

Cocoa consumption dose-dependently improves flow-mediated dilation and arterial stiffness decreasing blood pressure in healthy individuals

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Background: Cocoa flavonoids exert beneficial vascular effects and reduce the risk of cardiovascular morbidity and mortality. Nevertheless, the involved mechanisms have not been clarified and no study has yet focused on the dose–response effects.

Objectives: We aimed to investigate the effects of different doses of cocoa flavonoids on flow-mediated dilation (FMD), endothelin-1 (ET-1), pulse wave velocity (PWV), and SBP and DBP.

Design: According to a randomized, double-blind, controlled, cross-over design, 20 healthy volunteers (1.5% improvement in FMD in 20 individuals: 0.99 at $\alpha = 0.05$) were assigned to receive either five treatments with daily intake of 10 g cocoa (0, 80, 200, 500 and 800 mg cocoa flavonoids/day) in five periods lasting 1 week each.

Results: Cocoa dose-dependently increased FMD from 6.2% (control) to 7.3, 7.6, 8.1 and 8.2% after the different flavonoid doses, respectively ($P < 0.0001$). Compared with the control, even 80 mg cocoa flavonoids per day increased FMD ($P < 0.0001$). Cocoa dose-dependently decreased PWV ($P < 0.0001$). Cocoa intake decreased office blood pressure (BP) (SBP: -4.8 ± 1.03 mmHg, $P < 0.0001$; DBP: -3.03 ± 1.07 mmHg, $P = 0.0011$). With respect to control, cocoa ingestion decreased 24-h ($P = 0.05$) and daytime ($P = 0.038$) SBP, and 24-h ($P = 0.0064$), daytime ($P = 0.0088$) and night-time ($P = 0.0352$) pulse pressure. Compared with the control, cocoa dose-dependently decreased ET-1 levels [from 17.1 (control) to 15.2, 14.5, 14.2 and 14.1 pg/ml, after the different flavonoid doses, respectively (P for treatment < 0.05)]. Compared with the control, significant changes were observed for all doses of flavonoids (ET-1; $P < 0.05$).

Conclusion: Our study showed for the first time that cocoa dose-dependently improved FMD and decreased PWV and ET-1 also by ameliorating office and monitored BP. Our findings are clinically relevant, suggesting cocoa, with very low calorie intake, might be reasonably incorporated into a dietary approach, representing a consistent tool in cardiovascular prevention.

Keywords: arterial function, blood pressure, cocoa, endothelial function, flavonoids

Abbreviations: BP, blood pressure; ET-1, endothelin-1; FMD, flow-mediated dilation; PWV, pulse wave velocity

INTRODUCTION

Epidemiologic studies indicate that diets rich in fruit and vegetables are associated with a decreased incidence of cardiovascular events, such as coronary artery disease and stroke [1–3]. This effect was ascribed, at least in part, to the high content of polyphenolic compounds, such as flavonoids, in plant-based foods [3–5]. In this context, a number of studies showed that flavonoids – a major class of polyphenols acting as important bioactive constituents of cocoa, some chocolates, red wine and tea – received much attention. Indeed, they may be the causal relationship between cocoa consumption and improvements in cardiovascular function [5–7]. Cocoa is particularly rich in flavonoids, and the total flavonoid content of cocoa beans has been estimated to be 6–8% by weight of the dry bean [5–8]. Recent studies have demonstrated a potential and, to some extent, unanticipated and unexpected role of cocoa in ‘promoting health’ [5–7]. Indeed, a large body of evidence supports dietary intake of flavonoids, and the specific class of flavanols from cocoa might exert some beneficial vascular effects, reduce the risk of cardiovascular morbidity and mortality, and contribute to the prevention of other chronic diseases [5–8]. Expanding on this, we demonstrated that flavanol-rich dark chocolate administration significantly increased the endothelium-dependent flow-mediated dilation (FMD) of the brachial artery also positively affecting additional cardiovascular risk factors in healthy individuals [9], as well as in hypertensive patients

Journal of Hypertension 2015, 33:294–303

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Received 4 March 2014 **Revised** 8 September 2014 **Accepted** 8 September 2014
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DOI:10.1097/HJH.0000000000000412

with and without glucose intolerance [10,11]. Further, we also showed that (–)-epicatechin, the flavanol mostly represented in cocoa, induced a dose-dependent nitric oxide-mediated vasorelaxation in isolated rat aortic rings precontracted by phenylephrine [7]. However, a meta-analysis by Hooper *et al.* [12], aiming to systematically review the effectiveness of different flavonoid subclasses and flavonoid-rich food sources on cardiovascular disease and endothelial function, reported that only the group represented by chocolate or cocoa was able to show significant effects, both acutely and chronically, on FMD. Considering differences in chemical structures and range of doses, they also observed significant heterogeneity between different flavonoid subgroups [12]. According to this, all the recent meta-analyses suggested that although data from literature seem to indicate that flavonoids present with all the biological potential to positively affect vascular function via direct and indirect actions, the possible mechanisms involved should be yet clarified. Further, studies specifically focusing on dose–response effects are lacking [12–16]. Conflicting results on the effects of flavonoids on vascular function and blood pressure (BP) levels have been reported [12–16]. Part of this inconsistency may be due to the amount of cocoa flavonoids tested; accordingly, the dose–effect relation has never been evaluated [12–16].

Thus, the aim of the present study was to assess the effects of a range of doses of cocoa flavonoids on endothelium-dependent FMD, arterial stiffness, endothelin-1 (ET-1) levels, office and monitored BP and insulin sensitivity, according to a randomized, controlled, double-blind, cross-over study in healthy individuals.

