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ORIGINAL CONTRIBUTION

The effect of acute pre-exercise dark chocolate consumption on plasma antioxidant status, oxidative stress and immunoendocrine responses to prolonged exercise

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Abstract
Purpose Acute antioxidant supplementation may modulate oxidative stress and some immune perturbations that typically occur following prolonged exercise. The aims of the present study were to examine the effects of acutely consuming dark chocolate (high polyphenol content) on plasma antioxidant capacity, markers of oxidative stress and immunoendocrine responses to prolonged exercise.
Methods Fourteen healthy men cycled for 2.5 h at ~60% maximal oxygen uptake 2 h after consuming 100 g dark chocolate (DC), an isomacronutrient control bar (CC) or neither (BL) in a randomised-counterbalanced design.
Results DC enhanced pre-exercise antioxidant status ($P = 0.003$) and reduced by trend ($P = 0.088$) 1 h post-exercise plasma free [F_2-isoprostane] compared with CC (also, [F_2-isoprostane] increased post-exercise in CC and BL but not DC trials). Plasma insulin concentration was significantly higher pre-exercise ($P = 0.012$) and 1 h post-exercise ($P = 0.026$) in the DC compared with the CC trial. There was a better maintenance of plasma glucose concentration on the DC trial (2-way ANOVA trial × time interaction $P = 0.001$), which decreased post-exercise in all trials but was significantly higher 1 h post-exercise ($P = 0.039$) in the DC trial. There were no between trial differences in the temporal responses (trial × time interactions all $P > 0.05$) of hypothalamic–pituitary–adrenal axis stress hormones, plasma interleukin-6, the magnitude of leukocytosis and neutrophilia and changes in neutrophil function.
Conclusion Acute DC consumption may affect insulin, glucose, antioxidant status and oxidative stress responses, but has minimal effects on immunoendocrine responses, to prolonged exercise.

Keywords Polyphenols · Dark chocolate · Exercise · Neutrophil · Oxidative stress · Innate immune system

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Introduction

Prolonged exercise, which is often used as a model of physical stress, is an excellent, controllable, quantifiable and reproducible model with which to study the effects of stressors on oxidative stress and immunoendocrine responses, and possible nutritional interventions [1–7]. Supplementation with dietary antioxidants has proved effective in a number of studies [1–7] at reducing the magnitude of exercise-induced oxidative stress. However, other studies have observed no effects [8], or even negative effects [9], of dietary antioxidant supplementation on exercise-induced oxidative stress. Indeed, Nieman et al. [9]

found greater IL-6 and oxidative stress responses following a competitive 'ultraendurance' triathlon race in athletes who were consuming vitamin E, compared with placebo, supplements daily for 2 months prior to the event. However, one notable study by Alessio et al. [1] has demonstrated that acute (1 day) supplementation with vitamin C is more effective at blunting exercise-induced oxidative stress compared with a 2-week period of daily supplementation. Acute antioxidant supplementation may be more effective because prolonged supplementation could result in an adaptive decrease in other (endogenous) antioxidant defences in vivo [10]. The acute intake of antioxidants, such as vitamin C, has been shown to reduce the magnitude of the exercise-induced depression of neutrophil function, possibly by reducing exercise-induced oxidative stress, which may result in damage to these cells [7]. However, other studies have shown limited effects of acute vitamin C intake [9, 11] and also that oxidative stress has little influence on immunoendocrine changes during and after prolonged exercise [8, 9].

There has recently been a great deal of research interest in the potential beneficial effects of cocoa because of the potent antioxidant properties of polyphenols, of which cocoa is an abundant source. Polyphenols are amphipathic, exerting their antioxidant effects in both lipid and water milieu, and act in vivo by several mechanisms on cellular molecular targets. Therefore, they may be more effective at providing protection against a pro-oxidant challenge than other antioxidant compounds. Few studies have investigated the effects of cocoa or cocoa polyphenols on exercise-induced oxidative stress and immunoendocrine responses. In a recent study, Allgrove et al. [12] demonstrated that the daily consumption of dark chocolate (40 g per day) for 2 weeks was associated with a reduction in exercise-induced oxidative stress. If the mechanisms for such findings are due to polyphenols providing protection against a pro-oxidant challenge, then an acute increase in polyphenol availability in vivo should also be effective at reducing exercise-induced oxidative stress. Two studies have demonstrated significant blunting of exercise-induced oxidative stress when a high phenolic content antioxidant beverage [5] or a polyphenol containing sports drink [6] was consumed acutely before and during exercise. Non-exercise studies have also demonstrated that cocoa can acutely increase antioxidant capacity and protection against oxidative stress. For example, Kondo et al. [13] observed that acute consumption of delipidated cocoa (35 g) resulted in a decrease in plasma low density lipoprotein (LDL) susceptibility to oxidation.

In addition to the antioxidant effects of polyphenols, previous research has shown that the ingestion of polyphenols [14] or cocoa [15] may acutely increase the postprandial insulinaemic response. The significance of such findings is unclear at present but there may be some implications for the fields of nutritional immunology and exercise (stress) immunology. For example, as a model of physical stress, prolonged exercise has been consistently shown to cause a transient decrease in several immune cell functions, commonly termed exercise-induced immunodepression [16]. It has been shown that many neutrophil functions, including oxidative burst activity (OBA) and degranulation, are lowered after such exercise for up to 6 h or more [7, 10, 11, 17–22]. However, hyperinsulinaemia has been shown to enhance a number of neutrophil functions: either at rest when induced concurrent with a euglycaemic and aminoacidaemic clamp [23], or following an acute trauma/stressor, such as cardiac surgery [24]. Therefore, it is possible that foods high in cocoa may have a beneficial effect on neutrophil functions, via this polyphenol-mediated mechanism, over and above any effects caused by their antioxidant properties. However, there is currently no available literature on the effects of acute dark chocolate consumption (as a source of antioxidants/polyphenols) on oxidative stress and immunoendocrine responses to prolonged (>2-h duration) exercise. Hence, the aims of the present study were to examine the effects of consuming a single dose of dark chocolate, 2 h before prolonged exercise, on plasma antioxidant capacity, markers of oxidative stress and immunoendocrine responses.

