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The effect of polyphenol-rich dark chocolate on fasting capillary whole blood glucose, total cholesterol, blood pressure and glucocorticoids in healthy overweight and obese subjects

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Numerous studies indicate that polyphenol-rich chocolate reduces fasting blood glucose, blood pressure (BP) and total cholesterol in healthy individuals and hypertensives with or without glucose intolerance. The aim of the present study was to investigate the effect of two doses of polyphenol-rich dark chocolate (DC) on fasting capillary whole blood glucose, total cholesterol and BP and to examine whether improvements in these parameters are associated with changes in adrenocorticoid excretion in overweight and obese individuals. The study used a randomised, single-blind, cross-over design where fourteen overweight and obese subjects were randomised to either take 20 g DC with 500 mg polyphenols then 20 g DC with 1000 mg polyphenols or vice-versa. Participants followed each diet for 2 weeks separated by a 1-week washout period. It was observed that the 500 mg polyphenol dose was equally effective in reducing fasting blood glucose levels, systolic BP (SBP) and diastolic BP (DBP) as the 1000 mg polyphenol dose suggesting that a saturation effect might occur with increasing dose of polyphenols. There was also a trend towards a reduction in urinary free cortisone levels with both groups although it did not reach statistical significance. No changes in anthropometrical measurements were seen. We suggest that more research is required to investigate the mechanism(s) by which polyphenol-rich foods influence health.

Dark chocolate: Glucose: Blood pressure: Cholesterol: Glucocorticoids: Obesity

Epidemiological studies link high polyphenol intake with reduced risk of oxidative stress-related diseases like diabetes, hypertension and CVD^(1–3). In particular, consumption of cocoa and dark chocolate (DC) has been shown to improve endothelium function, insulin sensitivity, blood pressure (BP) in healthy individuals, hypertensives with or without glucose intolerance^(4–6) and obese subjects⁽⁷⁾. Cocoa and DC are rich sources of polyphenols providing on average more polyphenols per serving than red wine, green tea or black tea⁽⁸⁾. These polyphenols confer potent antioxidant properties to cocoa and DC^(8,9) in addition to the ability to regulate NO^(4–7).

Obesity is known to be associated with insulin resistance and elevated BP⁽¹⁰⁾. One of the underlying factors linked to these cardiovascular risk factors is abnormal cortisol metabolism^(11,12). Cortisol is a counterregulatory hormone that is essential in the long-term maintenance of blood glucose⁽¹³⁾ and which could also unfavourably influence BP and lipid profile^(12–15). When present in excess, cortisol induces overproduction of reactive oxygen species^(16,17) leading to reduced endothelial NO synthase expression⁽¹⁸⁾. In obesity, particularly abdominal obesity, postprandial hypercortisolism

and enhanced peripheral metabolism of cortisol, characterised by increased urinary cortisone-to-cortisol ratio, are observed which are linked to insulin resistance and increased fasting insulin⁽¹¹⁾. Increased expression of subcutaneous adipose tissue 11 β -hydroxysteroid dehydrogenase type 1 has also been reported, which is known to impair glucose-stimulated insulin secretion⁽¹⁹⁾. Since improved NO bioavailability is the main mechanism by which DC polyphenols reduce endothelium dysfunction, insulin resistance and hypertension^(4–7), this preliminary study aimed to assess and compare the effect of DC containing two different doses of polyphenols on fasting capillary whole blood glucose levels, total cholesterol, BP, urinary free cortisol and cortisone excretion in healthy overweight and obese subjects. The other objective was to observe whether improvements in fasting blood glucose, total cholesterol and BP could be correlated with changes in urinary free cortisol or cortisone excretion. A secondary objective was to monitor Mg intake and excretion since DC is known to contain large quantities of Mg, which, in turn, could influence BP, insulin action and the metabolic syndrome^(20–22).

Abbreviations: BP, blood pressure; DBP, diastolic BP; DC, dark chocolate; FG, fasting glucose; SBP, systolic BP.

