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**EFFECTS OF HIGH FLAVANOL DARK CHOCOLATE ON CARDIOVASCULAR
FUNCTION AND PLATELET AGGREGATION**

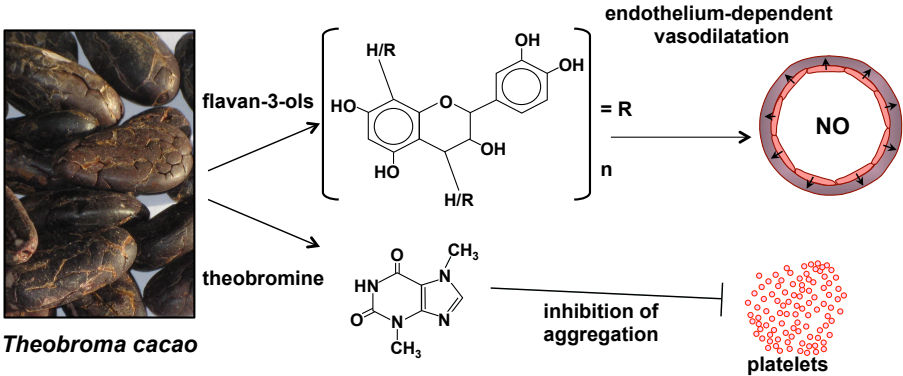
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Graphical Abstract



Abstract

Regular consumption of chocolate and cocoa products has been linked to reduced cardiovascular mortality. This study compared the effects of high flavanol dark chocolate (HFDC; 1064mg flavanols/day for 6 weeks) and low flavanol dark chocolate (LFDC; 88mg flavanols/day for 6 weeks) on blood pressure, heart rate, vascular function and platelet aggregation in men with pre-hypertension or mild hypertension. Vascular function was assessed by pulse wave analysis using radial artery applanation tonometry in combination with inhaled salbutamol (0.4 mg) to assess changes due to endothelium-dependent vasodilatation. HFDC did not significantly reduce blood pressure compared to baseline or LFDC. Heart rate was increased by LFDC compared to baseline, but not by HFDC. Vascular responses to salbutamol tended to be greater after HFDC. Platelet aggregation induced by collagen or the thromboxane analogue U46619 was unchanged after LFDC or HFDC, whereas both chocolates reduced responses to ADP and the thrombin receptor activator peptide, SFLLRNamide (TRAP6), relative to baseline. Pre-incubation of platelets with theobromine also attenuated platelet aggregation induced by ADP or TRAP6. We conclude that consumption of HFDC confers modest improvements in cardiovascular function. Platelet aggregation is modulated by a flavanol-independent mechanism that is likely due to theobromine.

Keywords: platelet, PDE inhibitor, blood pressure, heart rate, pulse pressure amplification ratio

Chemical compounds studied in this article:

theobromine (PubChem CID 5429); prostacyclin (PubChem CID 5280427)

1. Introduction

Regular consumption of dark chocolate has been linked to a lower incidence of myocardial infarction and stroke, and to an overall reduction in cardiovascular mortality [1-4]. Such observations are consistent with reports that cocoa products lower blood pressure [5-8], improve endothelium-dependent vasodilator responses [7-13], and inhibit platelet aggregation [12-18].

The actions of cocoa and dark chocolate have been attributed primarily to the high level of flavan-3-ols. However, the flavanol content of cocoa products is highly variable depending on post-harvest treatment of cocoa beans. Fermentation, drying and roasting reduce total flavanol content, with the result that commercial dark chocolate typically contain <1% flavanols by weight [19,20]. Cocoa flavanols are composed of monomers, primarily (-)-epicatechin (10 – 40% total flavanols), and procyanidins, which are short oligomers composed of epicatechin and catechin units, mainly dimer to decamer (20 – 80% total flavanols) [19,20]. Cocoa products also contain high levels of methylxanthines, theobromine (0.5 – 2.0%) and caffeine (0.03 – 0.2%) [20], which may act as phosphodiesterase inhibitors or adenosine receptor antagonists [21-23]. The specific contributions of flavanols and methylxanthines components to the cardioprotective effects of regular cocoa or dark chocolate consumption have yet to be fully defined.

Patient and volunteer studies of the effects of dark chocolate and cocoa on blood pressure, endothelial function, and platelet aggregation, have often lacked adequate blinding because inappropriate placebo products (such as white chocolate) were used, which lack flavanols and theobromine [5-7,9,12,14-18,24]. Here to investigate the role of flavanols in mediating the actions of dark chocolate on blood pressure and platelet function we compared responses to a high flavanol dark chocolate (HFDC) with a placebo low flavanol dark chocolate (LFDC) in a double-blind crossover study in men with pre-hypertension or mild hypertension.

2. Methods

2.1 Study Design and Participants

The study was a single centre randomized double-blind placebo controlled investigation with a crossover design. Ethical approval for the study was obtained from the NHS Research Ethics Service (East London and the City Research Ethics Committee; REC reference number: 07/Q0605/44). Volunteer participants were recruited through the east London GP network using a database search for men with recently diagnosed untreated mild hypertension (SBP 140 – 159 mmHg; DBP 90 – 99 mmHg) or prehypertension (SBP 120 – 139 mmHg; DBP 80 – 89 mmHg). Potential participants were sent a letter describing the study, which invited them to be screened for eligibility. Pre-recruitment screening involved clinic measurement of blood pressure, and 24 h ambulatory blood pressure measurement (ABPM) to verify that daytime blood pressure was consistent with prehypertension or mild hypertension. Additional criteria were aged >45 years and <70 years, otherwise healthy and free of other disease (confirmed by physical examination, 12-lead ECG, and routine blood chemistry including liver function tests).

