Methylxanthines enhance the effects of cocoa flavanols on cardiovascular function: randomized, double-masked controlled studies

Methylxanthines enhance the effects of cocoa flavanols on cardiovascular function: randomized, double-masked controlled studies[1]

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ABSTRACT

Background: Cocoa flavanol intake, especially that of (−)-epicatechin, has been linked to beneficial effects on human cardiovascular function. However, cocoa also contains the methylxanthines theobromine and caffeine, which may also affect vascular function.

Objective: We sought to determine whether an interaction between cocoa flavanols and methylxanthines exists that influences cocoa flavanol–dependent vascular effects.

Design: Test drinks that contained various amounts of cocoa flavanols (0–820 mg) and methylxanthines (0–220 mg), either together or individually, were consumed by healthy volunteers (n = 47) in 4 different clinical studies—3 with a randomized, double-masked crossover design and 1 with 4 parallel crossover studies. Vascular status was assessed by measuring flow-mediated vasodilation (FMD), brachial pulse wave velocity (bPWV), circulating angiogenic cells (CACs), and blood pressure before and 2 h after the ingestion of test drinks.

Results: Although cocoa flavanol intake increased FMD 2 h after intake, the consumption of cocoa flavanols with methylxanthines resulted in a greater enhancement of FMD. Methylxanthine intake alone did not result in statistically significant changes in FMD. Cocoa flavanol ingestion alone decreased bPWV and diastolic blood pressure and increased CACs. Each of these changes was more pronounced when cocoa flavanols and methylxanthines were ingested together. It is important to note that the area under the curve of the plasma concentration of (−)-epicatechin metabolites over time was higher after the co-ingestion of cocoa flavanols and methylxanthines than after the intake of cocoa flavanols alone. Similar results were obtained when pure (−)-epicatechin and the methylxanthines theobromine and caffeine were consumed together.

Conclusion: A substantial interaction between cocoa flavanols and methylxanthines exists at the level of absorption, in which the methylxanthines mediate an increased plasma concentration of (−)-epicatechin metabolites that coincides with enhanced vascular effects commonly ascribed to cocoa flavanol intake. This trial was registered at clinicaltrials.gov as NCT02149238. *Am J Clin Nutr* 2017;105:352–60.

Keywords: cocoa flavanols, methylxanthines, cardiovascular function, interaction, absorption

INTRODUCTION

Flavanols are plant-derived dietary bioactive compounds (1, 2) that have gained increasing attention because of their beneficial effects on human cardiovascular health, as demonstrated in short- to medium-term clinical dietary intervention studies (3, 4). Various controlled studies have demonstrated cocoa flavanol (CF)[7] intake–dependent improvements in endothelial function and other markers of vascular function, including blood pressure (BP), pulse wave velocity (PWV), and circulating angiogenic cells (CACs) (5–8). CACs have been implicated in vascular maintenance and repair and are a newly established biomarker of cardiovascular risk (9). Furthermore, it has been shown that the beneficial vascular effects observed after the consumption of foods containing CFs can at least partly be attributed to (−)-epicatechin (5, 10). However, in addition to flavanols, other compounds present in foods that contain flavanol could potentially affect vascular functions either by themselves or by modulating flavanol-related effects. In this context, 2 of the most important sources of flavanols in the diet—tea and cocoa-derived products—contain substantial amounts of methylxanthines (5). The main methylxanthine in cocoa is theobromine, whereas caffeine predominates in tea. However, smaller

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[7] Abbreviations used: BP, blood pressure; bPWV, brachial pulse wave velocity; BW, body weight; CAC, circulating angiogenic cell; CF, cocoa flavanol; C_{max}, peak plasma concentration; ED_{50}, intake amount to achieve a half-maximal effect; FMD, flow-mediated vasodilation; HHUD, Heinrich-Heine University Düsseldorf; KDR, kinase insert domain receptor; PBS, phosphate-buffered saline; PWV, pulse wave velocity; SREM, structurally related (−)-epicatechin metabolite; ΔFMD_{max}, maximum flow-mediated vasodilation increase.

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amounts of other methylxanthines can also be found in these food products (11).

