3^{d}	3.4±0.2^{d}	7.7±0.5^{a}	6.3±0.6^{b}	5.9±0.3^{b}	5.2±0.3^{bc}	<.0001	<.0001	.18
Abdominal circumference, cm (n=10)	20.0±0.2^{c}	19.5±0.3^{c}	19.0±0.3^{c}	18.2±0.2^{d}	22.7±0.5^{a}	22.1±0.3^{ab}	21.5±0.1^{b}	20.4±0.1^{c}	<.0001	<.0001	.82
Body mass index, kg/m^2 (n=8–10)	5.2±0.1^{c}	5.0±0.1^{c}	4.6±0.1^{c}	4.9±0.1^{c}	6.2±0.3^{a}	5.8±0.2^{ab}	5.1±0.1^{c}	5.5±0.1^{bc}	<.0001	<.0001	.42
Total body fat mass, g (n=8–10)	85.6±7.8^{c}	113.5±9.5^{c}	89.3±8.0^{c}	85.5±6.5^{c}	158.6±5.7^{b}	207.1±15.9^{a}	150.9±5.1^{b}	164.0±4.4^{b}	<.0001	<.0001	.34
Total body lean mass, g (n=8–10)	306.2±3.2^{a}	247.0±4.8^{c}	284.3±3.6^{b}	276.4±7.4^{bc}	304.8±15.0^{a}	250.9±8.8^{c}	260.5±4.2^{bc}	255.1±4.9^{bc}	.06	<.0001	.2
Bone mineral content, g (n=8–10)	13.6±0.3^{bc}	13.1±0.3^{cd}	13.1±0.4^{cd}	12.2±0.3^{d}	14.4±0.4^{b}	16.0±0.4^{a}	13.7±0.2^{bc}	15.4±0.1^{a}	<.0001	.0062	<.0001
Tissue wet weights, *mg/mm tibial length* (n=10)											
Retroperitoneal adipose tissue	169.4±12.6^{d}	121.7±13.0^{de}	131.0±12.9^{de}	106.4±9.7^{e}	387.6±26.1^{a}	312.4±19.9^{b}	300.5±14.7^{b}	226.7±10.8^{c}	<.0001	<.0001	.0216
Epididymal adipose tissue	105.3±7.0^{c}	98.4±8.3^{c}	105.0±7.3^{c}	91.1±4.8^{c}	192.8±13.5^{a}	189.8±19.6^{a}	178.6±12.0^{ab}	154.5±12.0^{b}	<.0001	.12	.6
Omental adipose tissue	102.8±11.4^{bc}	68.8±10.3^{cd}	46.7±6.3^{d}	67.2±5.5^{cd}	175.0±10.3^{a}	150.0±19.4^{a}	113.0±6.8^{b}	104.6±7.0^{bc}	<.0001	<.0001	.19
Liver	204.3±22.2^{c}	196.1±11.1^{c}	209.6±9.5^{c}	244.2±16.5^{bc}	318.5±11.0^{a}	317.9±15.1^{a}	283.3±14.6^{ab}	257.2±9.0^{bc}	<.0001	.73	.0009
Heart	22.3±0.4^{bc}	20.2±1.0^{c}	17.8±0.5^{c}	22.7±0.8^{bc}	25.7±1.0^{a}	22.9±1.0^{bc}	17.9±0.4^{c}	23.2±0.3^{ab}	.0019	<.0001	.07
Plasma total cholesterol, mmol/L (n=10)	1.8±0.1^{b}	1.3±0.1^{d}	1.9±0.1^{ab}	1.6±0.1^{b}	2.2±0.1^{a}	1.7±0.0^{b}	1.9±0.1^{ab}	1.9±0.1^{ab}	.1148	<.0001	<.0001
Plasma triglyceride, mmol/L (n=10)	0.5±0.1^{b}	0.5±0.1^{b}	0.3±0.04^{b}	0.4±0.04^{b}	1.1±0.1^{a}	1.0±0.1^{a}	0.5±0.1^{b}	0.4±0.03^{b}	.0011	<.0001	<.0001
Plasma NEFA, mmol/L (n=10)	1.3±0.1^{b}	1.8±0.2^{b}	1.3±0.1^{b}	1.3±0.2^{b}	2.5±0.4^{a}	2.6±0.2^{a}	1.7±0.2^{b}	1.1±0.1^{b}	.0099	<.0001	.0004
Plasma insulin, μmol/L (n=8–10)	1.0±0.1^{bc}	0.3 ±0.05^{c}	1.1 ±0.3^{bc}	1.9±0.3^{bc}	4.0±0.7^{a}	1.6±0.9^{bc}	2.0 ±0.4^{bc}	2.3±0.3^{b}	<.0001	.0052	.0348
Basal blood glucose, mmol/L (n=9–10)	2.8±0.1^{c}	4.9±0.1^{a}	3.8±0.1^{b}	2.4±0.1^{c}	3.7±0.3^{b}	5.0±0.2^{a}	5.1±0.1^{a}	4.3±0.2^{b}	<.0001	<.0001	<.0001
OGTT–AUC, mmol/L·min (n=9–10)	483.1±29.7^{c}	645.0±10.6^{b}	676.1±20.5^{b}	530.3±22.4^{c}	624.6±30.9^{b}	680.7±18.5^{b}	785.8±13.7^{a}	545.2±13.9^{c}	<.0001	<.0001	.0137
Plasma fatty acid, g/100 g of total fatty acid content (n=6)											
C16:0	26.6±2.7^{d}	56.5±5.5^{a}	48.9±3.9^{ab}	46.8±2.4^{abc}	23.1±2.0^{d}	42.4±1.4^{bc}	40.3±1.9^{bc}	37.0±1.9^{c}	<.0001	<.0001	.36
C16:1n-7	31.0±1.4^{a}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}	27.7±3.0^{a}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}	.34	<.0001	.44
C18:0	12.9±1.5^{b}	14.7±1.4^{ab}	17.1±1.0^{ab}	16.9±1.1^{ab}	8.9±1.7^{c}	13.5±0.8^{ab}	18.4±1.4^{a}	19.7±0.4^{a}	.76	<.0001	.05
C18:1n-9	7.6±1.3^{b}	10.9±1.8^{ab}	7.6±0.6^{b}	11.2±1.4^{ab}	10.5±2.0^{ab}	14.3±1.4^{a}	12.9±0.9^{ab}	15.8±1.3^{a}	.0003	.0143	.82
C18:1*trans*-7	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}	14.2±4.8^{a}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}	.0075	.0003	.0003
C18:2n-6	8.1±3.1^{ab}	6.1±1.5^{b}	9.8±0.7^{ab}	14.7±2.2^{a}	7.1±1.6^{b}	14.6±0.3^{a}	11.1±1.1^{ab}	13.6±0.9^{ab}	.12	.0059	.0257
C20:4n-6	12.0±1.2^{abc}	7.7±0.7^{c}	16.7±2.5^{a}	10.5±1.4^{bc}	6.4±1.4^{c}	12.1±1.2^{abc}	17.2±1.3^{a}	14.3±0.6^{ab}	.43	<.0001	.0049
Total SFA	39.5±3.1^{c}	71.2±6.9^{a}	65.9±3.0^{ab}	63.7±2.4^{ab}	32.3±2.3^{c}	55.9±2.1^{b}	58.7±1.6^{b}	56.7±2.1^{b}	.0002	<.0001	.48
Total MUFA	40.5±1.9^{b}	10.9±1.8^{cd}	7.6±0.6^{d}	11.6±1.4^{cd}	54.2±2.3^{a}	14.3±1.4^{c}	12.9±0.9^{cd}	15.4±1.3^{c}	<.0001	<.0001	.0069
Total PUFA	20.0±±2.4^{abc}	17.9±5.0^{bc}	26.5±2.9^{ab}	25.1±2.9^{ab}	13.5±0.6^{c}	29.8±1.7^{a}	28.3±1.0^{ab}	27.9±1.2^{ab}	.15	.0003	.0063
n-3:n-6	0.0±0.0	0.2±0.2	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.1	0.0±0.0	0.0±0.0	.71	.07	.94
Stearoyl-CoA 9 desaturase index	1.2±0.1^{a}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}	1.2±0.1^{a}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}	.9	<.0001	1

Each value is a mean±S.E.M. Number of repetitive experiments indicated within parenthesis. Means within a row with unlike superscript differ; P<.05.
NEFA, nonesterified fatty acid; OGTT–AUC, oral glucose tolerance test–area under curve.

supplementation decreased feed conversion efficiency in CALA but not HALA groups, oleic acid supplementation increased the feed conversion efficiency in HOA group, and linoleic acid supplementation did not affect this variable compared to C and H rats (Table 2).