METHODS

Participants were recruited at the University of L'Aquila – Department of Internal Medicine and Public Health –

among healthy volunteers, between 18 and 70 years of age, with a sitting SBP/DBP less than 140/90 mmHg and BMI at least 19 and less than 30 kg/m². Volunteers were excluded if they had a chronic–acute disease, including any kind of metabolic abnormality or major cardiovascular risk factor, or both. Habitual smokers and users of prescribed medication and dietary supplement, including those who used any type of medication or supplement, or both, within the past 2 weeks before entering, were also excluded. To further avoid confounding factors, we also excluded individuals reporting daily intense sporting activities (>10 h/week), and/or intolerance/allergy to cocoa-based products, and/or weight change above 10% body weight within the past 6 months before entering the study, and/or were currently treated with a diet due to any reason, and/or reported participation in another study 3 months before the study was started. The study was approved by the responsible Ethics Committee and all participants gave written informed consent. The procedures followed were in accordance with the ethical standards of the responsible institutional or regional committee on human experimentation and in accordance with the Helsinki Declaration of 1975 as revised in 1983.

Study design

Participants received five 1-week periods of cocoa treatments according to a randomized, controlled, double-blind, cross-over design. Total flavonoid content of cocoa during the five 1-week periods was randomly changed from 0 mg (control) to 80, 200, 500 and 800 mg/day on each week. Treatment phases were separated by a 1-week washout period, during which volunteers consumed their habitual diets. Measurements were assessed on the 7th (24-h ambulatory BP) and 8th day of each intervention phase (Fig. 1). In order to avoid any possible stress-dependent cardiovascular effect, all vascular evaluations were performed in complete

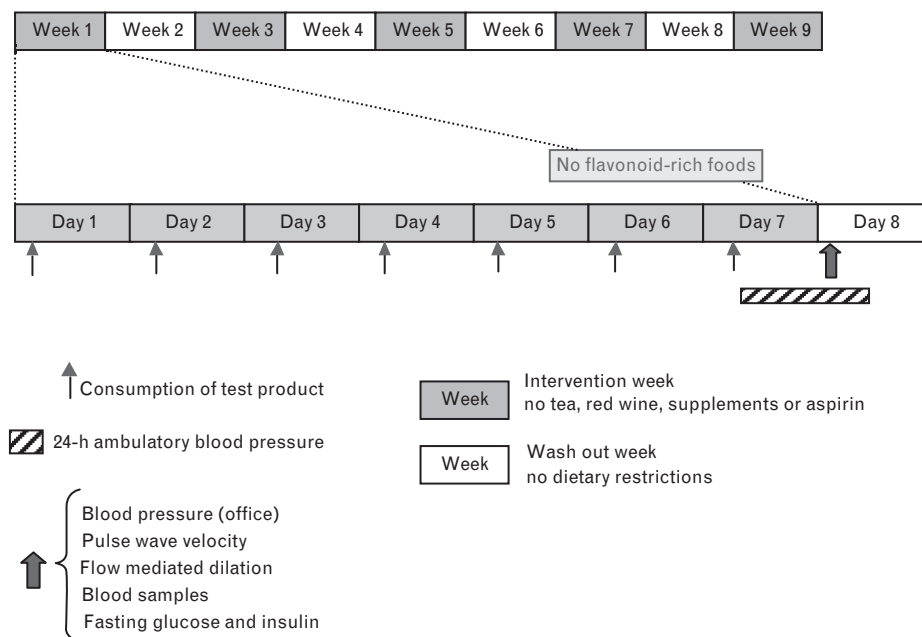


FIGURE 1 Study design.

relaxed behaviour (non-invasive devices, temperature-controlled room without noise and after at least 15 min of rest), with healthy individuals completely relaxed and without any condition of possible psychic or physical stress.

Individuals were instructed to maintain their usual diet and lifestyle, to avoid flavonoid-rich foods (tea, red wine and chocolate), dietary supplements or NSAIDs and to maintain their usual daily intake of fruits and vegetables.

To ensure blinding of the study, all cocoa doses for each day were provided in coded, non-transparent, identical sachets. Before the start of the study and at the four subsequent measurement visits, the volunteers received the intervention products for the next intervention week. The volunteers also received five additional cocoa powder sachets to correct mistakes and spillage. The intervention products were ambient stable. Volunteers returned additional sachets and empty sachets to check compliance. Adherence to the study protocol was confirmed at each visit by a checklist questionnaire of specified food items and by monitoring daily body weight and physical activity. The product was consumed dissolving the cocoa powder in 150 ml of hot water. Addition of milk and/or sugar was not allowed. Volunteers consumed one dose per day, fasting at least 3 h after breakfast.

Cocoa powder intake was of 10 g (including 0.2% sweetener, and 0.14% anti-caking agent), once a day, and the amount of flavonoids in the resulting cocoa was measured with the Folin Ciocalteu assay using gallic acid as a standard. Composition of the test product is shown in Table 1. Regardless of the flavonoid content, each sachet presented with similar colour, taste and flavour. Caffeine, theobromine, sodium, potassium and magnesium content was similar in all products.

