

## Effects of Cocoa Extract on Glucometabolism, Oxidative Stress, and Antioxidant Enzymes in Obese-Diabetic (Ob-db) Rats

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In this present study, we investigated the effects of cocoa extract containing polyphenols and methylxanthines prepared from cocoa powder on the biochemical parameters of obese-diabetic (Ob-db) rats. 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/day) was given to the rats for 4 weeks. The results indicated that there were no significant differences in fasting plasma glucose and insulin level after 4 weeks of cocoa extract 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. 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 nonsupplemented rats. However, no change was observed in catalase activity. The results showed that cocoa supplementation had an effect on postprandial glucose control but not for long term (4 weeks). Moreover, cocoa supplementation could reduce circulating plasma free fatty acid and 8-isoprostane and may enhance the antioxidant defense system.

**KEYWORDS:** Caffeine; catalase; catechin; cocoa; epicatechin; superoxide dismutase; theobromine

### INTRODUCTION

The practical use of *Theobroma cacao* had originated from Olmecs, Mayas, and Aztecs in South America. By the 16th and early 20th century in Europe and New Spain, over 100 medicinal uses of cocoa had been documented (1). In the years 1991–2008, more than 200 studies were reported on the bioactive compounds, chemical compositions, and health benefits of cocoa and cocoa products, namely, cocoa powder, cocoa liquor, and dark chocolate (2).

Numerous studies indicated that the health promoting properties of cocoa powder were attributed mainly to their polyphenolic compounds and methylxanthines. Cocoa comprises mainly of procyanidins monomers, namely, catechin and epicatechin,

dimer, trimer, tetramer, and up to tetradecamer (3, 4). In addition, methylxanthines, namely, caffeine, theobromine, and theophylline, had also been identified in cocoa (3). Because of the significant amount of bioactive compounds, the study of their contribution toward health benefits is an area of interest. Numerous publications were reported on the health promoting properties of cocoa polyphenols, which were based on *in vitro* and *in vivo* studies (5–9).

Recent work demonstrated that cocoa could suppress the development of atherosclerotic lesions (6), antiheptocarcinogenesis (8), and protect against prostate carcinogenesis (9). In addition, our previous studies indicated that cocoa supplementation possessed hypoglycemic and hypocholesterolemic properties in diabetic-induced rats (7, 10, 11). A study by Tomaru et al. (12) indicated cocoa has the ability to prevent the development of diabetes in genetically inherited diabetic rats. To a greater extent, it was reported that cocoa supplementation could reduce lipid profiles of normo- and hypercholesterolemic human subjects (13). These studies clearly indicated that cocoa could beneficially control blood glucose and reduce lipid profiles. However, to the best of our knowledge, limited studies are available on the effects of cocoa on a diabetes animal model

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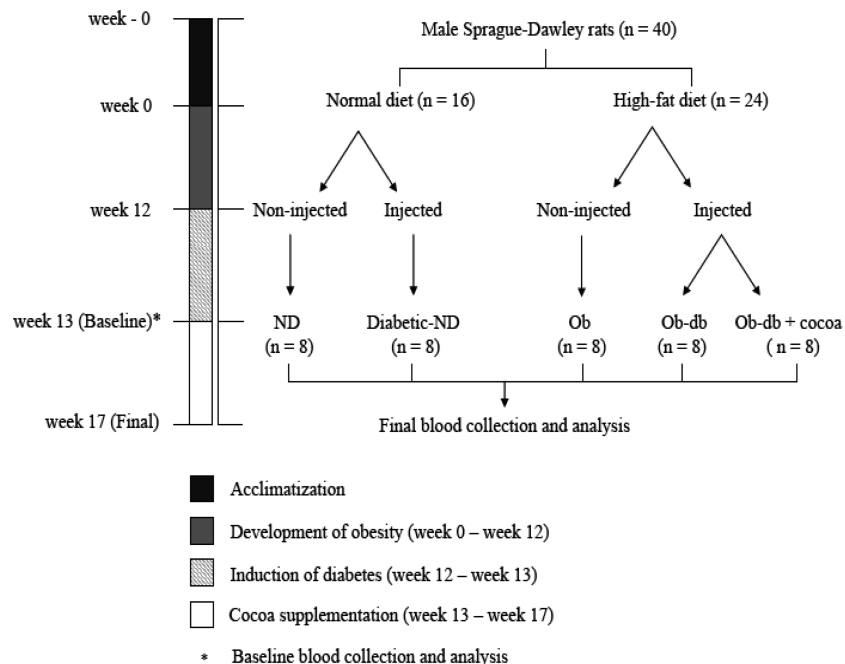


Figure 1. Schematic diagram of the experimental study.

that mimicks human diabetes syndrome. Hence, the present study was undertaken to determine the effects of cocoa extract containing polyphenols and methylxanthines on glucometabolism, oxidative stress, and antioxidant enzymes in obese-diabetic (Ob-db) rats.

## EXPERIMENTAL PROCEDURES

**Preparation of Standardized Cocoa Extract.** Cocoa extract was prepared from Malaysian cocoa powder (KL-Kepong Cocoa Products Sdn. Bhd., Port Klang, Selangor, Malaysia) according to our previous method (10). Briefly, cocoa extract was prepared by extracting defatted cocoa powder with 80% (v/v) 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 lyophilized using a freeze-dryer (The Virtis Company Inc., Gardiner, New York; –45 °C, 120 bar). The resulting extract was standardized according to a previous method (14). In standardizing the extract, 5 mL of cocoa extract was fractionated on a prepacked column (25 cm × 2.0 cm) with Sephadex LH 20 (Amersham Bioscience, Uppsala, Sweden). Stepwise increase in water–acetone ratio (85:15, 70:30, and 40:60) as elution medium and a flow rate of 2.0 mL/min was maintained along the procedure. The resulting fraction 1 (F1), fraction 2 (F2), and fraction 3 (F3) were used for the identification of bioactive compounds.

