

# Effects of cocoa extract containing polyphenols and methylxanthines on biochemical parameters of obese-diabetic rats

Abbe Maleyki Mhd Jalil,<sup>a</sup> Amin Ismail,<sup>a\*</sup> Pei Pei Chong,<sup>b</sup> Muhajir Hamid<sup>c</sup> and Syed Hasbullah Syed Kamaruddin<sup>a</sup>

## Abstract

**BACKGROUND:** Previous studies have indicated that cocoa extract possesses hypoglycaemic and hypocholesterolaemic properties in streptozotocin-induced diabetic rats. However, there has been limited research on the effects of cocoa extract on obese-diabetic (Ob-db) rats that mimic human diabetes syndrome. Hence this study was initiated to determine the effect of cocoa extract containing polyphenols and methylxanthines on several biochemical parameters, namely glucose level, insulin sensitivity and lipid profiles of Ob-db rats.

**RESULTS:** Intake of cocoa extract supplemented with polyphenols (2.17 mg epicatechin, 1.52 mg catechin, 0.25 mg dimer and 0.13 mg trimer g<sup>-1</sup> cocoa extract) and methylxanthines (3.55 mg caffeine and 2.22 mg theobromine g<sup>-1</sup> cocoa extract) for 4 weeks 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). Short-term (acute) supplementation of cocoa extract significantly ( $P < 0.05$ ) reduced the plasma glucose level at 60 and 90 min compared with untreated rats as assessed by the oral glucose tolerance test. However, no significant differences were observed in plasma glucose level, insulin level and insulin sensitivity after chronic (4 weeks) cocoa extract supplementation.

**CONCLUSION:** The results of this study suggest that cocoa extract possesses hypocholesterolaemic properties and can exert a transient glucose-lowering effect but not long-term glucose control.

© 2008 Society of Chemical Industry

**Keywords:** cocoa extract; obese-diabetic (Ob-db); lipid profiles; insulin sensitivity

## INTRODUCTION

Numerous studies have been reported on the bioactive compounds of cocoa and cocoa products, namely cocoa powder, dark chocolate and cocoa liquor. Cocoa is rich in polyphenolic compounds, predominantly procyanidin monomers, namely catechin and epicatechin, dimer, trimer, tetramer and up to tetradecamer.<sup>1-4</sup> In addition, methylxanthines, namely caffeine, theobromine and theophylline, have also been identified in cocoa.<sup>3,5</sup> Owing to the significant amount of bioactive compounds, the study of their contribution towards health benefits is an area of interest. The health-promoting properties of cocoa polyphenols have been reported both *in vitro* and *in vivo*.<sup>1,6</sup>

In recent years, cocoa and cocoa products have been shown to suppress the development of atherosclerosis and hepatocarcinogenesis, increase dermal blood flow, reverse endothelial dysfunction and inhibit the proliferation of human breast cancer cells.<sup>7-11</sup> To a greater extent, cocoa-based products might possess hypocholesterolaemic and insulinaemic properties in normal, hypercholesterolaemic and hypertensive human subjects.<sup>12-15</sup>

Our previous studies showed that cocoa polyphenol extract exerted hypoglycaemic and hypocholesterolaemic properties in streptozotocin (STZ)-induced diabetic rats.<sup>16,17</sup> Moreover, a recent

study demonstrated that cocoa supplementation could prevent hyperglycaemia in diabetic-obese mice.<sup>18</sup> However, these studies were focused on the effects of cocoa on the glucometabolism of STZ-induced diabetic rats and genetically inherited obese-diabetic mice. Although cocoa could possess hypoglycaemic and hypocholesterolaemic effects, limited research has been conducted on the effects of cocoa extract on a diabetic model that imitates human diabetes syndrome. Therefore the present study was initiated to determine the effects of cocoa extract on obese-diabetic rats. We postulated that cocoa extract might potentially

\* Correspondence to: Amin Ismail, Department of Nutrition and Dietetics, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia. E-mail: amin@medic.upm.edu.my

a Department of Nutrition and Dietetics, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

b Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

c Department of Microbiology, Faculty of Biotechnology and Molecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

reduce blood glucose and improve lipid profiles of obese-diabetic rats.

## MATERIALS AND METHODS

### Preparation of standardised cocoa extract

Cocoa extract was prepared according to our previous method.<sup>17</sup> Malaysian cocoa powder was purchased from KL-Kepong Cocoa Products Sdn. Bhd. (Port Klang, Selangor, Malaysia). In brief, cocoa extract was prepared by extracting defatted cocoa powder with 800 mL L<sup>-1</sup> ethanol for 2 h. The ethanol was removed from the extract using a rotary evaporator (Buchi Rotavor R-200, Flawil, Switzerland) for 40 min at 55 °C. The resulting extract was then kept at -80 °C and lyophilised using a freeze-dryer (The Virtis Company Inc., Gardiner, NY, USA) at -45 °C and 120 bar.<sup>18</sup>

### Identification of bioactive compounds

Bioactive compounds of the extract were identified using liquid chromatography/mass spectrometry (LC/MS; Finnigan LCQ Deca, San Jose, CA, USA) with slight modification of the LC/MS conditions.<sup>4</sup> A reverse phase Licosphere C<sub>18</sub> column (250 mm × 4 mm, 5 µm i.d.; Alltech, Deerfield, Illinois, USA) and gradient elution with water/trifluoroacetic acid (99.9:0.1 v/v) and acetonitrile/trifluoroacetic acid (99.9:0.1 v/v) at a flow rate of 0.8 mL min<sup>-1</sup> were used for the separation of polyphenolic compounds. For LC/MS a protonated molecular ion ([M + H]<sup>+</sup>) mass spectrometer with electrospray ionisation at a capillary temperature of 275 °C and a capillary voltage of 31 V was used. The full mass scan covered the range *m/z* 200–2000. Data acquisition was performed using Finnigan XCalibur Version 1.4. The amounts of polyphenols (catechin and epicatechin) and methylxanthines (caffeine and theobromine) were quantified based on external standards (100–1000 µg mL<sup>-1</sup>). Owing to limited commercial availability of standards, dimer and trimer were quantified based on catechin equivalent.