Endothelial function

On day 8 of each week of active treatment, FMD of the brachial artery was assessed after 15 min of rest. FMD was always determined by the same physician, who was blinded to the study design and objectives. FMD of the brachial artery of the dominant arm was measured by ultrasonography (Vivid E; GE Healthcare, Milano, Italy). The transducer was held at the same point throughout the scan by a stereotactic clamp. The arterial diameter was measured at approximately 5–10 cm above the elbow. After a 1-min baseline measurement, a cuff placed at the forearm below the elbow was inflated at 300 mmHg for 5 min and then released, resulting in a brief period of reactive hyperaemia. The maximal dilation of the brachial artery was

measured. The brachial artery diameter changes in response to increased blood flow were assessed for a further 3 min after cuff deflation. Using FMDStudio system (QUIPU, Pisa, Italy) [17–19], the approximate position of the edges of the vessel was manually located before starting the examination. After this procedure, an automatic mathematical contour tracking operator [17–19] locates and tracks the edges, supplying information about the quality and time course of measurements in real time [17–19]. On completion of the analysis, the device automatically generates a report, with all the recorded measurements. The FMDStudio system is composed of a special-purpose hardware/software device [17–19], which can be directly connected with any ultrasound equipment, enabling the FMD assessment in real time. FMD is expressed as percentage change from the baseline diameter.

Blood pressure measurements

From day 7 to day 8 of each active treatment week, 24-h ambulatory blood pressure monitoring (ABPM) was recorded by a non-invasive oscillometric device (Medical 90217–30; Spacelabs, Inc., Redmond, Washington, USA). BP was recorded for a period of 24 h at 15-min intervals (daytime 0600 to 2200 h) or 20-min intervals (night-time 2200 to 0600 h) under free-living conditions. The first hour was not considered and the mean of the remaining 24-h, daytime and night-time BP was calculated for statistical evaluation. Office BP measurements were also recorded after intervention phases in a quiet, temperature-controlled (24°C) room by trained, certified staff, who were blind about the study protocol, by the use of a validated oscillometric device with appropriately sized cuffs (Omron HEM-705 CP; Omron Matsusaka Co. Ltd., Matsusaka City, Japan) on the non-dominant upper arm. For this purpose, participants rested for 15 min in a seated position. Then, the first BP measurement was discarded and the subsequent three consecutive BP readings, taken at 5-min intervals, were recorded. The average of these latter measures was considered for statistical analysis.

Arterial stiffness

Carotid–femoral pulse wave velocity (cfPWV) was measured by the ‘gold standard’ [20] technique, with the individual in a quiet, temperature-controlled room (24°C) after 15 min in the supine position. Arterial tonometry was performed by one trained operator according to the international recommendations [20]. A hand-held probe was placed on the artery and 10–15 subsequent images were recorded. Aortic PWV was assessed (SphygmoCor; AtCor Medical, Sydney, Australia) by recording waveforms at the femoral and carotid site sequentially as distance-to-time ratio. Surface distance between the two recording sites and simultaneously recorded ECGs were used to calculate wave transit time. Three consecutive recordings were obtained, and measurements were repeated if the waveforms did not pass the automatic quality controls by the software. During each measurement, BP was also assessed.

Assessment of insulin sensitivity

After the intervention phases, after a 10–14-h overnight fast, the blood samples for plasma glucose and insulin assays

TABLE 1. Composition of the intervention products

Treatments	1	2	3	4	5
Energy (kcal)	38.4	38.4	38.4	38.4	38.4
Total flavanols (DP1-10) (mg)	0	80	200	500	800
Epicatechin (mg)	0	17	42	105	168
Theobromine (mg)	329	329	329	329	329
Caffeine (mg)	25	25	25	25	25
Na (mg)	3	3	3	3	3
K(mg)	713	680	647	348	240
Mg (mg)	68	68	68	68	68

K, potassium; Mg, magnesium; Na, sodium. Bold values indicate the only difference in flavanols and epicatechin between the treatments.

were drawn from an antecubital vein. Plasma glucose and insulin values were used to calculate the index of insulin resistance, homeostasis model assessment of insulin resistance (HOMA-IR) [21] and the indices of insulin sensitivity, quantitative insulin sensitivity check index (QUICKI) [22] and of insulin secretion HOMA-derived β -cell function (HOMA-% β) [22].

Endothelin-1, blood lipids and vascular inflammation

After each intervention phase, blood samplings were also performed to evaluate the circulating levels of ET-1 (ET-1 ELISA; R&D Systems, Minneapolis, Minnesota, USA), high-sensitive C-reactive protein (hs-CRP) (Cobas Integra 700; Roche Diagnostics, Indianapolis, Indiana, USA). Further, in all individuals, a routine haematochemical check was performed by standard methods before and after each active treatment phase. Serum total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol and triglyceride levels were assessed in the clinical chemistry laboratory using routine procedures.

Statistical analysis

Data are expressed as least square means $\pm$ sSEM. Statistical analyses and power calculation were performed with SAS (version 9.1.3, 2004; SAS Institute Inc., Cary, North Carolina, USA).

Statistical power was based on results obtained in similar studies with healthy individuals who consumed a chocolate bar containing 500 mg polyphenols [11]. In that study, the mean ambulatory BP decreased by 6.5 mmHg at a SD of 5.8 mmHg. On the basis of these data, a mean difference of 4 mmHg should be detected in a group of 20 individuals (two-sided, $\alpha=0.05$ and power=0.8). In the same study, FMD increased by 1.9% at a SD of 1.6% after cocoa polyphenol consumption. On the basis of these data, a 1.5% improvement in FMD could be detected in 20 individuals with a very high power (0.99 at $\alpha=0.05$). Random allocation sequence was generated by a Random Number Generator.

Results were evaluated using a linear-mixed model with Proc Mixed Procedure, with patients treated as a random factor, and treatment and sequence as fixed factors. Estimates for differences between mean responses at each dose [0 (control), 80, 200, 500 and 800 mg tea flavonoids per day] were computed using Dunnett–Hsu correction for multiple comparisons.