Methods

This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures were approved by the Loughborough University (UK) Research Ethics Committee, which is where the work was undertaken. Written informed consent was obtained from all subjects. Subjects also completed a pre-exercise screening questionnaire (Physical Activity Readiness Questionnaire: PAR-Q) before participating in each test.

Participants

Fourteen healthy men (age 22 ± 1 years, body mass 71.6 ± 1.6 kg, maximal oxygen uptake ($\dot{V}O_2$max) 53.1 ± 1.9 ml kg^{-1} min^{-1}, power output at $\dot{V}O_2$max 300 ± 12 W; means ± SEM) participated in this study. All participants completed 5 exercise bouts: 2 preliminary trials ($\dot{V}O_2$max determination and habituation) and 3 main trials. All trials were separated by 1 week.

Main trials

Participants exercised under 3 different conditions: BL (fasted) trial, DC (dark chocolate) trial or CC (control bar)

trial in a randomised-counterbalanced manner. They arrived at the laboratory at 08:30 on the morning of the main trials after an overnight fast (>10 h). Participants consumed the DC or CC (or nothing in the BL trial) and 200 ml of water 2 h prior to beginning exercise. Additionally, 2.5 $ml.kg^{-1}$ body mass of water was consumed at the onset, every 15 min during, and on completion of the exercise bout.

Venous blood samples were taken before bar consumption (Rest), immediately before beginning exercise (Pre-Ex), immediately after completing the bout (Post-Ex) and after 1 h of recovery (1 h Post-Ex). On each occasion, 25-ml blood was obtained from an antecubital vein, with minimal stasis, using a 21-gauge butterfly needle and syringe. Blood was then carefully dispensed into three vacutainer tubes (Becton–Dickinson, Oxford, UK): two K_3EDTA tubes and one Heparin tube. Expired gas was collected into Douglas bags (1-min sample), during the 15th and 30th min and every 30 min thereafter during exercise, for analysis of $\dot{V}O_2$ and respiratory exchange ratio (RER). Heart rate and rating of perceived exertion (RPE) were recorded every 15 min during exercise.

Participants completed a food record diary for the 48-h period before the first trial (habituation) and were required to follow the same diet during the 48 h prior to each main trial. The dietary records were analysed using computer dietary analysis software (CompEat Pro, Nutrition Systems, UK). All participants were non-smokers and were required to abstain from alcohol, caffeine and heavy exercise for 48 h prior to each trial and to have a rest day on the day immediately before each trial. It was also stipulated that participants should not take any mineral or vitamin supplement or any other antioxidant supplements during and for the 4 weeks before the study. Participants were provided with a food exclusion list to ensure that they avoided high polyphenol containing foods for the 48 h before each main trial.

Pre-exercise chocolate consumption

The order in which each participant undertook each condition (DC, CC or BL trials) was randomised and counterbalanced using a basic Latin Squares design. There are 6 different possible sequences in which the DC, CC and BL trials could be undertaken. Participants were assigned randomly to a sequence in blocks of 6 resulting in 2 participants in each of the possible sequences. Since there were 14 participants 2 out of the 6 sequences each contained an additional participant. It was not possible to blind participants from the trial order as the DC and CC bars were different in appearance, taste and mouth feel. However, participants consumed the bars or water only (BL trial) supervised by an independent non-interested party in a separate room, and the investigators were blinded until after completion of the study. Moreover, participants were not told until after the study was complete which was the experimental/treatment bar, just that the aims of the study were to determine the effects of 'different' types of chocolate.

Chocolate composition

The DC was a typical commercial 70% cocoa product (Nestlé Noir™ 70%) containing the ingredients cocoa liquor, sugar, cocoa butter, milk fat, lecithin and vanilla. The DC (100 g) had a total energy content of ~2.3 MJ (containing ~44 g fat and ~26 g carbohydrate). The polyphenol content was 39.1 mg catechin, 96.8 mg epicatechin, 58.4 mg Dimer B2, 7.3 mg Dimer B5, 34.7 mg Trimer C and 10.5 mg tetramer D. The CC was as closely matched as possible in terms of total energy, fat and carbohydrate content. The CC (71 g) contained all of the same ingredients except the cocoa liquor (0%) and therefore contained no polyphenols. The energy content of the CC was ~2.1 MJ (containing ~45 g fat and ~26 g carbohydrate).

Blood analysis

Blood from one of the K_3EDTA vacutainer tubes was used for haematological analysis including haemoglobin, haematocrit, and total and differential leucocyte counts using an automated haematology analyser (A^CT^{TM} 5diff analyser, Beckman Coulter, UK) and to determine changes in the plasma concentrations of hormones and interleukin (IL)-6. Plasma volume changes were estimated according to the methods described by Dill and Costill [25].

Assays of neutrophil function

From blood taken into a lithium heparin vacutainer, 1 ml was immediately added to an eppendorf tube (1.5 ml capacity) containing 50 μL of 10 mg mL^{-1} bacterial extract stimulant solution (840-15, Sigma, Poole, UK). Blood and stimulant were mixed by gentle inversion and then incubated for 1 h at 37 °C, with gentle mixing every 20 min. After incubation, the mixture was centrifuged for 2 min at 15,000×*g*. The supernatant was immediately stored at −80 °C prior to analysis of neutrophil elastase concentration using an ELISA method (Merck Calibiochem, Darmstadt, Germany). The amount of elastase released per neutrophil in response to bacterial stimulation was calculated according to Robson et al. [20].