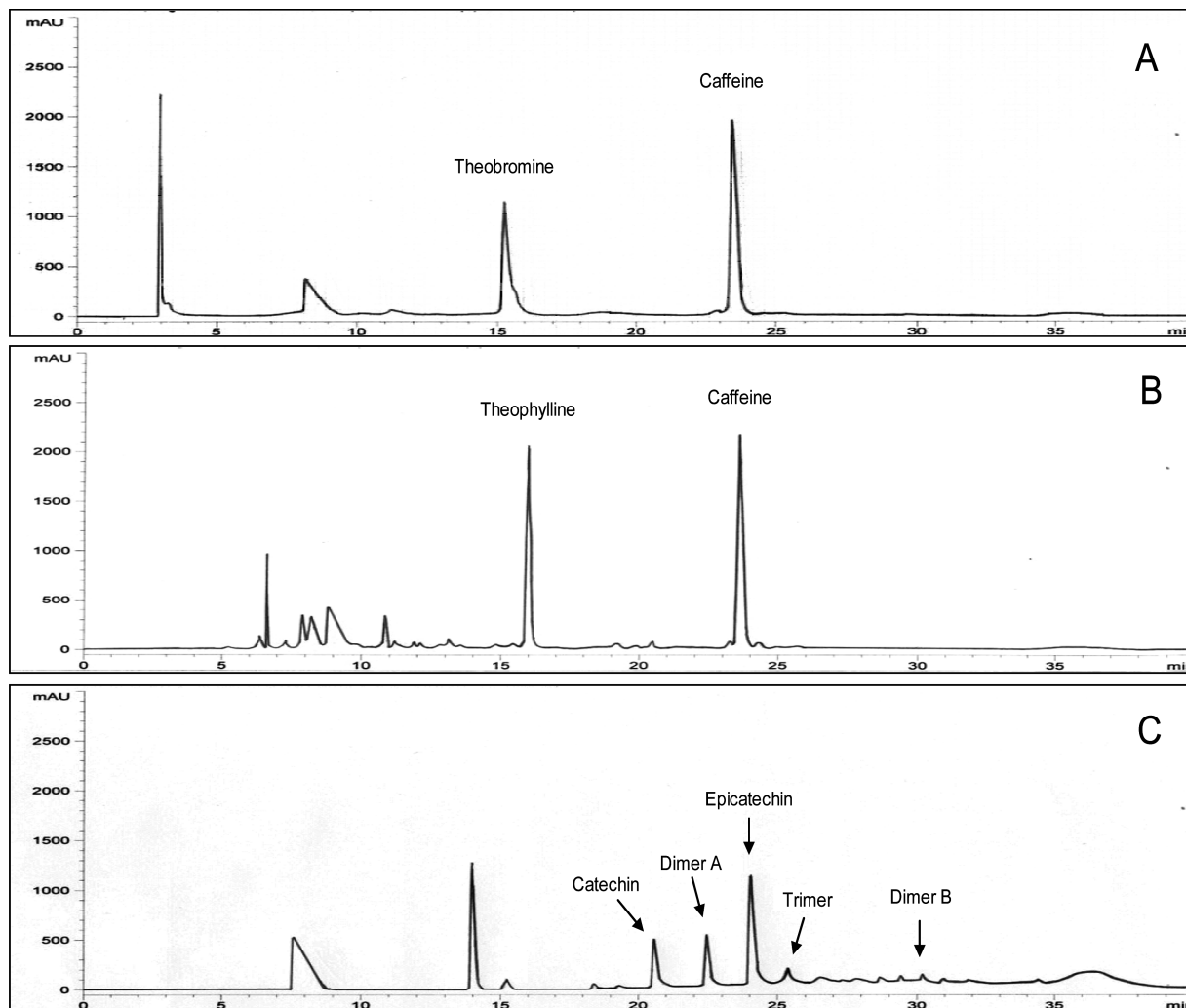
**Identification of Bioactive Compounds.** Bioactive compounds in each fraction were determined using high-performance liquid chromatography (HPLC) (Agilent 1100, Palo Alto, USA) equipped with a quaternary pump, auto injector, degasser, and DAD. A separation of bioactive compounds was done using a reversed-phase C18 column (Alltech, Licosphere, United States) (250 mm × 4 mm, 5 μm I.D) and gradient elution of (A) water–trifluoroacetic acid (99.9: 0.1, v/v) and (B) acetonitrile–trifluoroacetic acid (99.9: 0.1, v/v) (15). A linear gradient elution of 0–10% (A) for 5 min, 10–25% (A) for 25 min, and 25–100% (A) for 5 min with a flow rate of 0.8 mL/min was used for the analysis. The amount of catechin, epicatechin, caffeine, and theobromine (mg/g fraction) were quantified on the basis of external standards (100–1000 μg/mL). In this study, dimers (A and B) and a trimer were quantified on the basis of catechin equivalents (CE).