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Methods

Study design

The study used a randomised, cross-over design where each subject acted as their own control. Following a 1-week run-in phase, eligible subjects were randomly assigned to one of the two polyphenol doses: 500 mg polyphenols DC or 1000 mg polyphenols DC. Participants followed each intervention for 2 weeks, after which they were crossed-over to the next intervention separated by a 1-week washout period (Fig. 1). The study included healthy non-smoker volunteers, aged 19–50 years with BMI ≥ 25 kg/m²(2,3), no history of diabetes, hypertension or CVD. People taking dietary supplements, BP or cholesterol-lowering drugs, or those with soya and nut allergies were excluded. Smokers were excluded to minimise confounding factors since nicotine consumption is known to enhance hypothalamic–pituitary–adrenal axis activity, hence resulting in elevated cortisol levels(24,25). Participants gave written consent, completed a lifestyle questionnaire before being screened for fasting blood glucose, total cholesterol, BP and BMI to determine their eligibility. The study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures were approved by Queen Margaret University Ethics Committee.

Diet

Table 1 provides a summary of the nutrient composition of the two Acticoa DC used in the present study, which were kindly supplied by Barry Callebaut (Lebbeke, Belgium). The 500 mg dose was previously shown to reduce fasting glucose (FG) levels and BP by Grassi *et al.*(4,5) and Taubert *et al.*(26). However, due to the great variation in epicatechin and catechin levels between the chocolate used in the present study and the one used by Grassi *et al.*(4,5), a higher DC dose of 1000 mg was also chosen. This 1000 mg dose was selected to provide similar quantities of polyphenol to what is consumed by the Kuna population of Panama, who are known to consume large quantities of cocoa and to have low incidence of hypertension(27). This dose will also provide

Table 1. Nutritional composition of 20 g of 500 and 1000 mg polyphenol dark chocolate (DC)

Component	500 mg DC	1000 mg DC
Polyphenols (mg)	500	1000
Epicatechin and catechin (mg)	18.99	37.98
Energy (kJ)	425.8	425.8
Fat (g)	7.34	7.34
Protein (g)	1.34	1.34
Carbohydrate (g)	7.44	7.44
Mg (mg)	33.42	33.42
Na (mg)	1.4	1.4
K (mg)	168.42	168.42

about 43.2% of the epicatechin and catechin dose used by Grassi *et al.*(4,5). Subjects were instructed to distribute DC doses throughout the day to achieve a high steady-state concentration. They were also instructed to maintain their usual diet throughout the study but to refrain from polyphenol-rich foods and beverages that supply ≥ 15 mg/kg epicatechin and ≥ 4 mg/l epicatechin(28–30). Subjects completed a 3-d (two weekdays and one weekend) diet and physical activity(31) diary during the run-in phase and at the end of each dietary intervention. The Photographic Atlas of Food Portion Sizes was used to assist subjects in describing their portion sizes(32). Diet diaries were validated by interviewing the subjects using a validated questionnaire(33). The diet diaries were analysed and energy, fat, protein, carbohydrate and magnesium intake were estimated using Windiet software (Windiet Research, Univation Ltd, Robert Gordon University, Aberdeen, UK). Compliance with the study's protocol was assessed by direct interviewing, returning of empty chocolate foils and assessment of diet diaries.

Measurements

To measure fasting blood glucose and total cholesterol, 12-h fasting capillary whole blood samples were obtained and analysed using a calibrated Accutrend GC system (Roche diagnostics, Mannheim, Germany). Participants were instructed to consume the last DC dose 12 h before analyses(34), avoid heavy physical activity and alcohol intake 24 h before testing and consume the same diet the day before each test(30). Waist circumference, hip circumference and BMI were measured. Data on waist and hip circumference were used to calculate waist-to-hip ratio, where waist-to-hip ratio > 1.0 in men and > 0.85 in women indicate abdominal obesity(35). Both waist circumference and BMI serve as good indicators of the degree of insulin resistance in overweight and obese individuals(35) while waist-to-hip ratio serves as a predictor of hypertension(36) and hypothalamic–pituitary–adrenal axis hyperactivity, characterised by high baseline plasma cortisol and low 24-h urinary cortisol excretion in obese women(11). An automated A&D Medical UA-767 BP monitor (A&D Medical, San Jose, CA, USA) was used to measure BP according to Grassi *et al.*(5). This monitor was previously validated and was shown to achieve grade A for both systolic and diastolic BP according to the British Hypertension Society standard(37).