Specific exclusion criteria were: diabetes mellitus or raised fasting blood glucose; serum cholesterol >7 mmol/L; BMI >30 kg/m²; history of cardiac arrhythmias or ECG abnormalities at baseline; history of psychiatric or psychological illness; history of hypo- or hyperthyroidism; participation in another trial whether active or in follow-up; abnormal liver function tests or other routine blood chemistry; excessive alcohol consumption; regular medicines for any condition; regular use of herbal medicines or other alternative remedies. For those recruited to the study the following additional requirements were set: no red wine consumption during the study; no aspirin or other non-steroidal anti-inflammatory agents for 4 weeks before the study or during the study (allowable pain relief paracetamol); use of any medicines during the study to be recorded. Men meeting the recruitment criteria and electing

to participate in the study were randomized to consume HFDC (50 g/day; 25 g each morning and 25 g in the afternoon) or LFDC (50 g/day) of similar taste and appearance for 6 weeks. At the end of this period participants were crossed over to receive the other chocolate for 6 weeks.

A cohort size of 32 participants was chosen based on based on previous reports of blood pressure decreases of 5 mmHg after dark chocolate consumption [7]. With a crossover design this gave 97% power to measure a difference of 5 mmHg with a significance of 0.05 after HFDC compared to LFDC, and 80% power to detect a change in any variable by more than 0.5 of the standard deviation of its measurement.

2.2 Dark Chocolate Products and Randomization

HFDC and LFDC were from Barry Callebaut (Lebbeke-Wieze, Belgium). The compositions of the two chocolates are shown in table 1. The randomisation schedule was sent as a password-protected file to Barry Callebaut, who prepared separate patient coded boxes for each phase of the study (50 g bars x 42 per box, plain foil wrapped). The investigators were blinded to the randomisation schedule. Flavanol doses for LFDC (88 mg/day) and HFDC (1064 mg/day) were chosen based on published observations that ≈ 80 mg total flavanols/day was without significant effect on endothelial function in previous studies, whereas large doses of ≈ 900 mg/day produce a consistent and marked increase in endothelial function [10].

2.3 Study measurements

The main study measurements were made at baseline, and at 6 and 12 weeks – after completion of each phase of dark chocolate consumption. Blood samples were collected for full lipid profile at baseline, 3, 6, 9 and 12 weeks. Three-week intervals were used to ensure that any changes in plasma lipid profiles reached steady state [25]. Platelet aggregation responses were assessed at baseline, 6 and 12 weeks. Blood pressure and heart rate were recorded as the mean of three measurements using an Omron BP Monitor 705CP II at

baseline, 3, 6, 9 and 12 weeks. At 6 and 12 weeks pulse wave analysis (PWA) was undertaken by radial artery tonometry using a Millar tonometer in conjunction with SphygmoCor Px Model software analysis to derive haemodynamic indices. Pulse pressure amplification ratio (PPA) was expressed as the ratio of central pulse pressure (CPP) to peripheral (brachial) pulse pressure (PPP). Changes in vascular function as a result of endothelium-dependent vasodilatation were assessed by PWA at 10 and 15 min after inhalation of salbutamol (0.4 mg) [26]. ABPM recordings (24 h) were made during participant screening and at 6 and 12 weeks using a Spacelabs ABP monitor 90207 (Dolby UK, Stirling). Twenty-one subjects had ABPM recordings at both 6 and 12 weeks (reasons for missing recordings: failure to attend the clinic on the required day, or BP monitor recording failure at either 6 or 12 weeks). Caffeine and theobromine were measured by HPLC following published methodology [27]. Plasma lipids (total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides) were measured by the Pathology Laboratories at Barts Health NHS trust.

2.4 Platelet Function

Platelet aggregation with simultaneous measurement of responses to a range of agonist concentrations was performed as previously described using a 96-well plate methodology [28]. Blood was collected by venepuncture into tri-sodium citrate (3.2%, 1:9 v/v; Sigma, Poole, Dorset, U.K.) and centrifuged (175 x g, 15 min) to obtain platelet-rich plasma (PRP). At baseline, 6 and 12 weeks, platelet aggregation responses were assessed by transferring samples of PRP (100 µL/well) into a 96-well plate (Nunc, Lutterworth, Leicestershire, U.K.) containing 10 µL of vehicle or agonist: adenosine diphosphate (ADP; 0.1 – 30 µM; LabMedics, Salford, Manchester, U.K.), collagen (0.1-30 µg/ml; Nycomed, Linz, Austria), the PAR-1 activator peptide TRAP6 (SFLLRNamide; 0.1 – 30 µM; Bachem, Bubendorf, Switzerland), or the stable TXA₂-mimetic U46619 (0.1 – 30 µM; Cayman Chemical Company, Ann Arbor, MI, U.S.A.). The plate was placed into a 96-well plate reader (Tecan

Sunrise) at 37°C, and absorbance was measured at 595 nm every 15 s for 16 minutes with vigorous shaking between readings. Percentage aggregation was calculated with reference to the absorbance of platelet-poor plasma (obtained by centrifugation of PRP at 15,000 g for 2 min) as a surrogate for 100% aggregation. For studies of the effect of theobromine blood samples were collected from healthy volunteers. PRP was pre-incubated for 30 min with theobromine (Sigma, Poole, Dorset, U.K.) at the concentrations indicated. All graphs show aggregation responses at 10 min.

2.5 Statistical Analyses

Twenty-six subjects completed both phases of the 12-week study. Data were analyzed by using MiniTab or GraphPad Prism (GraphPad Software Inc, CA, USA) software. Results are reported as mean \pm SEM. Statistical differences for mean data obtained from PWA were analysed by two way ANOVA using MiniTab General Linear Model. Statistical analyses for platelet studies were conducted using GraphPad Prism. Concentration-response curves were compared by two way ANOVA with Bonferroni post-hoc tests.