### Animal study

#### Preparation of animals

Forty male Sprague-Dawley rats (each weighing 100–150 g) were purchased from the Institute for Medical Research (IMR), Kuala Lumpur, Malaysia. Approval for the animal study was obtained from the Animal Care and Use Committee (ACUC) of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (ACUC No. UPM/FPSK/PADS/BRUHH/00 180). The animals were housed individually in plastic cages with stainless steel covers. They were acclimatised for 1 week at room temperature (26–28 °C) under a 12/12 h light/dark cycle. All rats received normal rat chow (Gold Coin, Selangor, Malaysia) and distilled water *ad libitum* during acclimatisation. Following acclimatisation, the animals were divided randomly into five groups with eight rats (*n* = 8) per group as follows:

- group 1, normal diet (ND);
- group 2, normal diet + STZ injection (NDstz);
- group 3, high-fat diet (Ob);
- group 4, high-fat diet + STZ injection (Ob-db);
- group 5, high-fat diet + STZ injection + 600 mg cocoa kg<sup>-1</sup> body weight (Ob-db + cocoa).

### Induction of obesity

Obesity was induced using purified high-fat diet (HFD; 49% fat, 32% carbohydrate and 19% protein from total energy, kcal) containing fat from ghee (milk fat) and corn oil. Normal rats were given normal rat chow (Gold Coin; 14% fat, 61% carbohydrate and 25% protein from total energy, kcal). High-fat diet was given to the rats in groups 3–5 for 12 weeks. Body weight was determined weekly to confirm the development of obesity.

### Induction of diabetes

STZ was prepared according to Amin *et al.*<sup>16</sup> High-fat diet-fed rats were injected intravenously with a low dose of STZ (35 mg kg<sup>-1</sup> body weight) to induce diabetes. The combination of high-fat diet and STZ injection was applied in order to mimic human diabetes syndrome. Blood from the tail vein was collected 3 days after STZ injection and its glucose level was determined with a glucometer (Roche, Mannheim, Germany) to confirm the development of diabetes.

### Cocoa extract administration

Standardised cocoa extract at a dose of 600 mg kg<sup>-1</sup> body weight was given to Ob-db rats for 4 weeks (from week 13 to week 17). This dose was selected based on our previous study<sup>16</sup> and should be able to improve blood glucose metabolism in hyperglycaemic rats as evaluated by the oral glucose tolerance test (OGTT). Cocoa extract was suspended in 3 mL of 0.3 g L<sup>-1</sup> carboxymethyl cellulose (CMC) and given daily by gastric intubation using a force-feeding needle. Rats in the other groups received 0.3 g L<sup>-1</sup> CMC throughout the experiment.

### Blood collection for biochemical analysis

Blood was collected at week 12 after high-fat diet (Ob, Ob-db and Ob-db + cocoa) and normal diet (ND and NDstz), week 13 (after STZ injection in all groups except ND and Ob) and week 17 (after cocoa extract supplementation in Ob-db + cocoa) from the retro-orbital plexus under general anaesthetic. Samples of approximately 5 mL were collected in different blood collection tubes. Blood for plasma glucose was collected in tubes containing potassium oxalate as an antiglycolysis agent. Blood for insulin and lipid profiles was collected in tubes containing lithium heparin as an anticoagulant. Plasma was separated by centrifugation at 1500 × *g* for 15 min at 4 °C using a refrigerated centrifuge (Universal 32<sup>®</sup>, Hettich Zentrifugen, Föhrenstr, Tuttlingen, Germany) and stored at -80 °C for further analysis.

### Determination of plasma glucose and lipid profiles

Plasma glucose and lipid profiles were estimated using a commercial assay kit (Roche). An automatic chemical analyser (Hitachi 902, Hitachinaka, Tokyo, Japan) was used to determine glucose and lipid levels. Results were expressed as mmol L<sup>-1</sup>.

### Determination of plasma insulin level

Plasma insulin level was determined using a radioimmunoassay (RIA) kit (GE Healthcare, Uppsala, Sweden) according to the manufacturer's procedures. Results were expressed as mU L<sup>-1</sup>. Insulin sensitivity was estimated based on the fasting insulin (mU L<sup>-1</sup>)/glucose (mmol L<sup>-1</sup>) ratio.<sup>19</sup>

**Oral glucose tolerance test**

The OGTT was performed to determine the short-term effect of cocoa extract on Ob-db rats (group 5) at the end of the study (week-17). Rats were fasted overnight (10 h) and then administered 2 g glucose kg<sup>-1</sup> body weight.<sup>20</sup> Tail vein blood samples were withdrawn without anaesthetic before (0 min) and 15, 30, 60, 90 and 120 min after administration of glucose solution. Results were expressed as mmol L<sup>-1</sup> and the curve response of each group was plotted.

**Statistical analysis**

Data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA; SPSS Version 15.0, Chicago, Illinois) and the least significant difference (LSD) *post hoc* test were used to determine differences in means between groups. Comparisons between week 12 (after 12 weeks of HFD in all groups except ND and NDstz), week 13 (after STZ injection in all groups except ND and Ob) and week 17 (after 4 weeks of cocoa supplementation in Ob-db + cocoa) values were performed using paired sample *t* tests. Values were considered significantly different at the level of *P* < 0.05.