RESULTS

Baseline data

According to the inclusion/exclusion criteria, participants presented with normal ranges for BMI, lipid profiles, and gluco-insulin metabolism. The study was conducted between June 2008 and August 2011. The study was stopped from April 2009 to May 2010 in consequence of the earthquake which occurred in L'Aquila. The baseline clinical characteristics of the 20 volunteers are shown in Table 2. Twenty-three individuals were recruited; one smoker and two individuals reporting gastric disturbances were

TABLE 2. Baseline characteristics of the study population ($n=20$, 11 men, age 53.8 ± 8.9 years; mean $\pm$ SD)

Characteristic	Baseline
BMI (kg/m^2)	25.4 ± 2.4
SBP/DBP	$122.9 \pm 8.7/74.9 \pm 5.6$
LDL-cholesterol (mg/dl)	131.7 ± 28.4
HDL-cholesterol (mg/dl)	55.3 ± 15.5
Triglycerides (mg/dl)	114.0 ± 46.1
Plasma glucose (mg/dl)	86.1 ± 9.8

HDL, high-density lipoprotein; LDL, low-density lipoprotein.

excluded. All the 20 individuals enrolled completed all the study phases. No drop-outs were observed. Compliance was 100% in all the volunteers for all the study phases.

Endothelial function

Compared with control, all cocoa-active treatments significantly and dose-dependently increased FMD. The improvement was dose-dependent (Fig. 2). Even 80 mg cocoa flavonoids per day increased FMD by 1.07% when compared with control ($P<0.0001$). Furthermore, the change in FMD after consumption of 800 mg per day was not only significant when compared with control ($P<0.0001$) but also when compared with doses 80 mg/day ($P=0.0003$) and 200 mg/day ($P=0.05$). Furthermore, univariate testing between doses 500 and 800 mg did not yield a significant difference, which is in good agreement with the fit of the dosing–effect curve, reaching a plateau around 500 mg. Compared with control, cocoa flavonoids improved endothelial function ($P=0.0001$ for treatment).

Arterial stiffness

Compared with control, carotid–femoral artery PWV significantly (from 8.14 m/s after control to 7.39 m/s after 800 mg/day; $P<0.0001$) and dose-dependently decreased (0 vs. 200 mg, $P=0.0008$; 0 vs. 500 mg, $P<0.0001$; 0 vs. 800 mg, $P<0.0001$) (Fig. 3). Compared with control, cocoa flavonoids improved arterial stiffness ($P<0.0001$ for treatment).

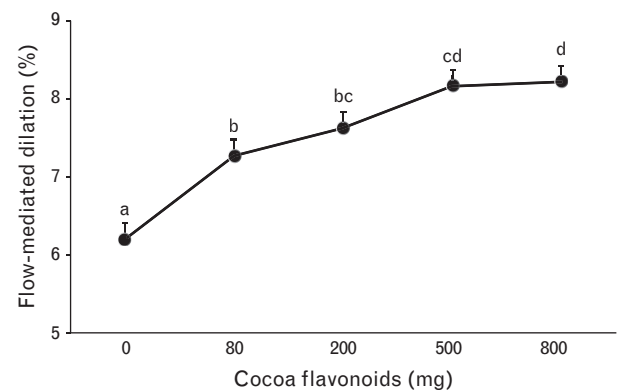


FIGURE 2 Effects of cocoa on endothelium-dependent flow-mediated dilation in 20 healthy volunteers. Data are expressed as least square means with standard error of the mean. Data points with different superscripts are significantly different. Differences are considered significant when P value is less than 0.05.

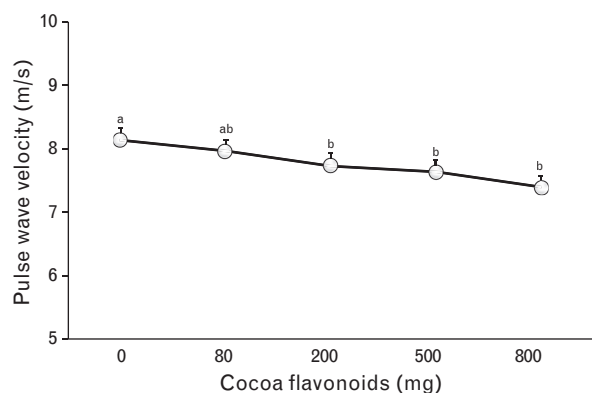


FIGURE 3 Effects of cocoa on carotid-femoral pulse wave velocity in 20 healthy volunteers. Data are expressed as least square means with standard error of the mean. Data points with different superscripts are significantly different. Differences are considered significant when P value is less than 0.05.

Office and 24-h ambulatory blood pressure monitoring

Compared with control, office SBP and DBP significantly decreased during each of the 4 weeks of active treatment (SBP: mean difference: -4.8 ± 1.03 mmHg, $P < 0.001$; DBP: mean difference: -3.03 ± 1.07 mmHg, $P < 0.001$); SBP, but not DBP, decreased dose-dependently (Fig. 4a and b). With respect to control, cocoa flavonoids decreased SBP ($P < 0.0001$ for treatment) and DBP ($P = 0.0011$ for treatment). Accordingly, with respect to control, 24-h (mean difference: -2.28 ± 1.22 mmHg) and daytime (mean difference: -2.45 ± 1.57 mmHg) ambulatory SBP significantly decreased after active treatments (Table 3), whereas no significant differences between treatments were observed for the monitored DBP levels. In addition, compared with control, 24-h, daytime and night-time pulse pressure (PP) and night-time heart rate significantly decreased after active

treatments, but the effects did not seem to be dose-dependent (Table 3).

Endothelin-1 levels

Compared with control, cocoa dose-dependently decreased ET-1 levels [from 17.1 (control) to 15.2, 14.5, 14.2 and 14.1 pg/ml; after the different flavonoid doses, respectively (P for treatment < 0.05)]. With respect to control, significant changes were observed for all doses of flavonoids (ET-1; $P < 0.05$) (Fig. 5).