**Animal Study.** Forty male Sprague–Dawley rats (weighing 100–150 g) were purchased from the Institute for Medical Research (IMR), Kuala Lumpur, Malaysia. The animal study approval was obtained from Animal Care and Use Committee (ACUC) of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (ACUC No: UPM/

FPSK/PADS/BRUHH/00180). The rats were housed individually in plastic cages with a stainless steel cover. They were acclimatized for 1 week at room temperature (26–28 °C) under a 12-h dark/12-h light cycle. All the rats were subjected to normal rat chow (Gold Coin, Selangor, Malaysia) and distilled water ad libitum during acclimatization. The rats were randomly divided into five groups with 8 rats (n = 8) per group after acclimatization. The experimental diagram is shown in Figure 1.

Obesity was developed using purified high-fat diet (HFD) (49% fat, 32% carbohydrate, and 19% protein from total energy, kcal) that consists of fat from ghee (milk fat) and corn oil. Normal rats were given normal rat chow (Gold Coin, Malaysia) (14% fat, 61% carbohydrate, and 25% protein from total energy, kcal). A high-fat diet was given to the rats in groups 3, 4, and 5 for 12 weeks. Body weight was determined weekly to ensure the development of obesity. Body mass index (BMI) was calculated on the basis of body weight (g) divided by the square of the anal–nasal length (cm). High-fat diet (HFD) fed rats were injected intravenously with a low-dose of streptozotocin (STZ) (35 mg STZ/kg body weight) to induce diabetes. Streptozotocin was prepared according to Ruzaidi et al. (10). A combination of HFD and STZ injection was done in order to mimic human diabetes syndrome. Blood was collected from the tail vein 3 days after STZ injection. Glucose levels were determined using a glucometer (Roche, Mannheim, Germany) to ensure that the rats developed diabetes. The standardized cocoa extract was given to obese-diabetic (Ob-db) rats for 4 weeks (week-13 to week-17) with a dose of 600 mg/kg body weight. The dose was selected on the basis of our previous study (11). This dose could improve blood glucose metabolism in hyperglycemic rats as evaluated by oral glucose tolerance test (OGTT). Cocoa extract was suspended in 3 mL of 0.03% (w/v) carboxymethyl cellulose (CMC) and was given daily by gastric intubation, using a force feeding needle. Rats in the control group (Ob-db) received 0.03% (w/v) CMC only throughout the experimental period.

**Blood Collection for Biochemical Analysis.** Blood was collected in all groups at baseline (at week-13, after 12 weeks of high-fat diet followed by low-dose of STZ injection) and after cocoa extract supplementation in Ob-db + cocoa at the final week (week-17) from the retro-orbital plexus under general anesthesia. Approximately 5 mL of blood was collected in different blood collection tubes containing potassium oxalate as an antiglycolysis agent (for glucose) and lithium heparin as an anticoagulant agent (for insulin, antioxidant enzymes, free fatty acid, and 8-isoprostane). Plasma was separated by centrifuga-



**Figure 2.** HPLC chromatograms of cocoa powder fractions. **A**, fraction 1; **B**, fraction 2; **C**, fraction 3.

tion at 1,500g for 15 min (4 °C) using a benchtop refrigerated centrifuge (Universal 32, Hettich Zentrifugen, Germany) and stored at -80 °C for further analysis.

**Determination of Plasma Glucose and Insulin Levels.** Plasma glucose was estimated using a commercially available kit (Roche, Mannheim, Germany). Plasma glucose was determined spectrometrically using Chemistry Analyzer (Hitachi 902 Automatic Analyzer, Hitachi, Tokyo, Japan). Plasma insulin level was determined using a radioimmunoassay (RIA) kit (GE Healthcare, Uppsala, Sweden).

**Oral Glucose Tolerance Test (OGTT).** Oral glucose tolerance test (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). Comparisons were made on the basis of the glucose response curve (0 to 120 min) and area under the curve (AUC) between each rat groups. Rats were fasted overnight (10 h) and administered with 2 g glucose/kg body weight. Tail vein blood samples were withdrawn without anesthesia before (0 min) and 15, 30, 60, 90, and 120 min after the administration of glucose solution. The curve response of each group was plotted. The incremental area under the curve (AUC) was calculated using a computer Calculator Software (kindly provided by Thomas Wolever, Department of Nutritional Sciences, University of Toronto, Toronto, Ontario, Canada).

**Determination of Plasma Free Fatty Acid.** Plasma free fatty acid (FFA) levels were determined using an ACS-ACOD method kit (Wako Diagnostics, Japan). The colorimetric reaction was estimated using Hitachi 902 Automatic Analyzer (Hitachi, Tokyo, Japan). Results were expressed as  $\mu\text{Eq/L}$ .

**Determination of Oxidative Stress.** Oxidative stress levels were measured in plasma using an 8-isoprostane (8-iso PGF<sub>2</sub> $\alpha$ ) Enzyme Immuno Assay (Cayman Chemicals, Ann Arbor, USA) kit. The op-

erating procedures were followed as described by the manufacturer. The absorbance values were read at 405 nm using an ELISA reader (Anthos 2020, Salzburg, Austria).

**Determination of Superoxide Dismutase and Catalase Activities.** Catalase (CAT) and superoxide dismutase (SOD) in plasma were determined using Catalase and Superoxide Dismutase assay kits (Cayman Chemicals, Ann Arbor, USA). The operational procedures were followed according to the manufacturer's instruction kit. The developed colored reaction of CAT and SOD were measured at 540 and 450 nm, respectively. The enzymes activities were expressed as U/mL.

**Statistical Analysis.** Data are presented as the mean  $\pm$  standard deviation. One-way ANOVA (SPSS version 15.0) and least significant difference (LSD) post hoc test were used to determine the mean differences between groups. Comparisons between baseline (at week-13, after 12 week of high-fat diet followed by low-dose of STZ injection) and final (week-17) values were performed using paired sample *t*-tests. Values are considered significantly different at the level of  $p < 0.05$ .