**RESULTS**

**Changes in body weight**

Similar body weight (115–140 g) was observed in all groups at the beginning of the experiment (Fig. 1). Rats administered high-fat diet (Ob, Ob-db, Ob-db + cocoa) showed a gradual increment

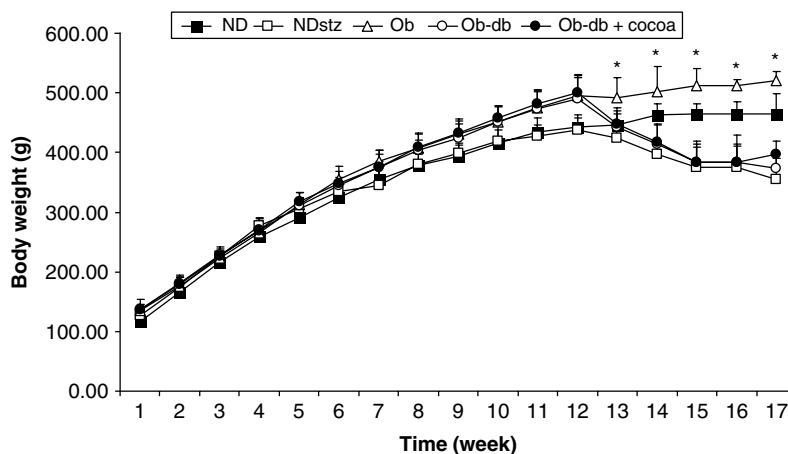
in body weight from week 1 (135.39 ± 6.52 g) to week 12 (494.49 ± 33.97 g). Normal diet (ND and NDstz) groups also showed a gradual increment in body weight from week 1 (116.82 ± 15.05 g) to week 12 (443.07 ± 19.76 g), but less pronounced than that of high-fat diet groups. A significant (*P* < 0.05) increase in body weight was observed in high-fat diet groups at week 11 compared with normal diet groups.

Body weight was significantly (*P* < 0.05) reduced after STZ injection (week 12) in NDstz, Ob-db and Ob-db + cocoa compared with non-injected (ND and Ob) groups. No significant difference in body weight was observed in rats supplemented with 600 mg cocoa kg<sup>-1</sup> body weight (Ob-db + cocoa) for 4 weeks (from week 13 to week 17) compared with Ob-db rats.

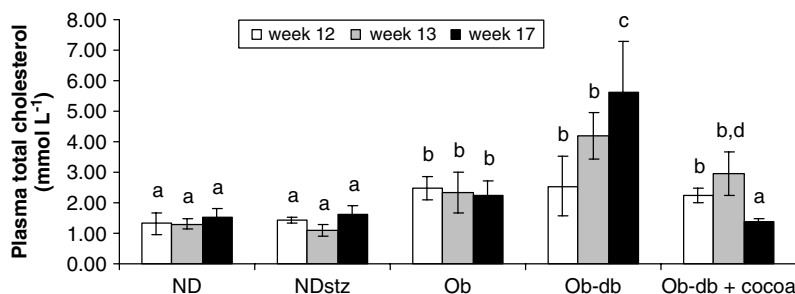
**Effect of cocoa extract on lipid profiles**

Plasma total cholesterol (TC) levels over the 17 week period are shown in Fig. 2. High-fat diet administration for 12 weeks significantly (*P* < 0.05) increased TC in Ob, Ob-db and Ob-db + cocoa compared with normal diet (ND and NDstz) groups, with levels of 2.23–2.94 and 1.32–1.44 mmol L<sup>-1</sup> respectively. STZ injection further increased (*P* < 0.05) TC in Ob-db compared with Ob rats. Cocoa extract supplementation for 4 weeks normalised the TC level at 1.38 mmol L<sup>-1</sup>.

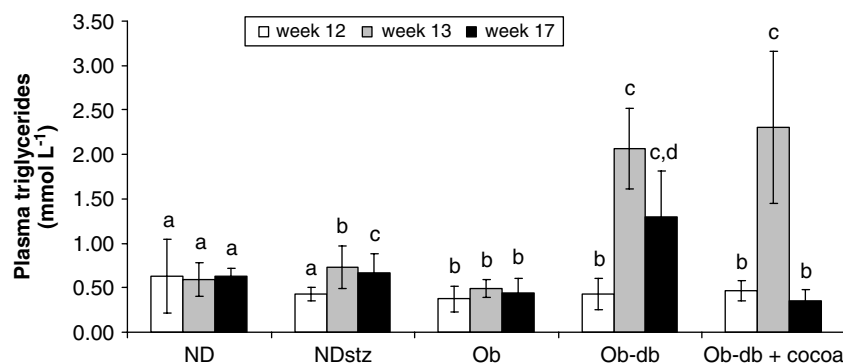
The level of plasma triglycerides (TGs) was similar in all groups after 12 weeks of high-fat or normal diet, with a range of 0.38–0.63 mmol L<sup>-1</sup> (Fig. 3). However, low-dose STZ injection significantly (*P* < 0.05) increased TGs in Ob-db and Ob-db + cocoa



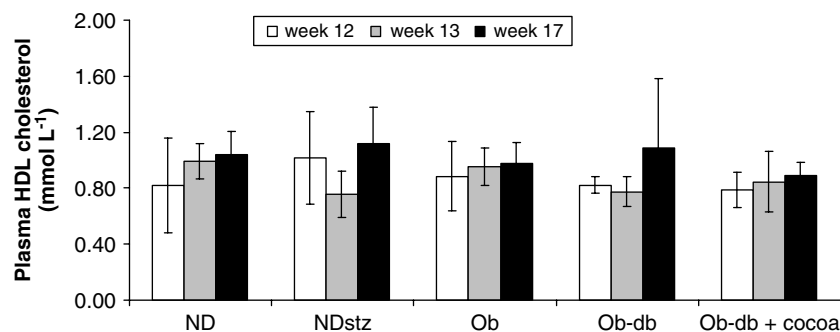
**Figure 1.** Changes in body weight of rats over 17 weeks. STZ was injected at week 12 in the NDstz, Ob-db and Ob-db + cocoa groups. Cocoa extract was supplemented for 4 weeks (week 13 to week 17). Each point is the mean of six rats; vertical bars show the standard deviation. \*Significant (*P* < 0.05) reduction in body weight of rats injected with STZ (NDstz, Ob-db and Ob-db + cocoa).