A microplate luminometer cell activation kit (Knight Scientific Limited, Plymouth, UK) was used to measure the phorbol-12-myristate-13-acetate (PMA)-stimulated

neutrophil oxidative burst activity in accordance with the manufacturer's instructions. Briefly, sample analysis was performed in duplicate as follows: 20 μL of K_3EDTA whole blood sample was added into a dilution tube with 2 ml of blood dilution buffer (HBSS without calcium and magnesium but with 20 mM HEPES, pH 7.4). A 20-μL aliquot of each diluted sample was then added into an opaque white microplate well. Ninety μL reconstitution and assay buffer (HBSS with 20 mM HEPES, pH 7.4) was then added into each well followed by the addition of 20 μL reconstituted Adjuvant-K™ and 50 μL Pholasin® (10 μg mL^{-1}). The microplate was placed into a luminometer (Anthos Lucy 1 Microplate Luminometer, Anthos Labtec Instruments, Austria) after adding 20 μL PMA into each well. After 1-min shaking and incubation at 37 °C, Pholasin®-enhanced chemiluminescence (CL) was measured at 1-min intervals for 30 min, and the incremental area under the curve (IAUC) was calculated. The oxidative burst activity per cell (pmaOBA) was calculated by dividing the IAUC by the number of neutrophils in each sample (well). The unstimulated CL was also recorded as an indicator of unstimulated/spontaneous ROS generation (spOBA).

The remaining K_3EDTA and heparinised whole blood samples were centrifuged at 1,500×*g* for 10 min in a refrigerated centrifuge at 4 °C within 10 min of sampling. The plasma obtained was immediately stored at –80 °C for later analysis.

Plasma vitamin C concentration and antioxidant capacity

Plasma vitamin C concentration was determined in plasma obtained from heparinised blood according to Liu et al. [26] using a specific spectrophotometric ascorbate oxidase (E 1.10.3.3) assay. Plasma Trolox equivalent antioxidant capacity (TEAC) was determined in plasma obtained from heparinised blood on an automated analyser (Cobas Mira Plus, Roche, Basle, Switzerland) using a commercially available kit (Randox, County Antrim, UK) for measuring the capacity of plasma to scavenge the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical cation. Trolox was used as the standard, and therefore this measure of total antioxidant status (TAS) was expressed in Trolox equivalent antioxidant units (μM).

Plasma polyphenol concentration

Aliquots of K_3EDTA plasma were analysed to determine the concentration of polyphenols (epicatechin and catechin) using HPLC. Briefly, thawed plasma was centrifuged at 4 °C at 2,000×*g* for 5 min, and 200 μL was mixed with 12 μL of 10% ascorbic acid-40 mM KH_2PO_4-0.1% EDTA, 20 μL of 50 mM potassium phosphate (pH 7.4), 20 μL of 1.0 μg/ml catechin gallate as internal standard, 500 units of *β*-D-glucuronidase type X-A from *E. coli* (Sigma Chemical Co, St Louise, MO, USA) and 4 units of sulfatase type VIII from abalone entrails (Sigma). The mixture was incubated at 37 °C for 45 min. The reaction was stopped by the addition of 2 ml of ethyl acetate followed by vigorous shaking for 20 min and centrifugation at 4 °C at 2,000 g for 5 min. The supernatant was transferred to a clean tube, and the ethyl acetate extraction was repeated. Ten μL of 0.02% ascorbic acid: 0.005% EDTA was added to the pooled supernatant fraction and vortex mixed. The supernatant was then evaporated to dryness with nitrogen for 2 h at room temperature. The samples were reconstituted in 200 μL of methanol/water (1:2 vol), vortexed well, sonicated for 10 min and centrifuged at 4 °C at 2,000×*g* for 5 min. Twenty μL of the supernatant was injected into the HPLC system, which consisted of an ESA Model 582 solvent delivery system, an ESA Model 542 autosampler, an ESA 5600 CoulArray electrochemical detector (ESA, Bedford, MA, USA) with CoulArrayWin Software 1.12, an Eppendorf CH-30 Column Heater, a C_{18} Phonomenex guard column (4.0 mm L × 3.0 mm) and a SUPELCO Ascentis RP-Amide column (15 cm × 4.6 mm i.d., 5 μm particles). Standard flavanol solutions and internal standard (catechin gallate) were prepared in a methanol and water (1:2 vol) solution and stored at −70 °C. The column was eluted at 35 °C starting at a flow rate of 1 ml/min with 70% buffer A (40 mM ammonia phosphate monobasic, pH adjusted to 3.0 with phosphoric acid) and 30% buffer B (40 mM ammonia phosphate monobasic 50%/L:50% acetonitrile/L, pH adjusted to 3.0 with phosphoric acid). After 1 min, the gradient was linearly changed with from 70% buffer A and 30% buffer B to 10% buffer A and 90% buffer B (1–10 min), 70% A/30% B (10–12 min). The eluent was monitored by electrochemical detection with potential settings at −30, 100, 230 and 400 mV. The dominant channel was at 230 mV.

Plasma free F_2-isoprostane concentration

For the determination of plasma free F_2-isoprostane, K_3EDTA plasma samples were stored at −80°C in the presence of 0.005% 3,5-di-tert-4butylhydroxytoluene, to prevent ex vivo formation, prior to analysis. Plasma free F_2-isoprostane concentration was determined using an ELISA kit according to the manufacturer's instructions (Cayman Chemical Co., MI, USA).