The decreases in the abdominal adipose tissues were associated with decreased plasma concentrations of triglycerides and nonesterified fatty acids in HLA and HALA rats, while plasma total cholesterol decreased in both COA and HOA groups compared to their respective controls (Table 2). Additionally, the plasma fatty acid profile suggests that MUFA, n-6 PUFA and n-3 PUFA supplementation inhibited the stearoyl-CoA desaturase-1-mediated desaturation of SFA (Table 2). C16:0 increased and C16:1n-7 decreased in all oil-supplemented groups compared to their respective controls (Table 2). C18:0 increased and C18:1*trans*-7 decreased in HOA, HLA and HALA groups and remained unchanged in COA, CLA and CALA groups (Table 2). Oleic acid remained unchanged in all oil-supplemented groups.

Table 3
Changes in cardiovascular structure, function and ventricular fatty acid composition in groups fed the C, COA, CLA, CALA, H, HOA, HLA and HALA diet

Variable	C	COA	CLA	CALA	H	HOA	HLA	HALA	P value		
									Diet	Treatment	Interaction
Heart rate, bpm (n=9–10)	242±6^{b}	252±11^{b}	265±14^{b}	248±14^{b}	233±8^{b}	289±12^{b}	394±33^{a}	290±22^{b}	<.0001	<.0001	.18
IVSd, mm (n=9–10)	1.7±0.1	1.7±0.1	1.8±0.0	1.8±0.0	1.7±0.1	1.7±0.0	1.9±0.1	1.9±0.0	.32	.0349	.8
LVIDd, mm (n=9–10)	6.3±0.2^{b}	7.9±0.2^{a}	8.0±0.3^{a}	6.1±0.2^{b}	7.7±0.3^{a}	8.5±0.2^{a}	7.7±0.2^{a}	6.7±0.3^{b}	.0015	<.0001	.0114
LVPWd, mm (n=9–10)	1.7±0.0	1.7±0.1	1.7±0.0	1.7±0.1	1.9±0.0	1.7±0.1	1.8±0.1	1.8±0.0	.0418	.54	.54
IVSs, mm (n=9–10)	2.9±0.1	3.0±0.1	3.2±0.1	3.0±0.1	2.9±0.2	2.9±0.1	3.4±0.1	3.2±0.1	.38	.0064	.51
LVIDs, mm (n=9–10)	3.4±0.2^{c}	4.4±0.2^{b}	4.2±0.2^{b}	2.5±0.2^{d}	4.3±0.2^{b}	5.0±0.2^{a}	3.3±0.1^{c}	3.5±0.2^{c}	.0049	<.0001	<.0001
LVPWs, mm (n=9–10)	2.6±0.1^{b}	2.6±0.1^{b}	3.0±0.1^{b}	2.7±0.1^{b}	2.8±0.1^{b}	2.7±0.1^{b}	3.3±0.1^{a}	3.0±0.2^{b}	.0101	.0004	.81
Relative wall thickness (n=9–10)	0.5±0.01^{ab}	0.4±0.02^{bc}	0.4±0.01^{bc}	0.6±0.01^{a}	0.5±0.02^{b}	0.4±0.02^{bc}	0.5±0.02^{b}	0.6±0.02^{a}	.42	<.0001	.0457
Fractional shortening, % (n=9–10)	60.2±1.5^{a}	43.4±1.5^{b}	47.7±2.5^{b}	59.4±2.3^{a}	45.1±1.6^{b}	41.4±1.4^{b}	56.8±1.4^{a}	62.9±2.3^{a}	.4	<.0001	<.0001
Diastolic volume, μl (n=9–10)	268.3±24.6^{b}	522.9±33.3^{a}	560.8±63.1^{a}	243.3±19.2^{b}	504.2±53.6^{a}	653.8±50.9^{a}	485.1±36.3^{a}	320.6±34.9^{b}	.0026	<.0001	.0041
Systolic volume, μl (n=9–10)	44.9±6.2^{c}	94.8±13.6^{b}	83.6±12.1^{b}	18.5±3.5^{c}	85.1±11.4^{b}	134.3±15.1^{a}	40.0±5.0^{c}	46.5±5.5^{c}	.0246	<.0001	<.0001
Stroke volume, μl (n=9–10)	223.4±21.9^{b}	428.1±22.8^{a}	477.2±58.6^{a}	224.8±17.3^{b}	419.1±46.9^{a}	519.5±41.3^{a}	445.2±33.8^{a}	274.1±34.4^{b}	.0047	<.0001	.0251
Cardiac output, ml (n=9–10)	54.1±5.8^{c}	107.2±6.0^{b}	131.4±23.2^{ab}	54.6±3.7^{c}	97.5±10.5^{b}	149.1±20.0^{ab}	172.2±16.7^{a}	83.3±13.4^{b}	<.0001	<.0001	.93
Ejection fraction, % (n=9–10)	84.9±1.8^{b}	82.3±1.6^{b}	84.4±1.8^{b}	92.7±1.2^{a}	82.6±1.6^{b}	79.6±1.4^{b}	91.8±0.8^{a}	83.8±2.4^{b}	.18	<.0001	.0001
Ejection time, ms (n=9–10)	89.7±2.9^{ab}	88.2±2.9^{ab}	85.9±2.1^{ab}	90.3±3.0^{ab}	93.1±1.9^{a}	84.3±1.6^{ab}	80.9±4.2^{b}	79.3±2.8^{b}	.037	.0273	.08
Estimated LV mass, g (n=9–10)	0.7±0.04^{b}	0.9±0.02^{a}	1.0±0.1^{a}	0.7±0.04^{b}	1.0±0.1^{a}	1.0±0.02^{a}	1.0±0.04^{a}	0.8±0.05^{b}	.0009	<.0001	.0344
LV developed pressure, mmHg (n=9–10)	68.7±4.4^{a}	41.2±3.4^{b}	60.5±5.8^{a}	43.8±3.8^{b}	42.8±3.4^{b}	48.2±7.4^{b}	56.5±5.5^{b}	43.1±3.6^{b}	.09	.0033	.0078
+dP/dt, mmHg·s^{-1} (n=9–10)	1248.1±67.5^{a}	751.9±74.1^{b}	1070.7±89.5^{a}	816.2±60.7^{b}	779.2±63.7^{b}	908.9±146.1^{b}	972.7±92.9^{a}	732.7±56.5^{b}	.0453	.007	.0056
−dP/dt, mmHg·s^{-1} (n=9–10)	−800.3±46.0^{a}	−427.3±35.6^{b}	−705.1±70.2^{a}	−415.8±29.2^{b}	−457.2±43.1^{b}	−400.2±67.5^{b}	−650.2±69.0^{a}	−433.6±41.0^{b}	.0073	<.0001	.0036
Diastolic stiffness constant (κ) (n=9–10)	22.1±0.7^{c}	25.1±1.4^{bc}	21.7±0.8^{c}	23.3±0.2^{c}	29.4±0.7^{a}	26.5±0.8^{ab}	24.8±0.7^{bc}	23.4±0.5^{c}	<.0001	.0007	.0002
Systolic blood pressure, mmHg (n=9–10)	127.3±0.7^{b}	127.1±1.7^{b}	131.1±3.2^{b}	123.7±0.9^{b}	159.7±2.1^{a}	132.8±2.3^{b}	157.1±4.8^{a}	121.1±2.3^{b}	<.0001	<.0001	<.0001
Systolic wall stress, mmHg (n=9–10)	84.5±6.1^{c}	111.0±8.9^{ab}	96.2±8.2^{bc}	57.8±4.5^{d}	123.2±7.3^{a}	124.8±8.9^{a}	81.1±6.7^{cd}	73.2±5.9^{cd}	.0111	<.0001	.0046
LV+septum wet weight,* mg/mm (n=10)	19.0±0.4^{abc}	16.6±0.7^{bc}	15.8±0.4^{c}	17.3±1.7^{bc}	21.4±1.0^{a}	19.8±0.9^{ab}	15.9±0.5^{c}	18.7±0.3^{abc}	.0054	<.0001	.33
Right ventricle wet weight,* mg/mm (n=10)	3.2±0.2^{ab}	3.5±0.4^{ab}	2.1±0.1^{b}	5.4±1.4^{a}	4.2±0.2^{ab}	3.1±0.2^{ab}	2.1±0.1^{b}	4.5±0.2^{a}	.9	<.0001	.38
Ventricular fatty acid, g/100 g of total fatty acid content (n=6)											
C16:0	2.1±0.1^{c}	29.3±0.6^{a}	25.4±1.2^{ab}	20.4±0.4^{b}	5.7±4.1^{c}	28.8±1.4^{a}	25.7±0.6^{ab}	19.3±0.8^{b}	.64	<.0001	.5
C16:1n-7	25.9±1.5^{a}	0.0±0.0^{b}	3.1±2.0^{b}	0.7±0.2^{b}	21.8±3.6^{a}	0.0±0.0^{b}	2.5±1.2^{b}	1.0±0.1^{b}	.34	<.0001	.5
C18:0	0.9±0.1^{d}	18.6±1.2^{c}	23.3±0.6^{a}	22.2±0.4^{ab}	1.5±0.1^{d}	19.7±1.1^{bc}	22.1±0.6^{ab}	19.2±1.0^{c}	.25	<.0001	.0394
C18:1n-9	0.9±0.1^{d}	16.5±2.2^{b}	9.3±0.9^{c}	7.3±0.5^{c}	0.6±0.1^{d}	16.8±4.0^{b}	11.8±0.9^{bc}	23.2±2.5^{a}	.0017	<.0001	.0003
C18:1*trans*-7	22.5±0.5^{b}	3.6±0.2^{c}	2.3±0.8^{c}	3.6±0.1^{c}	24.6±0.4^{a}	2.6±0.1^{c}	2.2±0.01^{c}	2.5±0.1^{c}	.93	<.0001	.0002
C18:2n-6	11.8±1.1^{cde}	13.8±0.5^{bc}	15.4±0.8^{b}	20.7±0.6^{a}	10.1±0.5^{e}	13.5±0.7^{bcd}	12.9±0.3^{bcd}	11.0±0.6^{de}	<.0001	<.0001	<.0001
C18:3n-3	0.0±0.0^{c}	0.0±0.0^{c}	0.0±0.0^{c}	0.6±0.1^{b}	1.7±1.7^{c}	0.0±0.0^{c}	0.0±0.0^{c}	1.0±0.1^{a}	.0027	<.0001	<.0001
C20:4n-6	19.6±0.7^{a}	13.2±0.9^{cd}	15.9±0.6^{bc}	13.6±0.3^{bcd}	19.5±0.4^{a}	13.0±1.5^{cd}	16.5±0.6^{b}	11.2±0.9^{d}	.35	<.0001	.32
C22:5n-3	0.1±0.1^{b}	0.0±0.0^{b}	0.0±0.0^{b}	2.7±0.1^{a}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}	2.7±0.2^{a}	.86	<.0001	.9
C22:6n-3	1.3±0.2^{c}	4.9±0.5^{b}	0.0±0.0^{c}	8.2±0.2^{a}	2.0±0.4^{c}	5.6±1.4^{b}	0.0±0.0^{c}	8.8±0.8^{a}	.28	<.0001	.94
C22:5n-6	0.0±0.0^{c}	0.0±0.0^{c}	5.1±0.4^{b}	0.0±0.0^{c}	0.0±0.0^{c}	0.0±0.0^{c}	6.3±0.1^{a}	0.0±0.0^{c}	.0036	<.0001	<.0001
Total SFA	3.5±0.3^{c}	47.9±1.0^{a}	48.7±0.9^{a}	42.6±0.3^{ab}	8.0±4.3^{c}	48.5±1.8^{a}	47.8±0.5^{a}	38.5±0.6^{b}	.99	<.0001	.12
Total MUFA	50.7±1.5^{a}	20.1±2.4^{bc}	14.8±1.5^{c}	11.6±0.7^{c}	48.6±4.0^{a}	19.4±4.0^{bc}	16.4±1.2^{c}	26.8±2.6^{b}	.06	<.0001	.0053
Total PUFA	45.8±1.4^{a}	32.0±1.5^{b}	36.5±0.9^{b}	45.8±0.7^{a}	43.4±0.6^{a}	32.1±2.9^{b}	35.8±0.8^{b}	34.7±2.2^{b}	.0032	<.0001	.0037
n-3:n-6	0.03±0.0^{de}	0.2±0.02^{cd}	0.0±0.0^{e}	0.3±0.01^{b}	0.1±0.05^{de}	0.2±0.05^{c}	0.0±0.0^{e}	0.6±0.03^{a}	.0003	<.0001	.0008
Stearoyl-CoA 9 desaturase index	12.4±0.9^{a}	0.0±0.0^{b}	0.1±0.1^{b}	0.03±0.01^{b}	13.5±2.9^{a}	0.0±0.0^{b}	0.1±0.05^{b}	0.05±0.01^{a}	.75	<.0001	.95