## RESULTS

**Bioactive Compounds in Cocoa Extract.** Methylxanthines, namely, caffeine, theobromine, and theophylline, were identified in cocoa fractions 1 and 2 (**Figure 2A** and **B**). Caffeine and theobromine contents were  $3.55 \pm 0.12$  and  $2.22 \pm 0.1$  mg/g cocoa extract, respectively. Epicatechin and catechin were quantified in fraction 3 with  $2.17 \pm 0.05$  and  $1.52 \pm 0.07$  mg/g cocoa, respectively (**Figure 2C**). Dimers and trimer were

**Table 1.** Characteristic of Experimental Rats<sup>a</sup>

rats	body weight (g)		body mass index (BMI) (g/cm <sup>2</sup> )		food intake (g/day)		energy intake (kcal/day)	
	baseline*	final**	baseline	final	baseline	final	baseline	final
ND	446.57 ± 18.64 a	465.35 ± 15.80 a	0.69 ± 0.03 a	0.71 ± 0.03 a	21.92 ± 1.5 a	23.15 ± 2.36 a,b	77.60 ± 5.35 a	81.95 ± 8.34 a
Diabetic-ND	423.83 ± 22.59 a	375.85 ± 32.52 d	0.68 ± 0.02 a	0.59 ± 0.04 b	23.04 ± 5.11 a,d	27.63 ± 2.52 b	81.56 ± 18.1 a	97.81 ± 8.91 a
Ob	492.58 ± 32.22 b,c	512.03 ± 29.65 c	0.74 ± 0.04 b,c	0.76 ± 0.06 a,c	16.74 ± 1.56 b	16.17 ± 3.67 b,c	79.01 ± 7.36 a	76.33 ± 17.30 a
Ob-db	435.12 ± 32.88 a	385.33 ± 35.14 d	0.69 ± 0.04 a	0.60 ± 0.09 b	16.64 ± 2.72 b,c	19.19 ± 1.91 c,d	78.55 ± 12.85 a	90.55 ± 9.03 a
Ob-db + cocoa	448.36 ± 20.84 a	383.55 ± 30.69 d	0.68 ± 0.02 a	0.60 ± 0.05 b	19.25 ± 3.24 b,d	17.21 ± 4.71 c,d	90.87 ± 15.30 a	81.24 ± 22.24 a

<sup>a</sup> Values are expressed as the mean ± SD. Values with different letters are significantly different between rows and columns at the level of  $p < 0.05$  (LSD post hoc test). Comparisons between baseline and final values were performed using paired sample *t*-tests. Single (\*) and double (\*\*) asterisks indicate baseline (week-13; after high-fat diet for 12 weeks followed by low-dose of STZ injection) and final (week-17) values, respectively. ND, normal diet; Diabetic-ND, normal diet + STZ injection; Ob, obese; Ob-db, obese-diabetic; Ob-db + cocoa, Ob-db + 600 mg cocoa/kg body weight.

quantified on the basis of catechin equivalents (CE). Dimers and trimer contents were  $0.25 \pm 0.05$  mg and  $0.13 \pm 0.00$  mg catechin equivalent/g cocoa extract, respectively (**Figure 2C**).

**Body Weight, BMI, Food Intake, and Energy Intake.** Body weight and body mass index (BMI), food intake, and energy intake of the experimental rats are depicted in **Table 1**. High-fat diet fed rats (obese, Ob) showed a significantly higher body weight at baseline (at week-13, after 12 week of high-fat diet followed by low-dose of STZ injection) compared to that of normal (ND). In addition, Ob rats showed significantly higher baseline body weight compared to that of normal diet + STZ (Diabetic-ND), obese-diabetic (Ob-db), and obese-diabetic + cocoa (Ob-db + cocoa). No significant decrease in body weight of Diabetic-ND, Ob-db, and Ob-db + cocoa after streptozotocin (STZ) injection at baseline was observed compared to that in ND rats. However, body weight significantly reduced ( $p < 0.05$ ) in Diabetic-ND compared to that in ND and Ob at the final week (week-17). Four weeks of cocoa supplementation did not improve the body weight of Ob-db + cocoa compared to that in Ob-db. Similar to body weight, high-fat diet significantly increased body mass index (BMI) of Ob compared to that of other groups at baseline. However, BMI was significantly ( $p < 0.05$ ) reduced in Diabetic-ND and Ob-db compared to that in ND and Ob groups at the final week (week-17). There was no significant increment in BMI of Ob-db + cocoa rats after 4 weeks of cocoa supplementation. Food intakes of Ob, Ob-db, and Ob-db + cocoa were significantly ( $p < 0.05$ ) higher at baseline and final compared to those of ND and Diabetic-ND in the range of 16 to 19 g/day. Although there were differences in food intake, energy intake (kcal) was similar between high-fat (Ob, Ob-db, Ob-db + cocoa) and normal (ND and Diabetic-ND) groups. This was due to the different energy levels of high-fat and normal diets. In this study, each gram of high-fat and normal diets provided 4.72 and 3.54 kcal, respectively.