Insulin resistance and lipid metabolism

Although insulin resistance tended to decrease also with a trend in reducing insulin levels, HOMA was not significantly different between treatments. Similarly, QUICKI and HOMA-% β did not significantly change after the different cocoa intervention phases (data not shown).

Similarly, lipid profile and vascular inflammation did not change after active treatments compared with control.

DISCUSSION

In this randomized, controlled, double-blind, cross-over study, we demonstrated for the first time that short-term cocoa administration dose-dependently improves endothelial function and ET-1 levels, and positively affects arterial stiffness and office and monitored BP in healthy individuals. Moreover, the present study is also the first suggesting that only 10 g of cocoa with a very low calorie (38 kcal) and flavanol (80 mg) intake per day is already significantly ameliorating vascular function. According to this, our findings are of clinical relevance and interest, and strongly support the epidemiological studies reporting an inverse relationship between cocoa and cardiovascular disease [3,5–7,23–25].

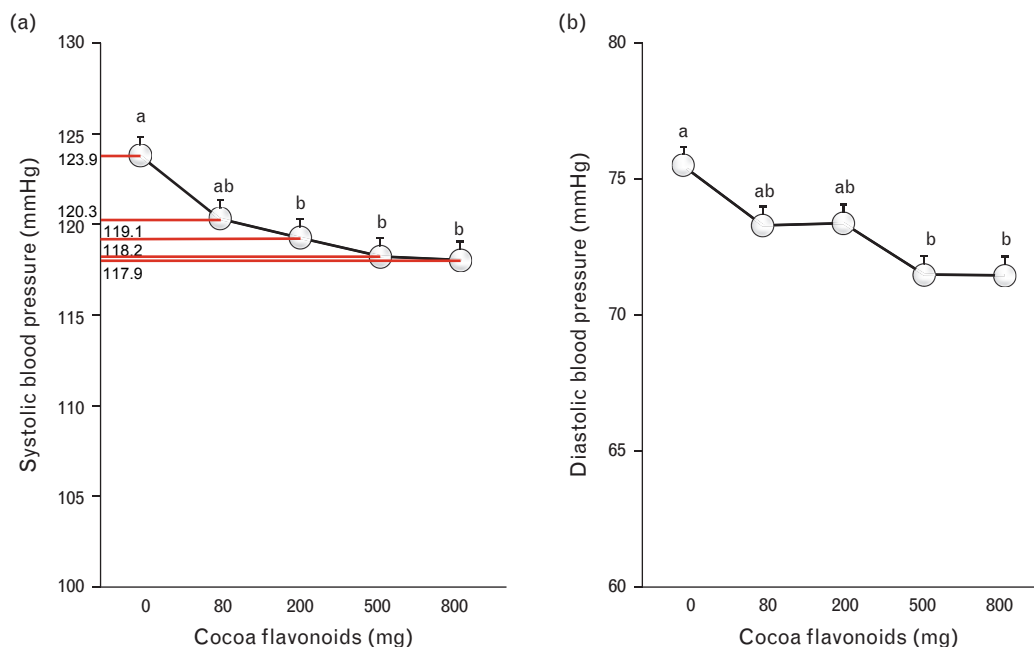


FIGURE 4 Effects of cocoa on office SBP and DBP in 20 healthy volunteers. Data are expressed as least square means with SEM. Data points with different superscripts are significantly different. Differences are considered significant when P value is less than 0.05.

TABLE 3. Least square means, SEM and *P* value in the analysis of variance of the ambulatory blood pressure monitoring as performed with the SAS linear model procedure

ABPM	0 mg control	80 mg	200 mg	500 mg	800 mg	SEM	<i>P</i>
SBP 24 h	117.7	118.3	114.7	115.5	114.3	1.60	0.05
SBP day	122.4	122.5	119.1	119.4	119.1	1.56	0.038
SBP night	108.7	110.1	106.6	107.8	106.3	1.33	NS
DBF 24 h	70.8	70.9	70.7	71.2	71.3	1.07	NS
DBP day	75.5	75.3	75.1	75.3	75.7	1.91	NS
DBP night	62.0	62.7	61.7	63.1	63.5	1.34	NS
HR 24 h	76.4	73.8	77.2	76.2	75.9	1.58	NS
HR day	79.2	77.5	81.5	80.3	80.2	1.94	NS
HR night	70.9	66.6*	68.7	68.5	67.5*	1.33	0.024
PP 24 h	47.0	47.5	43.8	44.6	43.1*	1.87	0.0064
PP day	47.2	47.5	43.4*	44.2	43.6*	1.81	0.0088
PP night	46.7	47.4	44.3	44.9	43.0	2.10	0.0352

ABPM, ambulatory blood pressure monitoring; HR, heart rate; NS, not significant. Bold values suggest the results are statistically significant.

