Investigation of Chinese Herbal Medicine in treatment of Metabolic Syndrome

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Declaration

I declare that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text. I also declare that this thesis has been written by me. Any help I received in my research and preparation of this thesis has been acknowledged.

Signature of Student

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John Shim

2008

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List of Abbreviations

ASCVD	Atherosclerotic cardiovascular disease
AUC	Area under curve
FFA	Free fatty acids
GLUT-4	Glucose transporter-4
HDL-C	High density lipoprotein cholesterol
HFD	High fat diet
IDF	International Diabetes Federation
i.p. GTT	Intra-peritoneal glucose tolerance test
i.p. ITT	Intra-peritoneal insulin tolerance test
LDL-C	Low density lipoprotein cholesterol
MetS	Metabolic syndrome
NCEP	National Cholesterol Education Program
NEFA	Non-esterified fatty acids
PI(3)k	Phosphatidylinositol 3-kinase
PPAR	Peroxisome proliferator-activated receptor
RAAS	Renin-angiotensin-aldosterone-system
SEM	Standard error of mean
STZ	Streptozotocin
T2DM	Type 2 diabetes mellitus
TNF-α	Tumour necrosis factor-α
TZD	Thiazolidinedione
VLDL-C	Very low density lipoprotein cholesterol
WHO	World Health Organisation

Abstract

Metabolic syndrome (MetS) is a clustering of atherosclerotic cardiovascular disease (ASCVD) risk factors including central obesity, insulin resistance, dyslipidaemia, hypertension, and pro-inflammatory and pro-thrombotic states. It is also a precursor of type 2 diabetes mellitus (T2DM). Research continues to uncover the mechanical links of the pathogenesis of MetS as well as to discover new drugs to target the multiple metabolic and haemodynamic abnormalities of MetS. The focus of this thesis is on the investigation of two Chinese herbal medicines, Sugarid and SK0504, in the treatment of MetS and T2DM induced in C57BL/6J mice by high fat diet (HFD) feeding with or without streptozotocin (STZ) injection.

The results described in Chapter 3 show that T2DM was induced in mice fed HFD when their blood glucose levels rose after the STZ injection. Treatment with a Chinese herbal product, Sugarid did not show any significant improvements of body and visceral fat weights, glucose metabolism, insulin sensitivity, and serum and liver lipid parameters in either MetS and T2DM models of mice. Therefore, the findings from this study do not support the notion of Sugarid being a potential drug in treatment of MetS and T2DM.

The purpose of Chapter 4 is to investigate the effects and mechanisms of a new herbal formula, SK0504 in HFD fed mice. Treatment with SK0504 showed significant improvements of visceral fat weight, insulin sensitivity and some biochemical parameters. The findings of this study showed potential beneficial effects of SK0504 on MetS by its ability to target central obesity, insulin resistance and hyperlipidaemia. However, the hypothesised effects of SK0504 in targeting the multiple metabolic

abnormalities of MetS were not obtained. The last chapter outlines possible avenues for further research in order to confirm any implications of the experimental findings reported in this thesis.

CHAPTER 1

General introduction

1.1 METABOLIC SYNDROME

1.1.1 Definition of MetS

MetS is a constellation of multiple disease states – central obesity, insulin resistance, hyperglycaemia, dyslipidaemia, hypertension, and pro-thrombotic and pro-inflammatory states – all of which are independent risk factors for acquiring atherosclerotic cardiovascular disease (ASCVD) (Alberti et al., 2005; Grundy et al., 2005; Moller & Kaufman, 2005). MetS is a precursor to T2DM, as people with the former have a five-fold risk of developing the latter compared to those without it (Grundy, 2006; Kahn, 2007). Insulin resistance, which is associated with a cluster of metabolic risk factors, precedes the onset of T2DM. There are many alternative names for MetS including insulin resistance syndrome, syndrome X, cardiovascular disease risk factors, and deadly quartet.

The exact clinical definition of MetS is to date unknown as there are still some uncertainties about its exact pathogenesis (Grundy et al., 2005; Moller & Kaufman, 2005). Several organisations, namely, the World Health Organisation (WHO), European Group for the Study of Insulin Resistance (EGIR), American Association of Clinical Endocrinologists (AACE), Adult Treatment Panel III (ATP III), and International Diabetes Federation (IDF) have attempted to formulate the exact clinical definition of MetS without much success. Although all the organisations defined MetS as a constellation of ASCVD risk factors such as central obesity, insulin resistance, dyslipidaemia, hypertension, and hyperglycaemia, each defined the syndrome slightly different from the others.

The early definitions of MetS were proposed by WHO and EGIR in 1999 (Magliano et al., 2006). The two organisations emphasised insulin resistance as the major component of MetS with obesity, dyslipidaemia, and hypertension as additional components. Microalbuminuria was also a component included by WHO, but not by EGIR, which

instead included raised fasting plasma glucose The latest definitions of MetS were set by ATP III and IDF in 2001 and 2005 (Grundy et al., 2005). The two groups' definitions of MetS were similar except for minor differences. ATP III proposed a diagnosis of MetS in a person who presented with any three or more of the following factors: central obesity, dyslipidaemia, hypertension and hyperglycaemia, whereas IDF decided that a person with MetS must present with central obesity plus any two of the other disease states (Zimmet et al., 2005; Table 1.1). Hence IDF placed a stronger emphasis on central obesity as the diagnostic component of MetS. Other differences in diagnostic criteria between ATP III and IDF included fasting plasma glucose levels and the range for waist circumferences. IDF set lower plasma glucose levels than ATP III (100 vs 110 mg/dL), as well as additional waist circumference ranges for Asians (Grundy et al., 2005). To this date, there are ongoing discussions between different organisations to determine the exact definition of MetS.

Table 1.1 Definition of MetS according to ATP III and IDF.

ATP III 2001	IDF 2005
Three or more of the following:	Central obesity Waist circumference (ethnicity specific)* Plus any two of the following:
1. Central obesity: waist circumference >102 cm (M), >88 cm (F)	1. Raised triglycerides ≥150 mg/dL (1.7 mmol/L) or specific treatment for this abnormality.
2. Hypertriglyceridaemia: triglycerides ≥ 150 mg/dL (1.7 mmol/L)	2. Reduced HDL-C <40 mg/dL (1.03 mmol/L) (M) <50 mg/dL (1.29 mmol/L) (F) or specific treatment for this abnormality
3. Low HDL-C: <40 mg/dL (1.03 mmol/L) (M), <50 mg/dL (1.29 mmol/L) (F)	3. Hypertension: blood pressure ≥ 130/85 mmHg or medication
4. Hypertension: blood pressure ≥ 130/85 mmHg or medication	4. Fasting plasma glucose ≥100 mg/dL (5.6 mmol/L) or previously diagnosed type 2 diabetes.
5. Fasting plasma glucose ≥ 110 mg/dL (6.1 mmol/L)	

*Europeans (≥102 cm (M), ≥88 cm (F)); Asians (≥90 cm (M), ≥80 cm (F)); Japanese (≥85 cm (M), ≥90 cm (F))

1.1.2 Causative factors of MetS

Although the exact cause of MetS is unknown, insulin resistance and central obesity are the most likely contributing factors. This statement is supported by the fact that the majority of organisations mentioned above stressed insulin resistance and central obesity as crucial components of MetS (Grundy et al., 2005). Nevertheless, there is so far no one major identifiable cause to the pathogenesis of MetS, but a cluster of closely related disease states exists (Alberti et al., 2006). Environmental factors, including lifestyle and diet, and hereditary factors have significant effects on the development of insulin resistance and central obesity.

1.1.2.1 Environmental factors

Sedentary lifestyle with reduced physical exercise is a well-known factor that causes obesity and ASCVD. In addition, insulin resistance seems to have its root in an obesity-associated sedentary lifestyle (Moller & Kaufman, 2005; Rao, 2001; Rupp, 2004). In the current era, new technology has brought many luxuries including foods rich in fat, simple sugars and salts that have been termed as 'atherogenic diet' (Matsuzawa et al., 2007). High intake of these foods has led to development of disease states such as obesity, dyslipidaemia and hypertension as well as ASCVD (Hill & Peters, 1998; Matsuzawa et al., 2007). A strong link between atherogenic diet and MetS is evident from many research studies revealing animal models of MetS or T2DM induced by high fat or sugar diet (Ahren & Scheurink, 1998; Hsu et al., 2007).

1.1.2.2 Genetic susceptibility to MetS

Genetic susceptibility to cardiovascular disease risk factors plays a strong role in the pathogenesis of MetS (Bays et al., 2005; Moller & Kaufman, 2005; Rao, 2001; Rupp, 2004). The impact of genetic susceptibility on the pathogenesis of MetS is described in the

statement that although insulin resistance is associated with obesity, not all obese people are insulin resistant and not all lean people are metabolically normal (Bays, 2005). However, it is unclear what the degree of influence genetic susceptibility has on the pathogenesis of MetS. Nevertheless, its influence is significant, particularly when it is combined with other factors such as atherogenic diet; as Bays et al. (2005) mentioned, adiposopathy was most likely to develop in genetically predisposed people on regular high fat diets.

1.1.3 Prevalence of MetS

Prevalence of MetS is at an epidemiological level, as is it closely associated with ASCVD and T2DM. It is known that ASCVD is the leading epidemiological disease in the world and T2DM is also growing (Rosamond et al., 2007). It is estimated that up to 20% of the adult population is affected by MetS globally (Dentali et al., 2007). In the United States alone, during the period from 1988 to 1994, the prevalence of MetS was 23.7% in adults aged ≥20 years old, and 43.5% and 42.1% in those aged 60 to 69 and >70 years old. Among ethnic groups, the highest prevalence of MetS was seen in Mexican Americans, making up 31.9% of the worldwide prevalence (Moller & Kaufman, 2005).

In conjunction with ASCVD, T2DM and other significant diseases such as obesity and polycystic ovarian syndrome (PCOS), the prevalence of MetS continues to grow (Bays et al., 2005; Simons et al., 2007). For example the prevalence of MetS in women with PCOS was more than two-fold compared to those without PCOS (Apridonidze et al., 2005). Interestingly, a relationship between PCOS and insulin resistance was established by Park et al. (2007) in showing that women with PCOS also presented with clinical features of increased hyperandrogenemia and lower serum sex hormone-binding globulin, which were related to insulin resistance.

1.1.4 T2DM in relation to MetS

T2DM is a metabolic disorder characterised by hyperglycaemia as a result of impaired secretion or action of insulin (Rao, 2001). The fasting plasma glucose (FPG) levels within ≥6.1 and <7 mmol/l (or blood glucose of ≥5.6 and <6.1mmol/l), which are slightly higher than the normal physiological level, are referred to as impaired fasting glucose (IFG) levels. The 2-hour post glucose load values of ≥7.8 and < 11.1 mmol/l for plasma (or ≥6.7 and <10 mmol/l for blood) are referred to as impaired glucose tolerance, which indicate insulin resistance. Finally, plasma glucose levels ≥7 mmol/l (or ≥6.1 mmol/l for blood) or the 2-hour oral glucose tolerance measurements of ≥11.1mmol/L for plasma (or ≥10mmol/l for blood) can be a diagnostic criterion for diabetes (American Diabetes Association, 2007; Henareh et al., 2004; Sacks et al., 2002; World Health Organisation, 1999). Although the exact cause of T2DM is unknown, the mechanisms of its pathogenesis relate closely to that of MetS, as the latter often precedes the former.

T2DM is an epidemiological disease that is growing rapidly in the United States and in many developing countries, including China and India. From 1935 to 1996, the prevalence of diagnosed T2DM climbed nearly 765%. The global figures are predicted to rise 46% from 150 million cases in 2000 to 221 million in 2010 (Basciano et al., 2005).

Table 1.2 Three ranges of hyperglycaemia, indicating impaired fasting glucose, impaired glucose tolerance, and diabetes

		FPG (mmol/l)	OGTT (2h post glucose load) (mmol/l)
Normoglycaemic		< 6.1	
Hyperglycaemic	Impaired fasting glucose	≥6.1 and <7	<7.8 (if measured)
	Impaired glucose tolerance	<7 (if measured)	≥7.8 and <11.1
	Diabetes	≥7	
			≥11.1

1.2 Mechanism of insulin resistance in the pathogenesis of MetS

1.2.1 Physiological functions of insulin

Insulin is an anabolic hormone secreted by the pancreas which maintains homeostasis and regulates metabolism (Saltiel & Kahn, 2001). Its main function is to increase energy storage while preventing its expenditure. Insulin is therefore essential for the growth of body tissues, such as body cell differentiations. Insulin increases energy storage by promoting lipogenesis, and glycogen and protein synthesis, meanwhile inhibiting lipolysis, glycogenolysis and protein breakdown. In glucose metabolism, insulin increases glucose uptake into the skeletal muscles and fats from the blood stream, as it inhibits hepatic glucose output to the blood stream (Saltiel & Kahn, 2001). Insulin also has haemodynamic effects as it regulates the vasodilative and vasoconstrictive actions of endothelium (Kim et al., 2006; Moller & Kaufman, 2005).

1.2.2 Definition and significance of insulin resistance

Insulin resistance refers to a reduced capacity for insulin to elicit increases in glucose uptake in target tissues such as adipose tissues and skeletal muscles (Tomas et al., 2002). Adipose tissues and skeletal muscles are the target tissues as they are most sensitive to the actions of insulin in the body (Boden & Shulman, 2002; Jellinger, 2007). Insulin resistance

is closely associated with other ASCVD risk factors of MetS, which are central obesity, hypertension, dyslipidaemia, and pro-inflammatory and pro-thrombotic states. For example, insulin resistance and obesity have a cause-effect relationship: as mentioned by Lamounier-Zepter (2006), insulin resistance increases with weight gain and decreases with weight loss. Insulin resistance may also be the underlying causative factor of hypertension (Kotchen, 1996). In the past, the two organisations, WHO and EGIR proposed that insulin resistance was an essential element in the diagnostic criteria of MetS (Grundy et al., 2005). Some propositions state that insulin resistance is the single most causative factor of MetS (Reaven, 1993).

1.2.3 Impairment of insulin signalling pathways

The mechanism of insulin action to elicit glucose uptake in cells of target tissues can be understood by studying the insulin signalling pathways. The first stage of insulin signalling pathways involves insulin binding to its receptors on cellular surfaces of the insulin sensitive tissues, such as skeletal muscles, adipose tissues and liver. Once insulin binds to the receptors, tyrosine phosphorylations of insulin receptor substrates 1 and 2 (IRS-1 and IRS-2) take place, leading to activation of the Phosphatidylinositol 3-kinase or PI(3)k pathway. Activation of PI(3)k initiates a cascade of serine/threonine kinase activations, where one kinase phosphorylates and activates the next kinase in sequence (Saltiel & Kahn, 2001). Of various serine/threonine kinases, protein kinase B (PKB/Akt) has been known to be the most important kinase in the regulation of glucose metabolism (Kurlawalla-Martinez et al., 2005; Tomas et al., 2002). Activations of serine/threonine kinases regulate insulin's anabolic functions of synthesis and storage of glucose in the form of glycogen, lipid and proteins, as well as promoting translocation of Glucose transporter-4 (GLUT-4) for glucose uptake (Saltiel & Kahn, 2001; Samuel et al., 2004).

During insulin resistance, insulin fails to bind to its receptors to activate IRS-1 and IRS-2 tyrosine phosphorylations and the subsequent kinases of the PI(3)k pathway. This leads to the reduced activity of GLUT-4 translocation for intracellular glucose uptake, and hyperglycaemia may occur (Saltiel & Kahn, 2001; Figure 1.1). There are various factors that may impair the insulin action of binding to its receptors such as adipocytokines and free fatty acids (FFAs) (Yu et al., 2002).

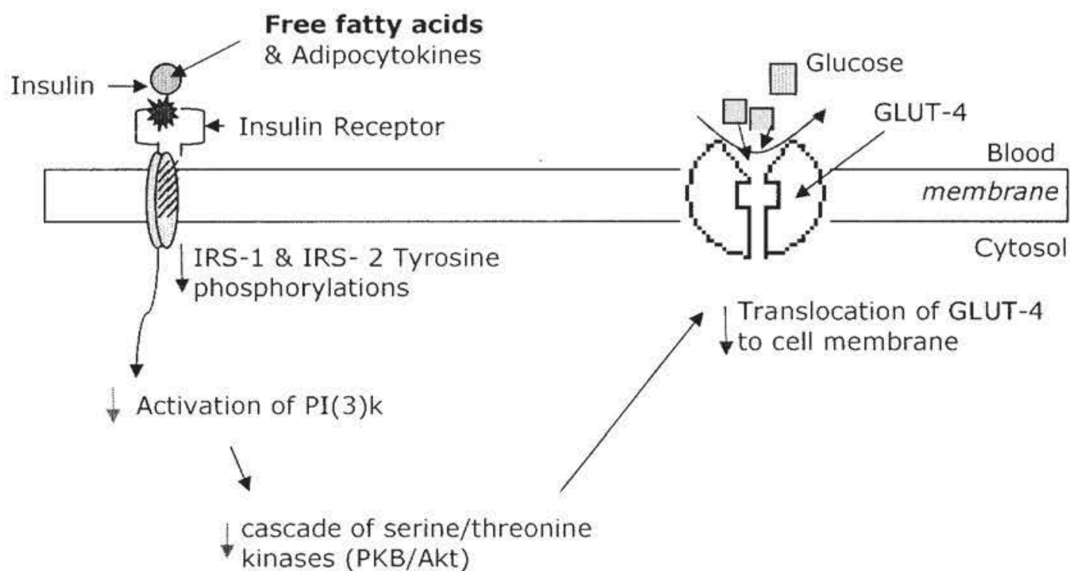


Figure 1.1 Altered insulin signalling pathways in insulin resistance. Various factors, such as free fatty acids and adipocytokines often associated with obesity, impair insulin binding to its receptor and activations of serine/threonine kinases of the PI(3)k pathway. This results in decreased translocation of GLUT-4 and inhibition of glucose uptake by tissue cells.

The role of GLUT-4, an isoform of glucose transporter proteins, is to trigger glucose influx or 'glucose uptake' into tissue cells. GLUT-4 proteins do this by attaching themselves to glucose on cellular membranes and transporting them to the cell interior. Initially, they reside within the intracellular fluid until insulin binds to its receptors, at which point they move out to cellular membranes. This process is known as translocation of GLUT-4. GLUT-4 is the most common glucose transporter of proteins in skeletal muscles and adipose tissues that induce glucose uptake (Saltiel & Kahn, 2001). Insulin resistance may

lead to decreased intrinsic activity or translocation of GLUT-4. Therefore, the level of GLUT-4 in adipose and muscle tissues can be measured to indicate the level of insulin resistance. For example, in an animal experiment by Chen et al. (2007), insulin resistant rats showed low expressions of GLUT-4 in adipose and muscle tissues, which led to low glucose influx into the cells.

It is known that most of the glucose taken up by GLUT-4 forms into glycogen and stored in liver. Shulman (2000) stated that glycogen synthesis accounted for most whole body glucose uptake and the majority of glycogen synthesis took place in the skeletal muscles. Shulman used nuclear magnetic resonance spectroscopy to reveal the steps involved in the formation of glycogen synthesis. Under insulin resistance, the spectroscopy showed that there was reduced concentration of intracellular glucose due to impairment of glucose uptake at the membrane of the cell (Shulman, 2000; Figure 1.2). Therefore, reduced glucose uptake due to decreased GLUT-4 translocation is the underlying mechanism of insulin resistance inhibiting glycogen synthesis.