A rich data set exists that describes methylxanthine bioactivity in humans, and several biological effects have been well described (12, 13). In addition to the more commonly referenced effects on the central nervous system, methylxanthines, particularly caffeine, have been demonstrated to directly modulate vascular functions, including BP, arterial stiffness, and endothelial function (14–16). In addition, co-ingestion can potentially affect the absorption and plasma concentration of other compounds. For instance, caffeine potentiates the analgesic and anti-inflammatory actions of aspirin and increases absorption and plasma concentration without influencing the volume of distribution and clearance (17, 18). Thus, based on the presence of both flavanols and methylxanthines in cocoa, we investigated the potential role of methylxanthines as modulators of the cardiovascular effects of CFs in humans. We hypothesized that an interaction between CFs and methylxanthines exists that modulates CF-dependent vascular effects. To investigate this hypothesis, we performed 4 studies, each of which aimed to answer a specific question.

TABLE 1
Composition of drink powder used to reconstitute the test drinks in study protocols 1–3[1]

	CF + MX	CF	MX
Serving size, g/75 kg BW	13.2	13.2	13.2
(−)-Epicatechin, mg/75 kg BW	111.8	112.8	ND
(+)-Catechin, mg/75 kg BW	3.2	3.8	ND
(−)-Catechin, mg/75 kg BW	11.6	35	ND
(+)-Epicatechin, mg/75 kg BW	ND	ND	ND
Total CFs, mg/75 kg BW	820	820	ND
Theobromine, mg/75 kg BW	111.0	1.6	112.8
Caffeine, mg/75 kg BW	11.4	1.2	10.2
Total MXs, mg/75 kg BW	122.4	2.8	123.0
Total calories, kcal/75 kg BW	35.4	34	37.4
Total fat, g/75 kg BW	0	0	0
Saturated fat, g/75 kg BW	0	0	0
Cholesterol, mg/75 kg BW	0	0	0
Total carbohydrates, g/75 kg BW	5.8	5.4	5.8
Sugars, g/75 kg BW	0	0	0
Protein, g/75 kg BW	0	0	0
Sodium, mg/75 kg BW	78	70	82

[1] BW, body weight; CF, cocoa flavanol; MX, methylxanthine; ND, below limit of detection.

METHODS

Subjects

Subjects for this study (NCT02149238) were recruited between 2011 February and 2014 December and consisted of healthy young men aged 25–30 y. All subjects with symptoms of cardiovascular disease, including coronary artery, peripheral artery, or cerebrovascular disease; acute inflammation (C-reactive protein >0.6 mg/dL); malignancies; arrhythmias (heart rhythm other than sinus); arterial hypertension (systolic and diastolic BP >140 and >90 mm Hg, respectively); diabetes (fasting glucose >126 mg/dL); and hypercholesterolemia (total cholesterol >200 mg/dL) were excluded.

Study design

Study protocol 1 was designed as the main proof-of-concept study and aimed at assessing potential interactions of CFs and methylxanthines with regard to the modulation of cardiovascular function, including flow-mediated vasodilation (FMD) (first endpoint), arterial stiffness, BP, and CACs (second endpoint). To accomplish this, an acute 3-arm, single-center, randomized, double-masked crossover study (n = 12) was carried out at Heinrich-Heine University Düsseldorf (HHUD). The 3 test drinks consumed by the volunteers provided CFs at 820 mg/75 kg body weight (BW) and cocoa methylxanthines at 125 mg/75 kg BW either together or individually. **Table 1** shows the composition of the base powder used to reconstitute the drinks. The total amount of CFs represents the sum of monomers and oligomers (procyanidins) with a degree of polymerization from 1 to 10. The predominant monomer in the test drinks was (−)-epicatechin; the (−)-epicatechin content of the test drinks was ∼112 mg/75 kg BW. All intake amounts were given on a per 75-kg BW basis (for clarity, applied amounts are only presented as milligrams henceforth). On the 3 study days, which were separated by a 1-wk washout, volunteers consumed the test drinks, and cardiovascular functions were assessed before (0 h) and 2 h (the time of peak FMD effects) after intake. Randomization and allocation to the 3 interventions were based on an electronic treatment plan generated by a GraphPad web application (www.graphpad.com; GraphPad Software Inc.).