Endothelial function and cardiovascular risk

In normal vascular physiology, nitric oxide plays a key role to maintain the vascular wall in a quiescent state by inhibiting inflammation, cellular proliferation and thrombosis [26–28]. The endothelium maintains vascular homeostasis through multiple complex interactions with cells in the vessel wall and lumen. It regulates vascular tone by balancing the production of vasodilators, most importantly nitric oxide, and vasoconstrictors. Therefore, a ‘healthy’ endothelium maintains vascular tone and structure by regulating the balance between vasodilation and vasoconstriction [26–28]. On the contrary, in pathologic conditions, particularly with the presence of cardiovascular risk factors, the endothelium undergoes functional and structural alterations, thus losing its protective role and becoming a pro-atherosclerotic structure [26–28]. In the earliest stages, the principal endothelial alteration is merely functional and is addressed as ‘endothelial dysfunction’. The fundamental feature of this condition is the impaired nitric oxide bioavailability. An impaired nitric oxide bioavailability is a common feature of the different endothelial dysfunctions [27–29] and an abnormal endothelium-dependent vasorelaxation has been associated with an increased risk of developing atherosclerosis [27–29]. It has been hypothesized that this endothelial-dependent vascular imbalance is

critical, not only in the initiation and progression of atherosclerosis, but also in the transition from a stable to an unstable disease state with the precipitation to acute vascular events [27–30]. According to this, it has been supposed that a dysfunctional endothelium may promote plaque activation, leading to a higher plaque vulnerability [27–31]. The magnitude of endothelial dysfunction is an important and independent predictor of both coronary and cerebrovascular events, and is linked to arterial wall hypertrophy and stiffening [26–35]. In turn, positive changes in nitric oxide bioavailability, by improving endothelium-dependent vasodilation and/or the correction of endothelial dysfunction, might protect against atherosclerosis [25,29].

Cocoa intake and cardiovascular protection

A recent study showed that acute cocoa ingestion dose-dependently increases brachial artery FMD in healthy older humans with a correlation of a serum marker of cocoa ingestion (total epicatechin) with increased FMD 1 and 2-h post-ingestion. Moreover, Shrimel *et al.* [36], aiming to perform a systematic review and meta-analysis of short-term randomized controlled trials performed in adults to examine the effects of flavonoid-rich cocoa on multiple markers of cardiovascular health, reported that a non-linear dose–response relationship exists between flavonoid-rich cocoa and FMD ($P=0.004$), with maximum effect observed at a flavonoid dose of 500 mg/day. Accordingly, in our short-term randomized, controlled, double-blind, crossover study, we found for the first time a dose-dependent effect of flavanol-rich cocoa on FMD. The maximum effect was observed at a flavonoid dose of 800 mg/day, but non-significant differences were observed between 500 and 800 mg intake.

Regarding our second finding, arterial stiffness describes the rigidity of the arterial walls and vascular stiffening is the consequence of complex interactions between stable and dynamic modifications involving structural and cellular elements of the vessel wall [37,38].

The structural and functional changes in the arterial circulation lead to disturbances in regional blood flow, progression of atherogenesis and microvascular changes occurring during senescence and in presence of cardiovascular risk factors [37–39]. However, arterial stiffness and

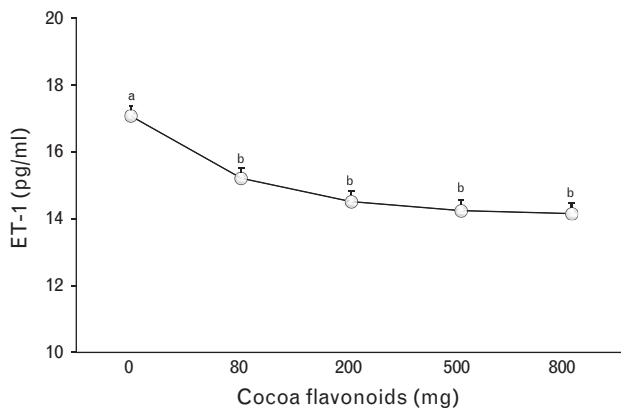


FIGURE 5 Effects of cocoa on endothelin-1 (ET-1) levels in 20 healthy volunteers. Data are expressed as least square means with standard error of the mean. Data points with different superscripts are significantly different. Differences are considered significant when *P* value is less than 0.05.

wave reflections are known to play a fundamental role in cardiovascular health and disease [37–39]. Indeed, in interplay with left ventricular ejection and the elastic properties of the aorta, wave reflections could specifically increase central PP, a recognized predictor of cardiovascular risk [37–39]. In this regard, our findings firstly suggest that cocoa dose-dependently decreases cPWV. Consistent with our data, Vlachopoulos *et al.* [40] showed that habitual higher cocoa intake was an independent determinant of low arterial stiffness and wave reflection indexes, and was also independently associated with significantly lower central (aortic) PP in healthy individuals.

Furthermore, our study showed lowering effect on both office SBP and DBP levels after cocoa ingestion, also confirmed by ABPM, with interesting observations on decreasing 24-h and daytime SBP, and 24-h, daytime and night-time PP after cocoa intake. The mechanism responsible for the observed BP-lowering effects of cocoa could be probably related to increased nitric oxide bioavailability. This latter mechanism could also explain the significant improvement in peripheral arterial distensibility and in cPWV we observed. Of note, we observed that 15 days of consumption of flavanol-rich, but not flavanol-free, chocolate was able to significantly lower both SBP and DBP in healthy individuals [9] and in hypertensive patients without (after dark chocolate: 24 h SBP -11.9 ± 7.7 mmHg, $P < 0.0001$; 24 h DBP -8.5 ± 5.0 mmHg, $P < 0.0001$) [11] and with [10] glucose intolerance. In hypertensive glucose-intolerant patients [10], we observed that monitored and clinical SBP and DBP significantly decreased ($P < 0.0001$) after flavanol-rich (SBP: -3.82 ± 2.40 mmHg; DBP: -3.92 ± 1.98 mmHg; 24 h SBP: -4.52 ± 3.94 mmHg; 24 h DBP: -4.17 ± 3.29 mmHg), but not after flavanol-free, chocolate ingestion [10]. Furthermore, the flavanol-rich chocolate administration significantly improved nitric oxide-dependent FMD ($P < 0.0001$) and interestingly the decrement in BP values was inversely correlated with the increase in FMD (SBP: $r = -0.547$, $P = 0.0004$; DBP: $r = -0.488$, $P = 0.001$; 24-h SBP: $r = -0.460$, $P = 0.003$; 24-h DBP: $r = -0.457$, $P = 0.003$), thus suggesting a possible pathophysiological correlation between the observed vascular and BP changes. According to this, more evidence on potential antihypertensive properties of cocoa was shown from a recently published interventional study that compared the long-term effect of dark compared with white chocolate consumption in patients with prehypertension or stage I hypertension [41]. In this interesting study, a small amount of dark chocolate daily ingestion for 18 weeks (6.3 g–30 kcal) significantly reduced mean SBP by -2.9 ± 1.6 mmHg and DBP by -1.9 ± 1.0 mmHg, with no changes in body weight, plasma lipid levels, glucose and 8-isoprostane. Furthermore, hypertension prevalence declined from 86 to 68%, and the BP reduction was accompanied by a sustained increase of S-nitrosoglutathione paralleled by appearance of cocoa phenols in plasma. These changes suggest an increase in nitric oxide production as a potential mechanism of the small reduction in BP seen with dark chocolate consumption [41]. Additionally, Davison *et al.* [42], aiming to evaluate the effect of three different doses of cocoa flavanols on 24-h mean arterial BP in untreated mild hypertensive patients, reported