3. Results

3.1 Blood pressure and heart rate changes

The study population was generally middle aged, and mainly pre-hypertensive based on daytime ambulatory blood pressure measurements (Table 2). No significant changes in systolic or diastolic blood pressure were observed after HFDC or LFDC, whether measured by 24 h ambulatory BP recording or in the clinic. There was a tendency for lower SBP and MAP (\approx -2 mmHg) after HFDC for both overall and daytime ambulatory BP measurements, but this did not reach significance. Heart rate was increased after LFDC by \approx 4 bpm compared to pre-study values. After HFDC it was not different from baseline and significantly less than after LFDC (Tables 3 and 4).

3.2 Pulse wave analyses and response to salbutamol

Basal measurements of peripheral and central SBP, DBP and PP showed no significant differences after HFDC compared to LFDC (Table 4 and Figure 1). Augmentation pressure (AP) and augmentation index (AI) were also not significantly different. Heart rate, difference between peripheral and central SBP (PSBP – CSBP), and PPA were all significantly lower after HFDC compared to values after LFDC (Table 4 and Figure 1). Salbutamol causes endothelium-dependent vasodilatation [26]. Consistent with this, peripheral DBP, and central SBP and DBP decreased significantly after salbutamol (Figure 1). Salbutamol tended to have greater effects on blood pressure measurements after HFDC but these changes did not reach significance (Figures 1 and 2). AP and AI were reduced by salbutamol but HFDC did not significantly modify these responses. Basal PSBP – CSBP and PPA were lower after HFDC compared to LFDC (Table 4). Salbutamol increased PSBP – CSBP and PPA with a marginally significant difference remaining after HFDC compared to LFDC (Figure 1). For PPA the average increases after salbutamol from basal (expressed as % change) were greater after HFDC (10 min and 15 min; HFDC: 6.06 ± 1.22 and 6.51 ± 1.20 ; LFDC: 3.81 ± 1.11 and 4.10 ± 1.20 ; $P = 0.005$) (Figure 2). Heart rate was unaffected by salbutamol and remained significantly lower after HFDC (Figure 1).

3.3 Platelet aggregation

Platelet aggregation responses after consuming HFDC for 6 weeks were not significantly different from those recorded after LFDC (Figure 3). However, while neither HFDC nor LFDC had any effect on the responses to collagen or U46619 compared to baseline (Figure 3a and 3b), both HFDC and LFDC suppressed significantly platelet aggregation induced by ADP ($P = 0.01$) and TRAP6 ($P = <0.001$) relative to baseline responses (Figure 3c and 3d). Based on the areas under the curve for 3, 10 and 30 μM agonist concentrations, HFDC and LFDC reduced ADP responses by $\approx 30\%$ and TRAP6 responses by $\approx 60\%$.

Both chocolates contained similar levels of theobromine and caffeine (Table 1), which can act as phosphodiesterase (PDE) inhibitors [21], a well-established mechanism for inhibiting platelet aggregation [29]. Theobromine plasma levels were comparable after HFDC and LFDC, and significantly greater than baseline (Table 5). To test whether the inhibitory effect of HFDC and LFDC on ADP and TRAP6 responses was due to actions of methylxanthines PRP was pre-incubated with theobromine before incubation with ADP and TRAP6. Theobromine (2 – 50 µg/ml) significantly reduced platelet aggregation observed with ADP ($P = <0.001$) but not with TRAP6 (Figure 4a and 4b). To test whether the anti-aggregatory effects of theobromine were mediated via cAMP the inhibitory effect of prostacyclin was assessed in the presence and absence of theobromine. Theobromine caused a significant leftward shift of the prostacyclin curve using ADP as the agonist for stimulating platelet aggregation ($P = 0.028$), representing an approximately 2-fold increase in the sensitivity to prostacyclin (Figure 4c). Similar results were observed using TRAP6 as the stimulus for platelet aggregation ($P = 0.005$) (Figure 4d).

3.4 Serum Lipids

No changes were observed in total cholesterol, LDL-cholesterol, HDL-cholesterol or triglycerides after HFDC or LFDC (Table 5).

4. Discussion

4.1 Overview

The age-related increased risk of an atherothrombotic event – myocardial infarction or stroke – is generally viewed as a complex relationship evolving over many years. It is frequently linked to deterioration in vascular function, increased prevalence of proinflammatory changes, raised blood pressure, dyslipidaemia, and platelet thrombogenicity. These are all changes that cocoa flavanols may modulate [1-18]. Here, HFDC tended to lower SBP and

improved vascular function, compared to LFDC, but the effects generally failed to reach significance. Increased flavanol intake reduced heart rate relative to LFDC – an effect that could potentially modify cardiovascular risk. Both HFDC and LFDC suppressed platelet aggregation, probably as a result of theobromine acting as a PDE inhibitor, rather than through an effect of flavanols.

4.2 Effects of flavanols on blood pressure and heart rate

A number of studies have investigated the effect of dark chocolate or cocoa on blood pressure. A meta-analysis of 20 studies of mainly healthy volunteers, reported a mean reduction in SBP of -2.77 mmHg (95% CI -4.72, -0.82; P = 0.005), and DBP of -2.20 mmHg (95% CI -3.46, -0.93; P = 0.006) [24]. The effect was greater for trials that compared active product to a flavanol free control product (SBP -3.70 mmHg, 95% CI -6.02, -1.36, P = 0.002; DBP -2.71 mmHg, 95% CI -4.26, -1.50, P = <0.001) [24]. But most investigations using flavanol free placebo products were poorly controlled, and not double-blinded. In ten studies where blinding was achieved by matching a low flavanol control product to the active high flavanol product, the meta-analysis showed no significant changes in blood pressure (SBP -0.71 mmHg, 95% CI -2.99, 1.57; DBP -0.78 mmHg, 95% CI -2.26, 0.70) [24]. This is consistent with results described here where a trend for SBP reduction of ≈ 2 mmHg was observed with HFDC compared to LFDC, and DBP was unchanged. A reduction in SBP of 2 mmHg would be medically important if sustained over time and reproducible across a larger population as this could equate to a reduction in stroke risk of $\approx 10\%$ [30].