**Figure 2.** Plasma total cholesterol of rats over 17 weeks. Values are expressed as mean ± SD. Week 12, after 12 weeks of high-fat diet in all groups except ND and NDstz; week 13, after STZ injection in all groups except ND and Ob; week 17, after 4 weeks of cocoa supplementation in Ob-db + cocoa. Values with different letters are significantly (*P* < 0.05) different between groups and time.



**Figure 3.** Plasma triglycerides of rats over 17 weeks. Values are expressed as mean  $\pm$  SD. Week 12, after 12 weeks of high-fat diet in all groups except ND and NDstz; week 13, after STZ injection in all groups except ND and Ob; week 17, after 4 weeks of cocoa supplementation in Ob-db + cocoa. Values with different letters are significantly ( $P < 0.05$ ) different between groups and time.



**Figure 4.** Plasma HDL cholesterol of rats over 17 weeks. Values are expressed as mean  $\pm$  SD. Week 12, after 12 weeks of high-fat diet in all groups except ND and NDstz; week 13, after STZ injection in all groups except ND and Ob; week 17, after 4 weeks of cocoa supplementation in Ob-db + cocoa.

(1.77–2.30 mmol L<sup>-1</sup>) compared with non-injected (ND and Ob) groups (0.49–0.73 mmol L<sup>-1</sup>). The plasma TG level was significantly ( $P < 0.05$ ) reduced after 4 weeks (from week 13 to week 17) of cocoa extract supplementation (0.35 mmol L<sup>-1</sup>) compared with Ob-db rats (2.30 mmol L<sup>-1</sup>).

Plasma high-density lipoprotein cholesterol (HDL-c) levels of the rats are shown in Fig. 4. No significant difference in plasma HDL-c was observed between high-fat (Ob, Ob-db and Ob-db + cocoa) and normal (ND and NDstz) diet groups at week 12. Moreover, no significant difference in plasma HDL-c was observed after low-dose STZ injection (week 13) in NDstz, Ob-db and Ob-db + cocoa compared with non-injected (ND and Ob) groups. The level remain unchanged after 4 weeks (from week 13 to week 17) of cocoa extract supplementation in Ob-db + cocoa compared with other groups.

Plasma low-density lipoprotein cholesterol (LDL-c) levels of the rats are shown in Fig. 5. Plasma LDL-c increased significantly ( $P < 0.05$ ) after 12 weeks in high-fat (Ob, Ob-db and Ob-db + cocoa) compared with normal (ND and NDstz) diet groups, with levels of 1.60–1.82 and 0.17–0.22 mmol L<sup>-1</sup> respectively. The plasma LDL-c level increased further from week 13 to week 17 in Ob-db compared with other groups. Four weeks of cocoa supplementation in Ob-db + cocoa rats significantly ( $P < 0.05$ ) reduced (96.04%) and normalised plasma LDL-c compared with Ob-db rats.

#### Effect of cocoa extract on plasma glucose, insulin level and insulin sensitivity

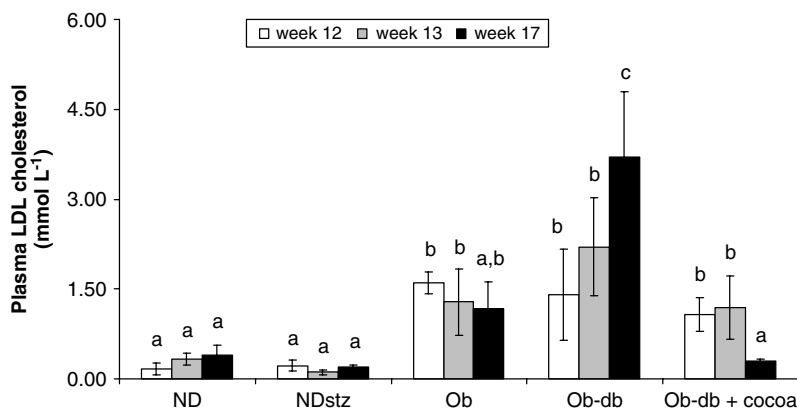
No significant difference in plasma glucose was observed in rats fed high-fat (Ob) or normal (ND) diet for 12 weeks, with levels of

7.09–8.12 mmol L<sup>-1</sup> (Table 1). In contrast, plasma glucose levels of rats injected with a low dose of STZ at week 12, namely NDstz, Ob-db and Ob-db + cocoa groups, were significantly ( $P < 0.05$ ) increased (14.91–19.93 mmol L<sup>-1</sup>) compared with control groups (ND and Ob). The levels remained higher (15.55–20.89 mmol L<sup>-1</sup>) at the end of the study (week 17) compared with non-injected groups. Furthermore, there was no significant reduction in plasma glucose after 4 weeks (from week 13 to week 17) of cocoa extract supplementation in Ob-db + cocoa compared with Ob-db rats.

Plasma insulin levels and insulin sensitivity of the rats were in the ranges 18.64–54.06 mU L<sup>-1</sup> and 0.96–6.61 mU mmol<sup>-1</sup> respectively (Table 1). High-fat diet rats (Ob) showed a significantly ( $P < 0.05$ ) higher insulin level (54.06 mU L<sup>-1</sup>) and insulin sensitivity (6.61 mU mmol<sup>-1</sup>) compared with other groups at week 12. Insulin levels and insulin sensitivity were significantly ( $P < 0.05$ ) reduced in rats injected with STZ at week 12 (NDstz, Ob-db and Ob-db + cocoa) compared with non-injected (ND and Ob) rats. Moreover, the levels remained significantly ( $P < 0.05$ ) lower at week 17 compared with ND and Ob groups. Four weeks of cocoa supplementation (from week 13 to week 17) did not significantly improve the insulin level and insulin sensitivity of Ob-db + cocoa compared with Ob-db rats.