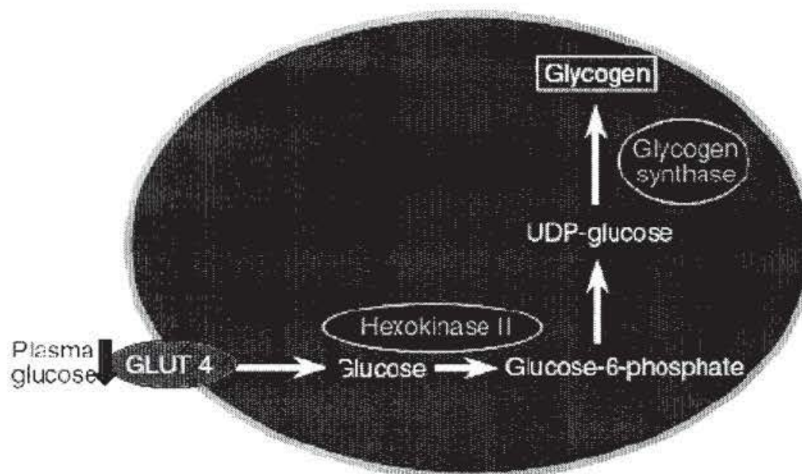


Figure 1.2 A brief outline of molecular steps in glycogen synthesis. Nuclear magnetic resonance spectroscopy showed reduced concentration of the intracellular glucose level due to decreased glucose uptake by GLUT-4.

1.3 Mechanisms of central obesity in the pathogenesis of MetS

1.3.1 Definition and significance of central obesity

Obesity refers to a condition of gaining excess body weight from over-accumulation of fats, and it is defined by having a body mass index (BMI) value of ≥ 30 kg/m^2, calculated by body weight (kg)/ height^2 (m^2). Over-accumulation of fats may be due to an increase in number, size or both of the adipocytes (Hopkins et al., 1997; Villena et al., 2004). According to WHO, over one billion adults are overweight on a worldwide basis, with at least 300 million being obese. The prevalence of obesity is also increasing in adolescents and children (Moller & Kaufman, 2005; Bays et al., 2005).

Central obesity, as one of the major causative factors of MetS, is an important clinical feature of obesity. It is defined by the waist girth or circumference of ≥ 102 cm in men and ≥ 88 cm in women (NCEP, 2002). These criteria were subject to variability as people of non-European ethnicities such as south Asians and Japanese presented with lower waist circumferences (Grundy et al., 2005). The waist circumference can be measured using a measuring tape around the smallest circumference of the waist, which is located below the rib cage and above the belly button. The significance of central obesity lies in the fact that insulin sensitivity is inversely correlated to the amount of central fats present (Coon et al., 1992; Moller & Kaufman, 2005). According to some propositions, central obesity exerts the strongest influence on the development of insulin resistance, MetS and ASCVD (Despres & Lemieux, 2006; Gastaldelli et al., 2002; Serrano Rios, 1998).

1.3.2 Possible causes of central obesity

Adipose tissue in the past has been thought of as a mere storage site for lipids. It is now well-known that adipose tissue is an endocrine organ that secretes various hormones, protein molecules, and cytokines (Kershaw & Flier, 2004). Adiposopathy is a condition

where adipose tissues lose their ability to store energy as fat depots, and their secretions become altered (Bays, 2005). Loss of the storing function of adipose tissues results in accumulation of lipids in the central abdomen and in other tissues such as liver and skeletal muscles. The altered adipose tissue secretions of various molecules and cytokines leads to insulin resistance, and pro-inflammatory and pro-thrombotic states associated with MetS (Schaffler et al., 2006). It has been proposed that obesity may be the very causative factor of adiposopathy, especially when its genetic predisposition is a combined factor (Bays et al., 2004). This indicates that the genetic factor is also a significant contributor to the mechanism of central obesity.

Loss in the energy storing function of adipose tissue may be due to reduction in glucose uptake in adipose tissues due to insulin resistance (Bays, 2005). Lack of energy from glucose may cause failure of differentiation of preadipocytes into mature adipocytes in tissues. Over the long term, this may result in atrophy of adipose tissues, which is a condition known as lipodystrophy (Despres & Lemieux, 2006). When adipose tissues lose their abilities to store energy, improper accumulation of lipids in the body may result. That is, instead of lipids residing in normal adipose tissues such as subcutaneous adipose tissue, they may accumulate in locations such as skeletal muscles, liver, heart and pancreatic beta-cells, which is a condition known as ectopic fat deposition (Boden & Shulman, 2002; Despres & Lemieux, 2006; Figure 1.4). Lipids may also accumulate in the viscera of abdomen, which causes central obesity (Bays, 2005). In the paper by Hegarty et al. (2003), mice fed with high fat diet (HFD) presented increased levels of free fatty acids (FFAs) in their skeletal muscles.

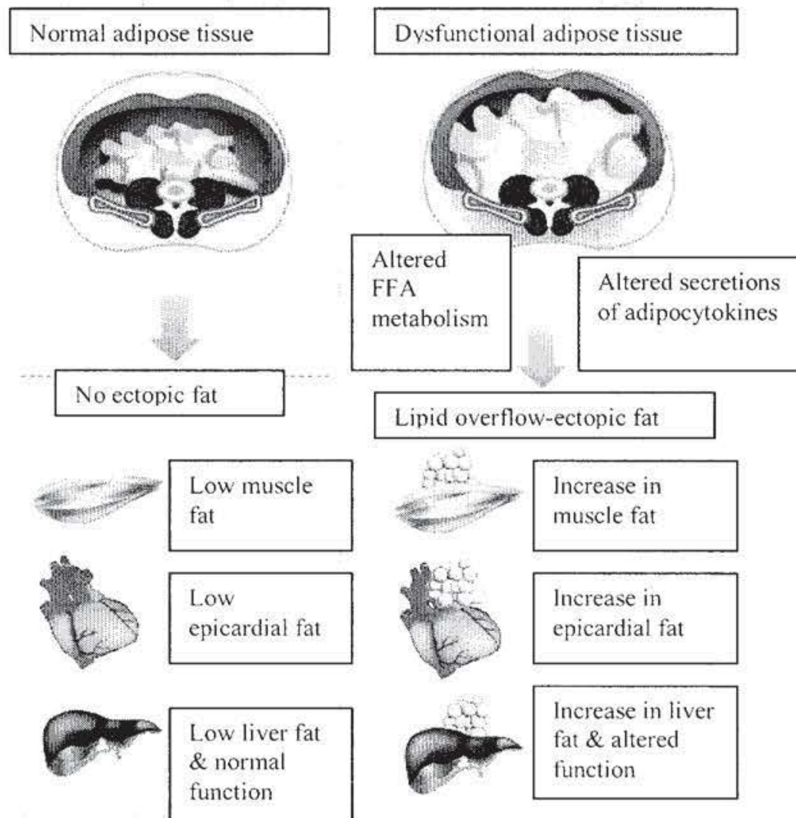


Figure 1.3 A diagram depicting the pathogenesis of central obesity in association with adiposopathy.

1.3.3 Central obesity, FFAs and insulin resistance

As mentioned earlier, central obesity and insulin resistance are closely associated. This is because central adipose tissue is the most resistant tissue to insulin action of all the body tissues (Haffner, 2006). As an anabolic hormone, insulin functions to store energy in fat depots by preventing the breakdown of lipids, a process known as lipolysis (Saltiel & Kahn, 2001). Therefore, insulin resistance produces the opposite effect of promoting lipolysis (Moller & Kaufman, 2005). For this reason, during insulin resistance, central adipose tissues actively undergo lipolysis to form fatty acids (Landin et al., 1990). As fatty acids are released into the bloodstream, they circulate freely and these are called FFAs (Meek et al., 1999). FFAs are essential source of energy for body, and they are formed by lipolysis, in times of starvation or exercise. During lipolysis, lipids in the form of triglycerides are

hydrolysed to liberate fatty acids, which are released into blood stream as FFAs. FFAs circulate throughout the body to places such as muscles, heart and liver, where they are oxidised for energy supply (Koutsari & Jensen, 2006).

In central obesity, FFAs are formed excessively due to increased lipolysis from insulin resistance. Excessive FFAs may be transported to the liver via the portal vein, where their accumulation results in the formation of triglycerides, which in turn causes an increase in hepatic glucose output (Meek et al., 1999). Excess FFAs may also circulate in the blood stream to cause peripheral insulin resistance or they may form into triglycerides and long-chain fatty acyl-CoA esters in non-adipose tissues such as skeletal muscles (Griffin et al., 1999; Kusunoki et al., 2006; Meek et al., 1999).

Increased accumulation of triglycerides in the liver leads to increased production of very low density lipoprotein-cholesterols (VLDL-Cs), which leads to dyslipidaemia (Chapman & Sposito, 2008). This is because triglycerides make up the majority of atherogenic lipoproteins such as VLDL-Cs (Howard & Howard, 1994; NCEP 2002). Triglycerides may also cause blunting of insulin-stimulated IRS-1 and IRS-2 tyrosine phosphorylations and subsequent serine/kinase activations in the PI-3k pathway in the liver, thereby impairing glycogen synthesis and causing hyperglycaemia. Increased FFAs in the liver also promote a process called, gluconeogenesis (Samuel et al., 2004). Gluconeogensis refers to a process of conversion of non-carbohydrate sources like amino acids and glycerol in fat, into glucose. When there are excess triglycerides in the liver, gluconeogenesis is promoted by the body, as a way to reduce the amount of lipids. This results in increased hepatic glucose output to the blood stream, which leads to hyperglycaemia (Boden & Shulman, 2002).

Excess FFAs or other fatty acid-derived metabolites in skeletal muscles may inhibit insulin-stimulated glucose uptake (Kim et al., 2001). The mechanism of this action is that increased FFAs activate protein kinase Cθ, a serine kinase, which in turn decreases insulin stimulated IRS-1 and IRS-2 tyrosine phosphorylations and subsequent activations of the PI(3)k pathway (Hegarty et al., 2003). As already explained, inactivation of the PI(3)k pathway leads to a reduction in the activity of GLUT-4 translocation. FFAs may also directly affect GLUT-4 proteins by altering their trafficking, budding or fusion (Boden & Shulman, 2002; Shulman, 2000).

1.4 Mechanism of pro-inflammatory and pro-thrombotic states in the pathogenesis of MetS

Pro-inflammatory and pro-thrombotic states of MetS lead to inflammatory and thrombotic states, endothelial dysfunction, and metabolic abnormalities such as insulin resistance and dyslipidaemia (Hall et al., 2006; Schaffler et al., 2006). Pro-inflammatory and pro-thrombotic states occur mainly due to the abnormal functions of adipose tissues, otherwise known as adiposopathy. As mentioned previously, adipose tissues are endocrine organs capable of secreting various hormones, proteins, cytokines and other bioactive molecules, called adipocytokines. Adipocytokines are important regulators of physiology, but in individuals with adiposopathy, the secretion of these adipocytokines from adipose tissues become altered. Adipocytokines secreted abnormally may cause inflammation, thrombosis and other metabolic disorders (Schaffler et al., 2006).

Adiposopathy causes increased production of adipocytokines; this causes pro-inflammatory and pro-thrombotic states associated with MetS and raises the risk of acquiring ASCVD. It is this anomaly that links obesity directly to insulin resistance, inflammatory and thrombotic

states, hypertension and dyslipidaemia (Chapman & Sposito, 2008; Odrowaz-Sypniewska 2007).

1.4.1 Pro-inflammatory and pro-thrombotic adipocytokines

Many pro-inflammatory and pro-thrombotic adipocytokines are secreted from macrophages located within adipose tissues (Matsuzawa et al., 2004; Permana et al., 2006). Examples of pro-inflammatory and pro-thrombotic adipocytokines include the tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), C- reactive protein (CRP), monocyte chemoattractant protein-1 (MCP-1), retinal binding protein-4 (RBP-4), resistin, and plasminogen activator inhibitor-1 (PAI-1). The majority of these adipocytokines have direct effects in causing insulin resistance (Tomas et al., 2002).

In central obesity, increased adipose tissues cause an increase in secretions of TNF-α (Matsuzawa et al., 2004). There are many studies about both in vitro and in vivo experiments that show TNF-α as a significant causative factor of obesity-related insulin resistance (Feinstein et al., 1993; Hotamisligil & Spiegelman, 1994; Wellen & Hotamisligil, 2005). TNF-α also causes adhesion of molecules in the endothelium, which contributes to atherosclerosis (Maeda et al., 2002).

Similar to TNF-α, IL-6 is secreted mostly from central adipose tissues, and it is also a causative factor of insulin resistance. IL-6 also stimulates production of CRP from the liver, which is an inflammatory marker that has a very close association with ASCVD (Pradhan et al., 2001). A high level of CRP causes low grade inflammation that is often present in obese individuals (Cancello & Clement, 2006). A predictive relationship between increased CRP production and future atherothrombotic events was established by Pepys and Hirschfield (2003). Furthermore, an increase in levels of CRP was associated with a high prevalence of

patients with MetS and increased risk for T2DM (Dehghan et al., 2007; Lizardi-Cervera et al., 2007).

MCP-1 is an inflammatory adipocytokine, the amount of which is proportional to the degree of insulin resistance. In-vitro studies by Sartipy and Loskutoff (2003) showed that there was a relative reduction in glucose uptake in 3T3-L1 adipocytes incubated with MCP-1 compared to the control adipocytes. Similarly, RBP-4 may cause insulin resistance and glucose intolerance: as Tamori (2006) showed, RBP-4 decreased the expression of GLUT-4 and lowered glucose uptake in adipocytes. RBP-4 also decreases glucose uptake by skeletal muscles while it increases glucose output by the liver, which leads to hyperglycaemia (Yoshikazu, 2006).

Resistin causes insulin resistance as well as inflammatory responses, by producing other pro-inflammatory adipocytokines such as TNF-α and IL-6 (Chapman & Sposito, 2008). In an experiment, rodents treated with exogenous resistin became insulin resistant with increased circulating glucose levels and hepatic glucose production; whereas the vehicle-treated animals showed low glucose levels (Moller & Kaufman, 2005). The mechanism for this action of resistin was that it impaired insulin-stimulated phosphorylation of insulin receptor substrates (Nakatani et al., 2005).

PAI-1 is a pro-thrombotic adipocytokine that inhibits fibrinolysis and conversion of plasminogen, which leads to increased formation of fibrinogen and blood clots. Increase in PAI-1 raises a person's susceptibility to coagulation of blood and formation of thrombosis thereby causing ASCVD. The amount of PAI-1 is also proportional to the degree of insulin resistance (Serrano Rios, 1998).

1.5 Additional metabolic and haemodynamic abnormalities of MetS

Dyslipidaemia and hypertension are additional metabolic and haemodynamic abnormalities of MetS, and they are by no means any less significant than insulin resistance and central obesity in contributing to the pathogenesis of MetS.

1.5.1 Dyslipidaemia

Dyslipidaemia is defined as abnormal lipid profiles in blood characterised by high levels of triglycerides and low levels of high density lipoprotein-cholesterols (HDL-Cs) (American Diabetes Association, 2004; Schwartz, 2006). The diagnostic values of dyslipidaemia are $\geq$1.7mmol/L for triglycerides, and $<$1.03 mmol/L (in males) or $<$1.29 mmol/L (in females) for HDL-Cs. In dyslipidaemia, triglyceride-rich VLDL-Cs and low density lipoprotein-cholesterols (LDL-Cs) increase, while protein-rich HDL-Cs decrease in numbers (Superko et al., 2002).

Large high density lipoproteins (HDLs) contain rich amounts of proteins with a small amount of cholesterols. A high level of HDL-Cs is associated with antioxidant, anti-inflammatory, and anti-atherosclerotic effects, but a low level increases risks for both ASCVD and coronary heart disease (Bruce et al., 1998; NCEP, 2002). Low density lipoproteins (LDLs) are small, dense and rich in cholesterols, taking up to 60 to70% of total serum cholesterols in body. Dyslipidaemia is often termed as atherogenic dyslipidaemia due to the high prevalence of ASCVD in people with increased LDL-Cs (Grundy, 1997; Lamarche et al., 1997; NCEP, 2002).

In the normal course of physiology, atherogenic lipoproteins such as very low density lipoproteins (VLDLs) and LDLs are cleared in liver and peripheral tissues by being bound to receptors called, B/E receptors. Insulin mediates the binding of VLDLs and LDLs to B/E

receptors. Alternatively they may be cleared by scavenger receptors on tissue macrophages (Howard & Howard, 1994). However, under insulin resistance, the binding of VLDLs and LDLs to B/E receptors is prevented, which leads to an accumulation of atherogenic lipoprotein cholesterols in the body. Increased VLDLs in the body trigger the transfer of cholesterol esters from HDL-Cs and LDL-Cs to VLDL-Cs in exchange for VLDL triglycerides, which is a process mediated by cholesterol ester transfer protein (Chapman & Sposito, 2008). This results in increased formations of triglycerides with decreased level of HDL-Cs, which characterise dyslipidaemia (Howard & Howard 1994; Taskinen, 2003).

Excessive productions of VLDLs in the liver, and the impaired suppression of VLDL release into the blood stream due to insulin resistance, may also cause dyslipidaemia. Excessive productions of VLDLs result from the increased flux of FFAs into the liver from central fats, as explained in Section 1.3.2. Impaired suppression of VLDL release into blood stream is due to insulin resistance, which results in excess levels of circulating triglycerides in blood. The elevation of VLDLs, in turn increases the formation of small and dense LDLs (Taskinen, 2003).

1.5.2 Hypertension

MetS is characterised by haemodynamic abnormalities including raised blood pressure or hypertension. Hypertension is defined as consistent elevation of systemic arterial blood pressure with systolic reading of ≥140 mmHg and diastolic reading of ≥90 mmHg (≥130/85 mmHg for diagnosis of MetS) (Moller & Kaufman, 2005). The prevalence of hypertensive adults during 1999 to 2002 was estimated to be 25.5% and was higher in African-Americans (Hall et al., 2006). Although the exact cause of hypertension is unknown, the likely causative factors are obesity and insulin resistance (Weissberg, 2000;

Matsuzawa et al., 2004). Obesity and insulin resistance may cause hypertension by activation of rennin-angiotensin-aldosterone-system (RAAS) and the sympathetic nervous system (Chapman & Sposito, 2008; Hall, 1994), and alteration of structures and functions of the kidneys (Narkiewicz, 2006).

Insulin resistance-associated obesity involves altered functions of adipose tissues, which leads to production of various adipocytokines including Angiotensin II. Angiotensin II causes elevation of RAAS, which in turn raises systemic blood pressure (Chapman & Sposito, 2008; Engeli et al., 2003; Kurtz, 2006). Angiotensin II also enhances sympathetic nervous activity, which raises blood pressure by increasing renal sodium and fluid retention, cardiac output and vascular resistance (Chapman & Sposito, 2008; Hall et al., 1998). Insulin resistance-associated obesity also involves accumulation of lipids in non-adipose sites such as liver, skeletal muscles, and kidneys, as described in Section 1.3.2 (Engeli et al., 2003). Excess fat accumulation surrounding kidneys may compress the organs and alter their structures. Altered renal structures result in an increase in sodium and water retention, tubular re-absorption, and glomerular hyperfiltration, which raises systemic blood pressure (Chapman & Sposito, 2008; Hall et al., 1998).