Urine samples were obtained for estimating 24-h urinary Mg excretion. Urinary Na and K excretion were also

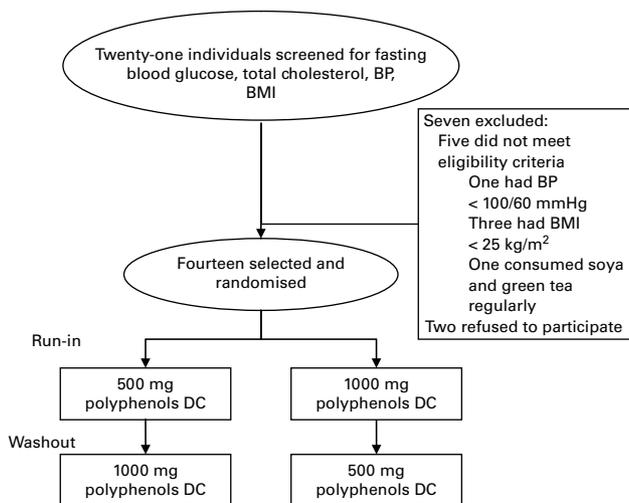


Fig. 1. Diagram showing random allocation of subjects into the different dietary interventions. BP, blood pressure; DC, dark chocolate.

monitored since they serve as direct measures of Na and K intake, which could act as confounding factors in relation to BP. The 24-h urine collections were validated by measuring creatinine excretion^(38,39). Analyses of urine Na, K and Mg concentrations were conducted using an automated platform (Olympus, Essex, UK) at the Clinical Biochemistry Laboratory, Royal Infirmary of Edinburgh, Scotland, UK. Urinary cortisol and cortisone levels were analysed in duplicates using ELISA according to the method described by Al-Dujaili & Bryant⁽⁴⁰⁾ and Al-Dujaili⁽⁴¹⁾. The data were then used to calculate urinary cortisol-to-cortisone ratio. This ratio serves as a measure of renal 11 β -hydroxysteroid dehydrogenase type 2 activity⁽⁴²⁾. Monitoring the activity of this enzyme helps detect changes in peripheral metabolism of cortisol⁽⁴²⁾. All tests were carried out at baseline, before and after each intervention.

Statistical methods

All data are expressed as means and standard deviations. Mixed between–within subjects ANOVA or split-plot ANOVA was performed for multiple comparison, where time (baseline, week 1, week 2) was the within-group variable and intervention group (500 mg, 1000 mg DC) was the between-group variable and the continuous variable were FG, SBP, DBP, BMI, weight, waist circumference, hip circumference and waist-to-hip circumference. A P -value ≤ 0.05 was considered statistically significant. Within each intervention group (500, 1000 mg DC), changes in fasting blood glucose levels, SBP and DBP were analysed using repeated measures ANOVA with Bonferroni *post hoc* tests. A separate split-plot ANOVA was also performed to detect any carry-over effects between the two interventions and to ensure changes in FG, SBP and DBP following each treatment were not affected by the sequence of DC administration (1000 mg followed by 500 mg *v.* 500 mg followed by 1000 mg). Two-tailed paired sample t tests were used to assess changes between baseline and post-intervention total cholesterol, urinary free cortisol or cortisone, urinary cortisol-to-cortisone ratio and mineral excretion. Similarly, differences in response to both DC doses among the various ethnic groups were assessed using one-way between-groups ANOVA with FG, SBP and DBP as the dependent variables and ethnicity as the factor. An independent sample t test was also used to compare the response to DC polyphenols between the abdominally obese individual and the peripherally obese individuals. The relationship between fasting blood glucose levels, total cholesterol, SBP, DBP, BMI, weight, waist circumference, hip circumference, waist-to-hip circumference, urinary Mg, Na and K levels, 24-h urinary free cortisol, cortisone and cortisol-to-cortisone ratio were assessed using Pearson product-moment correlation coefficient, r . The coefficient of determination was estimated by obtaining r^2 . All statistical analyses were performed using SPSS for Windows, version 16.0.0 (SPSS Inc., Chicago, IL, USA). The sample size was calculated using G-power software version 3.0.8 (Heinrich Heine University, Dusseldorf, Germany) to detect 0.3 mmol/l reduction in FG with baseline SD = 0.5 mmol/l and post-DC SD = 0.04 mmol/l, which is similar to the reduction reported by Grassi *et al.*⁽⁶⁾.