#### Oral glucose tolerance test

The OGTT was carried out to determine the effects of normal and high-fat diets and treatment with cocoa extract on glucose metabolism (Fig. 6). ND and Ob rats exhibited normal baseline glucose, with levels of 6.05 and 5.98 mmol L<sup>-1</sup> respectively. Glucose load (2 mg glucose kg<sup>-1</sup> body weight) significantly



**Figure 5.** Plasma LDL cholesterol of rats over 17 weeks. Values are expressed as mean  $\pm$  SD. Week 12, after 12 weeks of high-fat diet in all groups except ND and NDstz; week 13, after STZ injection in all groups except ND and Ob; week 17, after 4 weeks of cocoa supplementation in Ob-db + cocoa. Values with different letters are significantly ( $P < 0.05$ ) different between groups and time.

**Table 1.** Fasting plasma glucose, insulin level and insulin sensitivity of experimental rats

Parameter	ND	NDstz	Ob	Ob-db	Ob-db + cocoa
Fasting plasma glucose (mmol L <sup>-1</sup> )					
Baseline <sup>a</sup>	7.09 $\pm$ 0.78a	14.91 $\pm$ 3.81b	8.12 $\pm$ 0.86c	18.83 $\pm$ 1.42c	19.93 $\pm$ 1.05d
Final <sup>b</sup>	7.65 $\pm$ 0.55a	20.89 $\pm$ 3.47b	7.13 $\pm$ 0.53c	15.55 $\pm$ 3.06d	18.47 $\pm$ 2.37d
Insulin level (mU L <sup>-1</sup> )					
Baseline <sup>a</sup>	30.09 $\pm$ 8.77a	40.59 $\pm$ 21.53b	54.06 $\pm$ 17.52c	18.64 $\pm$ 4.21d	19.14 $\pm$ 7.29d
Final <sup>b</sup>	34.94 $\pm$ 6.13a	25.80 $\pm$ 8.66b	45.52 $\pm$ 15.32c	16.15 $\pm$ 5.88d	17.75 $\pm$ 8.23d
Insulin sensitivity <sup>c</sup> (mU mmol <sup>-1</sup> )					
Baseline <sup>a</sup>	4.21 $\pm$ 1.06a	2.63 $\pm$ 0.97b	6.61 $\pm$ 1.77c	0.99 $\pm$ 0.20d	0.96 $\pm$ 0.38d
Final <sup>b</sup>	4.61 $\pm$ 1.05a	1.25 $\pm$ 0.43b	6.52 $\pm$ 2.13c	1.14 $\pm$ 0.70d	0.98 $\pm$ 0.48d

Values are expressed as mean  $\pm$  SD. Means with different letters within a row are significantly ( $P < 0.05$ ) different.

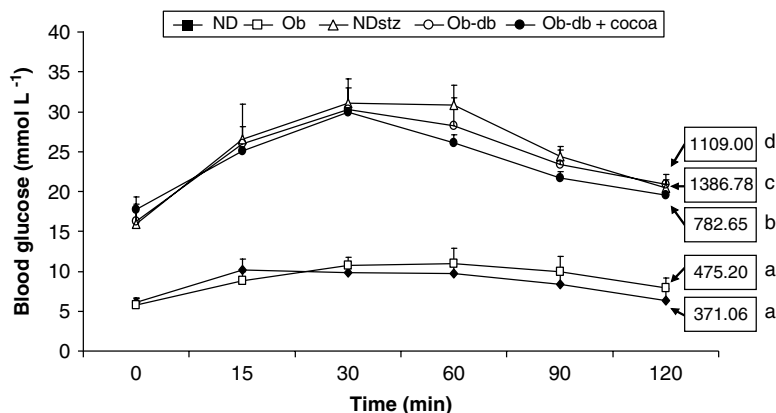
<sup>a</sup> Week 13 values.

<sup>b</sup> Week 17 values.

<sup>c</sup> Calculated based on insulin (mU L<sup>-1</sup>)/glucose (mmol L<sup>-1</sup>) ratio.

( $P < 0.05$ ) increased blood glucose at 15 min compared with baseline in both groups. Thereafter, blood glucose decreased gradually from 30 to 60 min and normalised at 120 min. There was a significant ( $P < 0.05$ ) increase in glucose in groups injected with STZ (NDstz, Ob-db and Ob-db + cocoa) compared with non-injected (ND and Ob) groups, with levels of 14.81–30.55 and

5.98–6.33 mmol L<sup>-1</sup> respectively. Ob-db rats showed a similar trend in glucose levels to that of NDstz rats. The area under the curve (AUC) was calculated for each OGTT to determine the increment in blood glucose from 0 to 120 min. There was no significant difference in AUC between ND and Ob groups. A significantly higher ( $P < 0.05$ ) AUC was observed in NDstz



**Figure 6.** Oral glucose tolerance test of experimental rats. Arrowed boxes indicate the area under the curve (AUC) from 0 to 120 min for each rat group. Values with different letters are significantly ( $P < 0.05$ ) different between groups.

(1386.78 mmol L<sup>-1</sup>) compared with Ob-db and Ob-db + cocoa rats. Cocoa supplementation significantly ( $P < 0.05$ ) reduced plasma AUC by 29% compared with non-supplemented Ob-db rats.