Each value is a mean±S.E.M. Number of repetitive experiments indicated within parenthesis. Means within a row with unlike superscript differ; P<.05. IVSd, interventricular septal thickness in diastole; LVIDd, left ventricular internal diameter in diastole; LVPWd, left ventricular posterior wall thickness in diastole; IVSs, interventricular septal thickness in systole; LVIDs, left ventricular internal diameter in systole; LVPWs, left ventricular posterior wall thickness in systole; LV, left ventricular.

* Normalized against tibial length.

Linoleic acid increased in HOA, and arachidonic acid increased in HLA and HALA. These n-6 series PUFAs were unaffected in COA, CLA and CALA groups. Consequently, the total SFA increased and the total MUFA decreased in all oil-supplemented groups with only HOA, HLA, and HALA showing increased total plasma PUFA (Table 2).

Plasma insulin concentrations almost quadrupled in H rats compared to C rats (Table 2). Plasma insulin concentrations were unaffected in COA, CLA and CALA rats but decreased in HOA, HLA and HALA rats when compared to their respective controls. However, oleic acid and linoleic acid supplementation increased basal plasma glucose concentrations in all groups (Table 2, Suppl. Figure 1). Increase in basal plasma glucose concentrations was associated with higher area under the curve of the oral glucose tolerance test in COA, CLA and HLA but not HOA groups (Table 2). HALA group showed improved glucose tolerance when compared to H rats and remained unchanged in CALA group when compared to C group (Table 2, Suppl. Figure 1).

3.2. Cardiovascular structure, function and fatty acid composition

Compared to C rats, H rats showed signs of eccentric hypertrophy, characteristic of increased preload, defined by increased left ventricular internal diameter in diastole without any changes to relative wall thickness (Table 3). Consequently, H rats showed impaired systolic function seen as decreased fractional shortening, increased wall stress, increased diastolic stiffness, decreased developed pressure and decreased dP/dt. Additionally, diastolic, systolic and stroke volumes, and cardiac output were elevated in H rats compared to C rats, without any changes in heart rate.

Oleic acid and linoleic acid supplementation did not change the left ventricular internal diameter in diastole in HOA and HLA groups but increased the dimension in COA and CLA groups (Table 3). Oleic or linoleic acid supplementation also increased left ventricular internal diameter in systole, except in HLA group where the reduced left ventricular internal diameter in systole was compensated by increased left ventricular posterior wall dimension in systole (Table 3). These effects were accompanied by reduced fractional shortening in COA and CLA groups but increased in HLA group (Table 3). Additionally, diastolic, systolic and stroke volumes, and cardiac output were elevated in COA and CLA groups compared to C rats (Table 3). While the diastolic volume, stroke volume and cardiac output remained unchanged in HOA and HLA group compared to H rats, systolic volume increased in HOA group and decreased in HLA group (Table 3). Additionally, the ejection time decreased and the ejection fraction increased in the HLA group. α-Linolenic acid-supplemented rats showed normalized left ventricular internal diameter in diastole and left ventricular internal diameter in systole, increased fractional shortening, decreased ejection time, and normalized volumes and diastolic stiffness constant compared to their respective controls (Table 3). Also, systolic blood pressure was normalized in HOA and HALA groups but not in HLA group