significant reductions in 24-h SBP (5.3 ± 5.1 mmHg; $P = 0.001$), DBP (3 ± 3.2 mmHg; $P = 0.002$) and mean arterial BP (3.8 ± 3.2 mmHg; $P = 0.0004$) at the 1052-mg dose cocoa flavanols, whereas no significant changes in BP were observed at any other dose. Furthermore, no evidence of dose–response was reported. Moreover, the same group [43] also observed the BP response to exercise was attenuated by high-flavanol compared with low-flavanol cocoa beverage. BP increases were 68% lower for DBP ($P = 0.03$) and 14% lower for mean BP ($P = 0.05$). Similarly, FMD measurements were higher after taking high-flavanol than after taking low-flavanol cocoa. This supports previous evidence for cocoa flavanols to lower BP also by facilitating vasodilation and attenuating exercise-induced increases in BP, thereby enhancing the cardiovascular benefits of moderate-intensity exercise in at-risk individuals.

Effects on blood pressure

In our short-term interventions, we observed a decrease in office SBP and DBP [significant effects for higher doses of flavonoids (>200 mg flavonoids and >42 mg of epicatechin), with a trend for dose-dependent effects for SBP] (Fig. 4). Consistent with our data, Shrimel *et al.* [36], in a meta-analysis evaluating 24 short-term studies, showed that in response to flavonoid-rich chocolate consumption, SBP, but not DBP, decreased by -1.63 mmHg ($P = 0.033$).

Finally, resuming all the findings regarding BP effects of cocoa and chocolate intake, an early meta-analysis by Hooper *et al.* [12] reported that chocolate and cocoa intake was able to significantly reduce SBP (-5.88 mmHg; -9.55 , -2.21 ; five studies) and DBP (-3.30 mmHg; -5.77 , -0.83 ; four studies) (effects appeared greater in studies with higher doses and shorter duration), whereas chronic intake of other flavanols (with ≥ 3 included studies) did not show any effect on BP. In agreement with our findings on BP, the same group, systematically reviewing 42 acute or short-term chronic (≤ 18 weeks) studies involving 1297 participants, recently reported chocolate and cocoa are able to reduce DBP and mean arterial pressure [48]. In particular, we observed a decrease in office DBP (mean difference: -3.03 ± 1.07 mmHg; $P < 0.001$), with doses of cocoa flavonoids higher than 500 mg and epicatechins higher than 105 mg. Consistent with our results on DBP, the above-cited meta-analysis showed significant reductions in DBP after cocoa and/or chocolate administration [-1.60 mmHg; 95% confidence interval (CI) -2.77 , -0.43 mmHg] considering 22 trials with 918 participants. Furthermore, in line with our study on dose–responses (significant effects in SBP for epicatechins higher than 42 mg and DBP higher than 105 mg), the meta-analysis showed (with different kinds of treatments: cocoa, chocolate or both) that doses above 50 mg epicatechin/day reduced SBP and DBP, whereas doses below 50 mg/day did not [48]. Although the BP reduction we observed was small, the effects might be clinically relevant. Indeed, it has been reported that 2–3 mmHg reduction in SBP would also reduce the risk of cardiovascular and all-cause mortality [44]. Moreover, it seems important to remark that a reduction of 5 mmHg in SBP and DBP has been shown to reduce the risk of cardiovascular events by about 20% over 5 years [45]. Therefore, the effect of cocoa in hypertensive patients is

comparable to other lifestyle modifications, such as moderate physical activity (30 min/day), which has been reported to decrease SBP by 4–9 mmHg [46,47].