## DISCUSSION

Numerous studies have been reported on animal models that mimic human diabetes. Generally, rats fed with high-fat diet and injected with STZ have been used to develop diabetes models.<sup>20–22</sup> However, the type and percentage of fat (% fat from total energy) used in the diet formulation differ from one study to another. In our study, Sprague-Dawley rats were fed with high-fat diet (49% fat from total energy) prepared from ghee (a type of animal fat) and corn oil for 3 months to develop obesity. These rats showed a significant increase in body weight and impaired insulin sensitivity as measured by the insulin/glucose ratio. The developed obese rats were injected with a low dose of STZ (35 mg kg<sup>-1</sup> body weight) to induce diabetes. Our rat model could imitate human diabetes in terms of obesity and impaired insulin sensitivity. Previously, it was widely reported that the development of insulin sensitivity was secondary to obesity.<sup>23,24</sup> Impaired insulin sensitivity plays a role in the development of diabetes.<sup>25</sup>

The hypocholesterolaemic properties of cocoa extract were previously observed in STZ-induced diabetic rats.<sup>17</sup> It was also found that cocoa supplementation could reduce LDL-c and TC levels in normo- and hypercholesterolaemic human subjects.<sup>12,13</sup> It was noted that the main cause of elevated cholesterol and TG levels could be insulin deficiency in STZ-induced diabetic rats. In addition, it is well known that, under normal circumstances, insulin activates the enzyme lipoprotein lipase (LpL) and then hydrolyses very-low-density lipoprotein-cholesterol (VLDL-c). However, in insulin-deficient diabetic rats it failed to activate LpL and caused hypercholesterolaemia and hypertriglyceridaemia.<sup>26</sup>

In our Ob-db model the cause of hypercholesterolaemia was different from that in STZ-induced diabetic rats. In Ob-db rats, hypercholesterolaemia was attributed to high-fat diet as indicated by elevated TC and LDL-c levels compared with normal diet (ND group). A significant increase in TG level was observed only after injection with a low dose of STZ. The results showed that the occurrence of hypercholesterolaemia may be different between STZ-induced diabetic and Ob-db rats. The present study showed that cocoa extract might have the potential to reduce TC, TG and LDL-c levels in Ob-db rats.

Although cocoa extract possessed hypocholesterolaemic properties, the exact mechanisms responsible for the observed effect remain elusive. Previous studies have indicated that polyphenols could exert their lipid-lowering properties through various mechanisms, namely by slowing down triacylglycerol absorption through inhibition of pancreatic lipase, increasing cholesterol excretion in faeces, attenuating hepatic lipid accumulation through activation of adenosine monophosphate (AMP)-activated protein kinase, suppressing hepatic secretion of apolipoprotein B100 and increasing expression of LDL receptors in the liver.<sup>27–31</sup> Investigation of these mechanisms could help to explain the hypocholesterolaemic properties of cocoa extract in Ob-db rats. Results from LC/MS confirmed that cocoa extract contained flavonoid monomers, namely epicatechin and catechin, with the same mass-to-charge ratio ( $m/z$ ). These compounds were identified as  $m/z$  291 at levels of  $2.17 \pm 0.05$  and  $1.52 \pm 0.07$  mg g<sup>-1</sup> cocoa extract respectively. Dimer and trimer were also identified in cocoa extract based on their  $m/z$  579 and 867 at levels of  $0.25 \pm 0.05$  and  $0.13 \pm 0.00$  mg

catechin equivalent g<sup>-1</sup> cocoa extract respectively. The dosage (600 mg kg<sup>-1</sup> body weight) used in this study is equivalent to 42 g for a 70 kg human and similar to those reported in human intervention studies.<sup>32</sup>

Our previous study indicated that supplementation with cocoa powder extract could reduce blood glucose in STZ-induced rats.<sup>16,17</sup> In the present study, no significant difference was observed in fasting plasma glucose, plasma insulin level and sensitivity after 4 weeks of cocoa extract supplementation compared with Ob-db alone. The results suggest that cocoa extract could not directly stimulate insulin secretion or insulin sensitivity. A study indicated that there was no significant improvement in insulin level in diabetic rats supplemented for 2 weeks with *Inula viscosa* L. (a medicinal plant commonly used in Morocco for treatment of diabetes).<sup>33</sup> Moreover, Ziai *et al.*<sup>34</sup> reported no significant improvement in insulin level in diabetic patients supplemented with psyllium seeds from *Plantago ovata* Forsk. Therefore it could be suggested that the hypoglycaemic properties of the extracts were not solely dependent on insulin action or secretion. Interestingly, previous studies reported that 5 mg caffeine kg<sup>-1</sup> body weight could reduce insulin-stimulated glucose uptake in type 2 diabetes mellitus (T2DM) and sedentary human subjects as measured by a hyperinsulinaemic-euglycaemic clamp procedure.<sup>5,35</sup> In our study, methylxanthines, namely caffeine and theobromine, were also identified based on their  $m/z$  195 and 181 respectively. Caffeine and theobromine contents were  $3.55 \pm 0.12$  and  $2.22 \pm 0.1$  mg g<sup>-1</sup> cocoa extract respectively, about 50% of the levels reported in the aforementioned studies. Thus it could be suggested that caffeine might also reduce glucose uptake and hence give a negative result on long-term glucose control. Matteucci and Giampietro<sup>36</sup> indicated that fasting plasma glucose could give ambiguous results. Instead, the OGTT was suggested as a sensitive tool to measure glucose disturbance.

In this study the OGTT was performed to determine short-term (acute) glucose control. The results demonstrated that cocoa extract could possess short-term glucose control. Cocoa extract supplementation significantly reduced the glucose level at 60–90 min compared with untreated Ob-db. Polyphenolic compounds present in cocoa extract could potentially be beneficial in reducing the glucose level. Although polyphenol metabolites in plasma of Ob-db rats were not determined here, a previous study reported that monomer epicatechin was maximally present in plasma at 2 h after consumption of polyphenol-rich chocolate.<sup>37</sup> Brand-Miller *et al.*<sup>14</sup> showed that cocoa-based product supplementation led to an increase in postprandial insulin secretion in normal subjects.