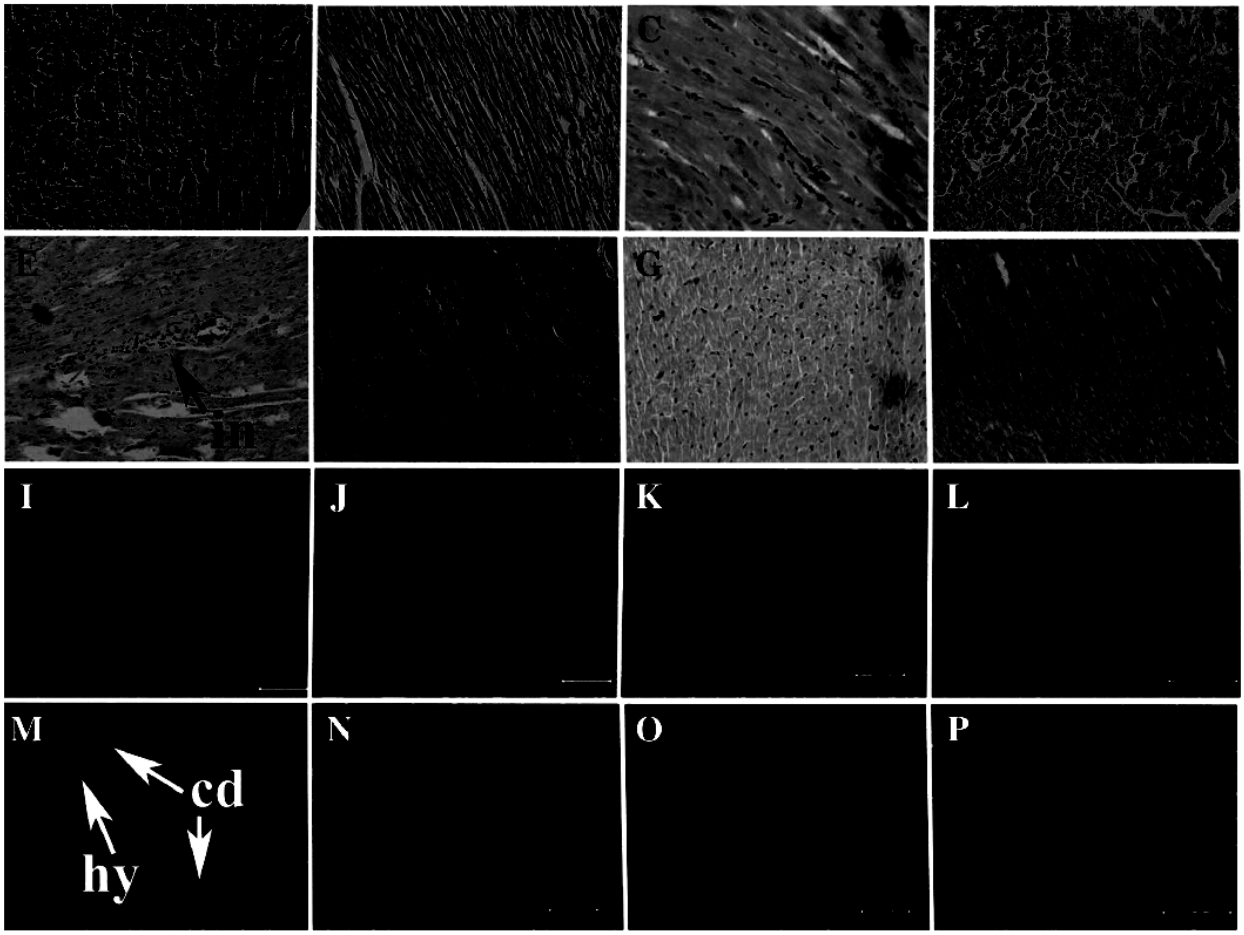

Fig. 1. Hematoxylin and eosin staining of left ventricle (×20) showing inflammatory cells (marked as "in") as dark spots outside the myocytes in rats fed the C (A), COA (B), CLA (C), CALA (D), H (E), HOA (F), HLA (G) and HALA (H) diet. Picrosirius red staining of left ventricular interstitial collagen deposition (×40) in rats fed the C (I), COA (J), CLA (K), CALA (L), H (M), HOA (N), HLA (O) and HALA (P) diet. Collagen deposition is marked as "cd," and hypertrophied cardiomyocytes are marked as "hy."

(Table 3). However, oleic acid supplementation increased systolic wall stress in COA and maintained the elevated wall stress in HOA group compared to C and H rats, respectively (Table 3). Wall stress decreased in CALA, HLA and HALA groups but was unchanged in CLA group (Table 3).

Histological evaluation of the left ventricle after 16 weeks showed greater infiltration by inflammatory cells (marked "in"; Fig. 1E) as well as increased interstitial collagen deposition (marked "cd"; Fig. 1M) in the H rats compared to C rats (Fig. 1A and I). Oleic acid supplementation did not alter the inflammatory state induced by H diet feeding in the HOA group but decreased collagen deposition (Fig. 1F and N). Linoleic acid and α-linolenic acid supplementation normalized the inflammatory state and markedly reduced collagen deposition in HLA (Fig. 1G and O) and HALA (Fig. 1H and P). No major changes were observed in COA (Fig. 1B and J), CLA (Fig. 1C and K) and CALA (Fig. 1D and L) rats, and the tissue morphology appeared normal.

H rats showed diminished α_1-adrenoceptor-mediated vascular contraction to noradrenaline in isolated thoracic aortic rings compared to C rats (Fig. 2A). Additionally, H rats showed decreased smooth muscle-dependent and endothelium-dependent relaxant responses to sodium nitroprusside and acetylcholine, respectively (Fig. 2B and C). All oil-supplemented groups showed improved vascular responses in isolated thoracic aortic rings (oleic acid<linoleic acid=α-linolenic acid) (Fig. 2A, B and C).

Similar to plasma fatty acid profile, all oil-supplemented groups showed reduced stearoyl-CoA desaturase-1-mediated desaturation in the heart with increased C16:0 and decreased C16:1n-7 compared to their respective controls (Table 3). C18:0 and C18:1n-9 increased and C18:1*trans*-7 decreased in all oil-supplemented groups (Table 3). C18:2n-6 increased in all oil-supplemented groups with the exception of COA, but C20:4n-6 decreased in all groups (α-linolenic acid= oleic acid<linoleic acid) (Table 3). Consistent with our hypothesis, C22:5n-3 and C22:6n-3 increased in CALA and HALA groups (Table 3); C22:6n-3 was also increased in COA and HOA groups. C22:5n-6 increased selectively in CLA and HLA groups (Table 3). Consequently, the total SFA increased and the total MUFA and PUFA decreased in all oil-supplemented groups (Table 3). Cardiac n-3:n-6 ratio was noticeably increased with α-linolenic acid supplementation (Table 3).

3.3. Hepatic structure, function and fatty acid composition

H rats showed elevated plasma markers of liver function in comparison to C rats (Table 4). Compared to H rats, HOA rats had decreased plasma activities of alanine transaminase, aspartate

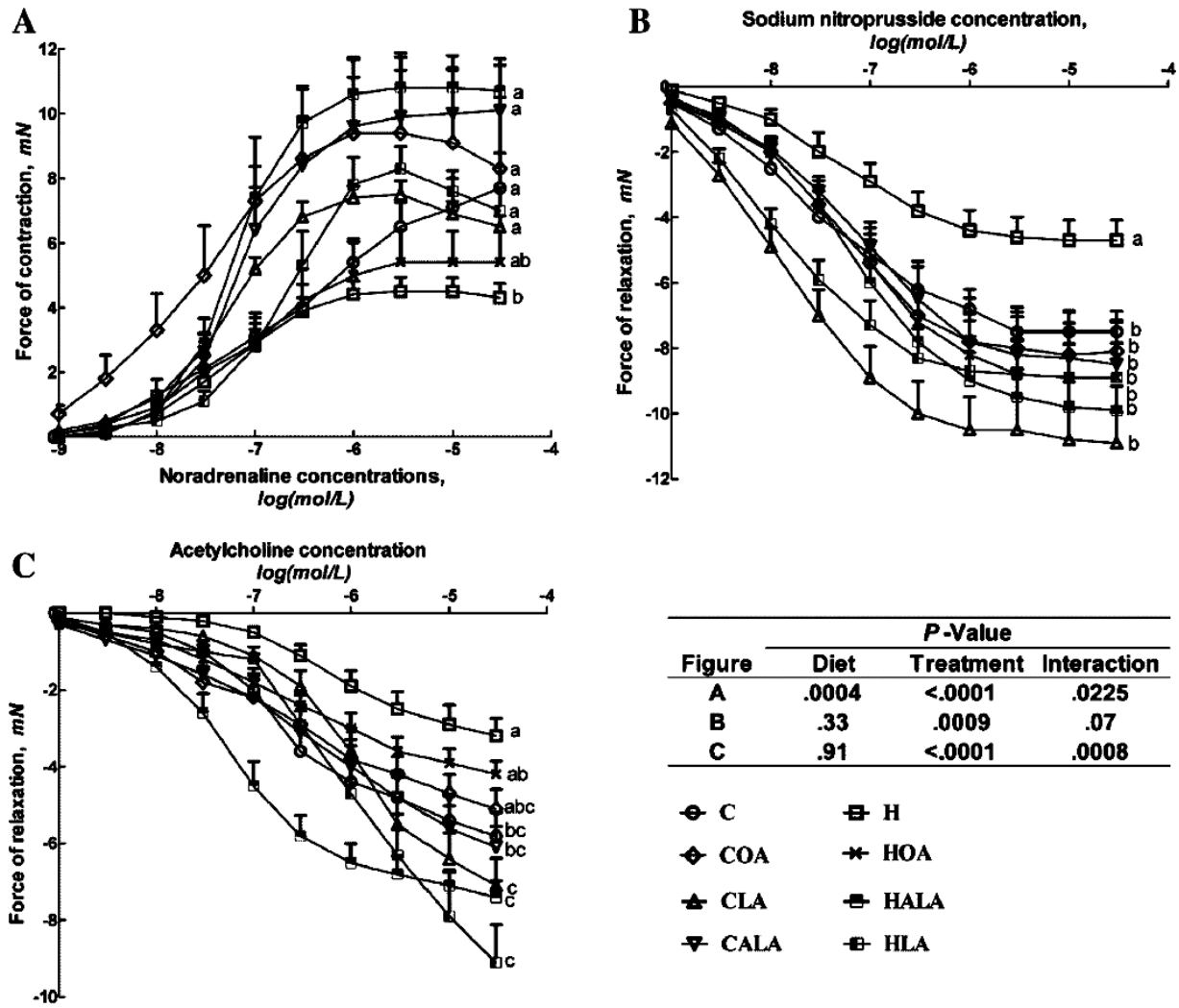

Figure	P-Value		
	Diet	Treatment	Interaction
A	.0004	<.0001	.0225
B	.33	.0009	.07
C	.91	<.0001	.0008

Fig. 2. Cumulative concentration–response curves for noradrenaline (A), sodium nitroprusside (B) and acetylcholine (C) in thoracic aortic rings from rats fed the C, COA, CLA, CALA, H, HOA, HLA and HALA diet. Data shown as means±S.E.M.; n=8–10/group. Endpoint means without a common alphabet in each data set significantly differ.