Effects on endothelial function

Furthermore, although the majority of available data supports the evidence that flavonoid-rich foods and beverages are able to improve nitric oxide bioavailability and thereby increase endothelium-dependent vasorelaxation in healthy individuals, as well as in various pathological conditions, a meta-analysis [12] reported that soy protein isolate, isoflavone extracts, as well as red wine or grape and other flavanols do not present with significant effects on endothelial function. On the contrary, the data suggested a beneficial effect for chocolate or cocoa. In particular, chocolate or cocoa increased FMD by 1.45% (95% CI 0.62, 2.28%; two studies). When data were available from at least three acute studies, only chocolate or cocoa significantly improved FMD (3.99%; 95% CI 2.86, 5.12; six studies). In addition, studies of red wine, or grape and black tea, suggested a modest benefit, although neither was statistically significant and both were significantly heterogeneous. For many of the sub-classes, including anthocyanins, flavanones, green tea and soyfoods, no published data on potential FMD effects were available. Only the group represented by chocolate or cocoa was able to show significant effects, both acutely and chronically, on FMD. Considering differences in chemical structures and range of doses, significant heterogeneity was observed between different flavonoid sub-groups (P for heterogeneity <0.01 , $I^2 = \approx 80\%$ in both acute and chronic studies). This confirmed that different flavonoid groups have different effects on FMD. Additionally, exactly supporting our findings on FMD in this study, the same group systematically reviewing the effects of chocolate, cocoa and flavan-3-ols on major cardiovascular risk factors, very recently reported consistent acute and chronic benefits of chocolate or cocoa on FMD, with chocolate or cocoa improving FMD regardless of the dose consumed [48].

Although not all the involved mechanisms have been exhaustively clarified, data from literature seem to suggest that flavonoids present with all the biological potential to positively affect vascular function via direct and indirect actions. They potentially could be considered as healthy compounds for diet supplementation.

Final comments and perspectives

As reported in all the recent systematic reviews and meta-analyses cited [12,14,15,36,48], our study satisfied the lacking information on the argument. Indeed, we observed for the first time that cocoa dose-dependently improved FMD and decreased PWV and ET-1 also by ameliorating office and monitored BP. Our findings are clinically relevant, suggesting cocoa, with very low calorie intake, might be reasonably incorporated into a dietary approach, representing a consistent tool in cardiovascular prevention. No studies reported dose–response effects on FMD, PWV and ET-1 – all fundamental markers of cardiovascular damage. Moreover, in this randomized, controlled, double-blind, cross-over study, we demonstrated for the first time that only 10 g of cocoa with a very low caloric

(38 kcal) and flavanol (80 mg) intake per day is already significantly ameliorating vascular function. Further, regardless of the flavonoid content, we administered cocoa presented with similar colour, taste, flavour and possible vasoactive compounds. As suggested in the above meta-analysis [48], our study also provided evidence on dose-dependent effects with respect to the epicatechin content. Specifically in agreement with this, the European Food Safety Authority (EFSA) for the first time concluded that a ‘cause and effect’ relationship has been established between the consumption of cocoa flavanols and maintenance of normal endothelium-dependent vasodilation [49]. EFSA conclusions are specifically based on our preliminary results on the dose-dependent effects on FMD we are reporting in this manuscript for the first time. Accordingly, EFSA clearly stated that: ‘In order to obtain the claimed effect, 200 mg of cocoa flavanols should be consumed daily. This amount could be provided by 2.5 g of high-flavanol cocoa powder or 10 g of high-flavanol dark chocolate. These amounts of cocoa powder or dark chocolate can be consumed in the context of a balanced diet. The target population is the general population’ [49].

Study limitations

Although short-term, our study design was extremely rigorous, and stringent inclusion and exclusion criteria avoided the confounding influence of medications, diet, lifestyle factors and concomitant diseases. Furthermore, for the first time, the different cocoa treatments differed only for flavanol content, being controlled for caffeine, theobromine, sodium, potassium and magnesium (all well known vasoactive substances).

In conclusion, our findings indicate that 10 g cocoa exerts significant effects on FMD, ET-1 levels, cfPWV, and office and monitored BP in healthy volunteers. As reported in all the recent systematic reviews and meta-analyses, our study on dose-dependent effects of cocoa flavanols satisfied the lacking information on the argument. The observed responses may help to explain associations between flavonoid and cocoa intake and cardiovascular risk in humans. Waiting for intervention studies testing long-term effects of cocoa intake, the robust nature of our randomized, controlled, double-blind, cross-over study design indicates cocoa, with very low calorie intake, can be reasonably incorporated into a dietary approach representing a consistent tool in cardiovascular prevention.

ACKNOWLEDGEMENTS

D.G., G.D. and C.F. designed research (project conception, development of overall research plan, and study oversight); D.G., G.D. and P.D. conducted research (hands-on conduct of the experiments and data collection); D.G. and R.B. provided essential reagents or provided essential materials (applies to authors who contributed by providing animals, constructs, databases, etc., necessary for the research); D.G., G.D. and S.N. analysed data or performed statistical analysis; D.G., G.D., L.A., H.B. and C.F. wrote the paper. All authors had primary responsibility for final content; all authors participated in drafting of the manuscript or revising it critically for important intellectual content; all the

authors participated for final approval of the manuscript submitted. The authors did not receive grants for the study. The study was supported by funding received from Barry-Callebaut, Belgium.

Conflicts of interest

L.A. and H.B. are employed by the Barry Callebaut, Lebbeke-Wieze, Belgium. D.G., G.D., S.N., P.D., F.M., R.M. and C.F. have no personal or financial conflicts of interest.

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Reviewers' Summary Evaluations

Reviewer 1

The study reinforces the fact recognized by the European Food Safety Authority (EFSA) that cocoa does provide benefits in improving hypertension. Daily doses of cocoa might be helpful in ameliorating the long-term effects of hypertension on ailments like heart failure and remote organ dysfunctions. Clearly, endothelium function is one of the possible beneficial mechanism but more investigation is needed to confirm other mechanisms, if any.

Finally, cocoa consumption and its benefits in patients with diabetes or high-lipids might need some confirmation.

Reviewer 2

The strength of the study is its design which renders the results very valuable and free of bias. In its design, the study is unique and confirms earlier but less well designed studies. The results also are of practical/clinical importance.

The study has no major weaknesses but a study of the same design done in subjects with impaired vascular function is needed for a wider clinical application.