Compared with baseline values, LFDC significantly increased heart rate. This is likely an effect of methylxanthines (theobromine and caffeine), which cause sympathetic activation primarily through adenosine receptor blockade [22,23]. Heart rate was not increased with HFDC even though it had similar methylxanthine content, indicating that flavanols modulate sympathetic activation. In agreement with this, treatment for six months with a diet enriched

in cocoa flavanols resulted in $\approx 25\%$ lower heart rate in a strain of rabbits, which are genetically prone to hypercholesterolaemia, compared to age-matched control rabbits of the same strain [31]. This difference in heart rate was attributed to cocoa flavanols preventing the decline in parasympathetic function observed in control hypercholesterolaemic animals [31]. Here, a flavanol-dependent increase in parasympathetic tone, suppressing methylxanthine-induced sympathetic activation, may explain the lower heart rate after HFDC compared with LFDC.

Elevated resting heart rate, with increased sympathetic activation, reduced vagal tone, and delayed heart rate recovery after exercise are associated with increased mortality particularly in patients with pre-existing CVD [32,33]. Hence, flavanol-induced improvement in sympathovagal balance may be a previously unrecognised beneficial effect associated with consumption of flavanol-rich cocoa products.

4.3 Pulse wave analysis

The difference between central and peripheral PP plays a critical role in cardiovascular risk [34,35]. Previous investigations of the effects of antihypertensive drugs on parameters derived from PWA indicate that reduced CSBP and CPP are associated with improved outcome [36]. Reduced risk after anti-hypertensive therapy is associated with increased PPA, mainly as a result of the vasodilator effects of antihypertensive agents. Here PPA was decreased, which might be considered detrimental. This was likely due to the reduction in heart rate as it leads to delayed timing of the reflected wave, which then merges with PP and increases AP [34]. Consistent with this PPA was inversely correlated with AP ($r^2 = 0.78$, data not shown). Summation of the reflected wave with central PP is thought to adversely affect cardiac function by prolonging ejection duration and increasing afterload [34]. However, the decrease in PPA after HFDC may be an anomalous observation. Athletes also have lower HR and reduced PPA, which is attributed to peripheral vasodilatation, reduced wave reflection and

lower pulse wave velocity [37,38]. Indeed, previous studies have reported a reduction in pulse wave velocity after dark chocolate [39,40], which in combination with lower heart rate could have contributed to the reduction in PPA after HFDC.

After HFDC salbutamol tended to have a greater effect on most parameters measured, but only PPA increased significantly more (Figure 2, $P = 0.005$). Nevertheless this indicates greater salbutamol-induced vasodilatation after HFDC. Based on previous studies and results described here, flavanols have only a modest effect on blood pressure, despite improving endothelium-dependent vasodilator responses (flow-mediated dilatation or salbutamol) [7-13], and reducing vascular stiffness (assessed by pulse wave velocity) [39,40]. These actions may have particular relevance for preventing cardiovascular aging, as reduced endothelium-dependent vasodilatation is associated with increased risk of hypertension in elderly subjects [41,42]. The degree to which cocoa flavanols increase vascular compliance and decrease cardiovascular disease risk, particularly in relation to increasing age, requires long-term studies in larger cohorts to assess whether such effects are sustained or develop further over time.

4.4 Platelet aggregation

ADP, collagen, the PAR-1 activator peptide TRAP6, and the stable TXA_2 -mimetic U46619 were used to stimulate platelet aggregation as these agonists are widely used for *in vitro* platelet testing [28]. Hypertension is frequently associated with a prothrombotic state with increased platelet thrombogenicity [43]. Previous studies of platelet aggregation after consumption of high flavanol chocolate and cocoa have ascribed anti-platelet effects to flavanols [13-18]. However, many of these studies were poorly controlled using low flavanol placebo products devoid of theobromine [14-18].

Here, platelet aggregation induced by thrombin-receptor activation or ADP was attenuated to a similar degree after consumption of LFDC or HFDC, compared to baseline responses.

Stimulation of platelets with ADP or thrombin inhibits adenylate cyclase activity and increases phosphodiesterase (PDE) activity in platelets, allowing these platelet agonists to override the inhibitory effect of cAMP [44]. Hence PDE inhibitors, including methylxanthines, reduce ADP and PAR1 responses and are potent suppressors of platelet aggregation [29]. As HFDC and LFDC contained comparable levels of theobromine, it seemed likely that PDE inhibition accounted for the decrease in platelet responses. However, addition of theobromine to platelet-rich plasma caused only modest reductions in ADP and TRAP6 responses, which only reached significance with ADP. Nevertheless, it augmented the effect of prostacyclin, confirming that theobromine promotes cAMP-dependent decreases in platelet aggregation via PDE inhibition. The inhibitory profile of theobromine is very similar to the nonselective PDE inhibitor pentoxifylline and the selective PDE3 inhibitor cilostazol, which have limited effect alone, yet show markedly increased inhibition of platelet aggregation in the presence of prostacyclin [29].

A number of other factors may have reduced the effectiveness of theobromine when added directly to PRP, compared to the observed reductions in ADP and TRAP6 responses after LFDC or HFDC consumption for 6 weeks. Theobromine has poor lipid solubility so that 30 min pre-incubation may be insufficient for transmembrane equilibrium to be reached.

Maximum PDE inhibition may require daily consumption of high levels of theobromine in order to reach high steady state levels in platelets, with high levels in portal blood after gastrointestinal absorption leading to greater changes in platelet function. Theobromine may also confer a greater anti-aggregatory action in the circulation, and in collected blood samples, by increasing and then preserving platelet cAMP and cGMP generated through exposure to endothelial prostacyclin and nitric oxide. This phenomenon has been observed with sildenafil, which has no effect when added directly to PRP, but significantly inhibits *ex vivo* platelet aggregation after oral dosing [29].

