UNIVERSITI PUTRA MALAYSIA

EFFECTS OF POLYPHENOLS FROM COCOA POWDER ON DIABETIC SYNDROME IN OBESE-DIABETIC RATS

ABBE MALEYKI BIN MHD JALIL

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MASTER OF SCIENCE

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2009

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By

ABBE MALEYKI BIN MHD JALIL

Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science
April 2009

DEDICATION

Specially dedicated to....

My beloved wife, Nadia Nazeerah Nasser
My mother, Hasnah Shariff
My father, Mhd Jalil Saad
My in-laws, Mohd Nasser Jaafar and Norehan Mohd Noor
My families,
My friends.

ABM 2009

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

EFFECTS OF POLYPHENOLS FROM COCOA POWDER ON DIABETIC SYNDROME IN OBESE-DIABETIC RATS

By

ABBE MALEYKI BIN MHD JALIL

April 2009

Chairman: Amin Ismail, PhD

Faculty: Medicine and Health Sciences

This study was carried out to determine the effects of polyphenols from cocoa powder on diabetic syndrome of obese-diabetic (Ob-db) rats. The studied diabetic syndrome are fasting plasma glucose level, oral glucose tolerance test, insulin level and insulin sensitivity, lipid profiles (total cholesterol, triglycerides, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and free fatty acids), oxidative stress biomarker (8-isoprostane), and antioxidant enzymes (superoxide dismutase and catalase). Obese-diabetic (Ob-db) rats were developed using a high-fat diet (49% fat, 32% carbohydrate, and 19% protein from total energy, kcal) for 3 months, followed by a low dose (35 mg/kg body weight) streptozotocin (STZ) injection. Cocoa extract (600 mg/kg body weight) containing both polyphenols (2.17mg epicatechin, 1.52 mg catechin, 0.25 mg dimer and 0.13 mg trimer g/cocoa extract) and methylxanthines (3.55 mg caffeine and 2.22 mg theobromine g/cocoa extract) was given to the rats for 4 weeks. The results indicated that there were no significant differences in

fasting plasma glucose, insulin level and insulin sensitivity after 4 weeks of cocoa extracts administration. Oral glucose tolerance test revealed that cocoa supplementation in Ob-db rats significantly ($p < 0.05$) reduced plasma glucose at 60 and 90 min compared to unsupplemented Ob-db rats. The supplementation significantly ($p < 0.05$) reduced the plasma total cholesterol, triglycerides and low-density lipoprotein cholesterol of obese-diabetic rats (Ob-db + cocoa) compared with non-supplemented animals (Ob-db). Plasma free fatty acid and oxidative stress biomarker (8-isoprostane) were significantly ($p < 0.05$) reduced after cocoa supplementation. Superoxide dismutase activity was enhanced in Ob-db compared to that in non-supplemented rats. However, no change was observed in catalase activity. Cocoa supplementation had an effect on postprandial glucose control but not for long-term glucose control (4 weeks). Four weeks of cocoa extract supplementation also possess hypocholesterolaemic properties. Moreover, cocoa supplementation could reduce circulating plasma free fatty acid and 8 isoprostane and may enhance the antioxidant defense system.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

KESAN POLIFENOL DARIPADA SERBUK KOKO TERHADAP SINDROM DIABETIK DI DALAM TIKUS OBESITI-DIABETIK

Oleh

ABBE MALEYKI BIN MHD JALIL

April 2009

Pengerusi: Amin Ismail, PhD

Fakulti: Perubatan dan Sains Kesihatan

Kajian ini dijalankan bagi menentukan kesan polifenol dari serbuk koko terhadap sindrom diabetik tikus obesiti-diabetik (Ob-db). Sindrom diabetik yang dikaji adalah paras glukosa plasma semasa puasa, ujian tolerans glukosa oral, paras insulin dan sensitiviti terhadap insulin, profit lipid (jumlah kolesterol, trigliserida, lipoprotein-kolesterol berketumpatan tinggi, dan lipoprotein-kolesterol berketumpatan rendah dan acid lemak bebas), penunjuk tekanan oksidatif *(8-isoprostane)*, enzim antioksidan (superoksida *dismutase* dan katalase). Tikus obesiti-diabetik (Ob-db) dibangunkan dengan memberi diet tinggi lemak (49% lemak, 32% karbohidrat, dan 19% protein daripada jumlah tenaga, kkal) selama 3 bulan, diikuti dengan suntikan *streptozotocin* (STZ) pada dos yang rendah (35 mg/kg berat badan). Ekstrak koko (600 mg/kg berat badan) yang mengandungi polifenol (2.17 mg epikatekin, 1.52 mg katekin, 0.25 mg dimer dan 0.13 mg trimer g/ekstrak koko) dan metilxantina (3.55 mg kafein dan 2.22 mg teobromina g/ekstrak koko) diberi selama 4 minggu. Keputusan menunjukkan