Study protocol 2 aimed at assessing the effect of methylxanthines consumed with CFs at different intake amounts. To accomplish this, a single-center (HHUD), randomized, double-masked 4-arm study (n = 24; n = 6/group) was conducted. Subjects were randomly assigned to the 4 parallel arms. In each arm, volunteers were given test drinks that were consumed during the 10 (arms 1 and 2) or 8 (arms 3 and 4) different study visits (crossover). The test drinks consisted of increasing amounts of CFs (0, 102, 205, 410, and 820 mg) (arm 1); increasing amounts of CFs (0, 102, 205, 410, and 820 mg) in the presence of 122 mg methylxanthines (arm 2); increasing amounts of methylxanthines (0, 61, 122, and 244 mg) (arm 3); and increasing amounts of methylxanthines (0, 61, 122, and 244 mg) in the presence of 820 mg CFs (arm 4). Volunteers consumed the test drinks on the study days, and FMD (first endpoint) measurements were taken before (0 h) and 2 h after ingestion.

Study protocol 3 investigated how methylxanthines might modulate the plasma concentration and urinary excretion of the structurally related (−)-epicatechin metabolites (SREMs) and the temporal association with FMD increases. This study was conducted at HHUD and followed a randomized, double-masked, 2-arm crossover design (n = 5). Volunteers were asked to consume the test drinks on the study day, and blood and 0- to 24-h urine samples were collected following previously described procedures (19). FMD (first endpoint) and blood sample measurements were taken to determine SREMs (second endpoint) and were performed before (0 h) and 1, 2, 3, 4, and 5 h after the ingestion of the test drink. The 2 test drinks consumed by the volunteers contained 820 mg CFs, including 112 mg (−)-epicatechin, in either the absence or presence of 122 mg methylxanthines comprising 111 mg theobromine and 11 mg caffeine (Table 1).

Study protocol 4 assessed the effects of pure theobromine and caffeine consumed simultaneously with (−)-epicatechin on plasma concentrations (first endpoints) and the urinary excretion of SREMs (second endpoint). To accomplish this, a single-center, randomized, double-masked crossover study (n = 6) was carried out at the University of California Davis, during which volunteers consumed 75 mg (−)-epicatechin either with or without 400 mg theobromine and 26 mg caffeine on 2 different days. On the study day, blood samples were drawn before (0 h) and 1, 2, and 4 h after the consumption of test drinks, and 0- to 24-h urine samples were collected following previously described procedures (19).

In all studies, volunteers were asked to follow a low-flavanol diet and to refrain from consuming alcohol, coffee, or other beverages that contain caffeine on the day before and during the study visits. Volunteers were also asked to fast for 12 h before each study day (water ad libitum). Study visits were scheduled ≥1 wk apart to avoid potential carryover effects. The study protocols were approved by the HHUD ethics committee and the University of California Davis institutional review board; all volunteers gave written informed consent.

Test drinks containing CFs and methylxanthines

Specifically designed test drinks that contained CFs and methylxanthines either together or individually were used in all studies. The amount of test drinks consumed was standardized on a per 75-kg BW basis, and regardless of the amount of CFs and methylxanthines, all test drinks used in the same study contained similar macro- and micronutrients, as well as very similar orosensory and physicochemical characteristics.

The test drinks used in study protocols 1–3 consisted of fruit-flavored nondairy drinks that contained CFs and the methylxanthines theobromine and caffeine either individually or together (Table 1). These test drinks were prepared for a 75-kg subject by reconstituting 13.2 g of a drink powder with 500 mL water to achieve a content of 820 mg CFs and/or 122 mg methylxanthines. Other intake amounts were achieved with the use of more or less of the identical drink powders in 500 mL water (e.g., 6.6 g to achieve 410 mg CFs). The source of CFs and methylxanthines in the test drinks that contained both groups of compounds simultaneously consisted of a standardized cocoa extract manufactured with the use of the Cocoapro process (Mars Inc.).

To prepare the drinks provided in study protocol 4, we used food-grade (−)-epicatechin isolated from cocoa (Mars Inc.); food-grade theobromine (AHD International LLC) and caffeine (Perrigo) were added to milk (1% fat) as a vehicle for delivering these compounds. The final composition of the test drinks was as follows: 75 mg (−)-epicatechin, 400 mg theobromine, and 26 mg caffeine dissolved in 375 g milk and 75 mg (−)-epicatechin dissolved in 375 g milk.