1.6 Animal models of MetS

Animal models of MetS can be established by several methods, including genetic modifications, use of chemical, dietary, surgical manipulations, or by combination thereof (Srinivasan & Ramarao, 2007).

1.6.1 Zucker fatty and Zucker fatty diabetic rats

Zucker fatty rats are genetically obese and they serve as a good model for MetS or the pre-diabetic stage of human disease. Thus, these rats are often used in research regarding the

prevention of transitions of T2DM from MetS (Marsh et al., 2007). The Zucker fatty rats are also both leptin deficient and resistant, which makes them hyperphagic (Bray, 1977).

Zucker fatty diabetic rats are good models for people with T2DM who are also obese. Genetically, Zucker fatty diabetic rats are similar to Zucker fatty rats but the former present with hyperglycaemia whereas the latter do not (Marsh et al., 2007).

1.6.2 ob/ob mice

ob/ob mice are genetically modified mice with obesity, dyslipidaemia, and insulin resistance associated with MetS. The genes of ob/ob mice are encoded in a way that causes deficiency of leptin production, a hormone responsible for inducing the satiety sensation. This causes mice to over indulge in food intake and become obese. In ob/ob mice, there is an increased level of TNF-α production in adipocytes, which also causes insulin resistance (Koteish & Maediehl, 2002).

1.6.3 Dietary factor-induced model

Diets rich in fructose or fat may be used to induce rodent models of MetS. Many papers recently have revealed that rodents fed a high fructose diet presented with insulin resistance and hypertension (Hsu et al., 2007; Li et al., 2000; Wang et al., 2003). In addition, high fat diet (HFD) feeding has been established as a good method to induce MetS in rodents (Luo et al., 1998). Surwit et al. (1997) showed that C57BL/6J mice became severely obese after HFD feeding, and the mice presented with clinical features of hyperinsulinemia, altered beta-cell function, and hypertension. Another study showed that HFD feeding in C57BL/6J mice induced hyperlipidaemia, hyperleptinemia and insulin resistance (Sumiyoshi et al., 2006).

HFD feeding to mice may also be incorporated to obtain a good model for T2DM. Surwit et al. (1988) showed that C57BL/6J mice developed T2DM when obesity was induced in them by HFD feeding. Several studies showed that feeding rodents a HFD induced hyperinsulinemia, and when streptozotocin (STZ) was injected into these mice, they developed hyperglycaemia. It is known that the murine models induced by STZ injection in combination with a high caloric diet possess the characteristic of insulin resistance (Chen et al., 2007). Many articles support the notion that a combination of HFD and STZ injection in mice can present a state that is equivalent to the human T2DM (Leng et al., 2004; Luo et al., 1998; Reed et al., 2000; Surwit et al.,1998).

Feeding HFD to C57BL/6J mice is an effective method to induce MetS because it is known that C57BL/6J mice have genes that make them prone to insulin resistance and hyperglycaemia (Coleman, 1982; Srinivasan & Ramarao, 2007). This can be seen in the experiment of Wencel et al. (1995), where two strains of mice, C57BL/6J and A/J were fed with the same diet but the former showed relatively lower glucose-stimulated insulin release by the pancreas cells. C57BL/6J mice also have pancreas cells that are tolerant to increased demand for insulin secretion under insulin resistance. In contrast, the pancreas cells of other strains such as C57BL/KsJ mice are unable to tolerate insulin resistance for a long period, resulting in death (Coleman, 1982). Therefore, C57BL/6J mice are good strains for the study of metabolic disorders because their genetic background appears to be relatively more susceptible to obesity and diabetes, compared to other strains.

The method of HFD feeding was developed in order to acquire more accurate models of MetS in mice to mirror the human disease condition. Even though the genetically inbred mice, such as ob/ob mice presented obesity and hyperinsulinaemia, the severity of these features was far greater than those normally presented in the corresponding human

condition. The clinical features of the HFD fed mice were similar to those of human, and compared to the genetically breeding method, the HFD feeding method was much easier in a practical sense (Luo et al., 1998).

1.7 Therapeutic interventions for MetS

The primary focus in the treatment of MetS is to minimise the risk of it developing into ASCVD. This is because the number of fatalities associated with MetS are principally due to ASCVD (Koren-Morag et al., 2005). The secondary focus in the treatment of MetS is on the risk of it developing to T2DM. People with MetS and T2DM are given priority consideration for treatment over those with MetS only, because having both conditions can lead to many serious complications. People with MetS who have a relatively low risk of developing ASCVD or T2DM are considered last for treatment (Grundy et al., 2005; International Diabetes Federation, 2005; Moller & Kaufman, 2005).

1.7.1 Lifestyle therapies

Lifestyle therapies concerning MetS include managing body weight, physical activity and diet. The patient is encouraged to make these the first choice of treatment intervention and pharmacotherapy is recommended only when this intervention is insufficient to achieve the desired therapeutic outcomes (Grundy et al., 2005; International Diabetes Federation, 2005; Moller & Kaufman, 2005).

Moderate exercise such as brisk walking for 30 to 60 minutes daily, or most days of the week, is a recommended plan for physical activity for the long-term prevention of ASCVD and T2DM. The goals for reducing abdominal fat and body weight are shown in Table 1.3. Diet therapy to control body weight aims to avoid atherogenic diet and reduce calorie intake,

as shown in Table 1.3 (Grundy et al., 2005; International Diabetes Federation, 2005; Moller & Kaufman, 2005).

Table 1.3: Recommendations for weight loss and diet

Risk factor	Goals
Calorie intake	Saturated fat (<7% of total calories); Total fat (25 to 35% of total calories).
Atherogenic diet	Avoid unsaturated fat and simple sugars.
Abdominal obesity	Achieve waist circumference of <40 inches (or <102cm) in men and <35 inches (or <89cm) in women.
Body weight	Lose 5 to 10% of body weight in first year.

1.7.2 Pharmacotherapy

Currently, there is no approved pharmaceutical drug that can target the entire metabolic and haemodynamic abnormalities of MetS. Appropriate drugs are available to target each specific abnormality of MetS. Research continues to discover and develop new pharmacological drugs to combat this complex disease syndrome (Grundy et al., 2005; International Diabetes Federation, 2005; Moller & Kaufman, 2005).

1.7.2.1 Drugs for T2DM

For T2DM, oral anti-hyperglycaemic agents used to lower glucose levels include sulfonlyureas (e.g. glyburide, glipizide, glimepiride), biguanides (e.g. metformin), α-glucosidase inhibitors (e.g. acarbose, miglitol), thiazolidinediones (TZDs), and glinides.

Development of TZDs dates back to 1982 with the discovery of ciglitazone, a thiazolidinedione derivative (Saltiel & Olefsky, 1996). Since then, development of various new TZDs has continued to this date. Some of the common TZDs used today include rosiglitazone, pioglitazone, and troglitazone, which all improve hyperglycemia and insulin resistance, as shown by many studies (Phillips et al., 2001; Rao, 2001; Wilding, 2006).

TZDs can also be beneficial in lowering plasma lipids including triglycerides, cholesterols and FFAs, as shown by a study that involved treating T2DM with troglitazone and pioglitazone in rats. The mechanism of the therapeutic actions of TZDs is that they bind to peroxisome proliferator-activated receptor (PPAR) γ, thereby promoting synthesis of glucose transporters and improving lipid metabolism (Iida, 2003).

Metformin is an anti-diabetic drug that belongs to the class of biguanides. Its action is to enhance peripheral insulin sensitivity and induce glucose uptake to lower blood glucose levels (Iida, 2003). Although metformin has been effective in the prevention of new-onset T2DM in people with MetS, its effectiveness was not so prominent in patients with MetS and T2DM with increased risks of acquiring ASCVD (Hall et al., 2006).

1.7.2.2 Drugs for obesity

Drugs for obesity should only be taken when the primary therapy of low calorie diet intake and increased physical activity has failed. The actions of drugs for obesity are to reduce energy intake and induce energy expenditure (Nisoli & Carruba, 2004). Kusunoki et al. (2006) showed that many drugs such as Acetyl-CoA carboxylase (ACC) inhibitors, fatty acid synthase (FAS) inhibitors, AMP Kinase activators, stearoyl-CoA desaturase inhibitors, hormone-sensitive lipase inhibitors, and diacylglycerol acyltransferase 1 inhibitors were effective in correcting the altered fatty acid metabolism. The actions of these drugs were mainly to increase energy expenditure by inducing fatty acid oxidation and reduce energy intake by preventing fatty acid accumulation.

Many drugs for obesity have been reported to cause side effects and may not be suitable for long term use. For example, serotoninergic agents such as fenfluramine suppress appetite, but were withdrawn from the market due to their potential adverse cardiovascular effects

(Glazer, 2000). Orlistat and sibutramine are drugs indicated for long term use, but detailed data regarding their safety is still lacking. For example, sibutramine may be used in conjunction with a reduced-calorie diet, but it may cause an increase in blood pressure and thus may not be suitable for patients with a history of hypertension, coronary heart disease, and heart failure (Padwal & Majumdar, 2007). A new class of appetite suppressants that has been investigated recently targets the endocannabinoid system. As cannabinoid type 1 receptors increase appetite, these may be blocked by drugs such as Rimonabant (Grundy, 2006).

1.7.2.3 Drugs for dyslipidaemia

Drugs for dyslipidaemia including fibrates, statins and nicotinic acids are primarily aimed at reducing LDL-Cs and raising HDL-Cs. Once LDL-C levels are controlled, the next goal is to reduce non-HDL-Cs such as VLDL-Cs, and increase HDL-Cs (Grundy et al., 2005; International Diabetes Federation, 2005; Moller & Kaufman, 2005).

Statins markedly reduce LDL-C levels and moderately improve triglyceride and HDL-C levels. Several recent clinical trials have confirmed the cardiovascular benefits of statins (Hall et al., 2006). Similar to statins, fibric acid derivatives decrease triglyceride and increase HDL-C levels. The two drugs are often used together in patients with MetS, who do not achieve the desired goal for the levels of triglycerides or HDL-Cs after a particular therapy. However, the combined use of these drugs increases the risk of acquiring myopathy (Bellosta et al., 2004).

1.7.2.4 Drugs for hypertension

Anti-hypertensive drugs should be administered to people with blood pressure of ≥140/90 mmHg (or ≥130/80 mmHg if person is diabetic) to prevent the risk of acquiring ASCVD.

These drugs include thiazide diuretics, β-blockers, angiotensin converting enzyme (ACE) inhibitors, and angiotensin II receptor blockers (ARBs) (Grundy et al., 2005; International Diabetes Federation, 2005; Moller & Kaufman, 2005). The older drugs, thiazide diuretics and β-blockers are now used less frequently as some of them, despite their blood pressure lowering effects, may exacerbate conditions of T2DM and ASCVD (van Zwieten & Mancia, 2006). They have been reported to increase the levels of LDL-Cs and triglycerides, and decrease insulin sensitivity (Kurtz, 2006).

The more recent drugs, ACE inhibitors and ARBs are the gold standard medications for hypertension-related heart failures (Australian Diabetes Society, 2008). These drugs also exert beneficial metabolic effects compared to the older drugs mentioned above. For example, telmisartan is a new ARB that has been shown to potentially activate PPAR-γ and enhance insulin sensitivity (Erbe et al., 2006; Kurtz, 2006).

1.7.2.5 Other drugs

PPAR agonists have been considered as a potential approach to target the multiple abnormalities of MetS. There are many types of PPAR agonists, including PPAR-γ (TZDs), PPAR-α (fibrates) and PPAR-δ. PPAR-γ agonists (TZDs), as explained previously improve insulin sensitivity and lower lipid accumulation. Both PPAR-α and PPAR-δ improve dyslipidaemia, where PPAR-α modulates expression of genes encoding regulators of fatty acid uptake and catabolism, as well as genes encoding key apolipoproteins, and PPAR-δ regulates fatty acid oxidation and energy homeostasis. A

recent drug discovery effort has focused on a new PPAR agonist made by combining PPAR-γ and PPAR-α agonists, which has been shown to be very effective in treating both dyslipidaemia and hyperglycaemia (Moller & Kaufman, 2005).

Currently there are no drugs available that directly target the pro-inflammatory states associated with MetS, but aspirin may be used to treat the pro-thrombotic states.

1.8 Discovery and development of new drugs

1.8.1 Adverse effects of current therapies

Unfortunately, although the majority of pharmaceutical drugs are effective in the treatment of a specific abnormality of MetS, there is an increasing concern for patients medicated with these drugs due to their potential adverse effects. For example, although TZDs work effectively to improve insulin sensitivity and lower lipid levels, they may also induce lipid accumulation by their stimulatory effect on adipocyte differentiation, thereby increasing body weight (Lee et al., 2004). Another potential adverse effect of TZDs is the retention of fluids, which may lead to development of heart failure in patients with T2DM (Tang & Maroo, 2006). In addition, increased incidences of hypoglycaemia, lactic acid intoxication, and gastrointestinal upset have been documented as common adverse effects due to anti-diabetic drugs (Jung et al., 2006).

Adverse effects of drugs are of significant concern when multiple drugs are administered in combination for a long period, which commonly occurs in patients with MetS (Grundy, 2006). For example, the long-term use of statins in combination with other drugs may elicit drug-to-drug interactions that may be harmful to the body (Bellosta et al., 2004). Therefore, there is an urgent need for the development of safer pharmaceutical drugs in the treatment of MetS. Moreover, a novel drug that can target the multiple abnormalities of MetS is needed to minimise the potential adverse effects of polypharmacy (Grundy 2006; Moller &

Kaufman 2005). In order to discover such a novel drug, research must focus on identifying the root cause of MetS in order to develop better diagnostic criteria and new pharmacological targets (Grundy et al., 2005).

1.8.2 Chinese herbal medicine

Chinese herbal medicine has been used for metabolic disorders for over 2000 years. The earliest classic text of Chinese medicine defines T2DM as 'xiao ke' or 'diabetes exhaustion' (Wang & Wylie-Rosett, 2008). The Chinese philosophy of diabetes involves heat-toxin, stasis-toxin and toxin as underlying mechanisms of hyperglycaemia and hyperlipidaemia (Chen, 2007). In the pathogenesis of MetS, the concept of insulin resistance is not significant in Chinese medical philosophy (Wang, 2003).

Today, the use of Chinese herbal medicine for treatments of MetS and T2DM is growing among health care consumers. This is largely the result of an awareness of the increased incidence of adverse effects related to pharmaceutical drugs (Hollander & Mechanick 2008; Jung et al., 2006). There are many studies that support the natural characteristics of Chinese herbal medicine with minimal adverse effects (Hollander & Mechanick 2008; Jung et al., 2006; Wang & Wylie-Rosett 2008). Furthermore, various studies have shown the multi-functional effects of Chinese herbal formulae on metabolic disorders in animal experiments (Li et al., 2000; Okumura et al., 2001; Wang et al., 2003). In addition, hundreds of Chinese herbal medicines of plant origin have been identified as being effective for diabetes mellitus due to their hypoglycaemic properties (Jia et al., 2003). A few examples of these herbs are listed in the *Appendix*. Nevertheless, there is still a shortage of detailed studies analysing the effects of Chinese herbal medicine in animal experiments. Moreover, scientific evidences of the molecular mechanisms by which Chinese herbal medicines exert their therapeutic effects are also lacking, and are much needed.

1.9 Aims of this thesis

As discussed in the literature review, MetS is a complex syndrome involving multiple disease states, namely, central obesity, insulin resistance, dyslipidaemia, hypertension, and pro-thrombotic and pro-inflammatory states. Its epidemiology as a global threat continues to expand due to the lack of knowledge about its exact pathogenesis, as well as a lack of more effective drugs with minimal adverse effects. Despite many unanswered questions, research continues to uncover the various mechanisms of the multiple disease states of MetS. For example, it is quite evident from the literature review that central obesity and insulin resistance play major roles in the pathogenesis of MetS. Chinese herbal medicine may be a potential novel pharmacotherapy, attributed for its natural characteristics and its ability to exert multi-functional effects. However, more studies investigating the effects of Chinese herbal medicine and mechanisms of their potential therapeutic effects are needed.

The aims of this thesis were initially to induce MetS and T2DM in C57BL/6J mice by feeding them HFD, with or without STZ injection. The secondary aim was to investigate the effects and mechanisms of two Chinese herbal medicines, Sugarid and SK0504. This was to test the hypothesis that Sugarid and SK0504 may potentially be novel drugs in targeting the multiple abnormalities of MetS and T2DM.

CHAPTER 2

Materials and methods

2.1 EXPERIMENTAL ANIMALS AND MATERIALS

2.1.1 Animals

The studies reported in this thesis were performed on male C57BL/6J mice. The animals were purchased from Gore-Hill (Sydney, Australia) at 8 weeks old, and they were kept in the animal facility of the University of Technology, Sydney. They were housed in individual plastic cages in a temperature-controlled room (22°C) with a 12 h light/dark cycle with lights on at 7 am. Unless stated otherwise, the mice were allowed free access to water and standard laboratory chow diet (Gordon's Specialty Stock Feed, Sydney, Australia). The composition of this diet was 49.5% carbohydrate, 25% protein, 5.7% fat (providing 15% total calories, where 1 calorie is approximately 4.184J), 11% moisture, 3.8% fibre and 5.1% ash.

2.1.2 Ethics approval

All experiments reported in this thesis were approved by the Animal Care and Ethics Committee (ACEC Protocol No. 0509-031A) of the University of Technology, Sydney and complied with the National Health and Medical Research Council's (Australia) guidelines for the care and use of animals for scientific purposes.

2.1.3 High fat diet

High fat diet (HFD) was prepared by mixing various ingredients of copha butter, bran, and cornflour (Coles Supermarkets Australia Pty Ltd., Victoria, Australia), pure safflower oil (Stoney Creek Oil Products Pty Ltd., Victoria, Australia), NZMP ALACID lactic casein (Fonterra, New Zealand), sucrose, trace minerals, choline bitartrate, mineral mix and AIN vitamins (MP Biomedicals, Inc., Ohio, U.S.), methionine

(MUSASHI, Victoria, Australia), and gelatine (GELITA NZ Ltd., Christchurch, New Zealand). The composition of this diet was 35.4% fat (providing 59% total calories), 28.9% carbohydrate, 28% protein, 5.9% mineral, and 1.8% vitamin. Mice were fed this diet for 12 weeks to induce a well-recognised syndrome of insulin resistance, hyperinsulinaemia, hyperlipidaemia and hyperglycaemia. The diet was replenished weekly to keep it fresh.