bahawa tiada kesan yang signifikan terhadap paras plasma glukosa puasa, paras insulin dan sensitiviti terhadap insulin selepas 4 minggu diberi ekstrak koko. Pemberian ekstrak koko pada tikus Ob-db menunjukkan penurunan glukosa pada minit 60 dan 90 yang signifikan di dalam ujian glukosa secara paksa jika dibandingkan dengan tikus Ob-db yang tidak diberi ekstrak koko. Pemberian itu juga menurunkan jumlah kolesterol plasma, trigliserida, dan lipoprotein-kolesterol berketumpatan rendah pada tahap yang signifikan ($p < 0.05$) terhadap tikus Ob-db jika dibandingkan dengan tikus yang tidak diberi ekstrak koko. Asid lemak bebas dan penunjuk tekanan oksidatif *(8-isoprostane)* menurun pada tahap yang signifikan *(p* <0.05) selepas pemberian ekstrak koko. Aktiviti enzim superokida *dismutase* diperkuatkan di dalam tikus Ob-db dibandingkan dengan tikus yang tidak diberi ekstrak koko. Walaubagaimanapun, tiada perubahan pada aktiviti enzim katalase. Keputusan menunjukkan pemberian koko mempunyai kesan terhadap kawalan glukosa selepas makan dan tidak untuk kawalan glukosa jangkamasa panjang (4 minggu). Pemberian ekstrak koko selama 4 minggu juga menunjukkan kesan hipokolesterolemia. Tambahan pula, pemberian koko boleh menurunkan asid lemak bebas dan *8-isoprostane* dan dapat memperkuatkan sistem pertahanan antioksidan.

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This thesis was submitted to the Senate of Universiti putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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DECLARATION

I hereby declare that the thesis is based om my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at other institutions.

_____

ABBE MALEYKI MHD JALIL

Date: 28 MAY 2009

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CHAPTER 1

INTRODUCTION

Background

Nowadays, diabetes mellitus has become a major health problem affecting more than 171,000,000 people worldwide (WHO, 2008a). Globally, total number of people with diabetes for the year 2030, as described by World Health Organization (WHO) is likely to be 366,000,000. In Malaysia, the number of people with diabetes for the year 2000 is 942,000, and is expected to be 2,479,000 in 2030 (WHO, 2008a). Generally, genetic, sedentary lifestyle, and obesity are factors directly related to diabetes. Specifically, it is well established that obesity and insulin resistance is a hallmark for the development of type 2 diabetes mellitus (T2DM). Insulin resistance could be defined as clinical stage in which a normal or elevated insulin level produces an inadequate biological activity, and can be considered as a risk factor for metabolic syndrome and T2DM (Hunter and Garvey, 1998).

Previous studies reported that many factors could exert the development of diabetes mellitus. According to Abou-Seif and Youssef (2004), oxidative stress is increased in both types of diabetes mellitus (Type 1 and 2), but the results were more pronounced in T2DM. A study also indicated that oxidative stress plays a major role in the development and progression of diabetes mellitus (Ferrari and Torres, 2003). Meigs et al. (2007) reported that there was a significant relationship between oxidative stress and insulin resistance.

Moreover, it has been reported that diabetic patients have significant defects in antioxidant protections that are important in the etiology of diabetic complications (Opara, 2002). Decreased in antioxidant defense such as superoxide dismutase (SOD), catalase (CAT), peroxidase (Px), ceruloplasmin (Cp) and glutathione peroxidase (GSH-Px) activities as well as a decrease in the GSH level and an increase in the concentration of glutathione disulfide (GSSG) were observed in erythrocytes of diabetic patients (Abou-Seif and Youssef, 2004).