Results

The study included fourteen healthy volunteers (eight males (five Caucasians, two Asians, one African) and six females (five Caucasians and one Hispanic)), 21–50 years old, mean age 26.4 (SD 11.5) years) with a BMI of 27.7 (SD 2.5) kg/m². Of these participants, thirteen were peripherally obese and one was abdominally obese (African).

Mixed between–within subjects ANOVA revealed a significant reduction in fasting capillary blood glucose concentrations ($P=0.002$), SBP ($P<0.0001$) and DBP ($P<0.0001$) following DC consumption. These effects were independent of the sequence of DC administration and no significant interaction between time, intervention group and sequence of DC administration was observed (FG $F(2,11) = 1.057$, $P=0.380$; SBP $F(2,11) = 0.431$, $P=0.660$; DBP $F(2,11) = 0.653$, $P=0.539$; Figs. 2 and 3). No significant differences between the effect of 1000 and 500 mg polyphenols DC on fasting capillary blood glucose ($P>0.05$) and BP ($P>0.05$) were observed indicating that both doses have a similar efficacy.

To explore the results further, a one-way repeated measures ANOVA was conducted to compare fasting blood glucose levels, SBP and DBP at baseline, week 1 and week 2 for each of the two dietary interventions. A significant effect of DC on fasting blood glucose levels ($F(2,12) = 4.305$, $P=0.039$), SBP ($F(2,12) = 12.330$, $P=0.001$) and DBP ($F(2,12) = 13.937$, $P=0.001$) was observed after consumption of 1000 mg DC. *Post hoc* comparisons using Bonferroni test indicated that mean fasting blood glucose levels and SBP at week 2 were significantly decreased after chocolate ingestion (FG 3.97 (SD 0.54) *v.* baseline 4.42 (SD 0.70) mmol/l; SBP 112.12 (SD 9.68) *v.* baseline 119.38 (SD 10.51) mmHg). Mean DBP levels were significantly lower at week 1 (74.45 (SD 7.17) mmHg) and week 2 (74.57 (SD 7.39) mmHg) compared to baseline (78.62 (SD 7.74) mmHg).

A significant effect of 500 mg DC on FG levels ($F(2,12) = 5.026$, $P=0.026$), SBP ($F(2,12) = 11.971$, $P=0.001$) and DBP ($F(2,12) = 7.709$, $P=0.007$) was also observed. *Post hoc* comparisons indicated that the mean FG levels at week 2 were significantly different from baseline (3.92 (SD 0.86) *v.* 4.42 (SD 0.30) mmol/l). Mean SBP was also reduced at the end of week 1 (114.24 (SD 9.53) mmHg)

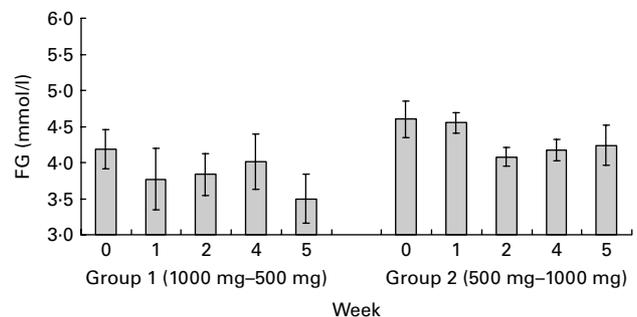


Fig. 2. Capillary fasting glucose (FG) levels at baseline (week 0), and at the end of 1 and 2 weeks of each of the polyphenols doses. Group 1 received 1000 mg polyphenols dark chocolate (weeks 1–2) followed by 500 mg polyphenols dark chocolate (weeks 4–5). Group 2 received 500 mg polyphenols dark chocolate (weeks 1–2) followed by 1000 mg polyphenols dark chocolate (weeks 4–5). Changes in FG were independent of the sequences of chocolate administration ($P>0.05$). Values are means with their standard errors represented by vertical bars.