**Fasting Plasma Glucose and Insulin Levels.** Fasting plasma glucose (FPG) is tabulated in **Table 2**. Normal diet (ND) and obese (Ob) rats had showed similar fasting plasma glucose (FPG) levels in the range of 7.1 to 8.2 and 7.1 to 7.7 mmol/L at baseline and final, respectively. Diabetic-ND, Ob-db, and Ob-db + cocoa rats had significantly ( $p < 0.05$ ) increased FPG at baseline with a range of 15 to 19 mmol/L compared to that of ND and Ob groups. No significant reduction in FPG was noted after 4 weeks (week-17) of cocoa supplementation in Ob-db + cocoa group as compared to Ob-db (nonsupplemented). Fasting plasma glucose of Diabetic-ND and Ob-db had remained high at the end of the experiment in contrast to that of the other groups. Normal (ND) rats showed a similar insulin level at baseline and final with a range of 209 to 243 pmol/L (**Table**

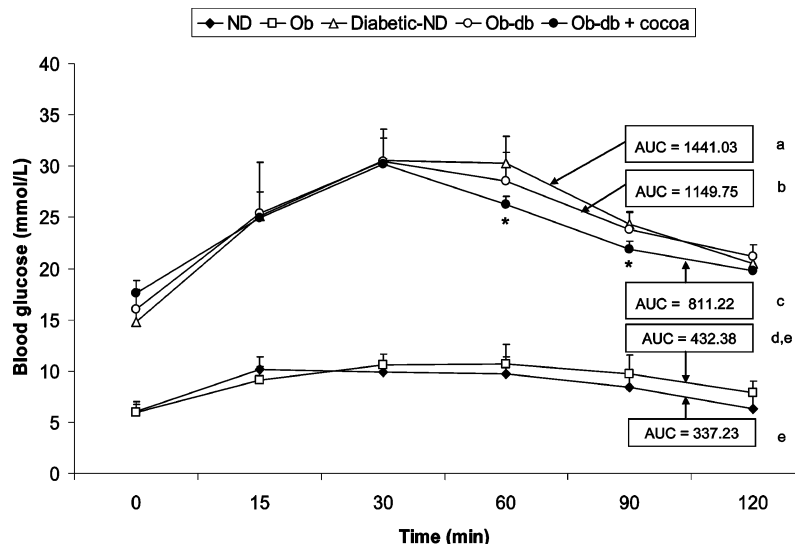
**Table 2.** Glucometabolism Parameters of Experimental Rats<sup>a</sup>

rats	glucose level (mmol/L)		insulin level (pmol/L)	
	baseline*	final**	baseline	final
ND	7.06 ± 0.78 a	7.68 ± 0.50 a	209.01 ± 60.3 a	242.65 ± 42.56 a
Diabetic-ND	14.93 ± 3.76 b	20.33 ± 3.39 b	281.87 ± 49.53 b	179.18 ± 60.17 c
Ob	8.16 ± 0.83 a	7.08 ± 0.49 a	375.46 ± 60.65 c,d	316.12 ± 70.12 d
Ob-db	18.41 ± 1.63 c	15.72 ± 3.07 c	129.45 ± 29.24 e	112.18 ± 40.82 e
Ob-db + cocoa	18.78 ± 1.19 c	16.21 ± 4.18 c	132.95 ± 50.60 e	123.29 ± 57.19 e

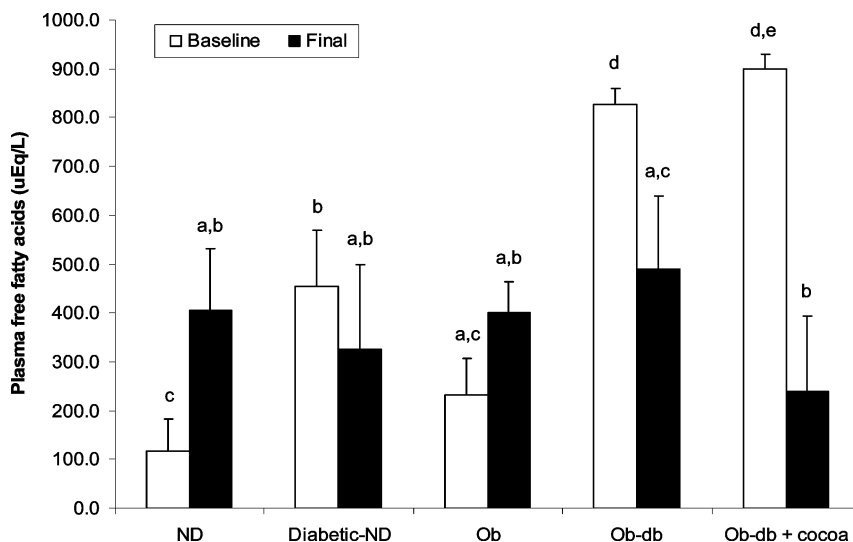
<sup>a</sup> Values are expressed as the mean ± SD. Values with different letters are significantly different between rows and columns at the level of  $p < 0.05$  (LSD post hoc test). Comparisons between baseline and final values were performed using paired sample *t*-tests. Single (\*) and double (\*\*) asterisks indicate baseline (week-13; after high-fat diet for 12 weeks followed by low-dose of STZ injection) and final (week-17) values, respectively. ND, normal diet; Diabetic-ND, normal diet + STZ injection; Ob, obese; Ob-db, obese-diabetic; Ob-db + cocoa, Ob-db + 600 mg cocoa/kg body weight.

2). In contrast, the obese (Ob) group showed significant ( $p < 0.05$ ) hyperinsulinemia at baseline and final compared to that of the ND group. Streptozotocin injection significantly ( $p < 0.05$ ) reduced the insulin level in Ob-db group from 129 pmol/L (baseline) to 112 pmol/L (final). The level of insulin in Ob-db + cocoa group was similar to that of Ob-db. No significant improvement in insulin level was observed in Ob-db + cocoa rats after 4-weeks of cocoa supplementation.