The study on cocoa polyphenols became more extensive with the discovery of major low molecular weight polyphenols in cocoa and cocoa products, namely catechin, epicatechin, dimers epicatechin-(4β→8)-catechin (procyanidin B1), epicatechin-(4β→8)-epicatechin (procyanidin B2), and trimer [epicatechin-(4β→8)]$_2$-epicatechin (procyanidin C1) (Thompson et al., 1972). Previous studies showed that monomeric polyphenols, namely epicatechin and catechin, dimer, trimer, and tetramer were detected by reverse-phase liquid chromatography mass spectometry (RP LC-MS) (Natsume et al., 2000). It is reported that flavonols (epicatechin and catechin) were predominant compounds in cocoa powder (Natsume et al., 2002). Recently, studies showed that chocolate is one of the most polyphenol-rich foods along with tea and wine (Arts et al., 1999; Arts et al., 2000).

Numerous studies have been reported on the health benefits of cocoa and their products on various types of diseases, namely, cardiovascular diseases, cancers, and diabetes (Wang et al., 2000; Osakabe et al., 2004; Taubert et al., 2007; Heiss

et al., 2005; Murphy et al., 2003; Amin et al. 2004; Bisson et al., 2007a; Bisson et al., 2008; Rozan et al., 2006).

Tomaru et al. (2007) reported that cocoa liquor dose-dependently prevents the development of hyperglycemia in diabetic obese mice. To a greater extent, previous studies indicated that 4 weeks of cocoa powder extract supplementation to diabetic animal model showed hypolipidemic and hypoglycaemic properties (Ruzaidi et al., 2005; Ruzaidi et al., 2008). In human subjects with hypertension, dark chocolate administration ameliorated insulin (Grassi et al., 2005a; Grassi et al., 2005b). Brand-Miller et al. (2003) reported that incorporation of cocoa powder as flavour in different foods increased postprandial insulin secretion.

Unlike cardiovascular diseases, there is still limited human study on the effects of cocoa polyphenols towards diabetes. More work is needed to explore the effects of cocoa polyphenols on diabetes risk. Currently, no other study has been carried out on the effects of cocoa extracts on diet-induced obese-diabetic (Ob-db) rats. Therefore, this study was carried out to study the effects of cocoa extract prepared from Malaysian cocoa powder on biochemical parameters of obese-diabetic rats.

Statement of Problem

Recently, there is limited study done on diabetic-induced rats using combination of high-fat diet and a low dose of streptozotocin (STZ) that mimick human diabetes syndrome with regards to obesity and insulin resistance. In addition,

previous studies used beef and lard as a source of fat, which are relatively expensive compared to ghee (a type of fat produce from animal milk) and corn oil. Previous studies indicated that cocoa extracts prepared from cocoa powder and cocoa beans possessed hypoglycaemic and hypolipidemic properties in streptozotocin-induced diabetic rats (Amin et al., 2004; Ruzaidi et al., 2005; Tomaru et al., 2007; Ruzaidi et al., 2008). However, study on the effect of cocoa extracts on obese-diabetic (Ob-db) rats that imitate human diabetes syndrome is limited.

Significance of Study

This study aims to develop obese-diabetic (Ob-db) rats using a combination of high-fat diet and low-dose of streptozotocin injection. The cocoa extracts supplementation (4 weeks period) is able to improve biochemical parameters, namely, fasting plasma glucose, insulin level and sensitivity, and lipid profiles. Moreover, the supplementation is expected to reduce oxidative stress biomarker and improve antioxidant enzymes regulation. The results from this study will also be used in future to determine the underlying mechanisms of the observed effects.

The growing numbers of functional foods is an area of interest owing to their significant health benefits. Recently in Japan, almost 49 Food for Specified Health Use (FOSHU) have been approved specifically for diabetes. In Malaysia, medicinal and complementary therapy (MCT) was established in selected government hospital as an alternative to conventional medicine. The

aforementioned situations showed that there is markedly increase in the need of functional foods as an adjunct for management of diabetes.

Objectives of Study

General Objective

To determine the effect of cocoa extracts on biochemical parameters in obese diabetic (Ob-db) rats.

Specific Objectives

1) To develop obese-diabetic (Ob-db) rats using combination of high-fat diet (49% fat, 32% carbohydrate, and 19% protein from total energy, kcal) and low-dose (35 mg/ kg body weight) of streptozotocin injection.

2) To determine the effect of cocoa extracts on glucometabolism (fasting plasma glucose, insulin level, insulin sensitivity, and oral glucose tolerance test) parameters in obese-diabetic (Ob-db) rats.