Plasma hormones, IL-6 and metabolite concentrations

Aliquots of K_3EDTA plasma were analysed to determine the concentrations of cortisol (DRG Diagnostics, Marburg/Lahn, Germany), ACTH (Biomercia, Ca, USA), insulin

(Ultrasensitive kit, DRG Diagnostics, Marburg/Lahn, Germany) and IL-6 (Diaclone, Besancon, France) using commercially available ELISA kits. Plasma glucose concentration was determined on an automated analyser (Cobas Mira Plus, Roche, Basle, Switzerland) using a colorimetric glucose oxidase-PAP kit (Randox, County Antrim, UK). Plasma lactate concentration was determined on a spectrophotometer at 340 nm using a standard enzymatic assay with lactate dehydrogenase and nicotinamide adenine dinucleotide (Sigma, Poole, UK). Plasma Non-Esterified Fatty Acids (NEFA) analysis was performed using the Wako 999-75406 NEFA-C kit (Alpha Laboratories, Eastleigh, UK).

Data analysis

To compare the 3 separate trials, DC, CC and BL, a 2-way repeated measures ANOVA (trial (3) × time (4)) was used for data with normally distributed residuals (normal data). Non-normal data were normalised with log (Vitamin C, free F_2-isoprostane, glucose, leucocyte count, neutrophil count) or square root (spOBA, NEFA) transformation and analysed with 2-way repeated measures ANOVA. When such transformations were unable to normalise the data (catechin, epicatechin, insulin, cortisol, ACTH, IL-6, pmaOBA), non-parametric Friedman tests were used. The Greenhouse–Geisser correction was applied to ANOVA P values, as appropriate, to correct for violations of the assumption of sphericity. In this case, P values were denoted with the letters GH in subscript to indicate this: e.g. $P_{GH} = 0.024$ indicates that the Greenhouse–Geisser correction has been applied). For non-parametric data, Friedman tests were applied to compare the 3 trials overall (equivalent to the main effect of trial comparison in 2-way ANOVA); and to compare the 4 time points overall (equivalent to the main effect of time comparison in 2-way ANOVA). Friedman tests were also applied on the differences from Rest to Pre-Ex, Rest to Post-Ex and Rest to 1 h Post-Ex between trials (with appropriate Holm–Bonferroni correction) to determine whether the temporal responses were different between trials (equivalent to the trial × time interaction comparison in 2-way ANOVA). *Post hoc* analysis was carried out, where appropriate, using paired samples t tests (for ANOVA) or Wilcoxon tests (for Friedman tests) with the Holm–Bonferroni correction. When a significant trial × time interaction or different temporal responses were evident between trials (indicating different temporal responses depending on trial), then 1-way repeated measures ANOVA of Friedman tests were also used, as appropriate, to determine the temporal response in each trial independently. Planned 'paired' comparisons were also carried out between the DC and CC trials at each specific time point.

Results

Diet composition

The average dietary composition for the 48-h period prior to each main trial was as follows: total daily energy intake of 11.2 ± 0.5 MJ, percentage energy derived from carbohydrate, protein and fat was 55 ± 3%, 16 ± 1% and 29 ± 3%, respectively. This was equivalent to an average daily intake of 5.4 ± 0.4 g kg^{-1} body mass of carbohydrate and 1.6 ± 0.1 g kg^{-1} body mass of protein. The average daily dietary vitamin A, vitamin C and vitamin E intake was 577 ± 126 μg, 68 ± 18 mg and 8.5 ± 1.6 mg, respectively. This is equivalent to 72 ± 16%, 113 ± 30% and 85 ± 16% of the current UK RDA, respectively.

Physiological demand of the exercise

There was no difference between trials for any of the variables related to the physiological demand of the exercise (1-way repeated measures ANOVA all $P > 0.05$). There were no differences in mean heart rate (overall mean, 142 ± 13 bpm), subjective rating of perceived exertion (RPE; overall, 12 ± 1), mean percentage of $\dot{V}O_2$max (overall, 57 ± 4%) or mean respiratory exchange ratio (RER; overall, 0.93 ± 0.07) during the main trials.

Plasma volume changes

Estimations of plasma volume change post-exercise were similar on all 3 trials. Percentage decrease compared with samples taken at Rest was 5.0 ± 1.0% at Pre-Ex, 7.5 ± 1.2% at Post-Ex and 2.4 ± 1.2% at 1 h Post-Ex.

Markers of oxidative stress and antioxidant capacity

There was a significant DC-induced increase in plasma epicatechin, but not catechin, concentrations as shown in Fig. 1.

Significant trial × time interactions were observed for plasma total antioxidant status (TAS), spOBA per litre of whole blood and plasma free F_2-isoprostane concentration, indicating that these measures responded differently depending on trial. Results (and post hoc analyses) are shown in Table 1. There was a trend for plasma free F_2-isoprostane concentration to be lower on the DC, compared with CC, trial at 1 h post-exercise ($P = 0.088$). One-way ANOVA on each trial separately showed a post-exercise increase in this parameter for the CC and BL trials but there was no change in the DC trial (Table 1). There was a significant exercise-induced increase in plasma vitamin C concentration but this was not different between trials (Table 1).