Table 3. Results for 24-h urine collections (Mean values and standard deviations)

	1000 mg DC						500 mg DC					
	Baseline			Week 2			Baseline			Week 2		
	Mean	SD	P	n	df	P	Mean	SD	n	df	P	
Free cortisol (nmol/d)	77.33	27.09	0.477	13	12	0.477	86.83	44.05	14	13	0.620	
Free cortisone (nmol/d)	54.34	26.90	0.159	13	12	0.159	59.64	32.56	14	13	0.139	
Cortisol-to-cortisone ratio	1.6812	0.75	0.664	13	12	0.664	1.68	0.72	14	13	0.682	
Cortisone-to-cortisol ratio	0.71	0.32	0.744	13	12	0.744	0.71	0.31	14	13	0.455	
Free cortisol (nmol/kg per d)	0.98	0.37	0.400	13	12	0.400	1.0529	0.43	14	13	0.731	
Free cortisone (nmol/kg per d)	0.69	0.36	0.162	13	12	0.162	0.73	0.38	14	13	0.180	
Creatinine (mmol/l)	12.74	5.35	0.219	13	12	0.219	12.83	5.15	14	13	0.387	
Mg (mmol/l)	3.26	1.23	0.497	13	12	0.497	3.29	1.19	14	13	0.928	
Na (mmol/l)	122.07	57.90	0.802	13	12	0.802	124.35	56.28	14	13	0.644	
K (mmol/l)	58.20	22.12	0.770	13	12	0.770	61.19	24.03	14	13	0.377	

DC, dark chocolate.

Table 4. Pearson product-moment correlations between changes in urinary glucocorticoid levels and changes in selected parameters

Correlation pair	n	r	P
Δ Urinary free cortisol (nmol/kg per 24 h)			
Δ Urinary free cortisone excretion (nmol/kg per 24 h)	27	0.599	0.001
Δ 24 h urinary Na (mmol/g creatinine)	26	0.489	0.011
Δ Physical activity (kJ)	26	-0.384	0.053
Δ Urinary free cortisone (nmol/kg per 24 h)			
Δ Cortisol-to-cortisone ratio	27	-0.662	0.000
Δ Cortisone-to-cortisol ratio	27	0.628	0.000
Δ 24 h urinary Na (mmol/g creatinine)	26	0.478	0.014

significantly. Moreover, no significant correlations were found between changes in Mg intake or excretion and the reductions in fasting blood glucose and BP seen following DC consumption. Energy expenditure, energy, macronutrient and mineral intake did not change significantly through the study period (Fig. 4).

Discussion

The present study demonstrates that polyphenol-rich DC reduces fasting blood glucose levels and BP in overweight and obese individuals. These findings are consistent with previous observations that polyphenol-rich DC intake improved insulin resistance, insulin sensitivity, FG levels and BP in healthy individuals⁽⁴⁾, hypertensives⁽⁵⁾, glucose-intolerant hypertensives⁽⁶⁾ and obese subjects⁽⁷⁾. The results are also in agreement with studies on diabetic obese mice, where reductions in blood glucose and fructosamine levels were reported following consumption of cacao liquor procyanidins⁽⁴³⁾.

Enhanced vascular function is thought to be the main mechanism by which DC polyphenols improve glucose and BP homeostasis^(4-7,23,34,44-47), although other mechanisms like decreased and delayed carbohydrate digestion and absorption might also be involved^(48,49). The present study investigated whether polyphenol-rich DC could alter cortisol metabolism and whether improvements in glucose and BP seen in obese individuals following DC consumption are linked to improved cortisol metabolism. The hypothesis was based on that cortisol plays an important role in glucose and BP homeostasis, probably through a mechanism involving increased reactive oxygen species production and decreased NO bioavailability, and that in obesity several alteration in cortisol metabolism are observed, which are, in turn, linked to increased insulin resistance and hypertension. The study demonstrates that both 500 and 1000 mg polyphenol DC decrease 24-h urinary free cortisol and cortisone levels. However, these reductions were not significant and are not associated with reductions in fasting blood glucose or BP. Such findings differ from previous findings, wherein polyphenols increased⁽⁵⁰⁻⁵³⁾ or decreased^(54,55) cortisol levels⁽⁵⁶⁾. The lack of significance could be related to a number of factors. For instance, the sample size might have not been sufficiently large to detect a significant change. In this case, using several parameters of cortisol metabolism including its measurement in urine, saliva and blood might have helped detect any such effect. Additionally, the study population consisted mainly of