**Oral Glucose Tolerance Test (OGTT).** The pattern of plasma glucose of rats after oral glucose tolerance test is depicted in **Figure 3**. Normal (ND) and obese (Ob) rats showed similar baseline (0 min) blood glucose levels. In contrast, baseline blood glucose of Diabetic-ND, Ob-db, and Ob-db + cocoa were significantly ( $p < 0.05$ ) higher compared to that of ND and Ob with 14.82 to 17.62 mmol/L. Plasma glucose increased gradually and reached a plateau at 30 min in all groups. At 30 min, plasma glucose of Diabetic-ND, Ob-db, and Ob-db + cocoa groups were significantly ( $p < 0.05$ ) higher compared to that of ND and Ob groups. Plasma glucose levels started to decrease gradually after 60 min in all groups. Cocoa supplementation significantly ( $p < 0.05$ ) reduced blood glucose level at 60 and 90 min compared to that in the nonsupplemented group (Ob-db). Area under the curve (AUC) in **Figure 3** was calculated for each of the individual OGTT to determine the increment of blood glucose from 0 to 120 min. There was no significant difference in AUC between ND and Ob groups (**Figure 3**). A significant highest ( $p < 0.05$ ) AUC was observed in Diabetic-ND at 1441.03 mmol/L compared to that of Ob-db and Ob-db



**Figure 3.** Oral glucose tolerance test (OGTT) of the rats. Asterisk (\*) indicates that the values are significantly different at the level of  $p < 0.05$  (LSD post hoc test) compared to those for Ob-db. Area under the curve (AUC, mmol/L) values with different letters are significantly different at the level of  $p < 0.05$ . ND, normal diet; Diabetic-ND, normal diet + low-dose STZ injection; Ob, obese; Ob-db, obese-diabetic; Ob-db + cocoa, Ob-db + 600 mg cocoa/kg body weight.



**Figure 4.** Plasma free fatty acids of experimental rats. Values are expressed as the mean  $\pm$  standard deviation. Values with different superscripts are significantly different between groups and time at the level of  $p < 0.05$  (LSD post hoc test). Comparisons between baseline and final values were performed using paired sample  $t$ -tests. Baseline, week-13; after high-fat diet for 12 weeks followed by low-dose of STZ injection; Final, week-17. ND, normal diet; Diabetic-ND, normal diet + low-dose STZ injection; Ob, obese; Ob-db, obese-diabetic; Ob-db + cocoa, Ob-db + 600 mg cocoa/kg body weight.

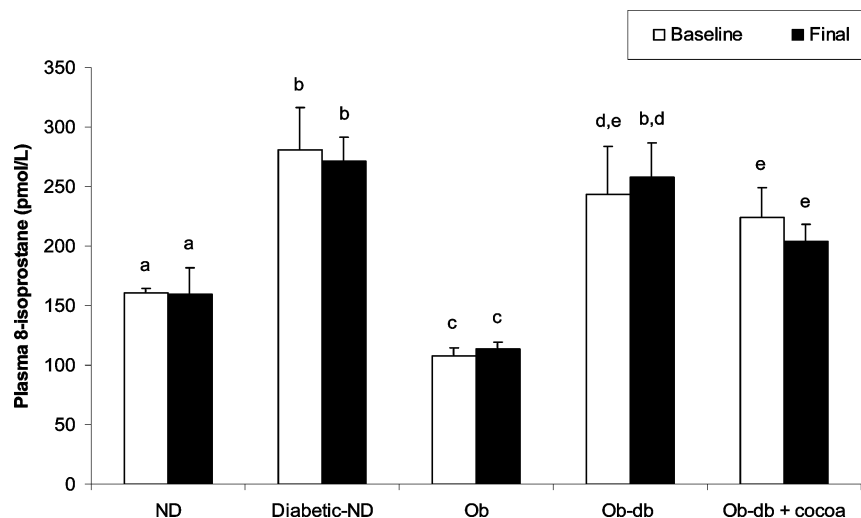
+ cocoa. Cocoa supplementation had significantly ( $p < 0.05$ ) reduced plasma AUC of Ob-db by 29% compared to that of nonsupplemented Ob-db.

**Plasma Free Fatty Acid.** Plasma circulating free fatty acid (FFA) of experimental rats is tabulated in **Figure 4**. At baseline (week-13), plasma FFA was significantly ( $p < 0.05$ ) higher in Diabetic-ND, Ob-db, and Ob-db + cocoa compared to that of ND and Ob. The FFA level was increased in ND and Ob to a level similar to that of Diabetic-ND and Ob-db at the end of the experiment. Four weeks of cocoa extract supplementation had significantly ( $p < 0.05$ ) reduced plasma FFA compared to baseline and Ob-db.

**Oxidative Stress (8-Isoprostane).** Plasma oxidative stress biomarker trend (8-isoprostane) at baseline (week-13) was similar to that of plasma FFA (**Figure 5**). Plasma 8-isoprostane had significantly ( $p < 0.05$ ) increased in Diabetic-ND, Ob-db, and Ob-db + cocoa compared to that in ND and Ob. The levels

remained high at the end of the experiment (week-17) in Diabetic-ND and Ob-db. Cocoa supplementation had significantly ( $p < 0.05$ ) reduced plasma 8-isoprostane in Ob-db + cocoa compared to that in the Ob-db group.