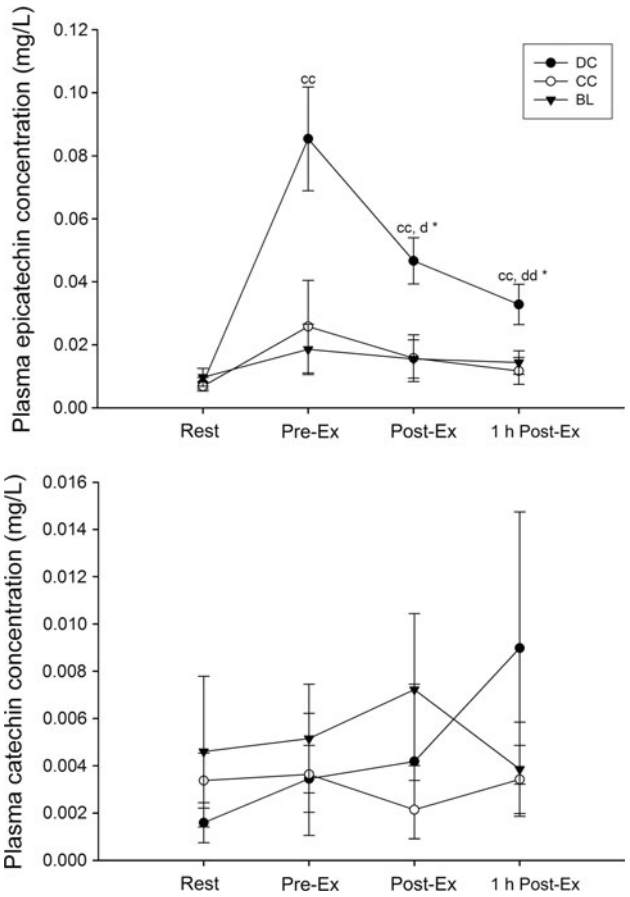

Fig. 1 Plasma Epicatechin (top panel) and Catechin (bottom panel) concentration. Values are means (±SEM). Epicatechin, main effects: trial (Friedman $P < 0.001$), time (Friedman $P = 0.016$), between trials temporal (Friedman Rest to Pre-Ex $P = 0.030$; to Post-Ex $P = 0.012$; to 1 h Post-Ex $P = 0.026$). Catechin, main effects: trial (Friedman $P = 0.389$), time (Friedman $P = 0.877$), between trials (Friedman Rest to Pre-Ex $P = 0.057$; to Post-Ex $P = 0.140$; to 1 h Post-Ex $P = 1.000$). Post hoc tests: Within-trial; mean values were significantly different from Rest ($^{c}P < 0.05$; $^{cc}P < 0.01$); mean values were significantly different from Pre-Ex ($^{d}P < 0.05$; $^{dd}P < 0.01$). Between trials; mean values were significantly different between DC and CC trials at the same time point ($^{*}P < 0.05$)

Plasma hormones, IL-6 and metabolites

There were significant overall effects of trial (Friedman $P = 0.001$) and time (Friedman $P < 0.001$) for plasma insulin concentration (Fig. 2). Plasma insulin concentration generally decreased post-exercise but the overall temporal response was different depending on the trial (Fig. 2).

Significant overall time effects were evident for plasma cortisol, ACTH, IL-6, NEFA and lactate concentrations (exercise-induced increase) but there were no differences in the temporal responses between trials (Table 2). For the analysis of NEFA, a number of samples were lost, and could not be replaced, during the analysis process. Incomplete data sets ($n = 3$) were removed from analysis leaving $n = 11$ for NEFA data. There was no significant trial × time interaction ($P = 0.455$) for plasma NEFA concentration although there was a trend ($P = 0.051$) for a main effect of trial and for NEFA concentration to be higher on the DC trial (Table 2).

There was a significant exercise-induced decrease in plasma glucose concentration, and the temporal response appeared different depending on trial (Table 2).

Blood leucocyte count and neutrophil function

Significant overall time effects were evident for circulating leucocyte and neutrophil counts (exercise-induced increase) but there were no differences, in the temporal responses, between trials (Table 3). Significant overall time effects were evident for in vitro-stimulated neutrophil degranulation and oxidative burst (exercise-induced decrease) but there were no differences, in the temporal responses, between trials (Table 3).

Discussion

The main findings of the present study are that DC consumption 2 h before an acute bout of prolonged exercise (2.5 h at ~60% $\dot{V}O_2$max) resulted in a significant insulinaemia and differential plasma glucose response compared with the consumption of CC (with a similar macronutrient and energy content) or fasting (BL). Dark chocolate ingestion significantly elevated the plasma epicatechin concentration and slightly, but significantly, increased plasma antioxidant capacity. Plasma free F_2-isoprostane concentration was elevated post-exercise in the CC and BL trials but there was no significant change observed in the DC trial (Table 1). However, there were no significant effects of acute DC ingestion on any of the measured immunoendocrine responses to exercise.

The dark chocolate also contained an appreciable amount of caffeine and theobromine (DC: 104 mg caffeine and 668 mg theobromine compared with CC: 17 mg caffeine and 11 mg theobromine per bar). These substances and their metabolites have some antioxidant activity [27], which could have contributed to the increase in TAS observed, but we cannot discriminate between the contributions of different components of cocoa. However, the chocolate used in the present study (DC) contains ~2.5-fold more (−)-epicatechin than (+)-catechin, and plasma concentrations of (−)-epicatechin after consumption were more than an order of magnitude higher than (+)-catechin. Similar findings have been reported after cocoa consumption in humans [28], demonstrating the significantly higher bioavailability of epicatechin compared with catechin.