FMD assessment

FMD was measured as previously described (7). Briefly, the diameter and flow velocity of the brachial artery was measured with the use of a Vivid I 10-MHz transducer (General Electric) and automatic edge-detection software (Brachial Analyzer version 5; Medical Imaging Applications) that yielded SDs of mean differences between repeated measurements of <1%. The brachial artery diameter was measured 2 cm proximal to the elbow. Reactive hyperemia was induced by 5 min of distal lower-arm occlusion with a sphygmomanometric cuff inflated to 250 mm Hg. After cuff deflation (20, 40, 60, and 80 s), the diameter was assessed, and the FMD was calculated as the maximal relative diameter gain relative to the baseline. Sublingual nitroglycerin (0.4 mg) was administered, and endothelium-independent nitroglycerin-mediated dilation was assessed at 4 min. The FMD was expressed as $(\text{diameter}_{max} - \text{diameter}_{baseline})/\text{diameter}_{baseline} \times 100$.

FMD was defined as the primary outcome (studies 1–3); thus, power calculations were based on FMD. The intra- and interindividual variability for FMD measurements established in our laboratory are 0.9% (SDs of differences between repeated FMD measurements in 20 healthy subjects) (C Heiss, unpublished results, 2015) and 1% (SDs within a group of healthy subjects), respectively. Therefore, a crossover study with 12, 6, or 5 subjects receiving treatments on 2 separate days would provide sufficient power to detect an absolute change in FMD of 0.8%, 1.3%, or 1.5% (2-sided α = 0.05; power = 0.80). For parallel independent group comparisons in study 2, 6 subjects receiving different treatments would provide sufficient power to detect absolute differences in FMD mean values of 1.8% (2-sided α = 0.05; power = 0.80).

Arterial stiffness assessment

Arterial stiffness was determined by assessing the brachial PWV (bPWV) before and 2 h after administering the test drinks in study 1. The bPWV was calculated by dividing the distance between the suprasternal notch to the cubital fossa by the pulse transit time. The pulse transit time from the heart to the brachial artery was calculated as the time delay between the R wave on an electrocardiogram and the beginning of the systolic Doppler flow velocity taken during brachial artery ultrasound exams. The distance between the suprasternal notch via the shoulder to the cubital fossa was determined with measuring tape.

BP measurements

Office BP was measured in study 1 before and 2 h after administering the test drinks in study 1 with the use of an automated clinical digital sphygmomanometer (Dynamap) at the upper left arm in the supine position after 10 min of rest in a quiet room with the arm at heart level before blood was drawn and FMD was measured. We took 3 measurements and discarded the first and calculated the mean values for the second and third for further analysis.

CAC quantification with the use of flow cytometry

CACs were isolated and quantitated with the use of flow cytometry as described previously (20). Briefly, blood was drawn in study 1 in heparinized vacutainer tubes before and 2 h after the ingestion of the 3 drinks, and the number of CACs in whole blood was measured as CD34/kinase insert domain receptor (KDR) double-positive cells with the use of flow cytometry. Staining was performed after washing with phosphate-buffered saline (PBS) and Fc receptor blocking (Ig: 1 mg/mL; Zymed). Phycoerythrin (CD34) and allophycocyanin (KDR)-conjugated anti-human mouse antibodies (Pharmingen) or isotype controls were incubated with a 100-μL (10^6-mL) cell suspension with the use

of a fluorescence-activated cell-sorting buffer (1% bovine serum albumin/PBS) for 20 min. After lysing red blood cells and washing with the fluorescence-activated cell-sorting buffer twice, cells were fixed with 1% formaldehyde/PBS and stored at 4°C until analysis; 100,000 events were collected in the lymphocyte and monocyte gates (FACSCanto; Becton Dickinson).

Quantification of (−)-epicatechin metabolites in plasma and urine

The quantification of SREMs corresponding to the sum of sulfated, glucuronidated, and *O*-methylated (−)-epicatechin metabolites was assessed in plasma and urine with the use of HPLC. Samples were analyzed with the use of a reversed-phase chromatograph equipped with a 4.6-mm Luna C18 column (3-μm particle size; Phenomenex) and a Hewlett-Packard 1200-series chromatograph equipped with a fluorescence detector operating at 276 nm (excitation) and 316 nm (emission) as described previously (19). Authentic reference SREMs were provided by Mars Inc.