The mechanisms whereby polyphenols reduce plasma glucose have been widely established, particularly their effects on muscle and intestine. The mechanisms could be through glucose transporter isoform 1 (GLUT1) protein synthesis and activation of phosphatidylinositol 3-kinase (PI3K) in muscle cells and inhibition of facilitated glucose uptake and sodium-dependent glucose transporter (SGLT1) in intestine cell lines.<sup>38–40</sup> Apart from polyphenols, a previous study reported a significant reduction in glucose-6-phosphatase activity *in vivo* at 2 h after metformin (a conventional drug for treatment of diabetes) administration. The reduction in this enzyme level was in accordance with the suppression of mRNA levels of this gene.<sup>41</sup> Therefore investigation of postprandial insulinaemia and glucose uptake in muscle and intestine and determination of hepatic enzymes involved in glycolysis could partly explain the observed findings.

## CONCLUSIONS

High-fat diet followed by low-dose STZ injection can be used to develop obese-diabetic (Ob-db) rats that mimic human diabetes in terms of obesity and impaired insulin sensitivity. This study indicated that cocoa extract possessed hypocholesterolaemic properties and short-term glucose control. However, there were no significant reductions in long-term (4 weeks) glucose control, insulin level and insulin sensitivity after cocoa extract supplementation. The exact underlying mechanisms for the hypoglycaemic and hypocholesterolaemic properties of cocoa extract remain elusive and are currently being investigated.

## ACKNOWLEDGEMENT

The authors would like to acknowledge the assistance of the laboratory staff from the Department of Nutrition and Dietetics, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia.

## REFERENCES

- Adamson GE, Lazarus SA, Mitchell AE, Prior RL, Cao G, Jacobs PH, *et al*, HPLC method for the quantification of procyanidins in cocoa and chocolate samples and correlation to total antioxidant capacity. *J Agric Food Chem* **47**:4184–4188 (1999).
- Rios LY, Gonthier M-P, Remesy C, Mila I, Lapiere C, Lazarus SA, *et al*, Chocolate intake increases urinary excretion of polyphenol-derived phenolic acids in healthy human subjects. *Am J Clin Nutr* **77**:912–918 (2003).
- Kelm MA, Johnson JC, Robbins RJ, Hammerstone JF and Schmitz HH, High-performance liquid chromatography separation and purification of cacao (*Theobroma cacao* L.) procyanidins according to degree of polymerization using a diol stationary phase. *J Agric Food Chem* **54**:1571–1576 (2006).
- Tomas-Barberan FA, Cienfuegos-Jovellanos E, Marin A, Muguerza B, Gil-Izquierdo A, Cerdaa B, *et al*, A new process to develop a cocoa powder with higher flavonoid monomer content and enhanced bioavailability in healthy humans. *J Agric Food Chem* **55**:3926–3935 (2007).
- Greer F, Hudson R, Ross R and Graham T, Caffeine ingestion decreases glucose disposal during a hyperinsulinemic-euglycemic clamp in sedentary humans. *Diabetes* **50**:2349–2354 (2001).
- Vinson JA, Proch J and Zubik L, Phenol antioxidant quantity and quality in foods: cocoa, dark chocolate, and milk chocolate. *J Agric Food Chem* **47**:4821–4824 (1999).
- Vinson JA, Proch J, Bose P, Muchler S, Taffera P, Shuta D, *et al*, Chocolate is a powerful *ex vivo* and *in vivo* antioxidant, an antiatherosclerotic agent in an animal model, and a significant contributor to antioxidants in the European and American diets. *J Agric Food Chem* **54**:8071–8076 (2006).
- Amin I, Koh BK and Asmah R, Effect of cacao liquor extract on tumor marker enzymes during chemical hepatocarcinogenesis in rats. *J Med Food* **7**:7–12 (2004).
- Neukam K, Stahl W, Tronnier H, Sies H and Heinric U, Consumption of flavanol-rich cocoa acutely increases microcirculation in human skin. *Eur J Nutr* **46**:53–56 (2007).
- Heiss C, Finis D, Kleinbongard P, Hoffmann A, Rassaf T, Kelm M, *et al*, Sustained increase in flow-mediated dilation after daily intake of high-flavanol cocoa drink over 1 week. *J Cardiovasc Pharmacol* **49**:74–80 (2007).
- Ramljak D, Romanczyk LJ, Methney-Barlow LJ, Thompson N, Knezevic V, Galperin M, *et al*, Pentameric procyanidin from *Theobroma cacao* selectively inhibits growth of human breast cancer cells. *Mol Cancer Ther* **4**:537–546 (2005).
- Baba S, Osakabe N, Kato Y, Natsume M, Yasuda A, Kido T, *et al*, Continuous intake of polyphenolic compounds containing cocoa powder reduces LDL oxidative susceptibility and has beneficial effects on plasma HDL-cholesterol concentrations in human. *Am J Clin Nutr* **85**:709–717 (2007).
- Baba S, Natsume M, Yasuda A, Nakamura Y, Tamura T, Osakabe N, *et al*, Plasma LDL and HDL cholesterols and oxidized LDL concentrations are altered in normo- and hypercholesterolemic humans after intake of different levels of cocoa powder. *J Nutr* **137**:1436–1441 (2007).
- Brand-Miller J, Holt SHA, de Jong V and Petocz P, Cocoa powder increases postprandial insulinemia in lean young adults. *J Nutr* **133**:3149–3152 (2003).
- Grassi D, Necozione S, Lippi C, Croce G, Valeri L, Pasqualetti P, *et al*, Cocoa reduces blood pressure and insulin resistance and improves endothelium-dependent vasodilation in hypertensives. *Hypertension* **46**:398–405 (2005).
- Amin I, Faizul HA and Azli R, Effect of cocoa powder extract on plasma glucose levels in hyperglycemic rats. *Nutr Food Sci* **34**:116–121 (2004).
- Ruzaidi A, Amin I, Nawalyah AG, Hamid M and Faizul HA, The effect of Malaysian cocoa extract on glucose levels and lipid profiles in diabetic rats. *J Ethnopharmacol* **98**:55–60 (2005).
- Tomaru M, Takano H, Osakabe N, Yasuda A, Inouse K-I, Yangisawa R, *et al*, Dietary supplementation with cacao liquor proanthocyanidins prevents elevation of blood glucose levels in diabetic obese mice. *Nutrition* **23**:351–355 (2007).
- Najjar N, Adra N and Hwalla N, Glycemic and insulinemic responses to hot vs cooled potato in males with varied insulin sensitivity. *Nutr Res* **24**:993–1004 (2004).
- Reed MJ, Meszaros K, Entes LJ, Claypool MD, Pinkett JG, Gadbois TM, *et al*, A new rat model of type 2 diabetes: the fat-fed, streptozotocin-treated rat. *Metabolism* **49**:1390–1394 (2000).
- Zhang F, Ye C, Li G, Ding W, Zhou W, Zhu H, *et al*, The rat model of type 2 diabetes mellitus and its glycometabolism characters. *Exp Anim* **52**:401–407 (2003).
- Srinivasan K, Viswanad B, Asrat L, Kaul CL and Ramarao P, Combination of high-fat diet-fed and low-dose streptozotocin-treated rat: a model for type 2 diabetes and pharmacological screening. *Pharmacol Res* **52**:313–320 (2005).
- Weyer C, Borgadus C, Mott DM and Pratley RE, The natural history of insulin secretory dysfunction and insulin resistance in the pathogenesis of type 2 diabetes mellitus. *J Clin Invest* **104**:787–794 (1999).
- Weyer C, Tataranni PA and Pratley RE, Insulin resistance and insulin secretory dysfunction are independent predictors of worsening of glucose tolerance during each stage of type 2 diabetes development. *Diabetes Care* **24**:89–94 (2001).
- Buchanan TA, Pancreatic beta-cell loss and preservation in type 2 diabetes. *Clin Ther* **25**(Suppl B):B32–B46 (2003).
- Rensen PC and Van Berkel TJ, Apolipoprotein E effectively inhibits lipoprotein lipase-mediated lipolysis of chylomicron-like triglyceride-rich lipid emulsions *in vitro* and *in vivo*. *J Biol Chem* **271**:14791–14799 (1996).
- Ikeda I, Tsuda K, Suzuki Y, Kobayashi M, Unno T, Tomoyori H, *et al*, Tea catechins with a galloyl moiety suppress postprandial hypertriacylglycerolemia by delaying lymphatic transport of dietary fat in rats. *J Nutr* **135**:155–159 (2005).
- Chan PT, Fong WP, Cheung YL, Huang Y, Ho WKK and Chen Z-Y, Jasmine green tea epicatechins are hypolipidemic in hamsters (*Mesocricetus auratus*) fed a high fat diet. *J Nutr* **129**:1094–1101 (1999).
- Lin C-L, Huang H-C and Lin JK, Theaflavins attenuate hepatic lipid accumulation through activating AMPK in human HepG2 cells. *J Lipid Res* **48**:2334–2343 (2007).
- Wilcox LJ, Borradaile NM, de Dreu LE and Huff MW, Secretion of hepatocyte apoB is inhibited by the flavonoids, naringenin, and hesperitin, via reduced activity and expression of ACAT2 and MTP. *J Lipid Res* **42**:725–734 (2001).
- Pal S, Ho N, Santos C, Dubois P, Mamo J, Croft K, *et al*, Red wine polyphenolics increase LDL receptor expression and activity and suppress the secretion of ApoB100 from human HepG2 cells. *J Nutr* **133**:700–706 (2003).
- Cooper KA, Donovan JL, Waterhouse AL and Williamson G, Cocoa and health: a decade of research. *Br J Nutr* **99**:1–11 (2008).
- Zeggwagh N-A, Ouahidi M-L, Lemhadri A and Eddouks M, Study of hypoglycaemic and hypolipidemic effects of *Inula viscosa* L. aqueous extract in normal and diabetic rats. *J Ethnopharmacol* **108**:223–227 (2006).
- Ziai SA, Larijani B, Akhoondzadeh S, Fakhrazadeh H, Dastpak A, Bandarian F, *et al*, Psyllium decreased serum glucose and glycosylated hemoglobin significantly in diabetic outpatients. *J Ethnopharmacol* **102**:202–207 (2005).