**Antioxidant Enzymes.** Antioxidant enzymes activities, namely, superoxide dismutase (SOD) and catalase (CAT) regulations are tabulated in **Figure 6a** and **b**, respectively. Superoxide dismutase (SOD) activity was significantly ( $p < 0.05$ ) reduced in Ob-db and Ob-db + cocoa at baseline (week-13) compared to that in ND and Ob. After 4 weeks of cocoa supplementation, SOD activity in Ob-db + cocoa had increased 23% compared to the initial value. In contrast, initial catalase (CAT) activity was not significantly reduced in Diabetic-ND, Ob-db, and Ob-db + cocoa compared to that in Ob rats. In addition, cocoa administration had significantly ( $p < 0.05$ ) reduced CAT activity in Ob-db compared to that in nonsupplemented groups (Ob-db, Ob, and ND).



**Figure 5.** Plasma 8-isoprostane of experimental rats. Values are expressed as the mean  $\pm$  standard deviation. Values with different superscripts are significantly different between groups and time at the level of  $p < 0.05$  (LSD post hoc test). Comparisons between baseline and final values were performed using paired sample *t*-tests. Baseline, week-13; after high-fat diet for 12 weeks followed by low-dose of STZ injection; Final, week-17. ND, normal diet; Diabetic-ND, normal diet + low-dose STZ injection; Ob, obese; Ob-db, obese-diabetic; Ob-db + cocoa, Ob-db + 600 mg cocoa/kg body weight.

## DISCUSSION

To date, there are at least three types of diabetic animal models, namely, diet or nutrition induced, chemically induced, and spontaneous or genetically modified rats (16). However, these models have their own strengths and weaknesses. In the present study, we utilized both combinations of nutrition- and chemically-induced diabetes for the development of obese-diabetic (Ob-db) rats that mimic human syndromes. Principally, the animal model was developed using a high-fat diet (HFD) (49% fat, 32% carbohydrate, 19% protein from total energy, kcal) to induce obesity. The high-fat diet significantly increased the body weight of rats starting at week-11 compared to that with the normal diet. As a result, obese rats showed marked hyperinsulinemia as indicated by a significant increase in insulin levels. In addition, the rats showed marked hyperglycemia after 3 days of diabetes induction using a low dose of streptozotocin (STZ). Hence, a combination of HFD and a low dose of STZ injection may be used to develop a diabetic model associated with obesity.

Previous studies showed that cocoa could exert hypoglycemic properties in diabetes animal models. A study found that cocoa could prevent the development of diabetes in genetically inherited diabetic mice (12). Our previous studies showed that cocoa possessed long-term (4 weeks) and short-term (2-h) blood glucose control in streptozotocin-induced diabetic rats (10, 11). The present study investigated the effects of cocoa extract on obese-diabetic (Ob-db) rats that mimicked human syndromes. We found that cocoa supplementation did not affect long-term blood glucose control of obese-diabetic (Ob-db) rats. In addition, the insulin levels were not affected after 4 weeks of cocoa supplementation.

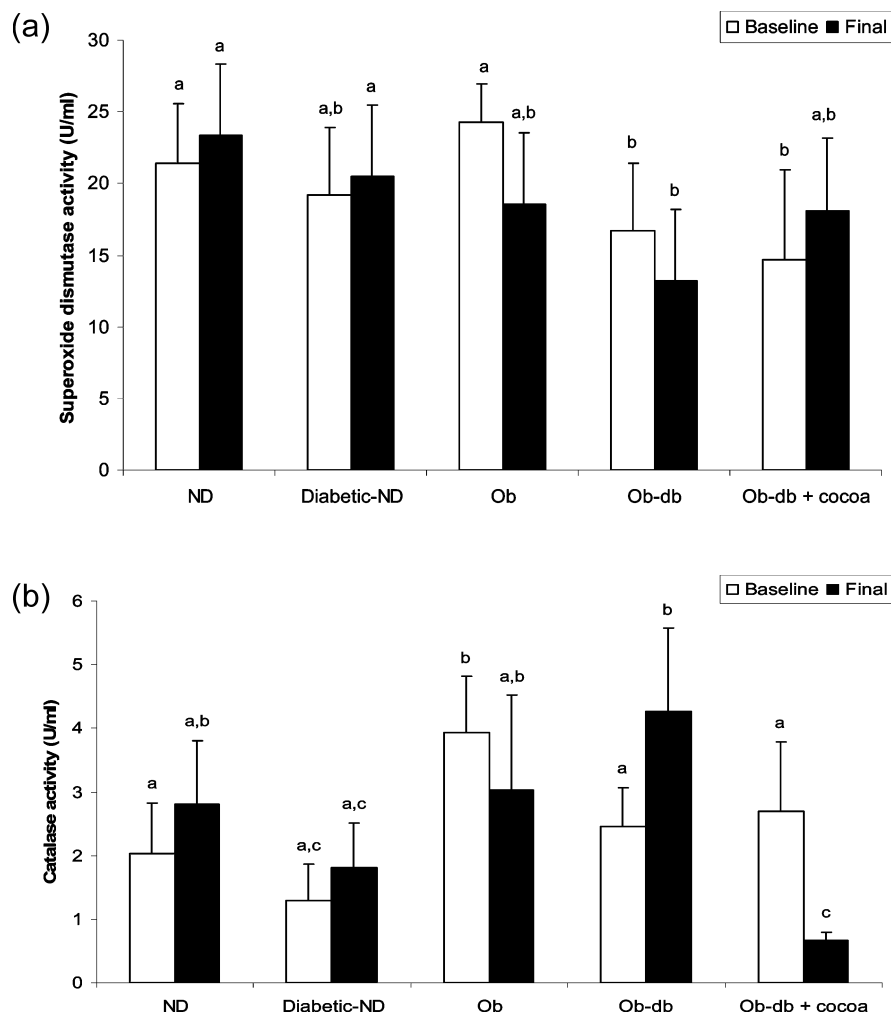
Glucagon-like peptide-1 (GLP-1) (mammalian incretin hormone) improved first-phase insulin secretion in type 2 diabetes subjects (17). Exenatide (incretin mimetic with 39-amino acid peptide that exhibits glucoregulatory activities similar to those of GLP-1A) was reported to improve first phase (0–0 min) and second phase (10–180 min) insulin secretion (18). Interestingly, our study demonstrated that cocoa supplementation could reduce short-term blood glucose as assessed by OGTT. Specifically, our results showed the cocoa extract had significantly reduced blood glucose of Ob-db at 60 and 90 min compared to that of