As published previously (21), the intraindividual variability in the peak plasma concentration (C_{max}) and 0- to 6-h AUC ($AUC_{0–6\ h}$) of total SREMs was 16% and 17%, respectively. The interindividual variability in the $AUC_{0–6\ h}$ and C_{max} of total SREMs was 38% and 39%, respectively. Plasma concentrations of SREMs were defined as the primary outcome of study 4; therefore, power calculations were based on SREMs. The intraindividual variability for the C_{max} of total SREMs established in our laboratory was 200 nmol/L (SDs of differences between repeated SREM measurements after acute consumption of identical drinks containing 10.3 mg/kg BW of CFs in 7 healthy subjects) (21). Therefore, our crossover studies with 6 subjects who received treatments on 2 separate days provided sufficient power to detect an absolute change in SREMs of 285 nmol/L (2-sided $\alpha = 0.05$; power = 0.80).

Statistical analyses

Results are expressed as means ± SDs. Groups were compared with the use of 2-factor repeated-measures ANOVA, and, if significant, a consecutive Bonferroni post hoc test was performed. The ANOVA in studies 1, 3, and 4 had the 2 within-subject factors (time and treatment). In study 2, we compared the changes in FMD at 2 h with the baseline between treatments. We performed 2 separate mixed-model 2-factor ANOVAs with 1 within-subject factor (methylxanthines: 0, 61, 122, and 244 mg; CFs: 0, 102, 205, 410, and 820 mg) and 1 between-subject factor (methylxanthines: 0 and 122 mg; CFs: 0 and 820 mg). Linear relations between continuous variables were expressed as Pearson's *r*. $P \leq 0.05$ was considered statistically significant. Statistical analyses were performed with the use of SPSS version 19 (IBM). Intake amount–response relations were analyzed with the use of a nonlinear curve fit with Graph Pad Prism 6, and intake amounts of zero were arbitrarily set to 1.

RESULTS

Baseline characteristics of study groups

The overall study population consisted of 47 young and healthy men. **Figure 1** shows the Consolidated Standards of Reporting Trials study flow diagram, distribution of subjects to studies, and individual study protocols. The characteristics of the study population are summarized in **Table 2**. No significant differences were observed between the volunteers in the individual studies, and all drinks were well tolerated. No adverse effects or side effects were observed in any of the studies.

Methylxanthines increase the effects of CFs on vascular biomarkers (study 1)

Study protocol 1 investigated whether the co-administration of methylxanthines and CFs would modulate CF-related effects on established biomarkers of vascular function, including FMD, BP, PWV, and CACs. The results showed that the ingestion of both test drinks containing CFs resulted in a significant increase in FMD 2 h after intake compared with the baseline (**Figure 2**A). Furthermore, the increase in FMD after the intake of the test drink containing both CFs and methylxanthines was greater than after the intake of the drink containing only CFs (1.7% ± 0.6% compared with 0.8% ± 0.3%, respectively; $P < 0.05$). No changes in FMD were observed with the intake of the test drink containing methylxanthines only (0.1% ± 0.5%). No statistically significant difference in nitroglycerin-mediated dilation at the end of each study visit was observed (after CF intake: 14.6% ± 1.0%;