2.1.4 Collection of Blood and Tissue Samples

At the end of each experiment, mice were sacrificed under anaesthesia with isoflurane after 12 h fasting and their blood, liver and visceral fat tissues were collected. 1mL of whole blood was collected from each mouse by heart puncture, using a Terumo syringe (Terumo Medical Corporation, USA). The collected blood was then centrifuged for 10 min at 5000 x*g* to isolate the serum from the whole blood, the former of which was stored at -20°C for biochemistry assays. Visceral fat and liver tissues were quickly excised, weighed, frozen in liquid nitrogen, and stored at -80°C for the measurements of their triglyceride concentrations, and histological assessment of their total lipid contents.

2.1.5 Chemicals

Chloroform, HCl, and glacial acetic acid were supplied by Chem-Supply (Gillman, Australia). Ethanol, methanol, Oil Red O, Mayer's haematoxylin solution, $NaCH_3COO$, and $MgSO_4$ were supplied by Sigma-Aldrich (MO, USA). Streptozotocin (STZ) and dextran sulphate were supplied by Sigma Chemical Co. (MO, USA). Formalin, NaCl, and $NaHCO_3$ were supplied by BDH AnalaR® Merck (Kilsyth, Australia). $CaCl_2$,

isopropanol and thymol blue crystals were supplied by Ajax Finechem Pty Ltd (NSW, Australia). ZYMED clearmount solution was supplied by Invitrogen (CA, USA). 100 IU/ml Humulin (insulin) was supplied by Eli Lilly Pty Ltd (NSW, Australia). 50% glucose solution was supplied by CSL Limited (Victoria, Australia). 0.9% NaCl Saline injection was supplied by Pfizer Pty Ltd (WA, Australia). 5000 units/5ml heparin sodium was supplied by Delta West (Perth, Australia).

2.2 IN VIVO MEASUREMENTS

2.2.1 Body weight and food intake

Body weights of mice were measured twice per week before the treatment period with Chinese herbal medicine. During the treatment period with Chinese herbal medicine, body weights of mice were measured on a daily basis. Mice were placed individually on a scale (Ohaus Corporation, Adventurer™ Pro AV2101, USA) for their body weight measurements. The amount of food consumed daily per mouse was calculated by measuring the amount of fresh food given, and then weighing the remaining food after 3 days.

2.2.2 Blood glucose test

Blood glucose test was performed in groups of conscious mice fasted for 12 h overnight, before and after treatment with Chinese herbal medicine. A puncture was made on the tail vein of mice to obtain a drop of blood, which was attached to a strip that had been inserted into an ACCU-CHECK GO glucometer (Roche Diagnostics, Germany), for the measurement of glucose concentration.

2.2.3 Intra-peritoneal glucose and insulin tolerance tests

Intra-peritoneal glucose tolerance test (i.p. GTT) was performed in groups of conscious mice fasted for 12 h overnight. Blood was obtained from the tail vein of mice by using steel blade (Swann-Morton Ltd., England) and blood clotting was prevented by application of 5000 units/5ml heparin sodium (Delta West, Perth, Australia). Blood glucose concentration was measured with an ACCU-CHEK GO glucometer as described above. Measurements were performed before and after i.p. injection of glucose (2g/kg) using 0.3mL insulin syringes (BD, New Jersey, USA) at 15, 30, 60, 90, and 120 minutes. Intra-peritoneal insulin tolerance test (i.p. ITT) was performed a week after i.p. GTT in the same mice fasted for 6 h. Blood was obtained from the tail vein of mice and glucose concentrations were measured before and after i.p. insulin injection (0.85 IU/kg) using 0.3mL insulin syringes (BD, New Jersey, USA) at 15, 30, 60, 90, and 120 minutes, in the same manner as described for i.p. GTT.

2.3 LABORATORY ASSAY OF SERUM SAMPLES

2.3.1 Triglyceride concentration

Serum triglyceride concentration was determined using the Triglycerides GPO-PAP reagent obtained from Roche (Australia) and 250mg/dL glycerol (Sigma, Australia). The principal of triglyceride assay is that triglyceride in serum is enzymatically hydrolysed by lipoprotein lipase to produce glycerol and fatty acids. The glycerol, in the presence of ATP, is then phosphorylated by glycerol kinase to form ADP and glycerol-1-phosphate. Glycerol-1-phosphate is then converted to dihydroxyacetone phosphate and hydrogen peroxide by oxidation. The hydrogen peroxide reacts with 4-aminophenazone and 4-chlorophenol under the catalytic action of peroxidise to form a

red dyestuff, the absorbance of which at 490 nm is proportional to the triglyceride concentration in the sample.

Into a 96-well microplate, 5μl of the glycerol standards 0, 1.4, 2.8, 4.2, 5.7, 8.5, and 11.3 mmol/L, and serum samples were delivered in duplicates. Triglycerides GPO-PAP reagent was then delivered to each of these wells to make up the total volume of 300μl per well. The mixed contents were incubated for 10 minutes at room temperature, allowing their colours to develop. The microplate was then shaken for 10 seconds before measuring the absorbance of each well against the reagent blank at a wavelength of 490 nm in Power Wave™ Microplate Spectrophotometer (BioTek, USA). The serum triglyceride concentration in each well could be read from the standard curve displayed by the spectrophotometer.

2.3.2 Non-esterified fatty acid concentration

Serum non-esterified fatty acid (NEFA) concentration was measured using the commercially available Wako NEFA C test kit (Wako Pure Chemical Industries, Ltd. Japan, 279-75401). In this enzymatic method, the acylation of coenzyme A (CoA) by the fatty acids in the presence of added acyl-CoA synthetase (ACS) produces acyl-CoA. The resultant acyl-CoA is oxidised by added acyl-CoA oxidase (ACOD) to form hydrogen peroxide. Hydrogen peroxide in the presence of peroxidase permits the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline (MEHA) with 4-aminoantipyrine to form a purple-coloured product with an absorption maximum at a wavelength of 550 nm.

Into a 96-well microplate, 5μl of the NEFA standards 0, 0.125, 0.25, 0.5, 1, and 2 mmol/l, and serum samples were delivered in duplicates. To each of the wells containing these contents, 100μl of the colour reagent A was added. The microplate was then incubated in 37°C for 10 minutes, after which 200μl of the colour reagent B was added to the same wells. The microplate was incubated in 37°C for 10 minutes for a second time, before measuring the absorbance of each well against the reagent blank at a wavelength of 550 nm in the Power Wave™ Microplate Spectrophotometer (BioTek, USA). Serum NEFA concentrations of each sample could be read from the standard curve revealed by the spectrophotometer.

2.3.3 Cholesterol concentration

Serum cholesterol concentration was measured using Cholesterol CHOD-PAP Single Reagent D95116, obtained from Dialab (Austria). The principal of this assay is that cholesterol ester is hydrolysed by addition of cholesterol esterase into cholesterol. Cholesterol is then oxidised by cholesterol oxidase to yield hydrogen peroxide. The hydrogen peroxide produced then reacts with 4-aminoantipyrine under the catalytic action of peroxidise to form a quinoneimine dye, the absorbance of which may be measured at a wavelength of 500 nm.

Into a 96-well microplate, 5μl of the cholesterol standards 0, 0.163, 0.325, 0.65, 1.3, 2.6, and 5 mmol/l, and serum samples were delivered in duplicates. To each of the wells containing these contents, 250μl of Cholesterol CHOD-PAP Single Reagent was added. The mixed contents of the wells were incubated at room temperature for 20 minutes,

before measuring the absorbance of each well against the reagent blank at a wavelength of 500 nm in Power Wave™ Microplate Spectrophotometer (BioTek, USA). Serum cholesterol concentrations of each sample could be read from the standard curve revealed by the spectrophotometer.

2.3.4 Insulin concentration

Circulating insulin concentrations were determined using a commercially available Rat Insulin RIA kit, based on a double antibody solid phase technique (Linco Research Inc., MO, USA). The principle of the assay is the ability of a limited quantity of antibody to bind a fixed amount of ^{125}I-insulin. The percentage of radiolabelled insulin bound to the antibody decreases as a function of the increasing unlabelled insulin in the test sample. After the reaction has reached a state of equilibrium, the bound and free insulin is separated in order to determine the quantity of unlabelled insulin. This separation can be accomplished by precipitation of the insulin-antibody complex by the addition of a second antibody directed against the immunoglobulin present in the original insulin. The quantity of unlabelled insulin in an unknown sample is then determined by comparing the distribution of radioactivity in the pellet, after centrifugation, with values established using known insulin standards in the same assay system.

Rat insulin standards were across the 0-10 ng/ml range (0.1, 0.2, 0.4, 1.0, 2.0, 5.0, 10.0 ng/ml) and stored at -20°C. On the day the assay was to be performed, one set of standards and the serum samples were thawed and thoroughly mixed with the aid of a vortex. 200μl and 100μl of Assay Buffer (0.05 M phosphosaline, pH 7.4, 0.025M EDTA, 0.08% NaN_3, 1% RIA grade BSA) were loaded to the Non-Specific Binding (NSB) tubes (3-4) and Reference (Bo) tubes (5-6) of 12 x 75 mm plastic tubes. 100 μl

duplicates of each standard, quality control and serum sample (50μl of sample added to 50μl of Assay Buffer due to limited sample size) were loaded to tubes 7 through the end of the assay, along with 100 μl of ^{125}I-insulin and 100 μl of rat insulin antibody (guinea pig anti-rat insulin). The ^{125}I-insulin was at a specific activity of 367 mCi/mg. 100 μl of ^{125}I-insulin was also loaded to Total Count (TC) tubes (1-2), and NSB and Bo tubes, while 100 μl of rat insulin antibody was loaded only to NSB and Bo tubes. The dispensed solutions were vortexed, covered and incubated overnight at 4°C.

The second antibody reaction involved the addition of 1 mL of Precipitating Reagent (goat anti-guinea pig IgG serum, 3% PEG and 0.05% Triton X-100 in 0.05M phosphosaline, 0.025M EDTA, 0.08% NaN_3) to all tubes except TC tubes, to precipitate the soluble insulin-antibody complex. After vortexing and further incubating at 4°C for 20 minutes, the precipitated insulin antibody complex was then separated from the dilute supernatant solution by centrifugation at 3000 x*g* for 20 minutes. The supernatant solution was then decanted and the pellets counted on a Wallac Wizard 1470 Automatic Gamma Counter (PerkinElmer Life And Analytical Sciences, Inc. Massachusetts, USA). Using the software available on the γ-counter, a four parameter logistic curve was found to provide the fit of the standard curve constructed from a plot of the B/Bo against the logarithm of the concentration of the standards. The insulin concentration was calculated in pmol of rat insulin per millilitre of unknown serum sample.

2.4 ANALYSES OF LIPIDS IN LIVER TISSUES

2.4.1 Hepatic triglyceride concentration

Total lipids were extracted from livers by a modified method of Folch et al. (1956). 30mg of liver samples were homogenised with 2mL of chloroform-methanol (2:1, v/v)

using Polytron (Ultra-Turrax T8, IKA-Labortechnik, Staufen, Germany) for 2 x 15 seconds (setting 5). The homogenate was vortexed and placed on the roundabout overnight. The next day, the homogenate was washed with 2mL of 0.6% Saline and centrifuged for 10 minutes at 4000 x*g* using Eppendorf 5810R Centrifuge (Global Medical Instrumentation, Inc., Minnesota, USA).

After centrifugation, two layers were formed: the bottom layer consisting of total lipids was saved, while the top layer consisting non-lipids was discarded. The bottom layer was then evaporated to dryness under N_2. The organic phase was recovered by adding pure ethanol to the dried content and its triglyceride concentration was measured using GPO Triglycerides reagent (Roche, Australia) and glycerol standard (Sigma, Australia), as described in Section 2.3.1.

2.4.2 Histological assessment of total lipid contents in liver

For gross inspection of the total lipid contents in livers, Oil Red O was used to stain the neutral lipids of the tissues. Frozen livers of 5x5x3 mm size were embedded in Tissue-Tek OCT compound (ProSci Tech, Queensland, Australia) and sectioned at 7 μm using Shandon Cryotome E (Thermo Electron Corporation, Cheshire, U.K.). The temperature of the knife used to section the frozen livers was set to -14°C. The sectioned livers were air dried for 1 hour, and then fixed on Menzel-Glaser microscopic slides (Gerhard Menzel, Glasbearbeitungswerk GmbH & Co., Braunschweig, Germany) by placing them in the formal calcium fixative solution, which consisted of 40% formalin, 10% $CaCl_2$ and distilled water (9:9:82, v/v/v) for 15 minutes.

Once the tissues were fixed on the slides, they were then rinsed in distilled water followed by 60% isopropanol. The slides were then stained in 60ml Oil Red O working solution, which consisted of 3.5 mg/mL Oil Red O stock solution and 1% dextran (3:2, v/v) for 15 minutes (Oil Red O stock solution was made by dissolving Oil Red O in 99% isopropanol). The slides were then rinsed in distilled water and counterstained in 60mL Mayer's haematoxylin solution for 30 seconds. The slides were washed in tap water until no more stain came out, before placing them in 60mL Scott's blue solution for 10 seconds. To make Scott's blue solution, pre-dissolved 4mM $NaHCO_3$ was made up to a volume to 60 mL with 166mM $MgSO_4$ and a few crystals of thymol blue (pH 9.2). The slides were then washed under running tap water for 7 minutes, and rinsed in distilled water. The tissues were then left to be dried, before being mounted with ZYMED clearmount solution (Invitrogenm, CA, USA). Photomicrographs of the stained tissues revealing total lipid droplets were taken by Olympus BX51 microscope (Olympus, USA) at 20x magnification, for gross inspection.

2.5 Statistical analysis

All results are presented as the mean ± the standard error of the mean (mean ± SEM).

Student's t test was used to test the statistical significance of differences between mean values obtained from two independent groups of samples (unpaired t-test). One-way analysis of variance (ANOVA) was used to test systematic differences among more than 2 means and significant differences between two groups were established using Dunnett's or Tukey's multiple comparison tests. Incremental areas under the curves (AUC) were calculated geometrically using the trapezoid rule.

Statistical significance was accepted at the $p < 0.05$ level. All statistical analyses were performed using Graph Pad Prism 4 (GraphPad Software Inc., CA, USA).

CHAPTER 3

Investigation of Sugarid in mice models of metabolic syndrome and type 2 diabetes

3.1 INTRODUCTION

MetS is a clustering of obesity-driven risk factors for atherosclerotic cardiovascular disease (ASCVD), as well as a precursor to T2DM. Currently, there is no single approved pharmaceutical drug capable of treating MetS or T2DM completely. A combination of different drugs is used to target the multiple abnormalities of MetS and T2DM, which often results in occurrences of adverse effects (Grundy, 2006; Moller & Kaufman 2005). In the search for a new novel drug with more effective and safe actions, research on natural substances such as Chinese herbal medicine has been extensively carried out (Jung et al., 2006; Lo et al., 2004). Nevertheless, there is still a lack of experimental studies investigating effects and mechanisms of Chinese herbal medicine (Yin et al., 2008). Therefore, in this study the effects and mechanisms of a Chinese herbal product called, Sugarid was investigated in mice models of MetS and T2DM.

Sugarid is a Chinese herbal product indicated for assisting blood circulation and controlling blood glucose level, and it has been approved by Therapeutic Goods Administration (TGA) in the Australian market (AUST L 77765). It is currently used as an adjuvant therapy to treat patients with T2DM in China, which means that it is used in conjunction with other conventional drugs. Implementing Chinese herbal medicine with conventional medicine is a common therapy in China. This approach is becoming increasingly popular as it has allowed patients to lower the dosages of their conventional medicines, thereby minimising any adverse effects (Jia et al., 2003). Since the aim of this study was to evaluate the efficacy of Sugarid, no conventional medicine was used with the herbal product for treatment given to mice.

For a detailed investigation, the effects of Sugarid in ameliorating insulin resistance-associated metabolic abnormalities including hyperglycaemia were evaluated. The herbal product was tested in two models of mice, MetS and T2DM. The two models were induced in mice by feeding them HFD with or without STZ injection. STZ is a diabetogenic agent, which selectively destroys the insulin producing pancreatic β-cells to induce hyperglycemia in animals (Szkudelski, 2001).

3.2 RESEARCH PLAN AND METHODS

Chinese herbal medicine

Sugarid (in capsules) was supplied by Shen Neng Pty. Ltd. (NSW, Australia). Each capsule contained herbal extracts equivalent to fagopyrum esculentum (11g), andrographis paniculate (383mg), and propolis (89mg). The shells of capsules were removed and their contents, in the form of powder, were mixed with the ingredients of the HFD (Section 2.1.3). The content of Sugarid in the HFD (≈5%) was calculated based on the dosage of the herbal product, which was 3.75g/kg/day, and the average daily food intake of mice.

Animals and treatment

Male C57BL/6J mice (n=42) were obtained at 8 weeks of age and housed under controlled temperature (21°C) and lighting (12 h light-dark cycle, lights on at 7 am) conditions with free access to water and standard laboratory chow diet (15% total calories from fat; Gordon's Specialty Stock Feed, Sydney, Australia). After adapting to this environment for 3 days, 8 mice were continuously fed with the chow diet; meanwhile, 34 mice were fed with HFD (59% total calories from fat; Section 2.1.3) for

12 weeks. At the end of week 12, the HFD fed mice were classified into the MetS group (n=18) or the T2DM group (n=16) according to their blood glucose levels after the streptozotocin (STZ) injections (except for 6 mice, all received injections). The MetS and T2DM groups were then further divided into the control (n=8 for both MetS and T2DM) or treatment (MetS, n=10 and T2DM, n=8) group by feeding HFD or Sugarid-supplemented HFD. At weeks 9 and 10 of treatment, mice were subjected to i.p. GTT and i.p. ITT. At the end of week 10, mice were sacrificed as described in Section 2.1.4.

STZ injection

Seventy five milligrams of STZ was freshly dissolved in 5mL of acetate buffer (0.1M glacial acetic acid and 2mM $NaCH_3COO$; pH5.2) on the day of injection. The dissolved content was filtered at 0.2µm just before the injection. To make the acetate buffer, 0.1M glacial acetic acid was firstly made by adding 588µl of pure acetic acid made up to 100ml of distilled water. 166 mg $NaCH_3COO$ was then added to 100 ml of 0.1M glacial acetic acid (pH 5.2).

According to the study by Manchem et al. (2001), T2DM model was achieved in the HFD fed mice receiving five daily injections of 35 mg/kg STZ. The non-fasting blood glucose levels of these mice ranged from 16.5 to 22 mmol/l. In the present study, 28 mice fed with HFD were injected with a single dose of 75 mg/kg STZ in the hope of achieving the T2DM model similar to that described in Manchem et al. (2001). Blood glucose test was performed to confirm if mice had reached the blood glucose range for T2DM. The mice that did not reach this range were injected with 75 mg/kg STZ for a second time. Finally, any mice that did not respond to the first injection were assigned

to the MetS group, together with the 6 other HFD fed mice that did not receive any STZ injections.

Measurements

Measurements of body weight and food intake were conducted throughout the experimental period, as described in Section 2.2.1. Experiments to assess glucose homeostasis and insulin sensitivity included blood glucose test, i.p. GTT and i.p. ITT (Sections 2.2.2 and 2.2.3). Serum triglyceride and cholesterol concentrations were measured with appropriate enzymatic assay kits (Section 2.3). Liver triglyceride concentration was determined by lipid extraction and enzymatic assay. Finally, histological assessment of total lipid contents in livers was conducted by the Oil Red O staining (Section 2.4).