Table 1 Markers of plasma antioxidant status and oxidative stress before and following exercise

	Rest	Pre-Ex	Post-Ex	1 h Post-Ex	Main Effects *P* values (trial; time; trial × time)
TAS (μM Trolox)					
DC	1103 (12)	1150 (12)^{cc}**	1165 (12)^{cc}	1149 (14)^{cc}	0.154; < 0.001$_{GH}$; 0.001
CC	1107 (11)	1119 (13)^{c}	1172 (14)$^{cc,\ dd}$	1149 (16)$^{cc,\ dd}$	
BL	1124 (9)	1131 (10)	1176 (12)$^{cc,\ dd}$	1163 (15)$^{cc,\ dd}$	
Vitamin C (μM)					
DC	57 (4)	55 (4)	69 (4)	60 (4)	0.158$_{GH}$; < 0.001; 0.453
CC	56 (4)	53 (5)	68 (4)	58 (5)	
BL	57 (4)	56 (3)	74 (6)	65 (5)	
F_2-isoprostane (pg ml^{-1})					
DC	14.4 (0.4)	14.8 (0.6)*	16.1 (0.9)	14.3 (0.5)	0.253$_{GH}$; < 0.001; 0.005
CC	14.1 (0.5)	13.2 (1.0)	16.0 (1.1)^{d}	15.5 (0.7)^{d}	
BL	13.3 (0.5)	12.6 (0.5)	15.7 (0.7)$^{cc,\ dd}$	15.8 (0.5)$^{cc,\ dd}$	
spOBA (RLU L blood^{-1})					
DC	491 (64)	418 (50)	438 (41)	365 (31)	0.325$_{GH}$; 0.118; 0.037$_{GH}$
CC	434 (43)	420 (36)	609 (70)	551 (72)	
BL	406 (33)	398 (30)	478 (42)	423 (38)	

Values are mean (SEM). CC (control bar trial), DC (dark chocolate trial), BL (fasting trial)

The DC was a typical commercial 70% cocoa product (Nestlé Noir^{TM} 70%) containing the ingredients cocoa liquor, sugar, cocoa butter, milk fat, lecithin and vanilla. The DC (100 g) had a total energy content of ~2.3 MJ. The CC was as closely matched as possible in terms of total energy (~2.1 MJ), fat and carbohydrate content and contained all of the same ingredients except the cocoa liquor

Results from 2-way ANOVA are presented in Main Effects *P* values column

spOBA: unstimulated/spontaneous oxidative burst activity

Post hoc analysis for time effects within each trial (when an interaction was observed): Mean values were significantly different from Rest ($^{c}P < 0.05$; $^{cc}P < 0.01$); mean values were significantly different from Pre-Ex ($^{d}P < 0.05$; $^{dd}P < 0.01$)

Post hoc analysis between trials for trial and/or trial × time interactions: Mean values were significantly different between DC and CC trials at the same time point (* $P < 0.05$; ** $P < 0.01$)

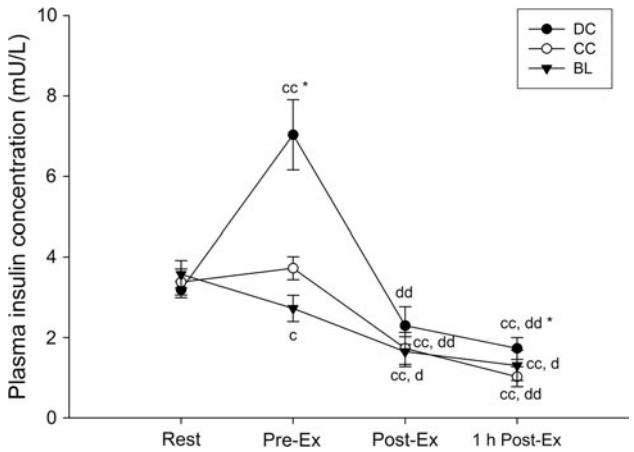

Fig. 2 Plasma insulin responses. Values are means (±SEM). Main effects: trial (Friedman $P = 0.001$), time (Friedman $P < 0.001$), between trials temporal (Friedman Rest to Pre-Ex $P = 0.003$; to Post-Ex $P = 0.218$; to 1 h Post-Ex $P = 0.168$). Post hoc tests: Within-trial: Mean values were significantly different from Rest ($^{c}P < 0.05$; $^{cc}P < 0.01$); mean values were significantly different from Pre-Ex ($^{d}P < 0.05$; $^{dd}P < 0.01$); Between trials: Mean values were significantly different between DC and CC trials at the same time point (* $P < 0.05$)

Therefore, epicatechin is likely to be the most relevant monomeric flavonoid in these experiments.

The aim of the present study was to determine whether acute dark chocolate consumption provides protection against exercise-induced oxidative stress (as has been observed with a 2-week period of daily DC consumption [12] and with acute ingestion of other dietary sources of polyphenols [5, 6]). The present findings are comparable to those observed by Allgrove et al. [12] after 2 weeks of supplementation, in that there were no meaningful effects on hormonal or immune responses but some effects on plasma polyphenol concentrations and antioxidant capacity, and plasma free F_2-isoprostane responses. Direct comparisons between the plasma free F_2-isoprostane results of the present study and many previous exercise and antioxidant supplementation studies [e.g. 3, 4] are not possible because of the variety of methods employed and wide variation in values reported between studies [10]. However, we are confident that the ELISA method for free F_2-isoprostane employed in the present study is acceptable for the intended purposes of comparing changes in response to exercise and between the 3 treatments within