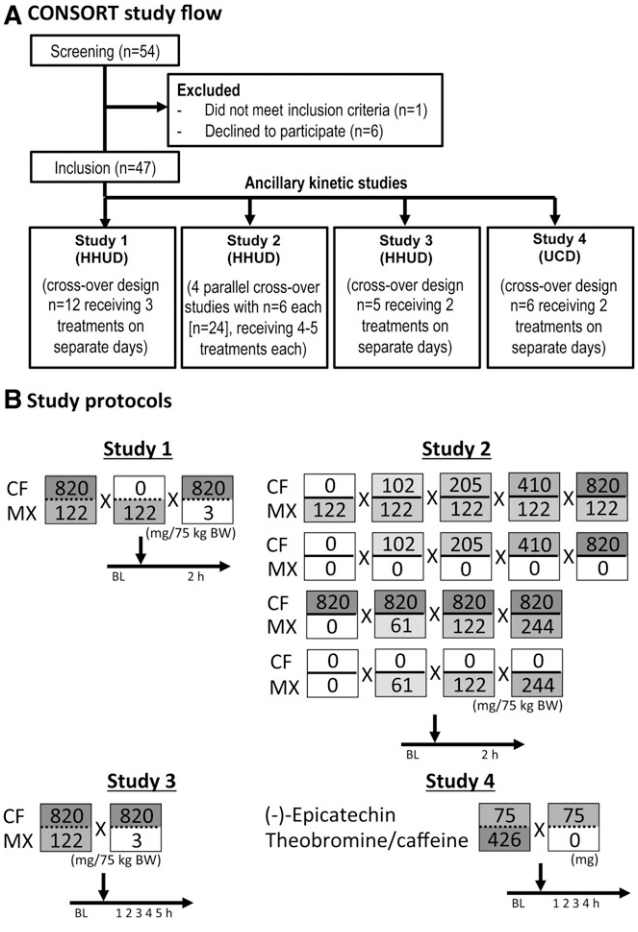

FIGURE 1 Study flow (CONSORT diagram) (A) and schematics of study protocols (B). BL, baseline; BW, body weight; CF, cocoa flavanol; CONSORT, Consolidated Standards of Reporting Trials; HHUD, Heinrich-Heine University Düsseldorf; MX, methylxanthine; UCD, University of California Davis.

TABLE 2
Characteristics of study population[1]

	Values
Men, *n*	47
Age, y	25.1 ± 2.3
BMI, kg/m^2	24.8 ± 2.8
Creatinine, mg/dL	0.9 ± 0.1
Smokers, *n*	5
Fasting glucose, mg/dL	89 ± 4
Total cholesterol, mg/dL	172 ± 25
Heart rate, beats/min	60 ± 8
SBP, mm Hg	121 ± 11
DBP, mm Hg	74 ± 9

[1] All values are means ± SDs unless otherwise indicated. DBP, diastolic blood pressure; SBP, systolic blood pressure.

CF and methylxanthine intake: 14.6% ± 2.5%; methylxanthine intake: 14.2% ± 1.0%; $P = 0.421$), demonstrating that none of the test drinks changed the endothelium-independent vasodilation response.

In addition to FMD, bPWV, BP, and the number of CACs were assessed. Only the ingestion of the test drink containing CFs resulted in significant changes in CD34$^+$/KDR$^+$ CACs (5 ± 4-fold increase; $P < 0.001$) as well as bPWV (−0.26 ± 0.14 m/s; $P = 0.011$), with no effect on diastolic BP (−2 ± 6 mm Hg; Figure 2B–D). The consumption of the test drink containing CFs and methylxanthines together also resulted in positive changes in CD34$^+$/KDR$^+$ CACs (12 ± 4-fold; $P < 0.001$) and decreased bPWV (−0.46 ± 0.22 m/s; $P = 0.011$) and diastolic BP (−6 ± 4 mm Hg; $P = 0.011$). Similar to the findings on FMD, however, the magnitude of the changes on CD34$^+$/KDR$^+$ CACs, bPWV, and diastolic BP after the intake of CFs and methylxanthines simultaneously were greater than after the intake of only CFs. No significant changes in the vascular parameters were observed after the intake of the test drink containing only methylxanthines. Systolic BP and heart rate were not significantly affected by the ingestion of any of the test drinks. In summary, the intake of CFs acutely improved multiple functional markers of vascular health, and, notably, the co-ingestions of CFs with methylxanthines enhanced the effect of CFs on the vascular function biomarkers investigated, namely FMD, CACs, bPWV, and diastolic BP.

Intake amount dependence of interaction between methylxanthines and CFs (study 2)

Study protocol 2 investigated changes in FMD after the intake of test drinks containing a range of intake amounts of CFs and methylxanthines. Volunteers who consumed increasing amounts of CFs ($n = 6$) showed a significant intake amount–dependent increase in FMD 2 h after the intake of >105 mg compared with the baseline (**Figure 3**A). This increase in FMD depended on the amount of CFs consumed, with a maximal FMD increase (ΔFMD_{max}) of 1.4% (95% CI: 1.2%, 1.7%) after 820 mg and an intake amount to achieve a half-maximal increase (ED_{50}) of 199 mg (95% CI: 150, 264 mg; $R^2 = 0.87$).