the nonsupplemented (Ob-db) ones. It was suggested that cocoa could at least play a role in second phase insulin secretion. Hence, determination of insulin response following a glucose tolerance test may partially answer this hypothesis. A study had indicated that cocoa supplementation possessed postprandial insulinemia in normal subjects (19).

Apart from glucometabolism parameters, free fatty acid (FFA) could play a role in the development of type 2 diabetes mellitus (T2DM). Individuals with a high level of FFA were at higher risk of T2DM (20). There was a significant correlation between FFA and lipoprotein lipase (LPL) activity (21). A decrease in FFA levels in serum and tissues was in accordance with a decrease in total lipoprotein lipase (LPL) activity (22). Our result indicated that the increment of FFA levels in Ob-db rats may be due to diabetic conditions. Cocoa supplementation for 4 weeks was able to reduce plasma FFA levels. Lecumberri et al. (23) demonstrated that cocoa fiber had not reduced plasma FFA in hypercholesterolemic rats. The conflicting result could be due to the different amounts of polyphenols present in cocoa extracts and cocoa fiber.

There is an association between oxidative stress and diabetes particularly through the generation of lipid peroxidation products, namely, 8-isoprostane and 8-hydroxy deoxyguanosine (8-OHdG) (24, 25). There was a significant increase in plasma 8-isoprostane of Ob-db compared to that in normal rats. Four weeks of cocoa supplementation reduced 8-isoprostane levels of Ob-db rats. This result suggested that cocoa extracts containing polyphenols may have protective effects on reducing lipid peroxidation levels. Yamagishi et al. (25) showed that cocoa liquor supplementation (containing about 50% of polyphenols) exerted protective effects against lipid peroxidation in rats fed with a vitamin E-deficient diet. However, the mechanisms underlying these effects are still unknown. In the present study, we determined the antioxidant enzyme, namely, superoxide dismutase (SOD) and catalase (CAT), to find out the possible mechanism involved in the observed results.

Our Ob-db rats showed impairment in SOD activity than normal ones. The enzyme had been reported to be dysregulated in diabetic patients (26). The present study showed cocoa supplementation had increased SOD activity of Ob-db. Ramiro-Puig et al. (27) reported that cocoa dose-dependently increased



**Figure 6.** (a) Superoxide dismutase activity of experimental rats. Values are expressed as the mean  $\pm$  standard deviation. Values with different superscripts are significantly different between groups and time at the level of  $p < 0.05$  (LSD post hoc test). Comparisons between baseline and final values were performed using paired sample *t*-tests. Baseline, week-13; after high-fat diet for 12 weeks followed by low-dose of STZ injection; Final, week-17. ND, normal diet; Diabetic-ND, normal diet + low-dose STZ injection; Ob, obese; Ob-db, obese-diabetic; Ob-db + cocoa, Ob-db + 600 mg cocoa/kg body weight. (b) Catalase activity of experimental rats. Values are expressed as the mean  $\pm$  standard deviation. Values with different superscripts are significantly different between groups and time at the level of  $p < 0.05$  (LSD post hoc test). Comparisons between baseline and final values were performed using paired sample *t*-tests. Baseline, week-13; after high-fat diet for 12 weeks followed by low-dose of STZ injection; Final, week-17. ND, normal diet; Diabetic-ND, normal diet + low-dose STZ injection; Ob, obese; Ob-db, obese-diabetic; Ob-db + cocoa, Ob-db + 600 mg cocoa/kg body weight.

SOD activity in the rat thymus. Superoxide dismutase is a metalloenzyme (copper, zinc, mangan, or iron may be located in the center of the enzyme structure) that is involved in the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide (28, 29). A study indicated that copper in cocoa and chocolate significantly contributed to the human diet (30). Thus, it is possible to postulate that copper may indirectly contribute to the present finding. In contrast to SOD, CAT activity was not improved after cocoa supplementation in Ob-db. This result indicated that cocoa could at least augment the antioxidant defense system through enhancement of SOD activity.

In conclusion, cocoa supplementation could augment post-prandial glucose metabolism and not long-term glucose control (4 weeks of cocoa supplementation). Moreover, cocoa may have protective effects against lipid peroxidation and concomitant increase in the antioxidant defense system. The health-promoting properties of cocoa extracts could be attributed to polyphenol compounds as well as methylxanthines (caffeine and theobromine) and minerals. The contribution of methylxanthines and minerals should also be considered in the health benefits of

cocoa extracts. However, the exact underlying mechanisms for the reduction in free fatty acid and lipid peroxidation, and the enhanced antioxidant enzyme activities of cocoa extracts remain elusive and are currently being investigated.

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