Based on results described here, evidence that chocolate consumption is associated with decreased risk of stroke may well be as much due to theobromine as to the presence of flavanols, since milk chocolate consumption is also associated with reduced cardiovascular mortality [4]. Indeed, PDE inhibition with theobromine may well be sufficient to reduce atherothrombotic events as the selective PDE3 inhibitor cilostazol reduces stroke risk [29]. Studies of chocolate consumption indicate regular daily intake of ≈ 10 g/day is sufficient to confer a benefit [1-4]. HFDC and LFDC both contained $\approx 0.8\%$ theobromine, which is comparable to other dark chocolates (0.5 – 1.6%) [20]. Milk chocolates also contain theobromine (0.1 – 0.2%) [20]. But the threshold daily amount of theobromine to modulate platelet function requires further investigation. Studies of coffee consumption, where caffeine is clearly a key bioactive, have also shown a reduced risk of CHD mortality and ischaemic stroke in habitual coffee drinkers [45-47]. When these findings are considered in conjunction with observations from chocolate studies, this suggests that daily intake of methylxanthines is a key dietary factor modulating platelet function.

4.5 Changes in plasma lipid profile

Cocoa fat is composed of approximately 33% stearic acid, 25% palmitic acid, and 33% monounsaturated fat, and has a largely neutral effect on cholesterol levels [48]. In agreement with this, total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides were unchanged during 12 weeks consumption of HFDC and LFDC.

4.6 Limitations of the study

The study was underpowered for confirming blood pressure lowering effects of flavanols. Measurement of pulse wave velocity as an index of vascular stiffness would have complemented PWA measurements after HFDC and LFDC, and following salbutamol challenge. Comparison of flow-mediated vasodilatation with responses to salbutamol would have provided additional insight into the changes in endothelial function after HFDC. The

effects of theobromine on heart rate, blood pressure, and platelet function are important confounders when attempting to discriminate the effects of the flavanol constituents of dark chocolate.

5. Conclusions

Clinical trials of blood pressure lowering drugs show consistent reductions in the incidence of stroke [49]. However, there is still scope for better treatment of hypertension, particularly in the elderly with systolic hypertension where endothelial dysfunction is an unmet clinical need [41,50]. In addition, exaggerated blood pressure responses during exercise with reduced heart rate recovery post exercise are associated with increased cardiovascular mortality, due to endothelial dysfunction and sympathetic activation [32,33]. Hence, new insights into the extent flavanols modify cardiovascular risk may be obtained by investigating effects on responses to exercise.

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Table 1. Composition of high and low flavanol dark chocolate bars

	HFDC	LFDC
Total cocoa solids (%)	65	65
Flavanols:		
monomers, mg	223	23
procyanidin dimers, mg	192	24
procyanidin trimers to decamers, mg	649	41
total flavanols (monomers to decamers), mg	1064	88
Theobromine, mg	395	420
Caffeine, mg	35	35
Total fat, g	19.9	18.8
saturated, g	12.4	11.7
monounsaturated, g	6.9	6.6
polyunsaturated, g	0.6	0.5
Total protein, g	2.8	2.9
Available carbohydrates, g	18.2	18.8
sugars, g	15.8	16.4
starch, g	2.4	2.4
Dietary fibre, g	3.0	3.1
Calories, kcal	264	257

Values are per 50 g bar (the daily amount consumed during the HFDC and LFDC phases of the study).

Table 2 Characteristics of the study population at recruitment

Age (years)	55.4 ± 1.5
Wt (kg)	80.1 ± 3.0
BMI (kg/m ²)	26.6 ± 0.5
Pre-/mild hypertension	20/6
SBP (mmHg)	
24 h	134.6 ± 1.4
day	137.2 ± 1.5
night	123.6 ± 1.7
DBP (mmHg)	
24 h	85.2 ± 1.1
day	87.2 ± 1.1
night	76.1 ± 1.5
MAP (mmHg)	
24 h	102.2 ± 1.0
day	104.4 ± 1.0
night	92.2 ± 1.5
HR (bpm)	
24 h	74.4 ± 1.9
day	75.4 ± 1.9
night	69.0 ± 2.5

Table 3 Comparison of 24 h ambulatory blood pressure and heart rate measurements after HFDC and LFDC compared to pre-study recordings (n = 21).

	Pre-Study	HFDC	LFDC	
SBP (mmHg)				
24-h	135.2 ± 1.7	133.1 ± 1.9	134.1 ± 1.9	
day	137.6 ± 1.6	135.4 ± 2.0	136.8 ± 1.9	
night	124.2 ± 2.0	124.1 ± 2.7	122.2 ± 2.2	
DBP (mmHg)				
24-h	85.1 ± 1.3	84.0 ± 2.0	84.9 ± 1.4	
day	87.1 ± 1.3	85.7 ± 2.0	87.0 ± 1.4	
night	76.3 ± 1.7	76.9 ± 2.2	75.0 ± 1.7	
MAP (mmHg)				
24-h	102.3 ± 1.3	100.5 ± 1.9	101.7 ± 1.4	
day	104.4 ± 1.2	102.3 ± 1.9	103.9 ± 1.4	
night	92.3 ± 1.7	93.0 ± 2.3	90.9 ± 1.8	
HR (bpm)				
24-h	72.9 ± 2.2	73.1 ± 2.1 ^a	77.2 ± 2.3 ^b	ANOVA P = 0.004
day	73.8 ± 2.0	75.1 ± 2.1 ^c	79.2 ± 2.1 ^d	ANOVA P = 0.001
night	67.5 ± 2.9	68.5 ± 2.8	69.7 ± 3.2	

Mean data from 21 subjects with ambulatory blood pressure measurements at all three time points.