Table 2 Circulating hormones, IL-6 and metabolites before and following exercise

	Rest	Pre-Ex	Post-Ex	1 h Post-Ex	Main Effects *P* values (trial; time; trial × time)
Cortisol (nM)					
DC	545 (45)	360 (35)	703 (80)	546 (75)	Friedman (trial; time) 0.259; <0.001
CC	539 (47)	346 (32)	665 (102)	552 (109)	Friedman change (Pre; Post; 1 h)
BL	541 (52)	366 (44)	737 (91)	610 (119)	0.807; 0.395; 0.223
ACTH (pg ml^{-1})					
DC	31 (3)	17 (2)	89 (19)	27 (5)	Friedman (trial; time) 0.575; <0.001
CC	33 (4)	20 (2)	81 (20)	23 (3)	Friedman change (Pre; Post; 1 h)
BL	35 (4)	20 (2)	81 (13)	29 (6)	0.395; 0.751; 0.062
IL-6 (pg ml^{-1})					
DC	1.9 (0.3)	2.0 (0.2)	4.1 (0.4)	4.3 (0.4)	Friedman (trial; time) 0.028; <0.001
CC	2.1 (0.5)	2.2 (0.4)	3.5 (0.4)	3.9 (0.6)	Friedman change (Pre; Post; 1 h)
BL	2.0 (0.4)	2.1 (0.4)	4.3 (0.7)	4.4 (0.6)	1.000; 0.06; 0.057
Lactate (mM)					
DC	1.2 (0.1)	1.1 (0.1)	2.0 (0.1)	1.1 (0.1)	0.921; <0.001; 0.758
CC	1.1 (0.1)	1.1 (0.1)	1.9 (0.1)	1.1 (0.1)	
BL	1.2 (0.1)	1.1 (0.1)	1.9 (0.1)	1.2 (0.1)	
Glucose (mM)					
DC	5.0 (0.1)	4.8 (0.1)	4.5 (0.2)^{cc}	4.3 (0.1)$^{cc,\ dd}$*	0.049; <0.001; 0.001
CC	5.0 (0.1)	4.6 (0.1)^{cc}	4.0 (0.1)$^{cc,\ dd}$	3.9 (0.1)$^{cc,\ dd}$	
BL	5.1 (0.1)	5,0 (0.1)	4.0 (0.2)$^{cc,\ dd}$	4.0 (0.1)$^{cc,\ dd}$	
NEFA (mM)					
DC	0.29 (0.03)	0.33 (0.03)	1.74 (0.11)	1.39 (0.13)	0.051; $<0.001_{GH}$; 0.455
CC	0.24 (0.03)	0.25 (0.05)	1.59 (0.09)	1.12 (0.15)	
BL	0.32 (0.05)	0.26 (0.03)	1.69 (0.12)	1.12 (0.12)	

Values are mean (SEM); $n = 14$ except for NEFA. CC (control bar trial), DC (dark chocolate trial), BL (fasting trial). Results from 2-way ANOVA and Friedman tests are presented in Main Effects *P* values column

Post hoc analysis for time effects within each trial (when an interaction was observed): Mean values were significantly different from Rest ($^{c}P < 0.05$; $^{cc}P < 0.01$); mean values were significantly different from Pre-Ex ($^{d}P < 0.05$; $^{dd}P < 0.01$)

Post hoc analysis between trials for trial and/or trial × time interactions: Mean values were significantly different between DC and CC trials at the same time point (* $P < 0.05$; ** $P < 0.01$)

the study, as was done in our previous study [10]. The effects in the present study are small, and it is not clear how meaningful these effects are physiologically. It should also be noted that food intake is regular and throughout life, so small nutritional changes such as observed here could, over a lifetime, be highly significant. Larger effects (in terms of blunting of oxidative stress) were evident in the study by Allgrove et al. [12] only after an exhaustive bout of exercise, which was performed following a 90-min bout of submaximal exercise. There were no differences at the end of the 90-min bout. The exercise bout employed in the present study was not exhaustive and did not induce a post-exercise increase in oxidative stress markers as large as that seen after the exhaustive bout in the study by Allgrove et al. [12]. However, the magnitude of the increase in pre-exercise antioxidant capacity induced by acute DC ingestion in the present study was similar to that reported in the study by Allgrove et al. [12]. It is possible, therefore, that greater effects on oxidative stress markers would have been evident in the present study if the pro-oxidant challenge (exercise) was of greater magnitude. It has also been demonstrated previously that a high phenolic content antioxidant beverage [5] and a polyphenol containing sports drink [6], when consumed acutely before and during exercise, significantly blunted exercise-induced oxidative stress markers (exercise was 90 min at $\sim$70% $\dot{V}O_2$max) despite minimal effects on total antioxidant capacity of plasma.

The absolute changes in plasma TAS in the present study are not surprising as the micromolar plasma polyphenol concentrations in plasma have a relatively small 'direct' effect on total antioxidant capacity, whereas the predominant antioxidant compounds in plasma, albumin and uric acid are at much higher concentrations. However,

Table 3 Circulating total leucocyte and neutrophil counts function before and following exercise

	Rest	Pre-Ex	Post-Ex	1 h Post-Ex	Main Effects *P* values (trial; time; trial × time)
Leucocyte count ($\times 10^9$ l^{-1})					
DC	5.3 (0.4)	5.9 (0.5)	16.0 (1.3)	13.4 (0.8)	0.946; <0.001$_{GH}$; 0.255$_{GH}$
CC	5.2 (0.5)	5.3 (0.5)	18.0 (1.7)	15.1 (1.5)	
BL	5.1 (0.5)	5.5 (0.5)	16.7 (1.4)	14.7 (1.1)	
Neutrophil count ($\times 10^9$ l^{-1})					
DC	2.7 (0.4)	3.4 (0.5)	11.0 (1.0)	10.5 (0.7)	0.935; <0.001$_{GH}$; 0.353$_{GH}$
CC	2.5 (0.4)	3.0 (0.4)	12.7 (1.4)	12.0 (1.3)	
BL	2.6 (0.4)	3.1 (0.4)	11.9 (1.2)	11.7 (1.0)	
Degranulation (fg neutrophil^{-1})					
DC	523 (36)	481 (37)	272 (23)	256 (30)	0.061; <0.001$_{GH}$; 0.294$_{GH}$
CC	421 (26)	429 (33)	219 (15)	242 (18)	
BL	492 (30)	459 (29)	263 (21)	246 (23)	
OBA (% of value at rest)					
DC	100 (−)	89 (4)	78 (6)	70 (6)	Friedman (trial; time) 0.807; <0.001
CC	100 (−)	96 (5)	73 (13)	77 (11)	Friedman change (Pre; Post; 1 h)
BL	100 (−)	89 (4)	67 (8)	71 (6)	0.807; 0.223; 0.807