Table 4
Changes in hepatic structure, function, and fatty acid composition in groups fed the C, COA, CLA, CALA, H, HOA, HLA and HALA diet

Variable	C	COA	CLA	CALA	H	HOA	HLA	HALA	*P* value		
									Diet	Treatment	Interaction
Plasma ALT, U/L (n=10)	27.6±2.2^{cd}	20.2±2.6^{d}	51.6±10.4^{a}	31.7±2.5^{bc}	46.5±5.2^{ab}	24.6±2.3^{d}	35.2±3.1^{abcd}	44.4±3.7^{abc}	.15	.0003	.0026
Plasma AST, U/L (n=10)	82.4±5.8^{b}	65.7±2.1^{b}	150.5±38.0^{a}	76.0±2.7^{b}	109.6±7.8^{a}	57.6±2.9^{b}	97.8±6.2^{a}	83.3±4.9^{a}	.52	.0004	.0434
Plasma LDH, U/L (n=10)	173.1±14.0^{c}	265.1±23.7^{c}	466.6±80.4^{ab}	351.8±51.0^{abc}	519.1±12.6^{a}	324.1±51.5^{bc}	478.4±59.0^{ab}	357.2±38.0^{abc}	.0021	.0028	.0011
Plasma ALP, U/L (n=10)	136.8±9.8^{bc}	95.8±11.0^{bc}	119.0±18.4^{bc}	135.5±16.7^{bc}	217.5±28.2^{a}	83.5±7.3^{c}	154.8±14.6^{b}	97.9±8.1^{bc}	.14	<.0001	.0016
Plasma bilirubin, μmol/L (n=10)	2.2±0.1^{a}	0.8±0.2^{b}	1.2±0.2^{b}	2.4±0.1^{a}	2.3±0.1^{a}	1.0±0.4^{b}	1.8±0.3^{a}	2.0±0.1^{a}	.41	<.0001	.15
Plasma CK, U/L (n=10)	165.3±13.2^{b}	107.8±19.1^{b}	303.5±65.9^{a}	135.7±13.7^{b}	225.5±38.2^{a}	131.3±28.8^{b}	166.2±28.3^{ab}	154.6±19.2^{b}	.71	.0034	.0173
Liver fatty acid, g/100 g of total fatty acid content (n=6)											
C14:0	7.1±1.2^{a}	0.0±0.0^{c}	0.0±0.0^{c}	0.7±0.2^{c}	3.1±0.6^{b}	0.8±0.04^{c}	1.2±0.1^{c}	0.6±0.1^{c}	.14	<.0001	<.0001
C14:1n-5	0.7±0.1^{a}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}	0.8±0.1^{a}	0.1±0.1^{b}	0.0±0.0^{b}	0.0±0.0^{b}	.27	<.0001	.61
C16:0	0.03±0.03^{c}	23.2±0.5^{a}	22.2±0.3^{ab}	23.7±0.9^{a}	0.2±0.05^{c}	23.4±0.2^{a}	22.9±0.5^{a}	21.3±0.3^{c}	.3	<.0001	.0054
C16:1n-7	26.3±1.0^{a}	2.4±0.2^{cd}	2.2±0.05^{cd}	3.8±1.4^{c}	23.3±0.7^{b}	1.7±0.1^{cd}	1.9±0.2^{cd}	0.8±0.1^{d}	.0005	<.0001	.07
C18:0	3.4±3.4^{c}	14.0±1.1^{ab}	13.6±0.7^{ab}	15.3±3.0^{a}	0.3±0.1^{c}	14.4±1.2^{ab}	8.0±0.5^{b}	16.3±1.4^{a}	.16	<.0001	.23
C18:1n-9	0.0±0.0^{c}	26.3±2.5^{b}	16.8±0.5^{b}	17.5±4.4^{b}	1.5±1.5^{c}	26.5±3.3^{b}	47.8±1.7^{a}	27.1±3.7^{b}	<.0001	<.0001	<.0001
C18:1*trans*-7	15.1±2.5^{a}	2.9±0.2^{b}	2.7±0.1^{b}	3.0 ± 0.5^{b}	10.9±3.4^{a}	2.1±0.1^{b}	1.6±0.1^{b}	1.5±0.04^{b}	.08	<.0001	.66
C18:2n-6	27.1±4.5^{b}	9.7±0.5^{c}	19.3±0.7^{c}	12.9±1.3^{c}	44.3±4.9^{a}	9.6±0.5^{c}	11.3±0.3^{c}	9.7±0.5^{c}	.39	<.0001	<.0001
C18:3n-6	0.0±0.0	0.0±0.0	0.5±0.03	0.2±0.1	0.9±0.9	0.0±0.0	0.6±0.1	0.0±0.0	.39	.23	.35
C18:3n-3	7.5±1.5^{a}	0.0±0.0^{c}	0.0±0.0^{c}	1.2±0.9^{c}	4.9±1.0^{b}	0.0±0.0^{c}	0.2±0.04^{c}	0.8±0.1^{c}	.18	<.0001	.23
C20:3n-6	0.7±0.1^{b}	0.0±0.0^{c}	0.0±0.0^{c}	1.3±0.3^{a}	0.7±0.1^{b}	1.1±0.1^{ab}	0.0±0.0^{c}	1.0±0.1^{ab}	.0334	<.0001	<.0001
C20:4n-6	10.9±2.9^{ab}	16.1±1.4^{a}	16.7±0.8^{a}	12.7±2.8^{ab}	8.0±2.3^{bc}	14.7±1.2^{ab}	3.4±0.5^{c}	11.8±1.4^{ab}	.0011	.0115	.0051
C20:5n-3	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}	0.9±0.2^{a}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}	0.9±0.1^{a}	.88	<.0001	.99
C22:5n-3	0.2±0.1^{c}	0.8±0.2^{b}	0.0±0.0^{c}	1.5±0.3^{a}	0.05±0.03^{c}	0.0±0.0^{c}	0.0±0.0^{c}	1.3±0.1^{a}	.0137	<.0001	.0311
C22:6n-3	0.3±0.2^{c}	4.5±0.4^{b}	0.0±0.0^{c}	4.8±1.0^{b}	0.03±0.03^{c}	4.6±0.3^{b}	0.0±0.0^{c}	6.4±0.6^{a}	.25	<.0001	.16
C22:4n-6	0.0±0.0^{b}	0.0±0.0^{b}	2.0±0.2^{a}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}	<.0001	<.0001	<.0001
C22:5n-6	0.0±0.0^{c}	0.0±0.0^{c}	2.9±0.2^{a}	0.0±0.0^{c}	0.0±0.0^{c}	0.0±0.0^{c}	1.1±0.2^{b}	0.0±0.0^{c}	<.0001	<.0001	<.0001
Total SFA	11.3±4.1^{b}	37.2±1.5^{a}	35.9±0.6^{a}	39.7±2.1^{a}	3.9±0.6^{c}	38.6±1.4^{a}	32.1±0.6^{a}	38.3±1.1^{a}	.0422	<.0001	.14
Total MUFA	42.1±2.4^{ab}	31.7±2.8^{bcd}	21.8±0.6^{d}	24.3±6.0^{cd}	36.7±1.8^{bc}	30.5±3.4^{bcd}	51.3 ± 1.7^{a}	29.4±3.7^{bcd}	.0037	.0017	<.0001
Total PUFA	46.6±6.4^{b}	31.1±1.4^{c}	42.4±0.9^{bc}	35.9±4.0^{bc}	59.4±2.1^{a}	30.9±2.1^{c}	16.6±1.1^{d}	32.3±2.6^{c}	.06	<.0001	<.0001
n-3:n-6	0.2±0.02^{c}	0.2±0.01^{c}	0.0 ± 0.0^{e}	0.3±0.03^{b}	0.1±0.02^{d}	0.2±0.0^{c}	0.01±0.0^{e}	0.4±0.01^{a}	.5	<.0001	<.0001
Stearoyl-CoA 9-desaturase index*	–	0.1±0.01	0.1±0.0	0.1±0.05	78.8±2.4	0.1±0.01	0.1±0.01	0.03±0.00	–	–	–

Each value is a mean±S.E.M. Number of repetitive experiments indicated within parenthesis. Means within a row with unlike superscript differ; P<.05. ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; CK, creatine kinase.
* Negligible C16:0 fatty acid detected in C diet-fed group, therefore showing a very high stearoyl-CoA 9 desaturation index in this group.

aminotransferase, alkaline phosphatase and creatine kinase with increased lactate dehydrogenase and alkaline phosphatase decreasing in both HLA and HALA groups and creatine kinase decreasing in HALA group (Table 4). None of the plasma enzyme activities was affected in COA or CALA groups, but all were elevated in CLA group. Both COA and HOA groups also had decreased plasma bilirubin concentrations (Table 4).