Statistical analysis

Two sets of comparisons for the measurements were performed by unpaired t-test for two groups of independent samples. The first comparison was for the HFD or the Sugarid-supplemented HFD fed mice in the MetS group. The second comparison was for the HFD or the Sugarid-supplemented HFD fed mice in the T2DM group. The area under the blood glucose concentration-time curves was calculated by the trapezoidal rule. All results are expressed as mean ± SEM. Statistical significance was accepted at the $p<0.05$ level.

3.3 RESULTS

3.3.1 Induction of MetS and T2DM in mice

There was a significant difference in body weights between the mice fed with HFD (n=34) and those fed with chow diet (n=8) after the 12 weeks feeding period (31 ± 0.5 vs 28.9 ± 0.61 g, $p<0.05$, Figure 3.1). This result was in correspondence to a study by Winzell and Ahren (2004), where C57BL/6J mice fed with HFD gained larger body weights over the first 12 weeks, followed by a slower weight gain during subsequent weeks. Of the 28 HFD fed mice injected with 75 mg/kg STZ, 16 mice responded with increased blood glucose levels 2 days after the injection, while the remaining 12 mice did not show any significant changes in their blood glucose levels. 5 out of 16 mice with relatively low blood glucose levels were chosen and injected with 75 mg/kg STZ for the second time. 3 days after the second injection, the mean blood glucose concentration of the 16 mice reached the range for T2DM (16.5 ± 1.1 mmol/l; Figure 3.2). Meanwhile, there were still no significant changes in the blood glucose levels of the 12 mice that did not respond to the initial STZ injection.

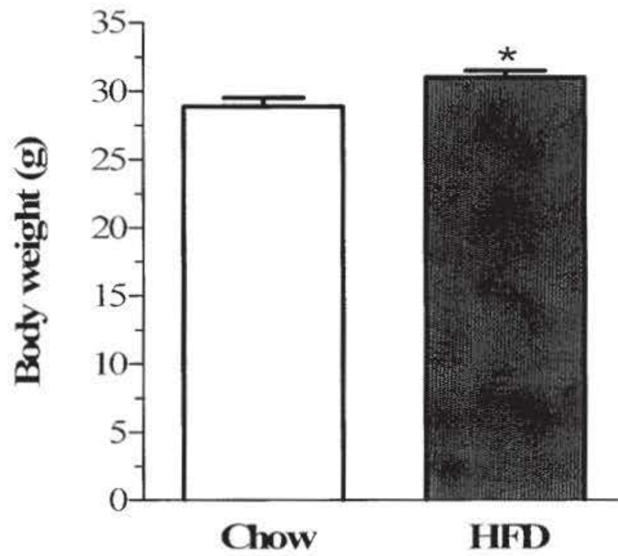


Figure 3.1 Body weights of the mice fed with chow diet (□, n=8) or HFD (▩, n=34) for 12 weeks. Data are mean ± SEM. *p<0.05 *vs* the chow diet fed mice.

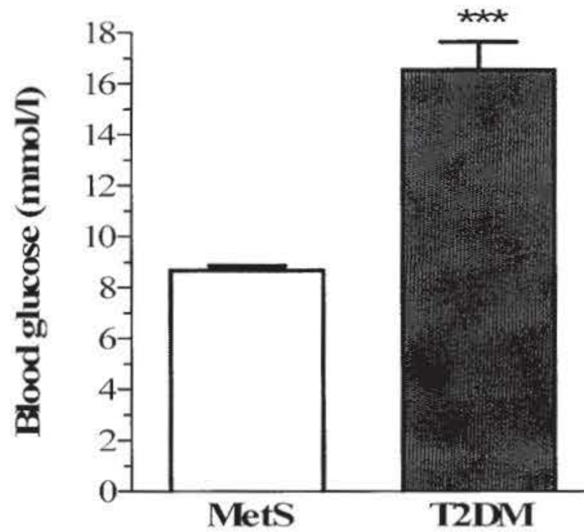
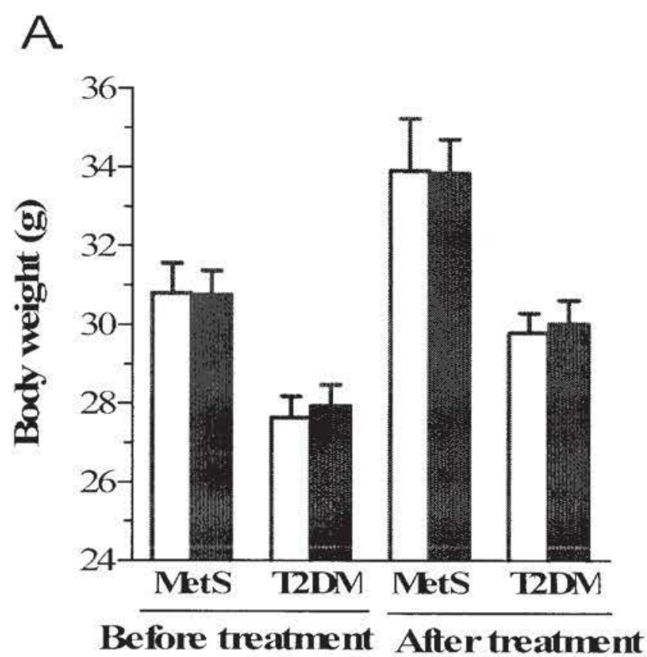


Figure 3.2 Non-fasting blood glucose concentrations of the mice in the MetS (□, n=12 given STZ injection; n=6 given no STZ injection) group and the T2DM (■, n=11 given STZ injection; n=5 given 2x STZ injections) group. Data are mean ± SEM. ***p<0.001 *vs* the MetS group.

3.3.2 Effects of Sugarid on the body and visceral fat weights

After 10 weeks' treatment, the body weights were not significantly different between the Sugarid-supplemented HFD fed mice and the HFD fed mice, in both the MetS (33.9 ± 1.3g vs 33.8 ± 0.9g, p=0.96, Figure 3.3A) and T2DM (29.8 ± 0.5g vs 30 ± 0.6g, p=0.76, Figure 3.3A) groups. A similar trend of result was revealed for the visceral fat weights of mice in both the MetS and T2DM groups (Figure 3.3B). Average daily food intake during the treatment period was slightly higher in the Sugarid-supplemented HFD fed mice, compared to the HFD fed mice in the T2DM group (2.54 ± 0.04 vs 2.28 ± 0.06 g/mouse/day, $p<0.01$, Figure 3.4), but not in the MetS group.



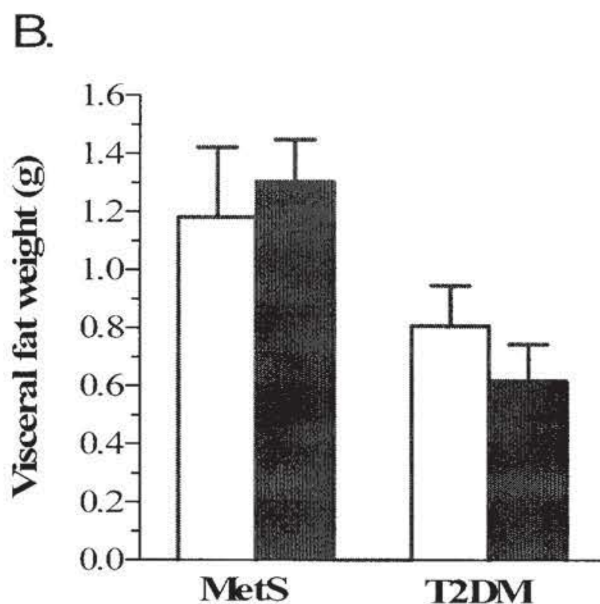


Figure 3.3 Effects of Sugarid on the body and visceral fat weights. Upper panel A: body weights before and after 10 weeks' treatment and lower panel B: visceral fat weights after 10 weeks' treatment of the mice fed with HFD (□, n=8) or the Sugarid-supplemented HFD (■, MetS, n=10; T2DM, n=8) in the MetS group and the T2DM group. Data are mean ± SEM.

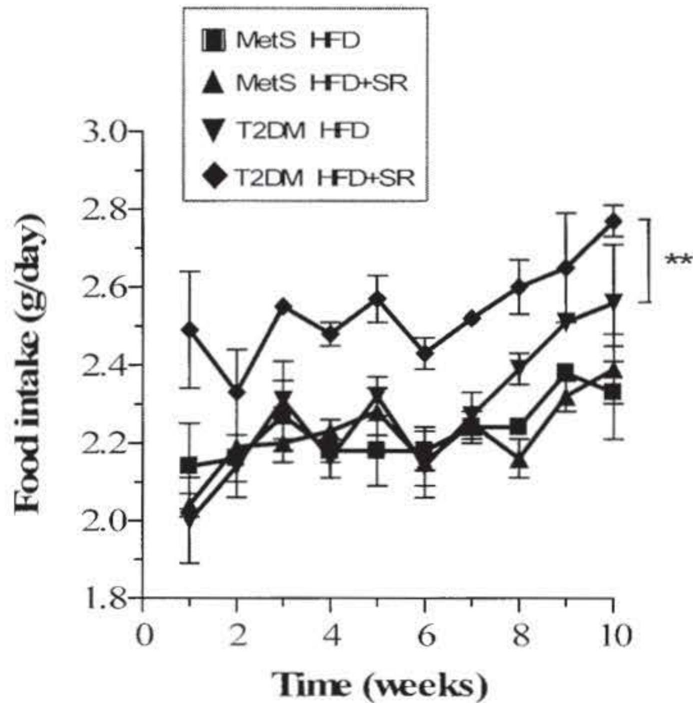


Figure 3.4 Daily food intake (g) per mouse for 10 weeks of the mice fed with HFD (n=8) or the Sugarid-supplemented HFD (MetS, n=10; T2DM, n=8), in the MetS group and the T2DM group. Each time point is the mean ± SEM. **p<0.01 *vs* the HFD fed mice.

3.3.3 Effects of Sugarid on glucose metabolism

After 9 weeks' treatment, there were no significant differences in the basal blood glucose concentrations between the Sugarid-supplemented HFD fed mice and the HFD fed mice in either the MetS (6.5 ± 0.4 vs 6.4 ± 0.2 mmol/l, p=0.43, Figure 3.5) or the T2DM (12.9 ± 2.1 vs 13.1 ± 1.4 mmol/l, p=0.45, Figure 3.5) groups.

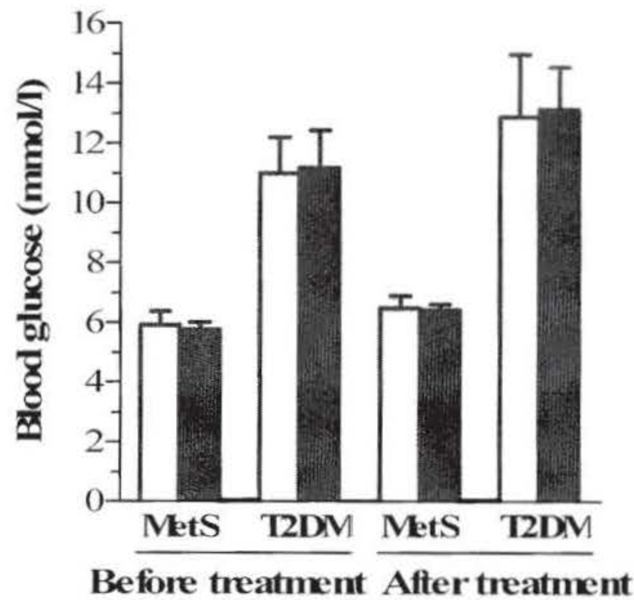


Figure 3.5 Effects of Sugarid on the blood glucose levels of mice fasted for 12 h. The mice in the MetS group and the T2DM group were fed with HFD (□, n=8) or the Sugarid-supplemented HFD (■, MetS, n=10; T2DM, n=8) for 9 weeks. Data are mean ± SEM.

i.p. GTT was performed to detect an imbalance in glucose homeostasis due to hyperglycaemia related to insulin resistance. There was no significant difference in blood glucose responses during the 2-hour i.p. GTT, between the Sugarid-supplemented HFD fed mice and the HFD fed mice in the MetS group (Figure 3.6). However, a significantly lower blood glucose concentration was observed at the 120 minutes time point in the Sugarid-supplemented HFD fed mice, compared to the HFD fed mice in the T2DM group (19.9 ± 2.9 vs 27.1 ± 2 mmol/l, $p<0.05$, Figure 3.6). A lower area under curve (AUC) of the blood glucose concentration-time profiles of the treatment group compared to the control group (2792.4 ± 290.1 vs 3423.3 ± 165.8 mmol·min/l, $p<0.05$, Figure 3.7) suggested that Sugarid attenuated impaired glucose tolerance in the T2DM group.

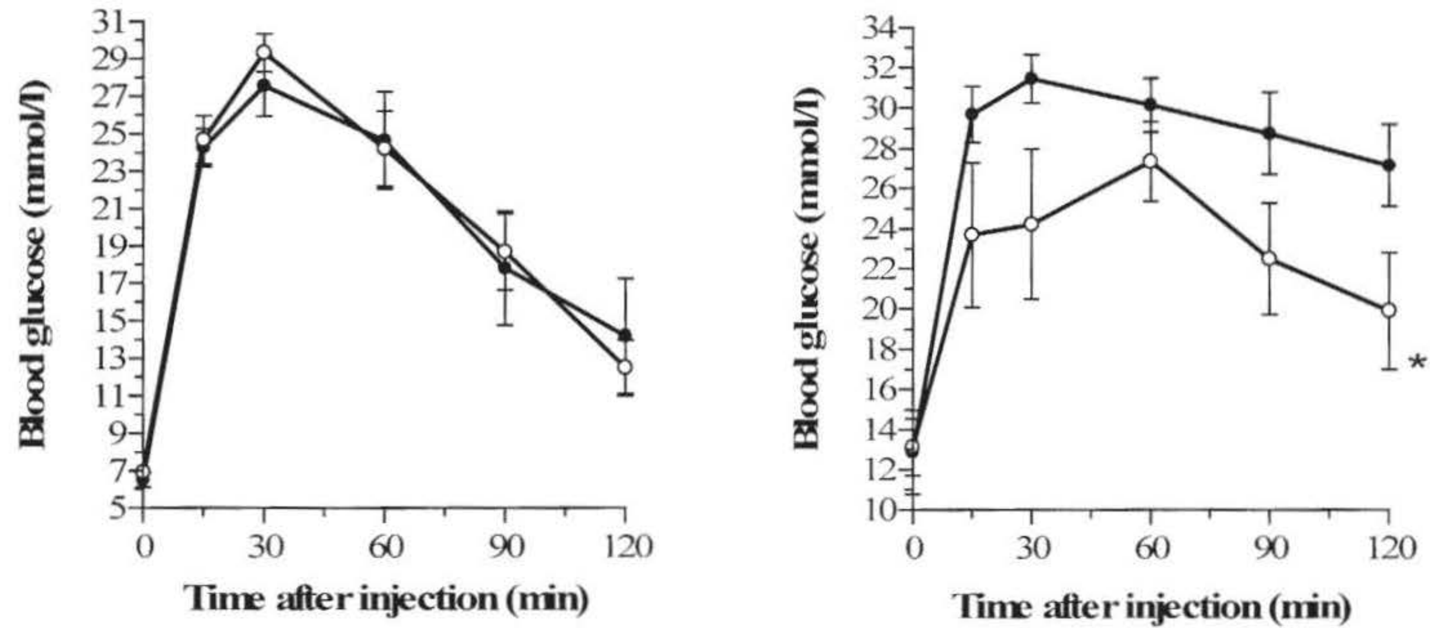


Figure 3.6 Effects of Sugarid on the blood glucose responses during the 2-h i.p. GTT of mice fasted for 12 h. The mice in the MetS group (left panel) and the T2DM group (right panel) were fed with HFD (○, n=7) or the Sugarid-supplemented HFD (●, MetS, n=10; T2DM, n=8) for 9 weeks. Blood glucose was measured from the tails at the indicated time points after i.p. injection of glucose (2g/kg). Each time point is the mean ± SEM. *$p<0.05$ *vs* the HFD fed mice.

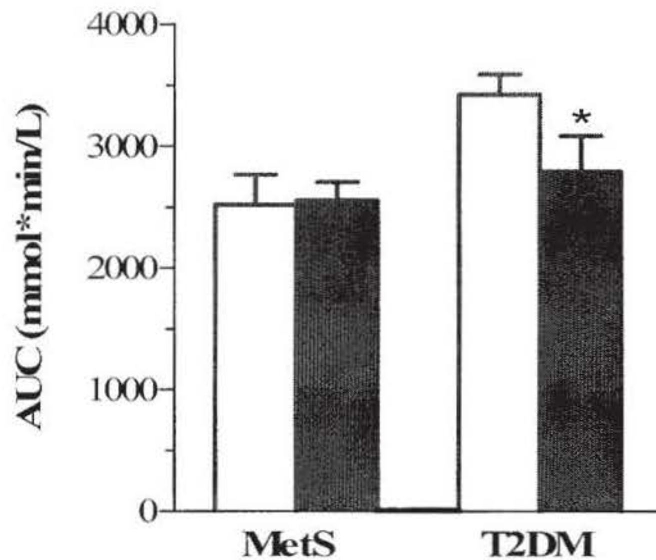


Figure 3.7 AUCs of the blood glucose concentration-time profiles following i.p. GTT in the mice fed with HFD (□, n=7) or the Sugarid-supplemented HFD (■, MetS, n=10; T2DM, n=8) in the MetS group and the T2DM group. *$p<0.05$ *vs* the HFD fed mice.

3.3.4 Effects of Sugarid on insulin sensitivity

i.p. ITT was performed to evaluate the effects of Sugarid on insulin sensitivity of mice fasted for 6 h. In response to the insulin-stimulated glucose disposal during i.p. ITT, there were no significant differences in blood glucose concentration-time profiles between the Sugarid-supplemented HFD fed mice and the HFD fed mice in either the MetS (AUC [Glu] 1103.3 ± 48.6 vs 1091.3 ± 58.1 mmol·min/l, Figure 3.8) or the T2DM (AUC [Glu] 2116.9 ± 299 vs 2187.7 ± 335.7 mmol·min/l, Figure 3.8) groups.