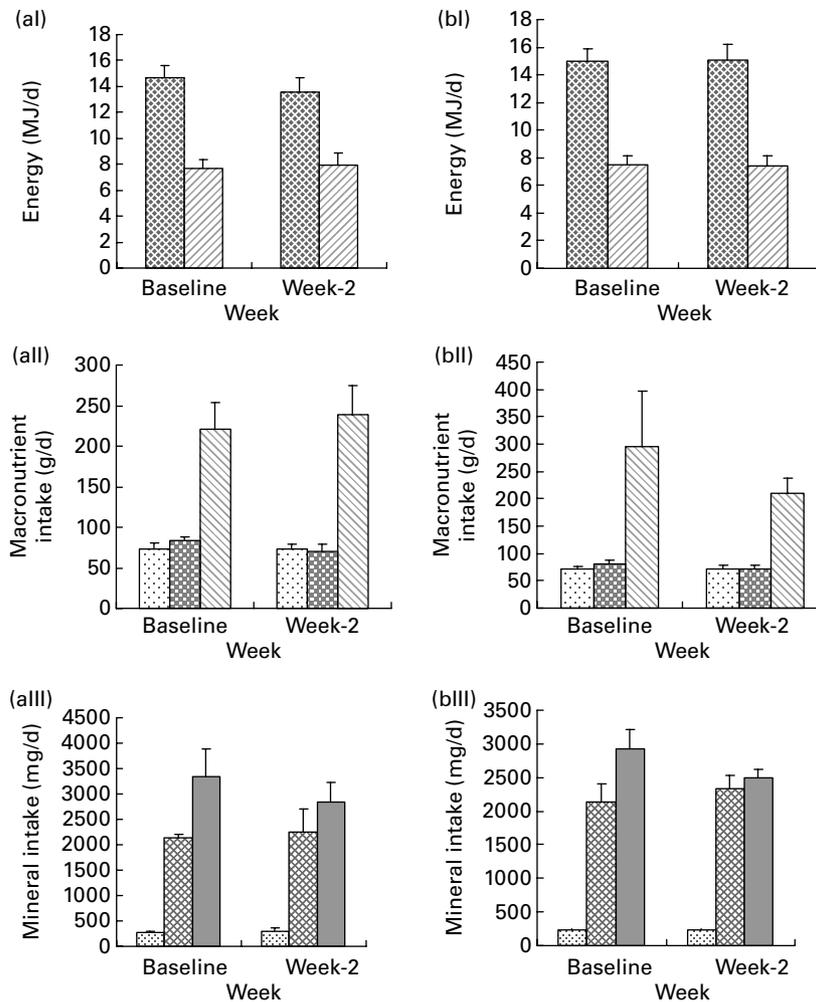


Fig. 4. Energy (I), macronutrient (II) and mineral intake (III) at baseline and at the end of each intervention: (a) 20 g dark chocolate with 1000 mg polyphenols and (b) 20 g dark chocolate with 500 mg polyphenols. Values are means with their standard errors represented by vertical bars. (▨), Energy expenditure; (▧) energy intake; (▩) fat intake; (▪) protein intake; (▫) carbohydrate intake; (▬) Mg intake; (▮) Na intake; (▯) K intake.

subjects with peripheral obesity rather than those with abdominal obesity, who exhibit more prominent abnormalities in cortisol metabolism as indicated by the association between high waist circumference or waist-to-hip ratio and high urinary cortisol or cortisone-to-cortisol ratio^(11,57). In addition, differences in Na intake were not controlled for and could have acted as confounding factors⁽⁵⁸⁾. This could be observed in the association between changes in urinary free cortisol or cortisone and changes in Na excretion, and the association between changes in Na intake and changes in cortisol-to-cortisone ratio. Dietary factors have been reported to influence cortisol metabolism. High-fat low-carbohydrate diets stimulate cortisol regeneration by 11 β -hydroxysteroid dehydrogenase type 1, while reducing cortisol inactivation in liver⁽⁵⁹⁾. Na loading, on the other hand, decreases plasma cortisol levels by enhancing cortisol elimination⁽⁶⁰⁾ possibly via a mechanism involving increased hepatic blood flow⁽⁶¹⁾. The latter may explain the association between increased urinary free cortisol excretion and urinary Na levels. However, subjects did not report significant changes in Na intake during the study, which overall suggest that DC polyphenols influence glucose and BP homeostasis mainly via the NO pathway.