^a P = 0.005 vs LFDC, ^b P = 0.008 vs pre-study, ^c P = 0.002 vs LFDC, ^d P = 0.001 vs pre-study.

Table 4 Basal brachial artery (peripheral) measurements and aortic (central) values derived from pulse wave analyses after 6 weeks of HFDC and LFDC prior to salbutamol administration (n = 26).

	HFDC	LFDC
Peripheral systolic blood pressure (PSBP, mmHg)	132.5 ± 2.0	133.4 ± 1.6
Peripheral diastolic blood pressure (PDBP, mmHg)	81.3 ± 1.3	80.9 ± 1.3
Peripheral pulse pressure (PPP, mmHg)	51.2 ± 1.5	52.5 ± 1.6
Peripheral mean artery blood pressure (PMAP, mmHg)	99.5 ± 1.6	99.7 ± 1.3
Central systolic blood pressure (CSBP, mmHg)	121.9 ± 2.1	121.5 ± 1.5
Central diastolic blood pressure (CDBP, mmHg)	82.4 ± 1.4	82.4 ± 1.3
Central pulse pressure (CPP, mmHg)	39.5 ± 1.6	39.1 ± 1.3
Heart Rate (HR, bpm)	67.3 ± 2.2 ^a	71.0 ± 2.4
Augmentation Pressure (AP, mmHg)	10.3 ± 1.1	9.4 ± 0.9
Augmentation Index (AI, %)	20.7 ± 2.1	21.5 ± 1.8
PSBP – CSBP (mmHg)	10.6 ± 0.6 ^b	11.9 ± 0.9
Pulse pressure amplification ratio (PPA)	1.31 ± 0.03 ^c	1.36 ± 0.03

Paired t-test comparison of basal values HFDC v LFDC: ^a P = 0.022; ^b P = 0.029; ^c P = 0.031

Table 5 Comparison of methylxanthine levels and lipid profiles at baseline with average values after HFDC and LFDC. Methylxanthine measurements were made on plasma samples collected after 6 weeks consumption of the respective products. Lipid values for HFDC and LFDC are the means of values obtained after 3 and 6 weeks consumption of each product

	Baseline	HFDC	LFDC
Theobromine ($\mu\text{g/ml}$)	0.77 ± 0.14	$4.49 \pm 0.48^{\text{a}}$	$4.34 \pm 0.51^{\text{a}}$
Caffeine ($\mu\text{g/ml}$)	1.24 ± 0.19	1.31 ± 0.20	1.28 ± 0.19
Cholesterol (mmol/L)	5.71 ± 0.19	5.60 ± 0.14	5.70 ± 0.16
LDL-C (mmol/L)	3.53 ± 0.15	3.44 ± 0.14	3.48 ± 0.13
HDL-C (mmol/L)	1.57 ± 0.06	1.56 ± 0.05	1.57 ± 0.06
Triglycerides (mmol/L)	1.36 ± 0.10	1.39 ± 0.11	1.45 ± 0.11