3) To determine the effect of cocoa extracts on lipid profiles (total cholesterol, total triglycerides, low-density lipoprotein-cholesterol, high-density lipoprotein cholesterol) and oxidative stress biomarker (8-isoprostane) in obese-diabetic (Ob-db) rats.

4) To determine the effect of cocoa extracts on antioxidant enzymes (superoxide dismutase and catalase) in obese-diabetic (Ob-db) rats.

CHAPTER 2

LITERATURE REVIEW

Diabetes Mellitus

The prevalence of diabetes worldwide is at an alarming state. In year 2000, the estimated prevalence of diabetes for all age-groups was 2.8% and the prevalence was expected to increase to 4.4% in 2030 (Wild et al., 2004). Countries with the highest diabetes prevalence among the adult population are Nauru (30.7%), United Arab Emirates (19.5%), Saudi Arabia (16.7%), Bahrain (15.2%), and Kuwait (14.4%) (IDF, 2008a). Men showed higher prevalence of diabetes, but increasingly more women are having diabetes. The increased in prevalence of diabetes is most likely due to ageing population, unhealthy diet, overweight and obesity and sedentary lifestyle (IDF, 2008b).

The total number of people with diabetes was 171 million in 2000 and is projected to be 366,000,000 in 2030 (Wild et al., 2004). Up to date, 5 countries with the largest numbers of people with diabetes are India (40.9 million), China (39.8 million), the United States (19.2 million), Russia (9.6 million) and Germany (7.4 million) (IDF, 2008a). In Malaysia, the number of people with diabetes in 2000 is 942,000 and is expected to be 2,479,000 in 2030 (WHO, 2008a).

In the year 2000, the excess global mortality attributable to diabetes was estimated to be 2.9 million deaths, equivalent to 5.2% of all deaths (Roglic et al., 2005). Poorest countries showed 2-3% of deaths attributable to diabetes and over 8% in the United States, Canada, and the Middle East. Between the ages of 35-64 years old, 6-27% of deaths are attributable to diabetes. Unfortunately, no available data on the mortality that was associated with diabetes in Malaysia.

The global mortality rate due to diabetes presents a high burden to individual, community, and government. It can be classified into direct medical (healthcare cost) and indirect productivity-related costs (disability and premature mortality) attributable to diabetes. In the United States, direct and indirect medical expenditures related to diabetes were 132 billion US Dollars (Hogan et al., 2003). However, the costs of diabetes are varied from one country to another, depending on the direct medical cost (hospital services, physician services, and medicines consumed by people with diabetes) and indirect cost (lost workdays, restricted activity days, prevalence of permanent disability, and mortality attributable to diabetes) (Hogan et al., 2003; Dawson et al., 2002).

Definition, Diagnosis, and Classification of Diabetes Mellitus

Diabetes comes from a Greek word which means "to siphon". Polyuria (excessive urination) is the commonest symptom of diabetes. Excessive water comes out of the body of a person as if it were being siphoned from the mouth through the urinary system out of the body (Medopedia, 2008). Mellitus is a Latin word that means "sweet like honey" (Merriam-Webmaster, 2008). Due to

high level of sugar in urine it is call Mellitus. In 600

BC, two Indian physicians, Chakrata and Susruta differentiated the two forms as disease although the description relate to what we know today as type 1 diabetes. Later during the eighteenth and nineteenth centuries, it was identified as less clinically asymptomatic, which is marked glycosuria, often detected in later life, and commonly associated with overweight (WHO, 2008b). It is also described as a metabolic disorder of multiple aetiology characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat, and protein metabolisms resulting from defects in insulin secretion, insulin action, or both (WHO, 2008b).

Nowadays, the diagnosis of diabetes mellitus is followed according to WHO (1999). The diagnosis criteria was made based on the values of venous or capillary blood glucose (mmol/l or mg/dl) (**Table 1.1**). Diabetes mellitus can be classified into the aetiology of disorders of glycaemia, as depicted in **Tables 1.2** and **1.3**.