The present study demonstrates that DC with 500 mg polyphenols is as effective in reducing fasting blood glucose levels in overweight and obese individuals as 1000 mg polyphenol DC with a similar macronutrient composition. Furthermore, the results indicate that DC polyphenols reduce blood glucose levels after 2 weeks of commencing a polyphenol-rich DC diet. These findings are important since in relation to glucose metabolism, inconsistencies still exist regarding the treatment duration and dose required to achieve a glucose-lowering effect. For example in their pilot study, Stote *et al.*⁽⁶²⁾ failed to show any significant improvement in glucose levels, insulin resistance and insulin sensitivity following 5 d of twice daily consumption of procyanidin-rich cocoa beverage containing 22–900 mg procyanidins by insulin-resistant men and women. Similarly, Taubert *et al.*⁽³⁴⁾ failed to demonstrate any improvement in glucose or insulin levels following 18 weeks of daily ingestion of 6.3 g DC containing 30 mg polyphenols. Conversely, Davison *et al.*⁽⁷⁾ showed reduced insulin resistance following consumption of a cocoa beverage containing 902 mg flavanols twice daily for 12 weeks in overweight and obese subjects. Together, these findings suggest that a longer duration and a higher dose of

polyphenols could be required to achieve a significant reduction in glucose levels. The present study reinforces this hypothesis while demonstrating that increasing polyphenol dose does not necessarily result in further reductions in glucose and BP levels since a saturation effect may occur with increasing DC polyphenol content⁽⁶³⁾. It also highlights the need to identify the minimum polyphenol dose at which maximal health benefits could be achieved, since a reduction in the polyphenol content of chocolate implies reduced bitterness^(64,65), which could render the chocolate more palatable and acceptable to the general population. In relation to BP, 20 g DC with 500 mg polyphenols reduced SBP and DBP to a similar extent as 20 g DC with 1000 mg polyphenol. Moreover, the reduction in BP observed following the 20 g DC (500 mg polyphenols) was comparable to the reduction reported in a previous study on normotensive subjects (7 and 3 mmHg reduction in SBP and DBP, respectively)⁽⁴⁾. This might suggest that reducing the portion of DC while maintaining a similar total phenol content results in equivalent reductions in BP. This could provide several advantages since reducing the portion of DC would permit delivery of high quantity of polyphenols in a less energy-dense form, which is essential if DC is to be included as part of a healthy balanced diet.

In contrast to Fraga *et al.*⁽⁶⁶⁾ and Grassi *et al.*⁽⁵⁾, we did not observe any significant changes in total cholesterol. Such results are to be expected since our subjects had normal baseline total blood cholesterol levels as compared to Grassi *et al.*⁽⁵⁾ (baseline total cholesterol = 5.4 (SD 0.6) mmol/l). Moreover, Grassi *et al.*⁽⁵⁾ suggested that both the catechin and the fat component of DC account for its beneficial effect on total cholesterol. Similar assumptions were made in relation to stearic acid in DC⁽⁶⁷⁾. Since in the present study a lower DC portion was used, the lack of significant change in total blood cholesterol could be related to the lower levels of linoleic, oleic and stearic acids present in this DC. There was also a lack of correlation between the reported energy intake and physical activity, which is similar to the findings of Davison *et al.*⁽⁷⁾, who argued that obese individuals might underreport energy intake and overreport physical activity.

In conclusion, the present study confirms previous reports of improved FG levels and BP following DC consumption. It also demonstrates that these effects do not appear to be mediated through changes in cortisol metabolism. Further studies are needed to identify the optimal dose of polyphenols required to improve glucose metabolism and to examine additional parameters that could be influenced by polyphenols.

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