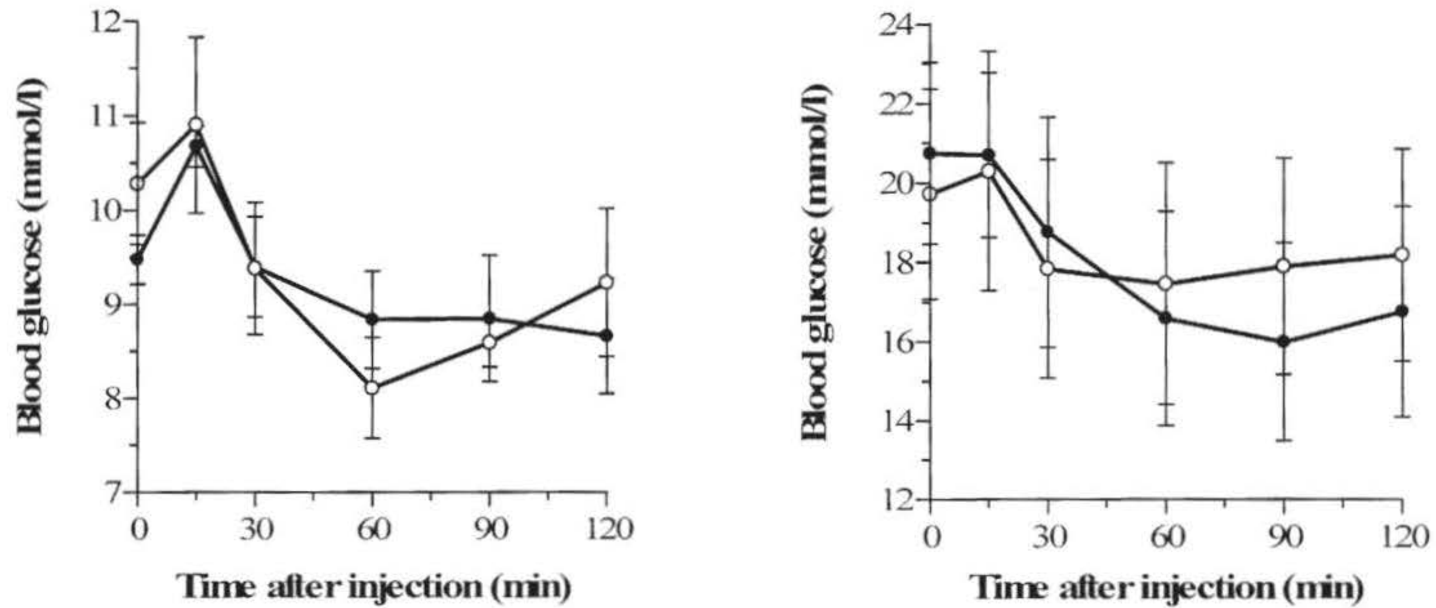


Figure 3.8 Effects of Sugarid on the blood glucose responses during the 2-h i.p. ITT of mice fasted for 6 h. The mice in the MetS group (left panel) and the T2DM group (right panel) were fed with HFD (○, n=7) or the Sugarid-supplemented HFD (●, MetS, n=10; T2DM, n=8) for 10 weeks. The blood glucose was measured from tails at the indicated time points after i.p. injection of insulin (0.85IU/kg). Each time point is the mean ± SEM. The AUCs of the blood glucose concentration-time profiles were not significantly different between the mice fed with HFD and those fed with Sugarid-supplemented HFD.

3.3.5 Effects of Sugarid on lipid metabolism

Measurements of serum lipid concentrations by enzymatic methods revealed that Sugarid did not improve dyslipidaemia associated with MetS and T2DM. For both serum triglyceride and cholesterol concentrations, no significant differences between the Sugarid-supplemented HFD and the HFD fed mice were observed in the MetS (triglyceride: 0.69 ± 0.1 vs 0.8 ± 0.09, p=0.19; cholesterol: 3.38 ± 0.21 vs 3.26 ± 0.31 mmol/l, p=0.37, Figure 3.9) and the T2DM (triglyceride: 1.23 ± 0.32 vs 1.23 ± 0.4, p=0.5; cholesterol: 4.07 ± 0.25 vs 4.01 ± 0.37 mmol/l, p=0.45, Figure 3.9) groups.

Similarly, the liver triglyceride concentrations were not significantly different between the Sugarid-supplemented HFD fed mice and the HFD fed mice, in the MetS (31.7 ± 4.6 vs 32.8 ± 4.2 μmol/g, p=0.43) and the T2DM (21.1 ± 3.9 vs 22.9 ± 7.5 μmol/g, p=0.41) groups (Figure 3.10). The photomicrographs of the Oil Red O-stained livers revealed that there were no differences in the size, number or distribution of lipid deposits between the treatment and control groups (Figure 3.11).

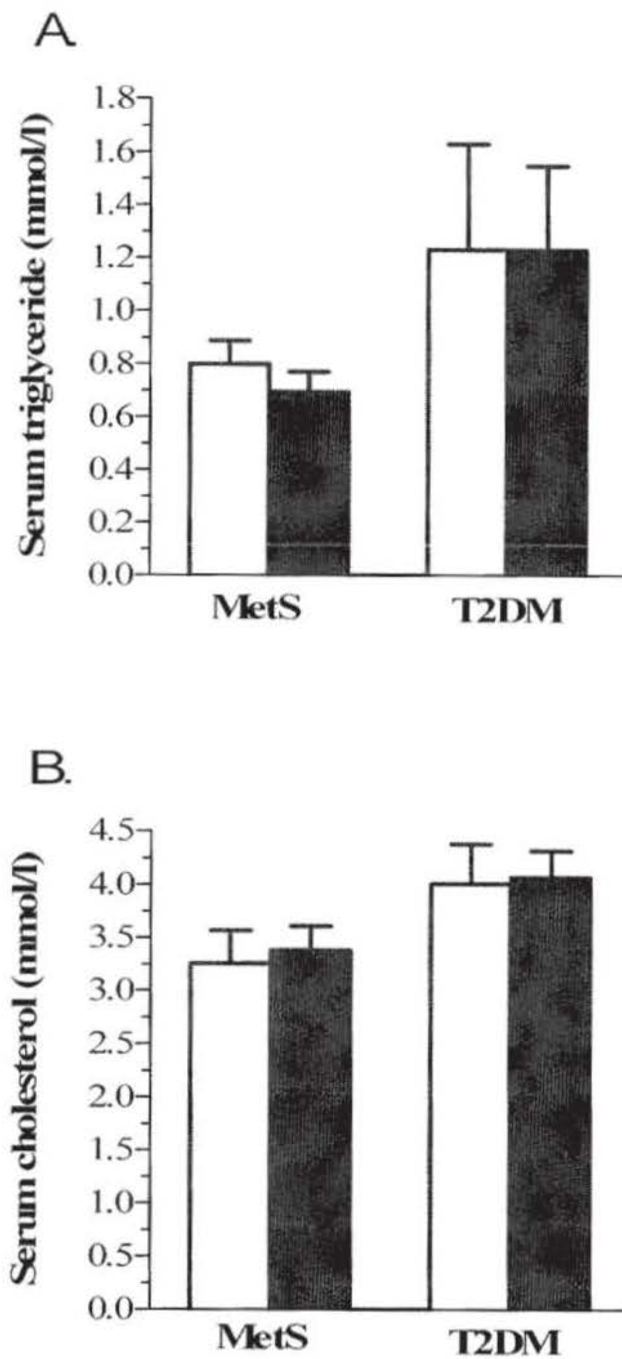


Figure 3.9 Effects of Sugarid on the serum triglyceride (A) and cholesterol (B) concentrations of mice fasted for 12 h. The mice in the MetS group and the T2DM group were fed with HFD (□, MetS, n=7; T2DM, n=6-7) or the Sugarid-supplemented HFD (■, MetS, n=8; T2DM, n=6-7) for 10 weeks. Data are mean ± SEM.

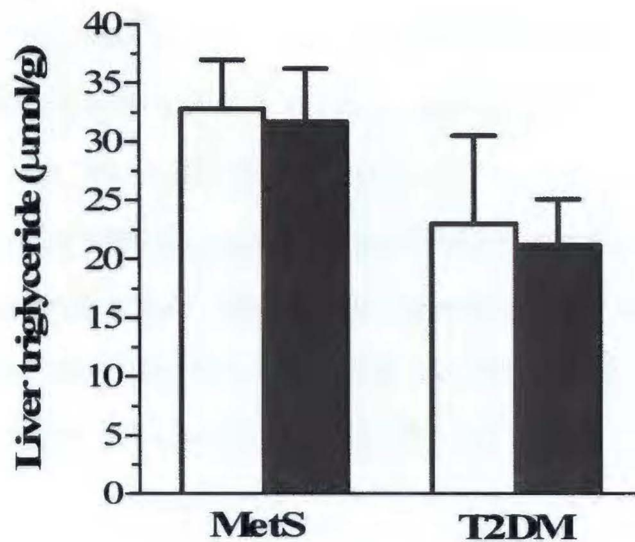


Figure 3.10 Effects of Sugarid on the liver triglyceride levels of mice fasted for 12 h. The mice in the MetS group and the T2DM group were fed with HFD (□, MetS, n=6; T2DM, n=4) or the Sugarid-supplemented HFD (■, MetS, n=8; T2DM, n=6) for 10 weeks. Data are mean ± SEM.

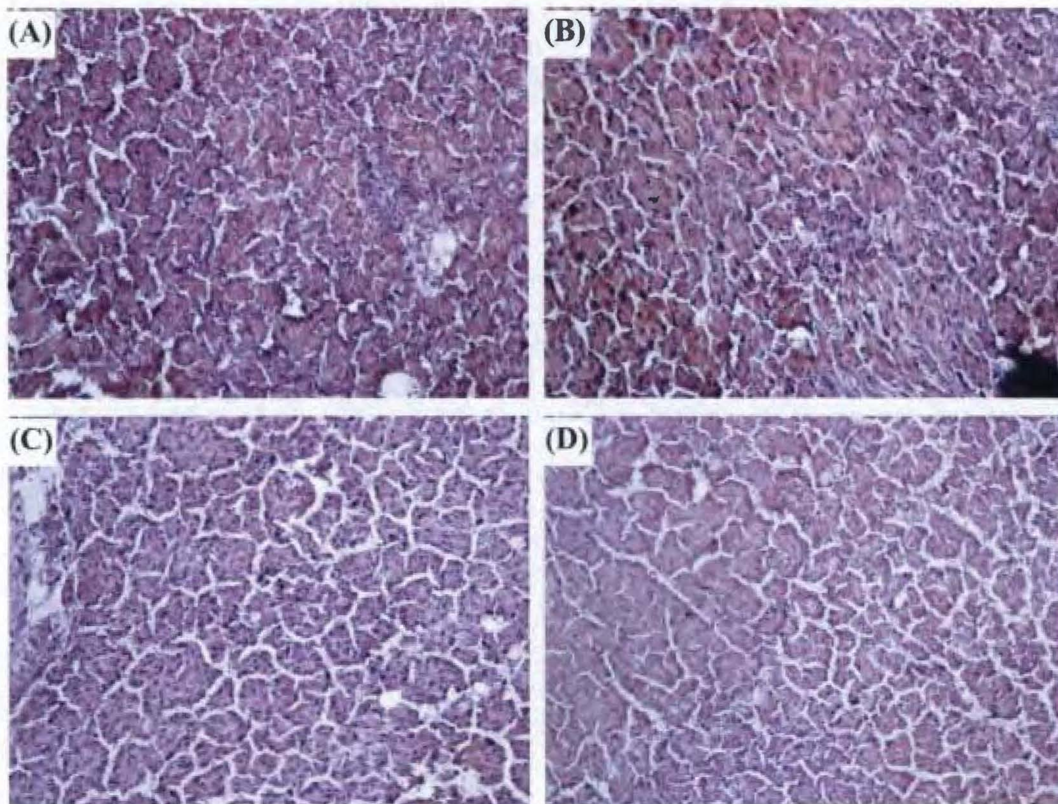


Figure 3.11 Histological assessment of the livers from mice. The photomicrographs are of the Oil Red O-stained frozen sections of livers from the HFD fed mice (A: the MetS group; C: the T2DM group) and the Sugarid supplemented-HFD fed mice (B: the MetS group; D: the T2DM group) after 10 weeks' treatment. Similar amounts of lipid deposits were found between the treatment and the control groups. All the photomicrographs are at 20x magnification.

3.4 DISCUSSION

In this study, the metabolic effects of Sugarid were investigated in mice models of MetS and T2DM. The body weights of mice increased significantly after 12 weeks of the HFD feeding. In one study, the plasma glucose levels of C57BL/6J mice rose with impaired glucose tolerance and impaired glucose-stimulated insulin secretion after 1 week of the HFD feeding. In addition, their body weights increased over 12 weeks of the feeding (Winzell & Ahren, 2004). The mice in this study also gained body weights and became insulin resistant, thereby becoming the models of MetS and T2DM.

It is known that hyperglycaemia, which characterises T2DM, develops after injection of STZ to the HFD fed mice (Srinivasan & Ramarao, 2007). It was documented in other studies that rodents fed with HFD and injected with small doses of STZ (50-100mg/kg) mimicked the human T2DM condition (Luo et al., 1998; Reed et al., 2000). These studies are in accordance with the present study, where a significant rise in blood glucose concentration was seen in mice injected with 75-150mg/kg STZ.

Despite the fact that the exact pathogeneses of MetS and T2DM are not completely clear to this date, many evidences indicate that central obesity and insulin resistance are two crucial elements (Appel et al., 2004; Eckel et al., 2005; Haffner 2003; Reaven & Chen 1988; Turkoglu et al., 2003). The present study also supports these evidences, as the mice with MetS and T2DM increased in their body weights concomitantly to their visceral fat weights. In addition, the results of i.p. GTT and i.p. ITT showed that these mice were glucose intolerant and insulin resistant.

Mice treated with Sugarid did not show significant improvements in body and visceral fat weights, blood glucose concentration, glucose tolerance, insulin sensitivity, and serum and

liver lipid concentrations, compared to those in the control group. The data shown in this study were not consistent with the findings of previous literatures on the individual ingredients of Sugarid, namely, andrographis paniculate, fagopyrum esculentum, and propolis. For example, Reyes et al. (2006) showed that the andrographis paniculate decoction lowered blood glucose levels of diabetic rats, but Sugarid was unable to lower blood glucose concentrations during the blood glucose test.

Fagopyrum esculentum was shown to reduce hyperglycaemia due to its active ingredient, D-chiro-inositol, which had a strong effect on insulin action (Fonteles et al., 2000; Ostlund et al., 1993), as well as lowering blood cholesterol concentration (Li & Zhang, 2001). However, neither insulin resistance nor lipid metabolism was improved by Sugarid in this study. Although, there was an improved glucose tolerance in the Sugarid-supplemented HFD fed mice of the T2DM group during i.p. GTT, the majority of results indicated that Sugarid was not effective in improving the glucose metabolism of mice in the study.

Propolis is known to contain over 300 active compounds with a wide range of effects including anti-hepatotoxic, anti-tumour, anti-oxidative, anti-microbial and anti-inflammatory effects (Banskota et al., 2001). The active ingredients of andrographis paniculate, Andrographolide and 14-deoxy-11, 12-didehydroandrographolide are also known to produce anti-thrombotic and anti-platelet effects (Thisoda et al., 2006). Whether these effects could be achieved by treatment with Sugarid is unknown, as no measurements to evaluate the haemodynamic abnormalities of MetS were implemented in this study.

The results of this study are in conflict with the fact that Sugarid is currently used as an adjuvant therapy for patients with T2DM in China. The findings of this study do not support Sugarid as a potential drug, at least in its individual form, for MetS or T2DM. The results are also inconsistent with the findings of previous studies carried out on the individual components of the herbal product. There may be several possible reasons to account for these contradictions, one of which may be that the dosage of Sugarid supplemented to mice was insufficient to cause significant improvements. Therefore, a stronger dosage of Sugarid should be incorporated into future animal studies, in order to confirm or rule out the beneficial effects of the herbal product on MetS and T2DM.

CHAPTER 4

Investigation of SK0504 in a mice model of metabolic syndrome

4.1 INTRODUCTION

As there is no approved pharmaceutical drug targeting all the clustering risk factors of ASCVD to date, research continues to discover and develop new medicinal agents from pure active principals, active fractions, and improved formulations of Chinese herbal medicines (Chen et al., 2006; Lee, 2000). Chinese herbal medicine may be credited for its ability to target multiple symptoms of disease syndromes in a single treatment (Lee, 2000). Traditionally, Chinese herbal medicines are administered by mixing several herbs together to form a herbal formula, where each herb contains numerous active principals and compounds to generate diverse effects (Chen et al., 2006; Dou et al., 2008).

A Chinese herbal formula comprises three groups of herbs: the principal group consists of herbs that provide the main therapeutic actions, the secondary group consists of herbs that assist the effects of the principal ones, and the last group consists of herbs that serve modulation roles such as treatment of accompanying symptoms, moderation of toxicity and harmonisation (Chen et al., 2006). Studies in the past have revealed the multi-functional effects of Chinese herbal formulae (Huo et al., 2003). For example, a herbal formula called Bofutsushosan (containing 18 herbs) was used to prevent obesity, hypertension, and insulin resistance (Akagiri et al., 2008). In addition, an anti-diabetes herbal formula, containing 8 single herbs was mixed into a special diabetes induction diet given to C57BL/6J mice in a study by Yushu Huo et al. (2003). Compared to the control groups fed with the diabetes induction diet only, the treatment groups fed with anti-diabetes herbal formula mixed into the diabetes induction diet presented with significantly lower body weights, blood glucose and serum insulin levels, insulin resistance, and smaller hepatic lipid vacuoles.

In the present study, a new herbal formula (coded as SK0504) was developed by mixing the extracts of four single Chinese herbs namely, Jiao Gu Lan (Gynostemma pentaphyllum), San Qi (Panax notoginseng), Huang Lian (Rhizoma Coptidis), and Dan Shen (Salvia miltiorrhiza). The four single herbs of SK0504 were selected based on the past studies of their effects on MetS, and on the hypothesis that when used together, they may exert synergistic effects by ameliorating multiple abnormalities of MetS. A well-known anti-hyperglycaemic drug called rosiglitazone that belongs to the thiazolidinedione (TZD) class was used as a positive control drug in the study. The aim of this study was to investigate the effects and mechanisms of SK0504 in a mice model of MetS induced by HFD feeding.

4.2 RESEARCH PLAN AND METHODS

Medicines

Herbal extracts of Jiao Gu Lan (Gynostemma pentaphyllum), San Qi (Panax notoginseng), Huang Lian (Rhizoma Coptidis), and Dan Shen (Salvia miltiorrhiza) were provided in powder form by Lian yun gang (Jiangsu, China). Equal proportions of each herbal extract were mixed together to form SK0504. Rosiglitazone was supplied by Avandia (NSW, Australia). For the preparation of SK0504, 4g of powders were dissolved in 5mL 0.9% NaCl saline. For the preparation of rosiglitazone, 10mg of the medicine was dissolved in 5mL 0.9% NaCl. Both SK0504 and rosiglitazone were freshly prepared at the beginning of each treatment week.

Animals and treatment

Male C57BL/6J mice (n=32) were obtained at 8 weeks of age and housed under controlled temperature (21°C) and lighting (12 h light-dark cycle, lights on at 7 am)

conditions with free access to water and standard laboratory chow diet (15% total calories from fat; Gordon's Specialty Stock Feed, Sydney, Australia). After adapting to the animal facility for 3 days, mice were divided into 2 groups receiving either HFD (59% total calories from fat; Section 2.1.3) or chow diet. After 12 weeks of feeding, HFD fed mice were further divided into 3 subgroups (n=8 each) and treated with SK0504 (daily dosage: 4g/5ml/kg), rosiglitazone (daily dosage: 10mg/5ml/kg) or 0.9% NaCl saline as vehicle (5ml/kg/day) for 5 weeks. Chow fed mice received 0.9% NaCl (5ml/kg/day) saline (n=8) as a control group. Daily treatment was given by oral gavage. At weeks 4 and 5, mice were subjected to i.p. GTT and i.p. ITT. At the end of experiment, mice were sacrificed as described in Section 2.1.4.