Values are mean (SEM). CC (control bar trial), DC (dark chocolate trial), BL (fasting trial). Results from 2-way ANOVA and Friedman tests are presented in Main Effects *P* values column

OBA: phorbol-12-myristate-13-acetate (PMA)-stimulated neutrophil oxidative burst activity

Post hoc analysis for time effects within each trial (when an interaction was observed): Mean values were significantly different from Rest ($^{c}P < 0.05$; $^{cc}P < 0.01$); mean values were significantly different from Pre-Ex ($^{d}P < 0.05$; $^{dd}P < 0.01$)

* There were no differences between trials

the amphipathic polyphenols may provide significant antioxidant-related effects in vivo, which could explain why similar absolute changes in plasma TAS observed in other studies [6, 12] were physiologically meaningful and significantly blunted exercise-induced oxidative stress. Therefore, we believe that the small effects observed here could be physiologically important, but arise from cocoa-induced metabolic changes leading to modulation of the major plasma constituents. In addition, DC was effective at blunting the exercise-induced increase in plasma TAS observed in the other trials (CC and BL), providing support for the idea that the elevated TAS on the DC trial has physiological significance. Another consideration is that it is not known whether plasma TAS in the present study reached a higher peak earlier during the 2 h post-prandial period after DC consumption. For example, Kondo et al. [13] observed that LDL susceptibility to oxidation was reduced 2 h after the ingestion of 35 g of delipidated cocoa in healthy male participants but this had returned to basal levels by 4 h post-ingestion. It is possible, therefore, that greater blunting of oxidative stress responses would be observed with a different timing and/or quantity of DC ingestion, although this will require further investigation.

It is unlikely that the differences in insulin and glucose responses observed in the present study are attributable to any slight differences that may exist in the glycaemic index (GI) between DC and CC bars. Although the GI of the bars was not directly measured in the present study, exercise was not commenced until 2 h post-ingestion, at a time when plasma glucose concentration was similar between trials. This suggests that some other mechanism(s) are responsible for the differential plasma insulin and glucose responses observed in the present study. It has been demonstrated that the acute ingestion of polyphenols [14] or the addition of cocoa to various foods [15] can stimulate increased insulin responses in the following post-prandial period, although the mechanisms are not well understood. Whether this is due to alterations in insulin activity and/or glucose metabolism and handling [14, 29, 30], interference with glucose uptake/clearance [31], the action of insulinogenic amino acids in the cocoa [15] or some other mechanism is not clear at present, and further work is required if this is to be determined in humans [30]. However, Brand-Miller et al. [15] showed that these effects were independent of total macronutrient composition or GI of the ingested foods. In the present study, the temporal response of plasma glucose concentration was different depending on the trial. However, it is clear that, in addition to the measurement times of the present study, more frequent blood sampling would be required throughout the

whole trial to gain further insight into the potential mechanisms by which DC consumption influences the plasma insulin and glucose responses as well as how meaningful these differences are. There was a significant exercise-induced increase in plasma NEFA (mobilisation) regardless of trial, and there was a tendency for higher pre- and post-exercise NEFA on the DC trial, which is likely related to the caffeine and theobromine content of DC.

Oxidative stress has been implicated in the depression of immune cell function that typically occurs following prolonged exercise, the magnitude of which may be decreased with appropriate pre-exercise antioxidant supplementation [7, 21, 32]. Acute supplementation with other types of dietary antioxidant has been shown to reduce exercise-induced oxidative stress [1, 2] and enhances neutrophil function following prolonged exercise but is not effective at reducing the hormonal or cytokine responses [7]. In line with these findings, acute DC consumption had no effect on plasma HPA axis stress hormones or IL-6 following prolonged exercise. There was also no effect of DC consumption on circulating neutrophil number or function, as measured by in vitro-stimulated OBA and degranulation, despite the increase in plasma antioxidant capacity and reduced exercise-induced oxidative stress. The lack of effect on neutrophil functions is similar to observations in previous studies [8, 11] that have suggested oxidative stress, and therefore antioxidant supplementation has little influence on immunoendocrine changes during and after prolonged exercise. These findings do not agree with those by Davison et al. [7]; however, in which acute vitamin C blunted the exercise-induced depression of neutrophil functions but antioxidants were also consumed during exercise in this study [7].

In conclusion, the present study demonstrates that DC consumption 2 h prior to prolonged exercise (when compared to the control and fasting conditions) leads to significantly higher pre-exercise plasma insulin and epicatechin concentrations, higher plasma antioxidant capacity and smaller perturbations in plasma glucose concentration. There was a post-exercise increase in the oxidative stress biomarker plasma free F_2-isoprostane concentration in the control and fasting conditions but this was not evident after DC consumption. However, there were no overall effects on the plasma concentration of HPA axis stress hormones, IL-6, total leucocyte or neutrophil trafficking and neutrophil function. These results with acute DC consumption are similar to those observed following 2 weeks of daily DC ingestion [12].

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