H rats (Fig. 3E and M) showed increased hepatic lipid deposition and inflammatory cell infiltration compared to C rats (Fig. 3A and I). α-Linolenic acid supplementation normalized and oleic acid supplementation markedly reduced the macrovesicular steatosis and portal inflammation in HALA (Fig. 3H and P) and HOA (Fig. 3F and N) rats, respectively. Linoleic acid supplementation further aggravated the macrovesicular steatosis and did not alter the inflammatory state in HLA rats (Fig. 3G and O) compared to H rats. Further, linoleic acid supplementation induced macrovesicular steatosis but not inflammation in CLA group (Fig. 3C and K). No changes were observed in COA (Fig. 3B and J) and CALA (Fig. 3D and L) rats, and the tissue morphology appeared normal.

C14:0 and C14:1n-5 decreased in the liver of all oil-supplemented groups (Table 4). Similar to the plasma and cardiac fatty acid profiles, all oil-supplemented groups showed reduced stearoyl-CoA desaturase-1-mediated desaturation in the liver with increased C16:0 and decreased C16:1n-7 compared to their respective controls (Table 4). C18:0 and C18:1n-9 increased and C18:1*trans*-7 and C18:2n-6 decreased in all oil-supplemented groups (Table 4). C20:4n-6 decreased in HLA group and remained unchanged in all other groups. Similar to the cardiac fatty acid profile, C22:6n-3 was increased in COA and HOA groups, and all the long-chain PUFAs of the n-3 series (C20:5n-3, C22:5n-3 and C22:6n-3) increased in CALA and HALA groups (Table 4). While C22:5n-6 increased in both CLA and HLA groups, C22:4n-6 increased in only CLA group (Table 4). Consequently, total SFA content increased in all oil-supplemented groups; total MUFA decreased in CLA, CALA and HALA but increased in HLA; and total PUFA decreased in COA, HOA, HLA and HALA (Table 4). All linoleic acid-supplemented groups had lower and α-linolenic acid-supplemented groups had higher n-3:n-6 ratio without the accumulation of either essential fatty acid (Table 4).

3.4. Skeletal muscle fatty acid composition

Similar to the liver, C14:0 and C14:1n-5 decreased in the skeletal muscle of all oil-supplemented groups (Table 5). The oil-supplemented groups showed reduced stearoyl-CoA desaturase-1-mediated desaturation in the liver with increased C16:0 and decreased C16:1n-7 compared to their respective controls (Table 5). C18:0 and C18:1n-9 increased and C18:2n-6 decreased in all oil-supplemented groups (Table 5). C18:1*trans*-7 increased in CLA group and remained unchanged in all other groups. C20:4n-6 increased in CLA and HLA groups and remained unchanged in all other groups. C18:3n-3, C22:5n-3 and C22:6n-3 increased in the skeletal muscle of CALA and HALA groups, with C22:6n-3 also increasing in COA and HOA groups (Table 5). C22:5n-6

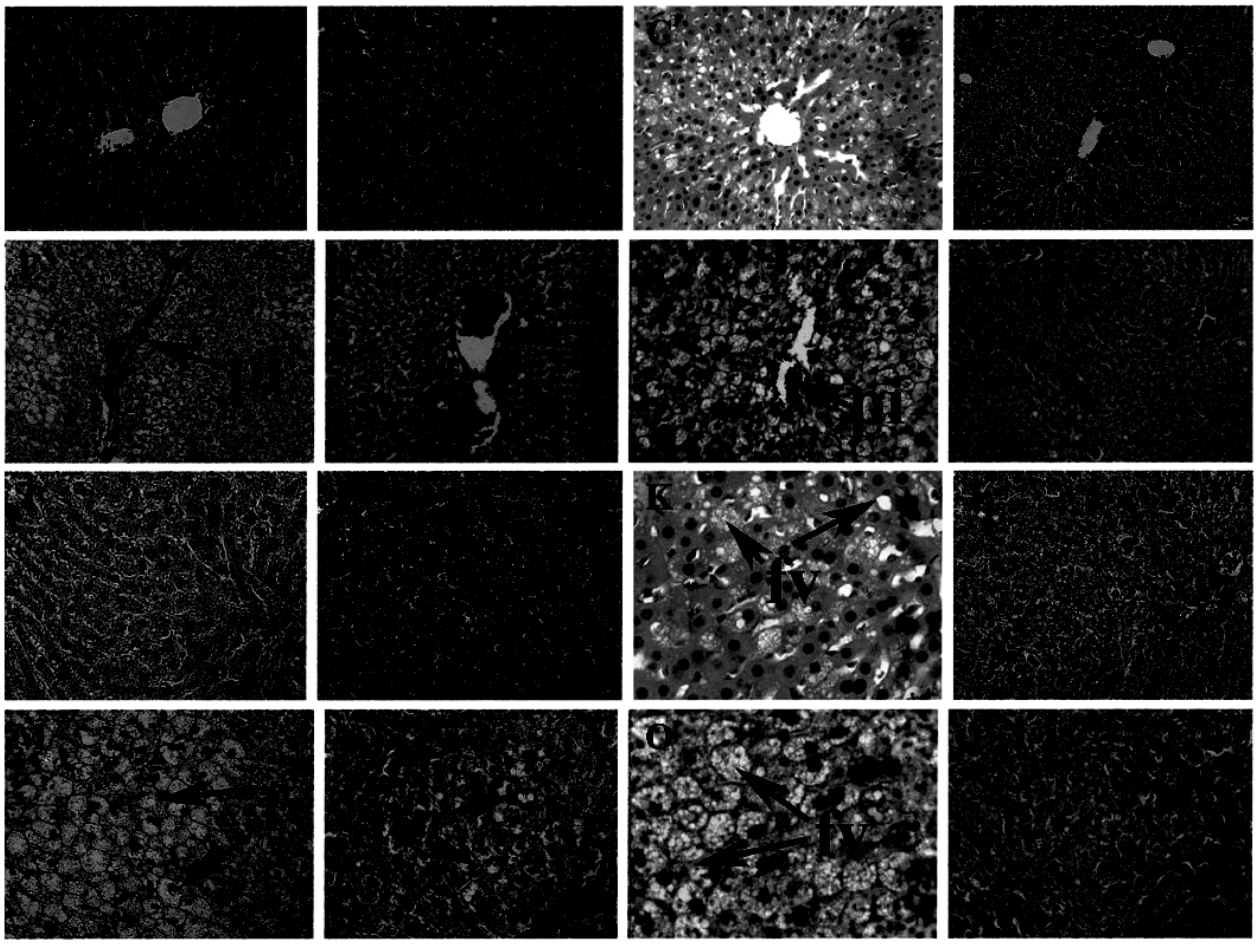

Fig. 3. Hematoxylin and eosin staining of hepatocytes showing inflammatory cells around the portal region (marked as "pi") (×20) in rats fed the C (A), COA (B), CLA (C), CALA (D), H (E), HOA (F), HLA (G), and HALA (H) diet and hepatocytes (×40) with enlarged fat vacuole (marked as "fv") in rats fed the C (I), COA (J), CLA (K), CALA (L), H (M), HOA (N), HLA (O), and HALA (P) diet.

increased in both CLA and HLA groups (Table 5). Consequently, total SFA increased and PUFA content decreased in all oil-supplemented groups, and total MUFA increased with oleic acid and α-linolenic acid but not linoleic acid supplementation (Table 5). All linoleic acid-supplemented groups had lower and oleic acid- and α-linolenic acid-supplemented groups had higher n-3:n-6 ratios (Table 5).

Table 5
Fatty acid profiles of skeletal muscle and desaturase index in groups fed the C, COA, CLA, CALA, H, HOA, HLA and HALA diet