Measurements

Food intake and body weight were recorded daily as described in Section 2.2.1. Blood glucose test, i.p. GTT, and i.p. ITT were performed to measure glucose tolerance and insulin sensitivity, as described in Sections 2.2.2 and 2.2.3. Visceral fat and liver tissues were weighed and stored as described in Section 2.1.4. Measurements of biochemical parameters including serum triglyceride, NEFA, cholesterol and insulin were conducted using appropriate assay kits, as described in Section 2.3. Finally, analyses of lipids in livers were conducted by methods of lipid extraction, triglyceride assay, and Oil Red O staining of neutral lipids, as described in Section 2.4.

Statistical analysis

Measurements of body and visceral fat weights, food intake, blood glucose, serum triglyceride, NEFA, cholesterol, and insulin, and liver triglyceride concentrations were analysed by one-way analysis of variance followed by Dunnett's or Tukey's multiple

comparison tests. The area under the blood glucose concentration-time profiles of i.p. GTT and i.p. ITT were calculated by the trapezoidal rule. All results are expressed as the mean ± SEM. Statistical significance was accepted at the $p<0.05$ level.

4.3 RESULTS

4.3.1 Effects of SK0504 on the body and visceral fat weights

Before the start of treatment, the body weights of mice fed with HFD were significantly greater than those of mice fed a chow diet (data not shown). After 5 weeks' treatment, the mean body weight of the SK0504-treated HFD fed mice was lower than that of the vehicle-treated HFD fed mice (29.07 ± 1.48 vs 32.1 ± 0.99 g, Figure 4.1), but not to the level of statistical significance ($p>0.05$). The mean visceral fat weight of the HFD fed mice treated with SK0504 was significantly lower than that of the control counterparts (0.68 ± 0.09 vs 1.17 ± 0.14 g, $p<0.05$, Figure 4.2). The differences in these figures were not due to differential energy input, as there were no significant differences in food intake among the HFD fed mice throughout the experimental period (data not shown).

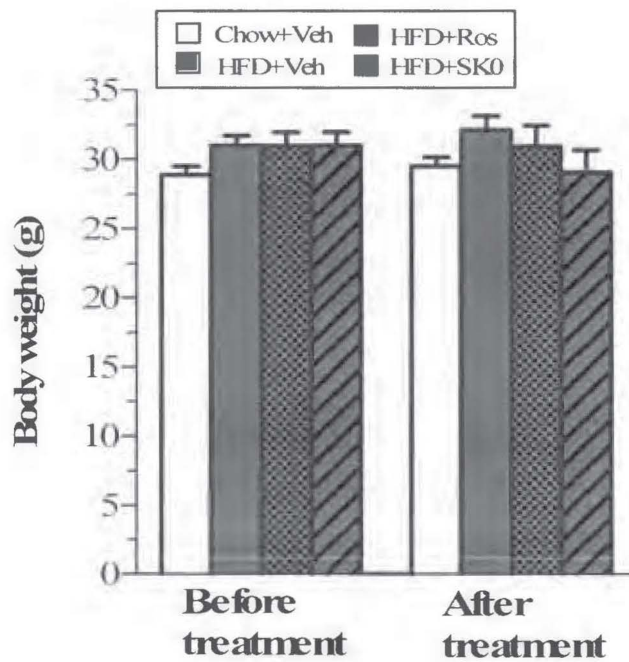


Figure 4.1 Effects of SK0504 on body weights of mice. The mice were divided into 4 groups receiving chow diet and vehicle (□, n=8), HFD and vehicle (■, n=8), HFD and rosiglitazone (■, n=8) or HFD and SK0504 (■, n=8) for 5 weeks. Data are mean ± SEM.

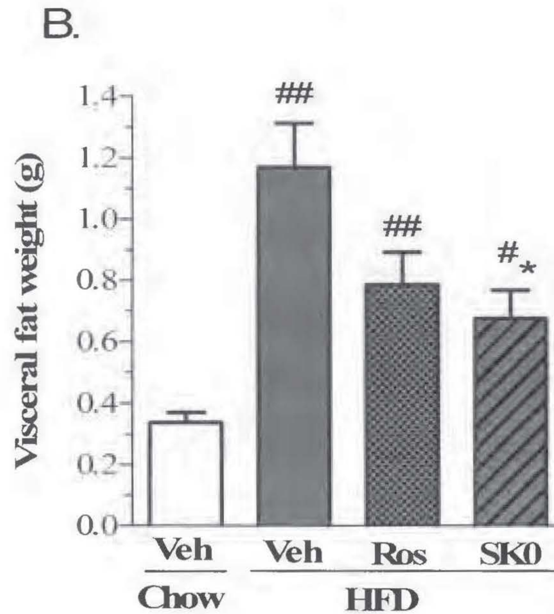


Figure 4.2 Effects of SK0504 on the visceral fat weights of mice. Mice were divided into 4 groups receiving chow diet and vehicle (□, n=8), HFD and vehicle (■, n=5), HFD and rosiglitazone (■, n=8) or HFD and SK0504 (■, n=6) for 5 weeks. Data are mean ± SEM. *$p<0.05$ *vs* the vehicle-treated HFD fed mice; #$p<0.05$, ##$p<0.01$ *vs* the vehicle-treated chow diet fed mice.

4.3.2 Effects of SK0504 on glucose metabolism

Blood glucose test and i.p. GTT were performed to assess the ability of SK0504 in ameliorating hyperglycaemia and improving glucose metabolism. Although the mean blood glucose concentration of the SK0504-treated mice was lower than that of the vehicle-treated mice, this difference did not reach the statistical significance (7.4 ± 0.62 vs 8.2 ± 0.4 mmol/l, $p>0.05$, Figure 4.3). Mice treated with rosiglitazone, on the other hand, showed a marked reduction in their mean blood glucose concentration compared to those treated with vehicle (6.57 ± 0.24 vs 8.2 ± 0.4 mmol/l, $p<0.05$, Figure 4.3).

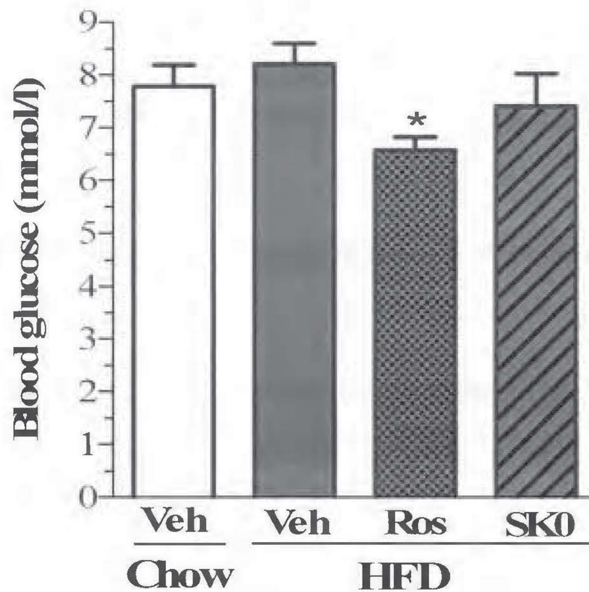
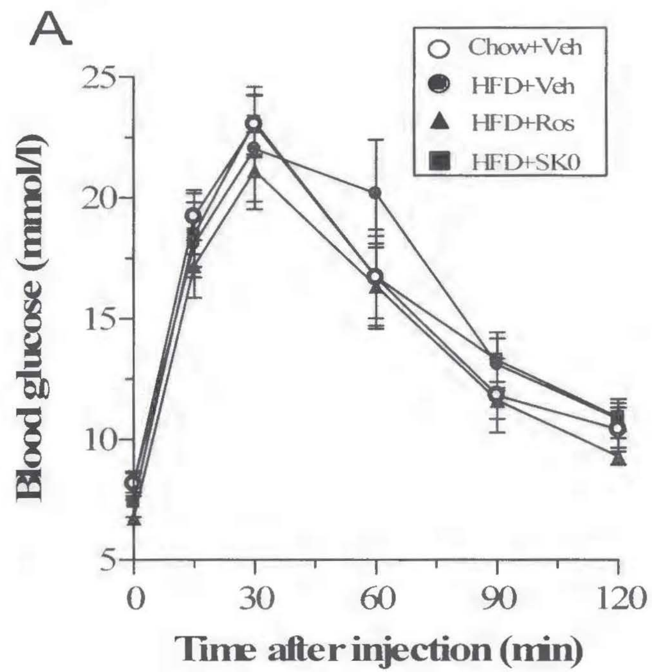


Figure 4.3 Effects of SK0504 on the blood glucose levels of the mice fasted for 12 h. The mice were divided into 4 groups receiving chow diet and vehicle (□, n=7), HFD and vehicle (■, n=7), HFD and rosiglitazone (■, n=7) or HFD and SK0504 (■, n=7) for 4 weeks. Data are mean ± SEM. *$p<0.05$ *vs* the vehicle-treated HFD fed mice.

The blood glucose disposal rates during the 2-h i.p. GTT were not significantly different between the SK0504-treated HFD fed mice and the vehicle-treated HFD fed mice (AUC [Glu] 1916.3 ± 122.6 vs 1989 ± 159.6 mmol·min/l, $p>0.05$; Figure 4.4). Although the blood glucose disposal rate of the rosiglitazone-treated HFD fed mice was faster than that of the vehicle-treated HFD fed mice, the difference did not reach the statistical significance (AUC [Glu] 1761.5 ± 106.5 vs 1989 ± 159.6 mmol·min/l, $p>0.05$; Figure 4.4).

4.3.3 Effects of SK0504 on insulin sensitivity

To elucidate the effects of SK0504 on insulin sensitivity, i.p. ITT was performed on all mice after 6 h fasting. The basal blood glucose levels after 6 h fasting were significantly lower in the HFD fed mice treated with SK0504 or rosiglitazone compared to those treated with the vehicle (7.7 ± 0.5 or 9 ± 0.2 vs 10.4 ± 0.5 mmol/l, $p<0.01$ or $p<0.05$, respectively; Figure 4.5A). During the 2-h i.p. ITT, an increased insulin-stimulated glucose disposal rate was promoted by treatment with SK0504 compared to the vehicle in the HFD fed mice (Figure 4.5A). The AUC of blood glucose concentration-time profile of the SK0504-treated mice was lowered by 24% that of the vehicle-treated mice (588.4 ± 18.3 vs 773.9 ± 41.7 mmol·min/l, $p<0.05$, Figure 4.5B). The circulating insulin levels of mice, measured by radioimmunoassay were slightly lower in the SK0504 treatment group compared to the vehicle treatment group (2.85 ± 0.26 vs 3.37 ± 0.54 nmol/l, $p>0.05$; Figure 4.6). These results implied that SK0504 enhanced insulin sensitivity of the HFD fed mice.



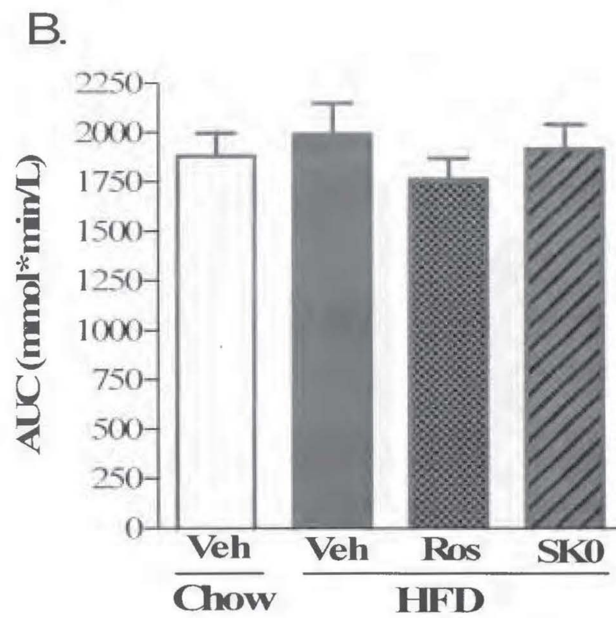


Figure 4.4 Effects of SK0504 on i.p. glucose tolerance. Upper panel (A): blood glucose responses during the 2-hour i.p. GTT of the mice fasted for 12 h, receiving chow diet and vehicle (○, n=8), HFD and vehicle (●, n=7), HFD and rosiglitazone (▲, n=8) or HFD and SK0504 (■, n=7). The blood glucose was measured from the tails at the indicated time points after i.p. injection of glucose (2g/kg). Each time point is the mean ± SEM. Lower panel (B): AUC of the blood glucose concentration-time profiles of the mice receiving chow diet and vehicle (□, n=8), HFD and vehicle (■, n=7), HFD and rosiglitazone (■, n=8) or HFD and SK0504 (■, n=7).

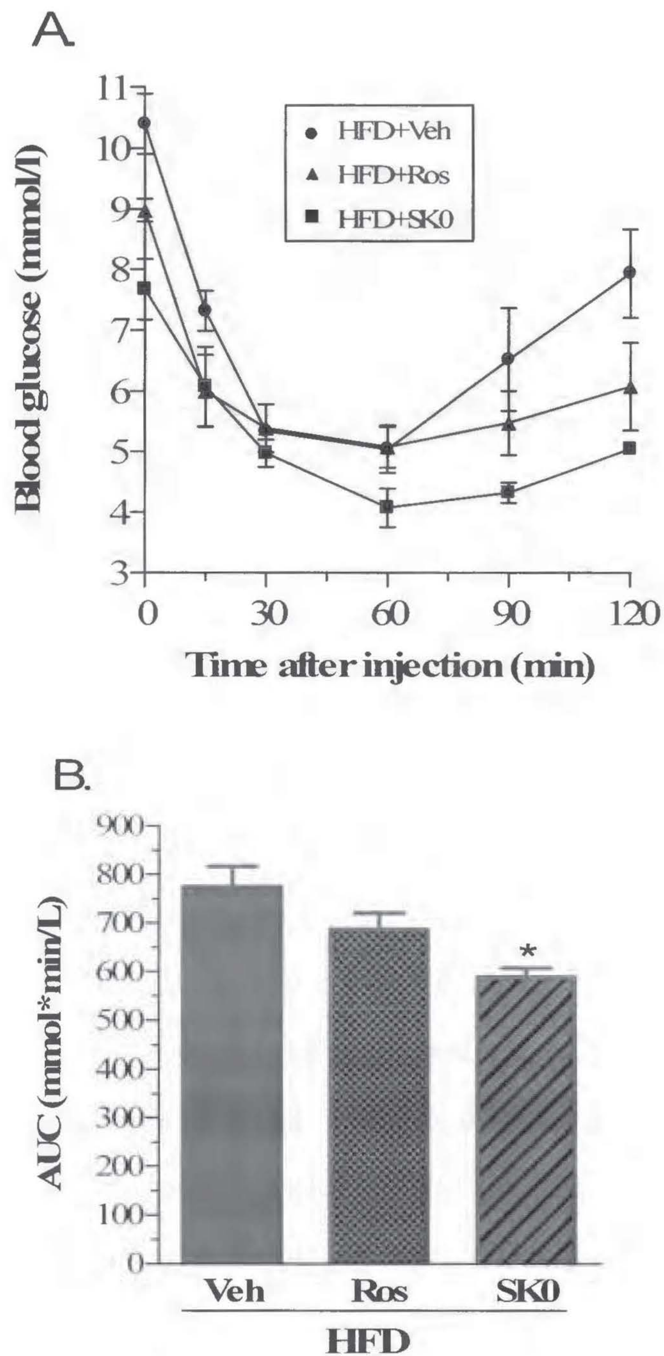


Figure 4.5 Effects of SK0504 on i.p. insulin tolerance. Upper panel (A): blood glucose responses during the 2-hour i.p. ITT of the HFD fed mice fasted for 6 h, receiving vehicle (●, n=5), rosiglitazone (▲, n=8) or SK0504 (■, n=4). Blood glucose was measured from the tail at the indicated time points after i.p. injection of insulin (0.85 IU/kg). Each time point is the mean ± SEM. Lower panel (B): AUC of the blood glucose concentration-time profiles of the HFD fed mice receiving vehicle (■, n=5), rosiglitazone (▩, n=8) or SK0504 (■, n=4). *$p<0.05$ *vs* the vehicle-treated HFD fed mice.

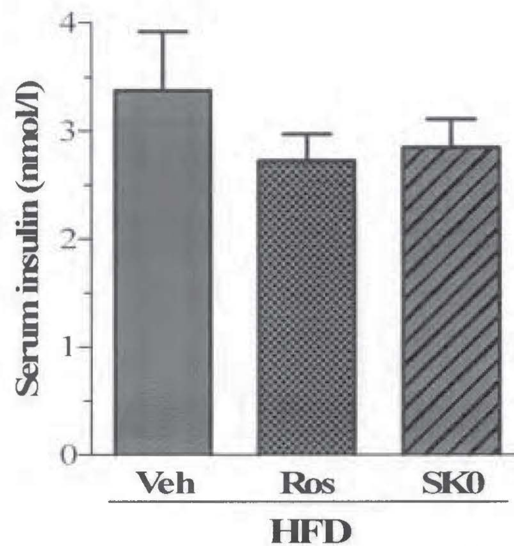
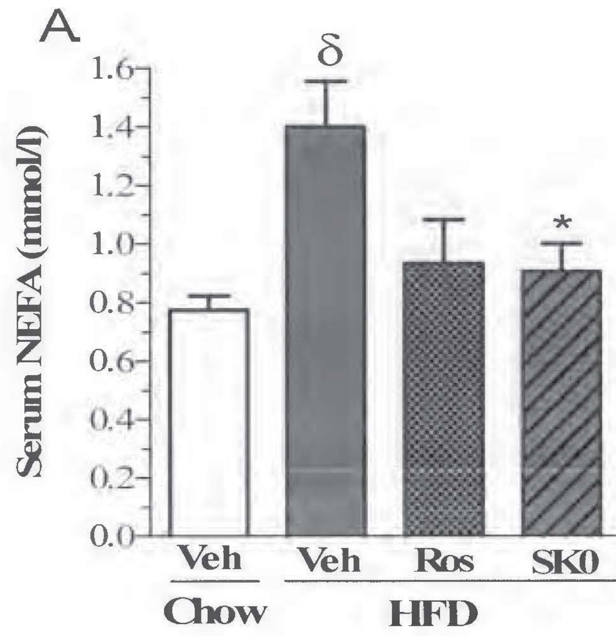
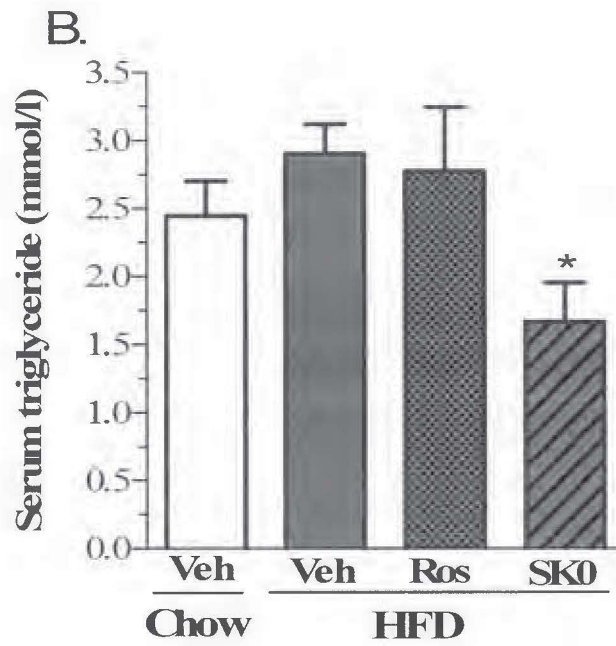


Figure 4.6 Effects of SK0504 on the serum insulin levels of the mice fasted for 12 h. The mice were fed with HFD and received vehicle (■, n=4), rosiglitazone (■, n=6) or SK0504 (■, n=4) for 5 weeks. Data are mean ± SEM.