^a, P = <0.001 compared to baseline

FIGURES

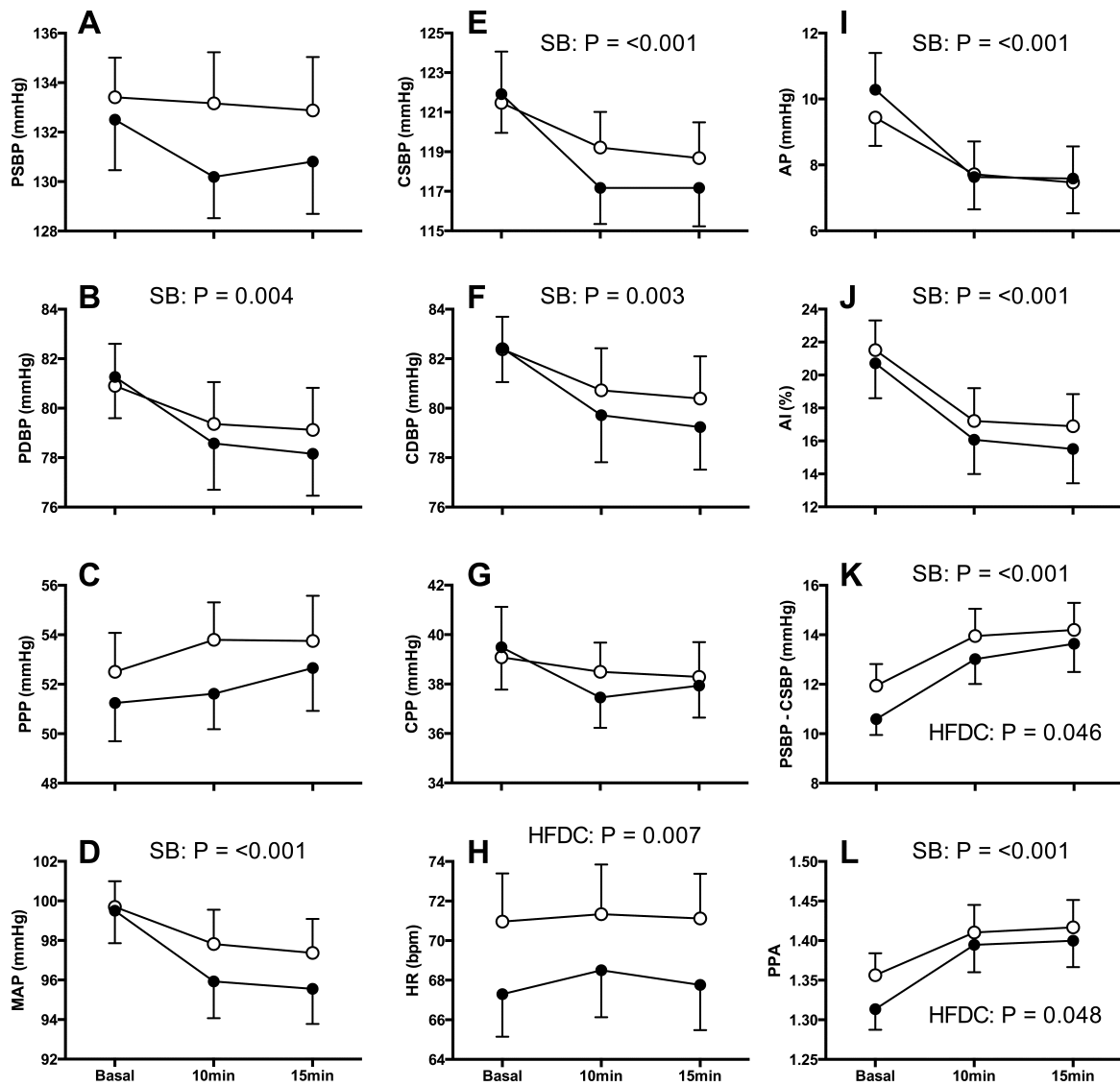


Figure 1 Effect of 6 weeks daily consumption of HFDC and LFDC on responses to salbutamol determined by pulse wave analysis (abbreviations as Table 4). P values: comparison of HFDC (solid circles) v LFDC (open circles); and comparison of salbutamol responses v basal values.

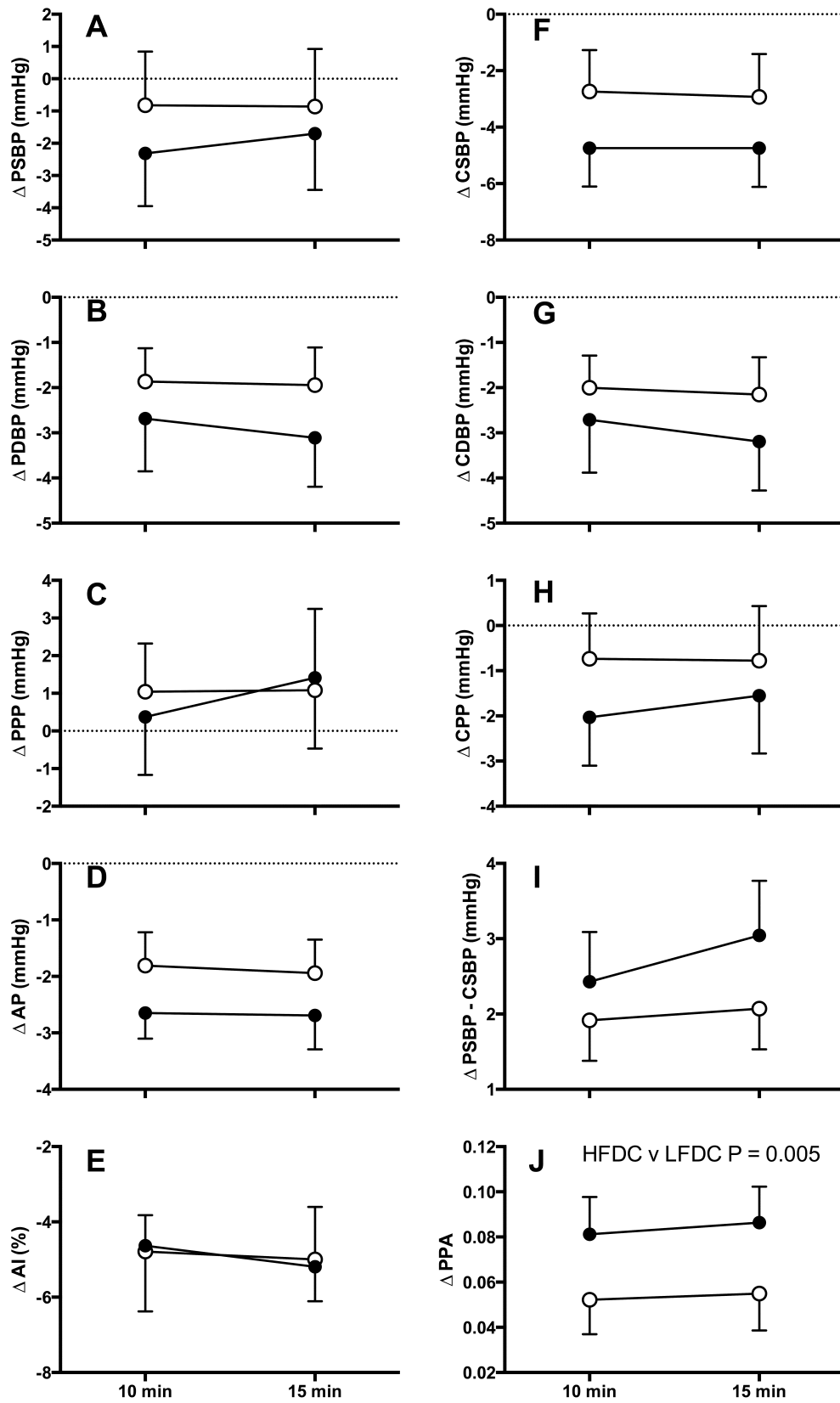


Figure 2 Mean changes from basal after salbutamol inhalation for parameters determined during pulse wave analyses, HFDC (solid circles) and LFDC (open circles).