Table 1.1. Values for Diagnosis of Diabetes and Other Categories of Hyperglycaemia

	Glucose concentration, mmol/l (mg/dl)			
	Whole blood		Plasma	
	Venous	*Capillary*	*Venous*	*Capillary*
Diabetes mellitus:				
Fasting or	≥ 6.1 (≥ 110)	≥ 6.1 (≥ 110)	≥ 7.0 (≥ 126)	≥ 7.0 (≥ 126)
2-hr post glucose load	≥ 10.0 (≥ 180)	≥ 11.1 (≥ 200)	≥ 11.1 (≥ 200)	≥ 12.2 (≥ 220)
Impaired Glucose Tolerance:				
Fasting (if measured) and	< 6.1 (< 110) and	< 6.1 (< 110) and	< 7.0 (< 126) and	< 7.0 (< 126) and
2-hr post glucose load	≥ 6.7 (≥ 120)	≥ 7.8 (≥ 140)	≥ 7.8 (≥ 140)	≥ 8.9 (≥ 160)
Impaired Fasting Glycaemia (IFG)				
Fasting	≥ 5.6 (≥ 100) and < 6.1 (< 110)	≥ 5.6 (≥ 100) and < 6.1 (< 110)	≥ 6.1 (≥ 110) and < 7.0 (< 126)	≥ 6.1 (≥ 110) and < 7.0 (< 126)
and (if measured)				
2-hr post glucose load	< 6.7 (< 120)	< 7.8 (< 140)	< 7.8 (< 140)	< 8.9 (< 160)

Source: WHO (1999)

Note: For epidemiological or population screening purposes, the fasting or 2-h value after 75 g oral glucose may be used alone. For clinical purpose, the diagnosis should always be confirmed by repeating the test on another day unless there in unequivocal hyperglycaemia with acute metabolic decompensation or obvious symptoms.

Table 1.2. Aetiological Classification of Disorders of Glycaemia

Type 1	Beta-cell destruction, usually leading to absolute insulin deficiency -Autoimmune -Idiopathic
Type 2	May range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with or without insulin resistance
Other specific types (see table 1.3)	-Genetic defects of beta-cell function -Genetic defects in insulin action -Diseases of the exocrine pancreas -Endocrinopathies -Drug- or chemical-induced -Uncommon forms of immune-mediated diabetes -Other genetic syndromes sometimes associated with diabetes

Source: WHO (1999)

Note: Asterisk (*) includes the former categories of gestational impaired glucose tolerance and gestational diabetes

Table 1.3. Other Specific Types of Diabetes Mellitus

Specific Types of Diabetes	Causes of diabetes
Genetic defects of β-cell function	Chromosome 20, HNF 2α (MODY 1)
	Chromosome 7, Glucokinase (MODY 2)
	Chromosome 12, HNF 1α (MODY 3)
	Chromosome 13, IPF-1 (MODY 4)
	Mitochondria DNA 3243 mutation
Genetic Defects in Insulin Action	Type A insulin resistance
	Leprechaunism
	Rabson-Mendenhall syndrome
	Lipoatrophic diabetes
Disease of The Exocrine Pancreas	Fibrocalculous pancreatopathy
	Pancreatitis
	Trauma/pancreatectomy
	Neoplasia
	Cystic fibrosis
	Haemochromatosis
Endocrinopathies	Cushing's syndrome
	Acromegaly
	Phaeochromocytoma
	Glucagonoma
	Hyperthyroidism
	Somatostatinoma
Infections	Congenital rubella
	Cytomegalovirus
Uncommon form of Immune-mediated Diabetes	Insulin autoimmune syndrome (antibodies to insulin)
	Anti-insulin receptor antibodies
	"Stiff Man" syndrome

Source: WHO (1999)

Risk Factors

Family History

Family history has been demonstrated to play a role in the development of diabetes mellitus. Silva et al. (2005) showed that insulin sensitivity and clearance was lower in white children with family history of diabetes mellitus compared to children without family history of diabetes mellitus. In African-American children, Kapriel et al. (1999) showed that family history of T2DM is a risk factor for insulin resistance. The important metabolic alterations are impaired insulin-stimulated total and nonoxidative glucose disposal early in the first decade of life. These studies showed that children with family history of diabetes had higher BMI and body fat distribution. Cruz et al. (2002) support the hypothesis in which specific accumulation of visceral fat in addition to overall adiposity in Hispanic children increases the risk of type 2 diabetes. Among Japanese-Americans population, women with family history showed a significantly higher incidence of T2DM compared to those without family history of diabetes. However, no significant result was observed for men. The result suggested that in Japanese people living in America increased westernised lifestyle, family history of diabetes still predict the incidence of T2DM especially among women (Nakanishi et al., 2003).

In a cross-sectional study of 3,068 men and women, aged between 20–65 years and without known diabetes, Van Dam et al. (2001) indicated that those with family history of diabetes had larger waist circumference and fasting plasma glucose. Moreover, the association between abdominal obesity and