Fatty acid, g/100 g of total fatty acid content (n=6)	C	COA	CLA	CALA	H	HOA	HLA	HALA	*P* value		
									Diet	Treatment	Interaction
C14:0	14.3±3.2^{a}	1.4±0.1^{bc}	0.0±0.0^{c}	1.3±0.04^{bc}	6.3±1.6^{b}	2.3±0.1^{bc}	0.3±0.3^{c}	2.4±0.1^{bc}	.12	<.0001	.0024
C14:1n-5	1.8±0.1^{b}	0.0±0.0^{c}	0.0±0.0^{c}	0.0±0.0^{c}	2.5±0.2^{a}	0.0±0.0^{c}	0.0±0.0^{c}	0.1±0.04^{c}	.0025	<.0001	.0004
C16:0	0.6±0.1^{e}	23.9±0.8^{c}	38.8±1.1^{a}	24.3±0.4^{c}	0.4±0.03^{e}	23.1±0.3^{c}	32.6±1.3^{b}	20.5±0.2^{d}	<.0001	<.0001	.0003
C16:1n-7	28.4±0.9^{a}	5.6±0.6^{c}	2.8±0.9^{d}	5.9±0.5^{c}	20.9±1.3^{b}	3.2±0.1^{d}	0.2±0.2^{e}	2.4±0.2^{d}	<.0001	<.0001	.0042
C18:0	0.3±0.1^{e}	6.3±0.3^{cd}	10.8±0.9^{b}	5.6±0.1^{d}	0.6±0.03^{e}	7.7±0.2^{c}	13.8±0.6^{a}	7.4±0.3^{c}	<.0001	<.0001	.0281
C18:1n-9	4.2±1.0^{e}	43.7±3.5^{b}	16.8±1.8^{d}	34.0±0.6^{c}	5.3±1.1^{e}	52.0±1.0^{a}	32.8±2.2^{c}	54.6±0.6^{a}	<.0001	<.0001	<.0001
C18:1*trans*-7	0.7±0.7^{b}	4.2±0.5^{b}	10.1±3.0^{a}	3.2±0.1^{b}	1.1±1.1^{b}	2.3±0.05^{b}	0.3±0.3^{b}	1.3±0.3^{b}	.0004	.0072	.0008
C18:2n-6	36.6±4.0^{b}	9.5±1.0^{c}	9.7±3.3^{c}	15.8±0.6^{c}	50.5±3.5^{a}	7.0±0.3^{b}	12.8±0.3^{b}	6.9±0.2^{b}	.41	<.0001	.0001
C18:3n-3	1.4±1.4^{bc}	0.0±0.0^{c}	0.0±0.0^{c}	4.7±0.4^{a}	0.0±0.0^{c}	0.0±0.0^{c}	0.0±0.0^{c}	2.5±0.1^{a}	.0189	<.0001	.1
C20:4n-6	2.9±0.7^{c}	3.0±0.7^{c}	7.0±0.8^{a}	1.5±0.3^{cd}	0.8±0.2^{d}	1.4±0.3^{cd}	4.9±0.6^{b}	0.8±0.1^{d}	<.0001	<.0001	.58
C22:5n-3	0.1±0.1^{c}	0.0±0.0^{c}	0.0±0.0^{c}	1.0±0.1^{b}	0.0±0.0^{c}	0.0±0.0^{c}	0.0±0.0^{c}	0.3±0.03^{b}	<.0001	<.0001	<.0001
C22:6n-3	0.3±0.1^{bc}	2.2±0.5^{a}	0.0±0.0^{c}	2.1±0.1^{a}	0.1±0.05^{c}	1.0±0.2^{b}	0.0±0.0^{c}	0.9±0.1^{b}	<.0001	<.0001	.0071
C22:5n-6	0.0±0.0^{c}	0.0±0.0^{c}	3.9±0.5^{b}	0.0±0.0^{c}	0.0±0.0^{c}	0.0±0.0^{c}	2.1±0.8^{c}	0.0±0.0^{c}	.07	<.0001	.0269
Total SFA	15.6±3.4^{c}	31.6±0.7^{b}	49.7±2.0^{a}	31.3±0.5^{b}	7.6±1.5^{d}	33.1±0.4^{b}	46.8±1.6^{a}	30.2±0.4^{b}	.0339	<.0001	.05
Total MUFA	35.3±1.2^{c}	53.6±2.6^{a}	29.7±0.8^{c}	43.1±0.9^{b}	30.1±1.6^{c}	57.5±1.1^{a}	33.3±2.4^{c}	58.4±0.6^{a}	.0004	<.0001	<.0001
Total PUFA	49.1±3.4^{b}	14.8±2.1^{de}	20.6±2.5^{cd}	25.6±0.9^{c}	62.4±2.4^{a}	9.4±0.8^{e}	19.9±1.5^{cd}	11.4±0.4^{e}	.24	<.0001	<.0001
n-3:n-6	0.04±0.03^{c}	0.1±0.03^{b}	0.0±0.0^{c}	0.5±0.04^{a}	0.0±0.0^{c}	0.1±0.02^{b}	0.0±0.0^{c}	0.5±0.01^{a}	.43	<.0001	.52
Stearoyl-CoA 9 desaturase index	63.1±13.7^{a}	0.2±0.02^{b}	0.1±0.03^{b}	0.2±0.02^{b}	49.1±1.2^{a}	0.1±0.0^{b}	0.01±0.01^{b}	0.1±0.01^{b}	.32	<.0001	.41

Each value is a mean±S.E.M. Number of repetitive experiments indicated within parenthesis. Means within a row with unlike superscript differ; P<.05.

Table 6
Fatty acid profiles of retroperitoneal adipose tissue and desaturase index in groups fed the C, COA, CLA, CALA, H, HOA, HLA and HALA diet

Fatty acid, g/100 g of total fatty acid content (n=6)	C	COA	CLA	CALA	H	HOA	HLA	HALA	*P* value		
									Diet	Treatment	Interaction
C14:0	0.0±0.0^{e}	1.4±0.1^{b}	1.4±0.03^{b}	1.5±0.04^{b}	0.0±0.0^{e}	2.2±0.05^{a}	2.3±0.1^{a}	2.3±0.05^{a}	<.0001	<.0001	<.0001
C14:1n-5	2.2±0.04^{b}	0.1±0.02^{e}	0.0±0.0^{e}	0.0±0.0^{e}	2.8±0.1^{a}	0.2±0.01^{c}	0.0±0.0^{e}	0.1±0.03^{e}	<.0001	<.0001	<.0001
C16:0	0.2±0.05^{d}	23.5±0.4^{b}	23.0±0.3^{b}	25.4±0.5^{a}	0.4±0.1^{d}	20.0±0.3^{c}	20.2±0.2^{c}	20.0±0.1^{c}	<.0001	<.0001	<.0001
C16:1n-7	31.5±1.1^{a}	6.9±0.3^{cd}	5.2±0.2^{d}	6.1±0.4^{c}	22.4±0.2^{b}	3.1±0.1^{e}	2.8±0.2^{e}	2.0±0.3^{e}	<.0001	<.0001	<.0001
C18:0	0.2±0.03^{e}	3.2±0.1^{c}	3.5±0.1^{bc}	3.7±0.1^{b}	0.6±0.02^{d}	6.4±0.2^{a}	6.8±0.2^{a}	6.8±0.2^{a}	<.0001	<.0001	<.0001
C18:1n-9	0.0±0.0^{f}	50.5±0.6^{c}	33.9±0.4^{e}	39.0±0.5^{d}	0.0±0.0^{f}	62.0±0.9^{a}	54.7±0.7^{b}	0.0±0.0^{f}	<.0001	<.0001	<.0001
C18:1*trans*-7	3.5±0.2^{cd}	4.4±0.2^{c}	3.8±0.1^{cd}	3.1±0.06^{d}	7.0±0.4^{b}	0.7±0.3^{f}	1.6±0.5^{e}	59.8±0.2^{a}	<.0001	<.0001	<.0001
C18:2n-6	51.2±1.0^{b}	8.8±0.4^{f}	27.4±0.4^{c}	15.3±0.6^{d}	60.8±0.5^{a}	5.0±0.2^{g}	10.6±0.3^{e}	6.1±0.2^{g}	<.0001	<.0001	<.0001
C18:3n-6	10.1±0.3^{a}	0.0±0.0^{d}	0.5±0.02^{c}	0.0±0.0^{d}	5.2±0.1^{b}	0.0±0.0^{d}	0.3±0.1^{cd}	0.0±0.0^{d}	<.0001	<.0001	<.0001
C18:3n-3	0.0±0.0^{e}	0.3±0.01^{c}	0.5±0.03^{c}	5.8±0.4^{a}	0.1±0.1^{c}	0.3±0.02^{c}	0.4±0.02^{c}	2.6±0.1^{b}	<.0001	<.0001	<.0001
Total SFA	1.1±0.1^{c}	28.4±0.5^{b}	28.0±0.3^{b}	30.6±0.5^{a}	1.5±0.1^{c}	28.7±0.5^{b}	29.3±0.4^{b}	29.3±0.3^{b}	.49	<.0001	.0123
Total MUFA	37.2±0.9^{f}	62.4±0.6^{b}	43.2±0.3^{e}	48.2±0.6^{d}	32.4±0.4^{g}	65.9±0.6^{a}	59.2±0.6^{c}	62.0±0.4^{b}	<.0001	<.0001	<.0001
Total PUFA	61.7±0.9^{b}	9.3±0.4^{f}	28.8±0.4^{c}	21.2±0.9^{d}	66.1±0.5^{a}	5.4±0.2^{g}	11.5±0.3^{e}	8.7±0.3^{f}	<.0001	<.0001	<.0001
n-3:n-6	0.0±0.0^{e}	0.04±0.0^{d}	0.02±0.0^{de}	0.4±0.02^{b}	0.0±0.0^{e}	0.1±0.0^{c}	0.04±0.0^{d}	0.4±0.01^{a}	.0002	<.0001	.09
Stearoyl-CoA 9 desaturase index	129.5±16.7^{a}	0.3±0.01^{c}	0.2±0.01^{c}	0.2±0.01^{c}	44.4±0.5^{b}	0.2±0.0^{c}	0.1±0.01^{c}	0.1±0.02^{c}	<.0001	<.0001	<.0001

Each value is a mean±S.E.M. Number of repetitive experiments indicated within parenthesis. Means within a row with unlike superscript differ; P<.05.