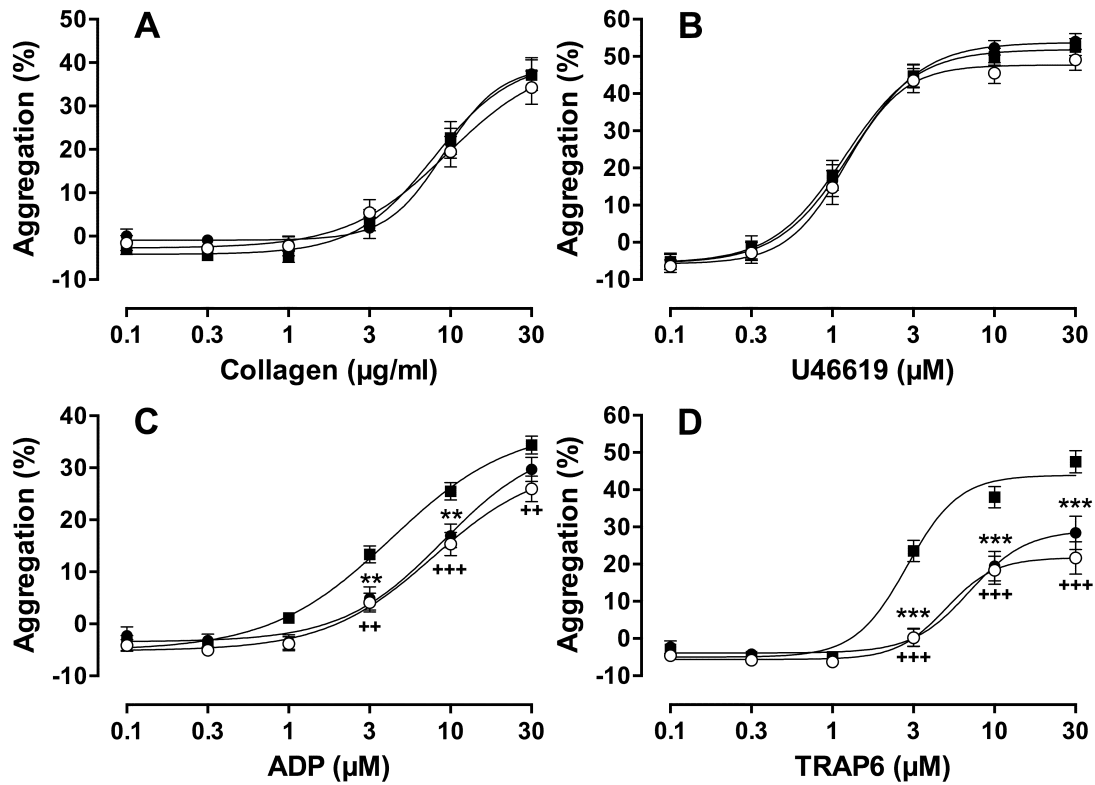


Figure 3 Comparative concentration response curves for platelet aggregation stimulated with (a) collagen, (b) U46619, (c) ADP and (d) TRAP6. Baseline responses prior to chocolate consumption (solid squares) compared with those after 6 weeks HFDC (solid circles) and 6 weeks LFDC (open circles). Data are from 22 subjects studied at all time points; **, ++ $P = <0.01$; and ***, +++ $P = <0.001$ versus baseline responses.

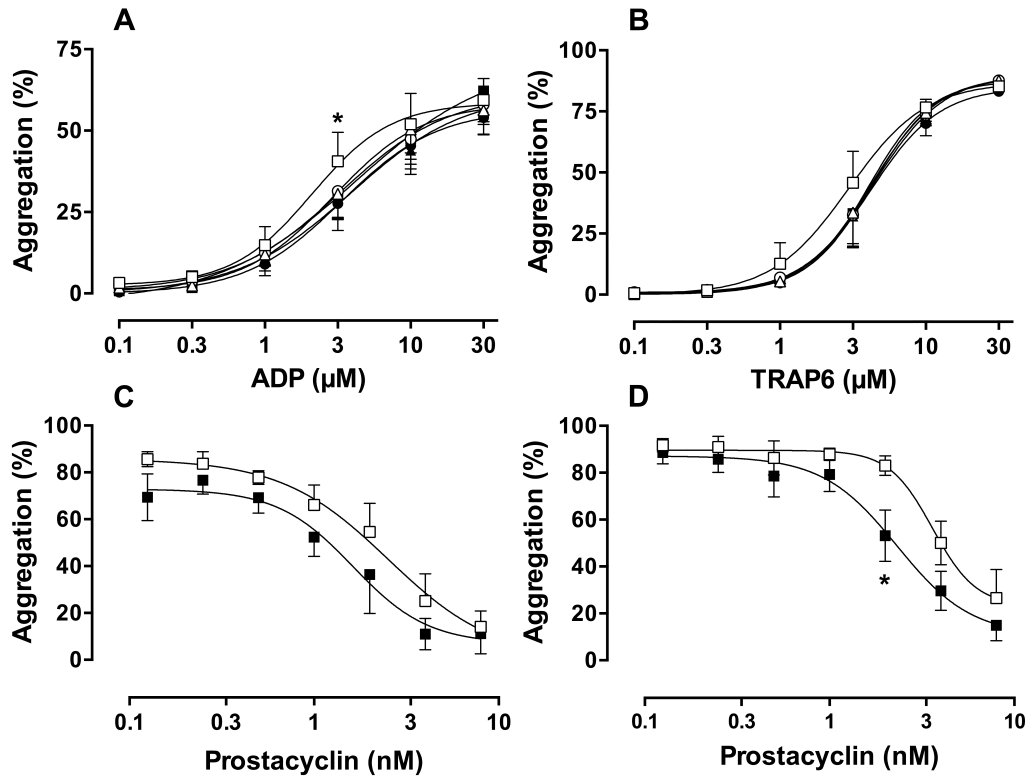


Figure 4 Effect of theobromine on platelet aggregation induced by (a) ADP and (b) TRAP6 (open squares vehicle control; theobromine: 2 μg/ml open triangles, 5 μg/ml open circles, 10 μg/ml solid triangles, 20 μg/ml solid squares, 50 μg/ml solid circles; n = 5 for each). Effect of prostacyclin on platelet aggregation induced with (c) 10 μM ADP, or (d) 10 μM TRAP6, either alone (open squares) or in the presence of 20 μg/ml theobromine (solid squares). *P = <0.05 versus response without theobromine.