4.3.4 Effects of SK0504 on lipid metabolism

In order to evaluate the effects of SK0504 on abnormal lipid metabolism related to insulin resistance and obesity, measurements of serum triglyceride, NEFA and cholesterol concentrations, and liver triglyceride and total lipid contents of mice were conducted. Compared to the chow diet fed mice, the HFD fed mice presented with significantly higher serum NEFA concentration (0.77 ± 0.05 vs 1.40 ± 0.16 mmol/l, $p<0.05$, Figure 4.7A). Compared to the vehicle, treatment with SK0504 significantly lowered serum NEFA (1.4 ± 0.16 vs 0.91 ± 0.1 mmol/l, $p<0.05$, Figure 4.7A) and triglyceride (2.9 ± 0.22 vs 1.66 ± 0.29 mmol/l, $p<0.05$, Figure 4.7B) concentrations but not serum cholesterol (3.09 ± 0.23 vs 3.13 ± 0.64 mmol/l, $p>0.05$, Figure 4.7C) concentration of the HFD fed mice.


A.
Serum NEFA (mmol/l)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
δ
*
Veh
Veh
Ros
SK0
Chow
HFD


B.
Serum triglyceride (mmol/l)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Veh
Veh
Ros
SK0
Chow
HFD

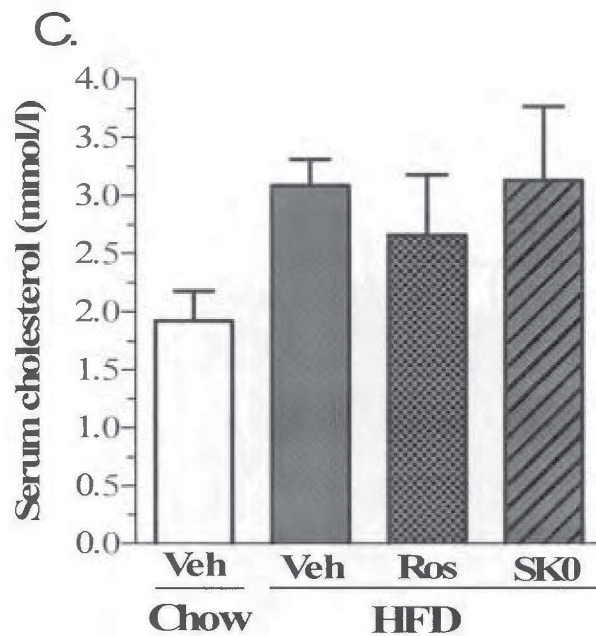


Figure 4.7 Effects of SK0504 on the serum (A) NEFA, (B) triglyceride, and (C) cholesterol concentrations of the mice fasted for 12 h. The mice were divided into 4 groups receiving chow diet and vehicle (□, n=4-7), HFD and vehicle (■, n=5-7), HFD and rosiglitazone (▩, n=5-8) or HFD and SK0504 (■, n=5-8) for 5 weeks. Data are mean ± SEM. δp<0.05 *vs* the vehicle-treated chow diet fed mice; *p<0.05 *vs* the vehicle-treated HFD fed mice.

Abnormal lipid metabolism related to insulin resistance results in accumulation of triglycerides in non-adipose tissues including liver. Therefore lipids were extracted from livers of mice and the assay was performed to measure the hepatic triglyceride concentrations. The SK0504-treated HFD fed mice showed slightly lowered hepatic triglyceride concentrations compared to the vehicle-treated HFD fed mice, but not to the level of statistical significance (11.99 ± 1.65 vs 12.73 ± 2.1 μmol/g, $p>0.05$, Figure 4.8). The gross inspection of the Oil Red O-stained tissues revealed a lesser number of total lipid deposits in the livers of the SK0504-treated mice compared to those of the vehicle-treated mice (Figure 4.9). Taken together, SK0504 lowered serum NEFA and

triglyceride concentrations, and reduced lipid deposition in the livers of the HFD fed mice (Figure 4.9).

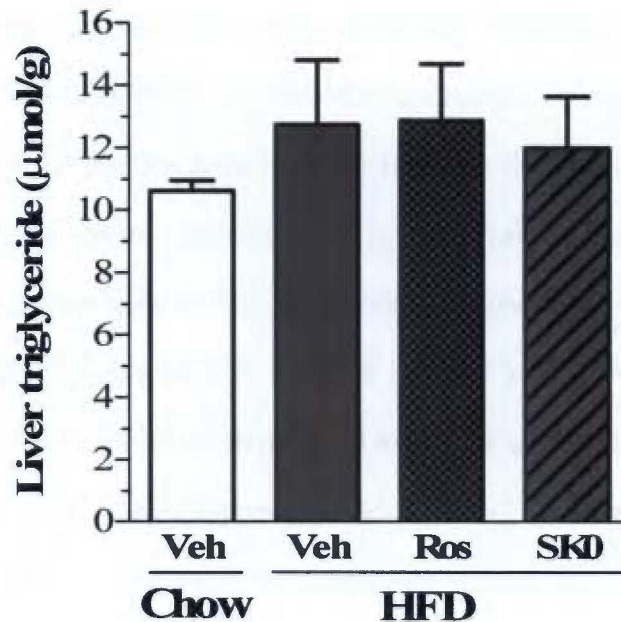


Figure 4.8 Effects of SK0504 on the liver triglyceride concentrations of the mice fasted for 12 h. The mice were divided into 4 groups receiving chow diet and vehicle (□, n=4), HFD and vehicle (■, n=7), HFD and rosiglitazone (■, n=8) or HFD and SK0504 (■, n=4) for 5 weeks. Data are mean ± SEM.

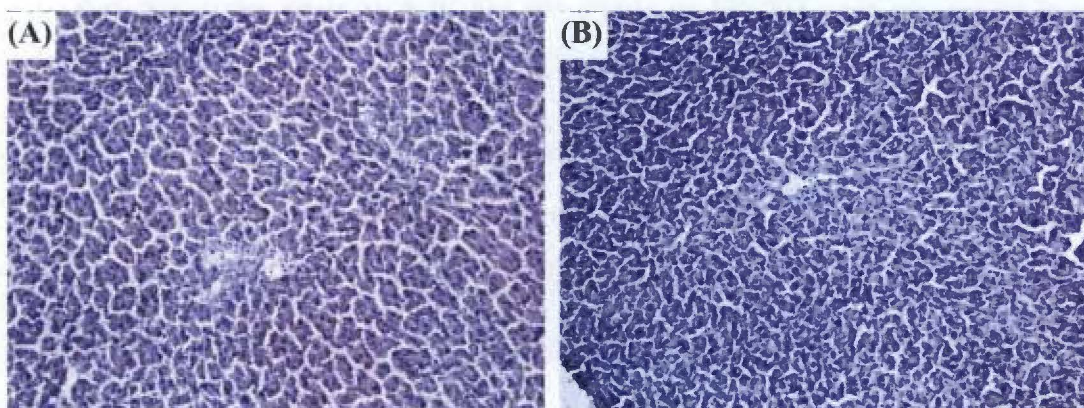


Figure 4.9 Histological assessments of the livers from mice. The photomicrographs are of the Oil Red O-stained frozen sections of livers from the vehicle-treated HFD fed mice (A) and the SK0504-treated HFD fed mice (B) after 5 weeks' treatment. Less number of lipid deposits was present in the SK0504-treated mice compared to the vehicle-treated mice. The photomicrographs are at 20x magnification.

4.4 Discussion

The major finding of this study was that treatment with SK0504 in a dose of 4g/kg/day was associated with metabolic effects in C57BL/6J mice fed with HFD and presenting insulin resistance, central obesity and dyslipidaemia. Mice fed with HFD presented with greater visceral fat weights and serum NEFA concentrations compared to those fed with chow diet. It is known that mice fed with HFD are characterised by marked obesity, insulin resistance, glucose intolerance and hyperglycaemia (Lin et al., 2000). This was shown in a previous study by Huo et al. (2003), where mice were fed with a diabetes induction diet containing 33.35% fat for 8 or more weeks. In addition, according to Oakes et al. (1997), HFD containing > 50 kcal% fat was sufficient to drive moderate obesity, glucose intolerance, hypertriglyceridaemia and hypertension in animals (Moller & Kaufman, 2005). In this study, mice were also fed with HFD containing 35.4% or 59 kcal % fat for 12 weeks to induce MetS.

SK0504 was hypothesised to be capable of exerting multi-functional effects. This is because it is known that synergistic effects can be achieved by the multiple ingredients of Chinese herbal formulae (Jia et al., 2004; Wang et al., 2003). However, as the results presented in the study showed that the effects of SK0504 were no greater than the effects of its individual herbs themselves, this hypothesis was rejected. For example, Huang Lian (Rhizoma Coptidis), which contains an active compound known as berberine, has been revealed in numerous publications to attenuate insulin resistance, obesity and abnormal glucose and lipid metabolisms in both *in vivo* and *in vitro* studies (Huang et al., 2006; Kong et al., 2004; Lee et al., 2006; Leng et al., 2004). In addition, Jiao Gu Lan (Gynostemma pentaphyllum) was shown to lower blood glucose concentration by 20% during the 2-hour glucose tolerance test in obese Zucker rats, compared to vehicle (Megalli et al., 2006). In the present study, although both Huang Lian and Jiao Gu Lan were

consisted in SK0504, the defect in i.p. GTT was not improved by treatment with the latter.

Studies by Megalli et al. (2005, 2006) revealed that Jiao Gu Lan administered to Sprague Dawley and obese Zucker Fatty rats was not only able to reduce excess formations of triglycerides, but also total cholesterols and VDL-Cs. In addition, a study by Ji & Gong (2007) revealed that an extract of San Qi (Panax notoginseng) called *n*-BuOH exerted lipid lowering effects in rats fed with high fat and cholesterol diet. Compared to the control group, the *n*-BuOH-treated group showed significantly decreased levels of serum total cholesterol, triglyceride, and LDL-Cs, as well as hepatic total cholesterol and triglyceride concentrations. In the present study however, SK0504 containing both Jiao Gu Lan and San Qi, was only able to significantly lower serum triglyceride and NEFA, but not serum cholesterol and hepatic triglyceride concentrations. Finally, Dan Shen (Salvia miltiorrhiza) was shown to exert anti-atherosclerotic effects and inhibit endothelial permeability (Chan et al., 2004), but the potential hameodynamic effects of SK0504 were not investigated in this study.

Despite the anti-hyperlipidaemic effects of SK0504, the hepatic triglyceride levels of the HFD fed mice were only slightly reduced by treatment with the herbal formula. It is possible that a stronger dosage of SK0504 or a longer length of treatment period may have significantly reduced their levels. Accumulation of triglycerides in the liver leads to formation of VLDLs, which in turn increases LDLs (Malmstrom et al., 1997; Taskinen, 2003). This accounts for a possible reason why serum total cholesterol concentrations were not reduced by treatment with SK0504. A further experiment to clarify the proportions of LDL-Cs and HDL-Cs in total cholesterols is required for validation of this statement. Consistent to the result of the hepatic triglyceride levels, gross inspection

of the total lipid contents revealed less lipid deposits in the livers of the treated mice compared to those of the control mice. Since this data was not quantitated, it remains inconclusive whether or not SK0504 significantly reduced total lipid contents of the liver.

Treatment with SK0504 significantly improved visceral fat weights, insulin sensitivity, and serum NEFA and triglyceride concentrations of the HFD fed mice. In contrast to these findings, treatment with rosiglitazone significantly attenuated hyperglycaemia only. Therefore, the advantages of SK0504 over rosiglitazone reside in the fact that the former produces anti-hyperlipidaemic effects. Rosiglitazone, on the other hand, has been documented in previous studies as being associated with weight gain (Malinowski & Bolesta 2000).

It is known that central obesity-associated insulin resistance leads to increases in lipolysis and formation of circulating FFAs, as well as hepatic triglyceride accumulations (Meek et al., 1999). Therefore, it is possible that the insulin sensitising and anti-hyperlipidaemic effects of SK0504 were in part secondary to the effects on visceral fat weights. Further studies are needed to elucidate the exact mechanisms of the insulin sensitising and anti-hyperlipidaemic effects of SK0504.

Treatment with SK0504 improved insulin resistance and lipid metabolism to a certain extent, in a dietary factor-induced model of MetS in mice presenting with central obesity, insulin resistance and dyslipidaemia.

CHAPTER 5

Conclusions and future directions

5.1 SUMMARY AND CONCLUSIONS

Chinese herbal medicine was studied in this thesis because there are emerging literature evidences about its safe and multi-functional effects on MetS. The two studies reported in this thesis were conducted because there is a lack of scientific experimental studies on the potential effects and mechanisms of Chinese herbal medicine. The purpose of these studies was to show that Chinese herbal medicines, Sugarid and SK0504, may be potential novel drugs for MetS or T2DM, by investigating their effects and mechanisms on the mice models of MetS or T2DM. In short, the hypothesised effects of Sugarid and SK0504 were not achieved, and the molecular mechanisms of their effects were not elucidated.

To account for the experimental results unsupportive of the hypothesised effects of the two Chinese herbal medicines, a couple of points may serve as justification. Firstly, the small sample sizes used in the studies may have led to large standard errors of the measured mean values. This may provide a possible explanation for the insignificant effects of treatment with Chinese herbal medicines, compared to the control in the two studies. Moreover, the Chinese herbal medicines used in the two studies might have produced significant effects on the metabolic abnormalities had stronger dosages been chosen for treatment of the mice.

Investigation of Sugarid in mice models of MetS and T2DM

The metabolic effects of a Chinese herbal product, Sugarid, were evaluated in mice models of MetS and T2DM. Both models of MetS and T2DM were successfully induced by HFD feeding and STZ injections, and there were significant increases in body weights and blood glucose concentrations of the mice. Treatment with Sugarid did

not reduce body and visceral weights, blood glucose, serum triglyceride and cholesterol, or liver triglyceride concentrations of the HFD fed mice. Similarly, no significant improvements of glucose tolerance and insulin sensitivity were achieved in i.p. GTT and i.p. ITT by treatment with Sugarid. The results of this study do not support the notion that Sugarid, at least in its individual form, may be a potential drug in the treatment of MetS and T2DM.

Investigation of SK0504 in a mice model of MetS

The metabolic effects of a Chinese herbal formula, SK0504, were evaluated in a mice model of MetS induced by HFD. SK0504 significantly reduced visceral fat weights, serum NEFA and triglyceride concentrations, and improved i.p. ITT. SK0504 ameliorated the effects of HFD feeding on NEFA and triglyceride levels, and improved the defect in i.p. ITT. Although some of these findings are far from conclusive, the results provide evidence that supports the notion that SK0504 may be a potential new drug in treatment of MetS. However, further studies are needed to elucidate the precise mechanisms of the anti-hyperlipidaemic and insulin sensitising effects of SK0504. In light of current literature supporting the multi-functional effects of Chinese herbal formulae, a hypothesis was stated in the introduction of Chapter 4. It was hypothesised that SK0504 may be able to target the multiple metabolic abnormalities of MetS. However, this hypothesis was rejected due to the insufficient number of positive results pertaining to the effects of SK0504.

5.2 FUTURE DIRECTIONS

Future study should focus on elucidating the molecular mechanisms behind the effects of SK0504 on glucose and lipid metabolisms discovered in Chapter 4. Studying the

molecular expressions of adiocytokines in adipose tissues and other tissue sites, for example, may be useful in understanding the mechanisms of the anti-hyperlipidaemic effects of SK0504.

In Chapter 1, the haemodynamic abnormalities of MetS were introduced but neither of the studies presented in this thesis explored the potential haemodynamic effects of Chinese herbal medicine. The reason for this is because there was a limitation of facilities during the study period. In light of existing literature, the potential anti-atherosclerotic and anti-thrombotic effects of SK0504 and its individual ingredients should be investigated. It is anticipated that an infrastructure grant will be obtained for blood pressure monitor to evaluate the effects of SK0504 in hypertension.

Finally, translation of the animal study of SK0504 into the clinical study in subjects with MetS should be carried out in the future.

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APPENDIX

Examples of Chinese herbal formulae: Yi-jin, Ke-le-nin, Yu-san-xiao, Qi-zhi, Shen-qi, Jin-qi, and Xiao-ke-an and their anti-diabetic effects.

Herb	Mechanism	Actions	Study
Yu-san-xiao	Contains polysaccharide and other ingredients.	Lowers fasting plasma glucose levels.	30 patients treated with Yu-san-xiao for 1 month. 5 showed no effect, while 12 showed significant improvement and 13 showed reasonable improvements.
Yi-Jin		Lowers blood glucose level. Lowers triglycerides, cholesterols, lactic acids in serum.	Used in alloxan-induced diabetic mice with Phenformin (DBI), as a positive control. Showed positive result as good as DBI.
Shen-qi	Unknown but predicted by others to contain polysaccharides to enhance and protect beta cell function and increase insulin secretion.	Lowers blood glucose, urine glucose levels, as well as cholesterol & triglyceride levels.	150 diabetic patients treated with Shen-qi. Blood glucose level, urine glucose level, cholesterol and triglyceride levels all improved significantly.
Ke-le-nin	Contains main extract *hirudin*, which is from Hirudo nipponia. Contains Rehmannia root.	Inhibits action of thrombin on fibrinogen; anti-coagulant effect. Rhmannia has been used for deficiency of body fluid, thirst, diabetes and constipation. Lowers glucose level.	Ke-le-nin administered to 30 T2DM patients for 90 days. Fasting glucose level reduced from 10.35mmol/l to 7.54mmol/l.
Qi-zhi	Contains Rehmannia root.	Lowers glucose level.	Qi-zhi tested on alloxan induced diabetic mice for 30 days was able to lower their blood glucose levels.
Xiao-ke-an	Contains main extract *hirudin*, which is from Hirudo nipponia.	Inhibits action of thrombin on fibrinogen, and has anti-coagulant effect.	Xiao-ke-an administered to alloxan induced diabetic mice showed hypoglycemic effects.

	Also contains polysaccharides and Pueraria lobata that has puerain.	Pueraria can promote blood circulation and it also has hypoglycaemic effects.	Blood glucose reduced from 8.21 to 4.69 mmol/l, and from 29.88 to 19.56mmol/l, in normal and streptozocin induced diabetic mice after xiao-ke-an administration.