3.5. Adipose tissue fatty acid composition

In contrast to other tissues, C14:0 increased and C14:1n-5 decreased in the adipose tissue of all oil-supplemented groups (Table 6). However, all oil-supplemented groups showed reduced desaturation of C16:0 with decreased C16:1n-7 compared to their respective controls (Table 6). C18:0 and C18:1n-9 increased in all oil-supplemented groups except HALA which had an equivalent increase in C18:1*trans*-7 instead of C18:1n-9 in the adipose tissue (Table 6). C18:2n-6 decreased in all oil-supplemented groups, and C18:3n-3 increased in CALA and HALA groups (Table 6). Consequently, total SFA and MUFA increased and total PUFA decreased in all oil-supplemented groups, with only α-linolenic acid-supplemented groups showing higher n-3:n-6 ratio (Table 6).

4. Discussion

Metabolic syndrome is a multifaceted disorder with complex interactions at tissue and cellular levels. Cardiovascular disease and insulin resistance are the major consequences of metabolic syndrome, with the major risk factors being abdominal obesity, dyslipidemia, impaired glucose tolerance, hypertension and fatty liver [20,21]. As dietary fat is a major component in the etiology of metabolic syndrome, it is important to evaluate the role of different dietary fats in health and disease. Dietary unsaturated fatty acids include both MUFA and PUFA, and their individual biological actions remain unclear [1] due to the paucity of comparative studies.

The benefits of MUFA-rich diets in attenuating the risk factors of metabolic syndrome are inconsistent [6]. Some studies report that MUFAs lower blood pressure, serum lipids, insulin resistance, fasting blood glucose and adiposity, and reduce the incidence of cardiovascular diseases, while other studies report a neutral response [6]. Moreover, most studies reporting the benefits of MUFA, including the landmark Seven Countries study, have used olive oil or have compared the responses of MUFA against SFA rather than PUFA [6,22]. There is a growing consensus that the cardioprotective effects of olive oil have been attributed to its high phenolic content, mainly as oleuropein and hydroxytyrosol [23].

Although PUFAs are generally considered to be cardioprotective, n-3 (α-linolenic acid) and n-6 (linoleic acid) PUFAs produce opposing responses [7,9,16]. The n-6 PUFAs may mitigate or worsen cardiovascular diseases [24–27]. Recently, similar inconsistencies in the cardioprotective effects of n-6 PUFAs have been highlighted [9]. Some studies attribute the cardioprotective effects of n-6 PUFAs to low-density lipoprotein cholesterol-lowering properties [26], while others disagree on the long-term implications of recommended dietary intake of linoleic acid because of the proinflammatory effects of eicosanoids derived from arachidonic acid, the major metabolite of linoleic acid [25,27]. Nevertheless, a lower dietary n-6:n-3 PUFA ratio has been associated with reduced risk of cardiovascular diseases and metabolic syndrome [28–30]. Epidemiological, human and animal studies suggest that α-linolenic acid lowers blood pressure, visceral adiposity, plasma lipids, insulin resistance, impaired glucose tolerance, hepatic steatosis, cardiac and hepatic inflammation and fibrosis, and improves cardiovascular structure and function [7,16,31–33]. However, independent effects of oleic acid, linoleic acid and α-linolenic acid on the risk factors of metabolic syndrome have been rarely compared.

Given the small number of comprehensive studies comparing the effects of the three principal C18 unsaturated fatty acids, the main aim of this study was to characterize systematically the effects of dietary supplementation of oleic, linoleic and α-linolenic acids in rats fed either a low-fat, high-starch diet as control or a high-fructose, high-tallow diet as a model of metabolic syndrome. Here, we have shown that dietary supplementation of C18 unsaturated fatty acids elicits different responses in adiposity, cardiovascular remodeling, plasma lipids, hepatic structure and function, and tissue fatty acid compositions. In particular, we have shown that oleic acid, linoleic acid and α-linolenic acid induced lipid redistribution away from the abdomen and that this effect is more pronounced with increasing degree of unsaturation. We have previously shown that α-linolenic acid-rich chia seed and oil but not eicosapentaenoic or docosahexaenoic acid-rich fish oil induce lipid redistribution in diet-induced obese rats [12,34]. These results suggest that the C18 unsaturated fatty acids favor lipid partitioning away from the abdomen. However, only linoleic and α-linolenic acids induced lipid partitioning without affecting whole-body adiposity, as oleic acid supplementation increased whole-body adiposity.

Additionally, oleic acid decreased total plasma cholesterol but did not change plasma triglycerides and nonesterified fatty acids, whereas the PUFAs (linoleic and α-linolenic acids) decreased plasma triglycerides and nonesterified fatty acids but not cholesterol. α-Linolenic acid supplementation improved left ventricular structure and function, diastolic stiffness and systolic blood pressure. Oleic or linoleic acid supplementation did not change the left ventricular remodeling induced by H-diet feeding, but both fatty acids induced dilation of the left ventricle and subsequent deterioration in function in the rats fed the low-fat (C) diet. However, oleic but not linoleic acid

supplementation normalized systolic blood pressure. Further, oleic acid supplementation was effective in reducing the plasma markers of liver damage, while linoleic and α-linolenic acids had minor effects.

Additionally, linoleic acid induced left ventricular adaptation to reduce the ventricular wall stress. We have previously shown that H-diet feeding induces a characteristic increase in preload and consequent eccentric hypertrophy of the left ventricle [18]. Linoleic acid supplementation increased the ejection fraction, ejection time and rate of contraction to eject the larger diastolic volume. However, since linoleic acid did not reduce blood pressure, the left ventricle had to eject a large proportion of its diastolic volume against higher arterial pressure. This effect led to structural adaptation in the chamber dimensions in systole, with decreased left ventricular internal diameter in systole and increased left ventricular posterior wall dimension in systole but not in diastole. In contrast, α-linolenic acid supplementation decreased chamber dimensions in diastole in response to decreased LV volumes and systolic blood pressure without affecting the dimensions in systole.

These effects were associated with unique tissue fatty acid profiles induced by oleic, linoleic and α-linolenic acids. While all three fatty acids inhibited the desaturation of C16:0 in all tissues, linoleic acid induced preferential oxidation of *trans* fat and therefore loss of vaccenic acid from all tissues; α-linolenic acid induced the selective storage of *trans* fat in the adipose tissue and oleic acid in the organs. These results expand our previous observations with dietary n-3 PUFA where we have shown that α-linolenic acid-, eicosapentaenoic acid- and docosahexaenoic acid-supplemented diets reduced tissue *trans* fatty acids in vital organs such as the heart, liver and skeletal muscle [12,35]. Higher dietary intake of *trans* fatty acids is an independent modifiable risk factor of metabolic syndrome and cardiovascular diseases. There is a growing consensus that dietary *trans* fatty acids trigger systemic inflammation; deteriorate endothelial function; and induce dyslipidemia, insulin resistance and obesity to increase the risk of cardiovascular diseases [35,36]. In addition to reducing tissue *trans* fatty acids, both linoleic and α-linolenic acids increased the accumulation of their respective C22 elongated products, while oleic acid supplementation showed an n-3 PUFA-sparing effect seen as a consistently increased proportion of docosahexaenoic acid in all tissue types.

Arachidonic acid, the product of linoleic acid metabolism, is considered to be the key regulator of inflammation due to production of proinflammatory eicosanoids by the action of lipoxygenase and cyclooxygenase [7]. However, in this study, linoleic acid supplementation did not aggravate the inflammatory response but attenuated it, at least in the heart. Contrary to our hypothesis, linoleic acid supplementation did not result in major accumulation of either linoleic or arachidonic acids in vital organs but resulted in accumulation of the long-chain metabolite, C22:5n-6. These results strongly suggest that the elongation of arachidonic acid is preferred over metabolism by lipoxygenase/cyclooxygenase, at least when the diet and/or tissues are low in long-chain PUFA. It is also plausible that the elongation is preferred over lipoxygenase/cyclooxygenase metabolism to limit eicosanoid-induced vasoconstriction, platelet aggregation and inflammation. Such a negative feedback mechanism has been previously described for at least arachidonic acid-derived leukotrienes [37]. The mechanism of partitioning of arachidonic acid between lipoxygenase/cyclooxygenase metabolism and elongation is currently unknown and warrants further investigation.

In conclusion, α-linolenic acid but not oleic acid and linoleic acid attenuated the risk factors of metabolic syndrome. Although oleic acid and linoleic acid were both associated with cardiovascular remodeling, oleic acid decreased blood pressure and improved liver structure and function, and linoleic acid only decreased plasma triglyceride concentrations without affecting other risk factors of metabolic syndrome. However, the three fatty acids were all associated with trafficking of fat away from the abdomen, proportional to the number of double bonds.

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