- 35 Lee S, Hudson R, Kilpatrick K and Graham TE, Caffeine ingestion is associated with reductions in glucose uptake independent of obesity and type 2 diabetes before and after exercise training. *Diabetes Care* **28**:566–572 (2005).
- 36 Matteucci E and Giampietro O, Proposal open for discussion: defining agreed diagnostic procedures in experimental diabetes research. *J Ethnopharmacol* **115**:163–172 (2008).
- 37 Rein D, Lotito S, Holt RR, Keen CL, Schmitz HH and Fraga CG, Epicatechin in human plasma: *in vivo* determination and effect of chocolate consumption on plasma antioxidant status. *J Nutr* **130**:2109S–2114S (2000).
- 38 Johnston K, Sharp P, Clifford M and Morgan L, Dietary polyphenols decrease glucose uptake by human intestinal Caco-2 cells. *FEBS Lett* **579**:1653–1657 (2005).
- 39 Purintrapiban J, Suttajit M and Forsberg NE, Differential activation of glucose transport in cultured muscle cells by polyphenolic compounds from *Canna indica* L. root. *Biol Pharmaceut Bull* **29**:1995–1998 (2006).
- 40 Kobayashi Y, Suzuki M, Satsu H, Arai S, Hara Y, Suzuki K, *et al*, Green tea polyphenols inhibit the sodium-dependent glucose transporter of intestinal epithelial cells by a competitive mechanism. *J Agric Food Chem* **48**:5618–5623 (2000).
- 41 Heishi M, Ichihara J, Teramoto R, Itakura Y, Hayashi K, Ishikawa H, *et al*, Global gene expression analysis in liver of obese diabetic db/db mice treated with metformin. *Diabetologia* **49**:1647–1655 (2006).