When volunteers consumed test drinks with increasing amounts of CFs in conjunction with 122 mg methylxanthines ($n = 6$), there was a CF intake–dependent increase in FMD 2 h after intake, with a ΔFMD_{max} of 2.5% (95% CI: 1.9%, 3.1%) after the ingestion of 820 mg CFs and an ED_{50} of 226 mg (95% CI: 152, 335 mg;

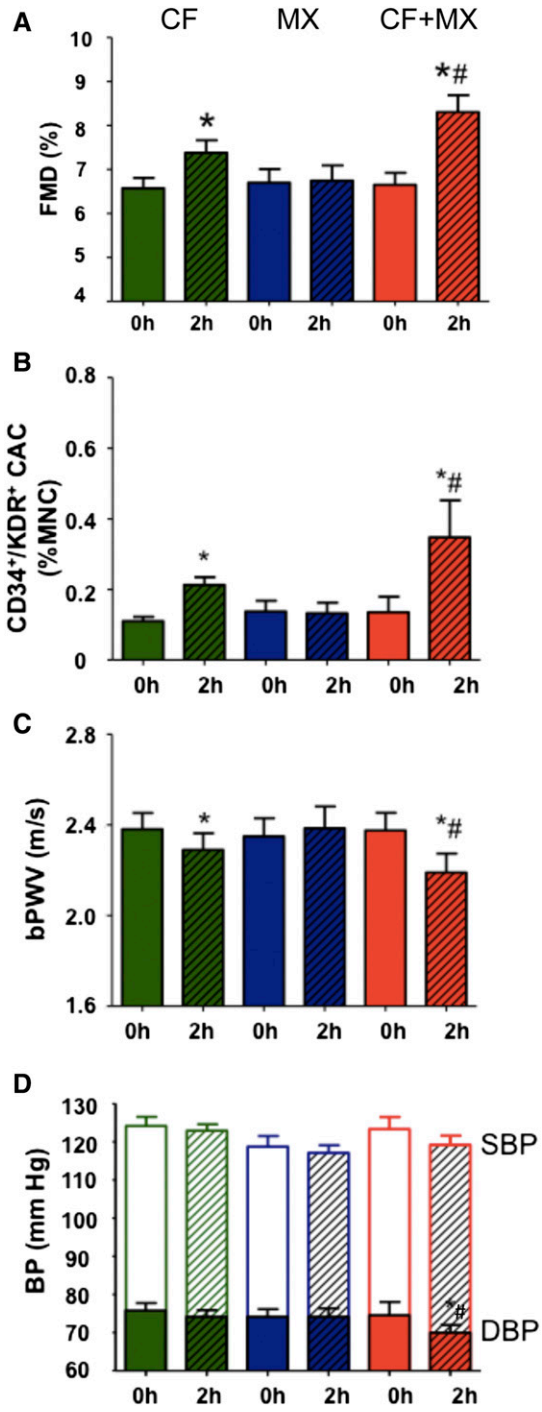

FIGURE 2 Impact of MXs on CF-related improvements in vascular parameters. (A) FMD at 0 and 2 h after the ingestion of the test drinks; (B) mobilization of CD34$^+$/KDR$^+$ CACs; (C) bPWV; and (D) SBP and DBP. Columns are mean values and error bars are SDs. Green columns show the drink containing CF only, blue columns show the drink containing MX only, and red columns show the drink containing both CF and MX ($n = 12$; 2-factor repeated-measures ANOVA; $P < 0.01$ for time-by-treatment interaction for all parameters). *$P < 0.05$ compared with respective baseline (0 h). #$P < 0.05$ compared with respective time point after CF. BP, blood pressure; bPWV, brachial pulse wave velocity; CAC, circulating angiogenic cell; CF, cocoa flavanol; DBP, diastolic blood pressure; FMD, flow-mediated vasodilation; KDR, kinase insert domain receptor; MNC, mononuclear cell; MX, methylxanthine; SBP, systolic blood pressure.

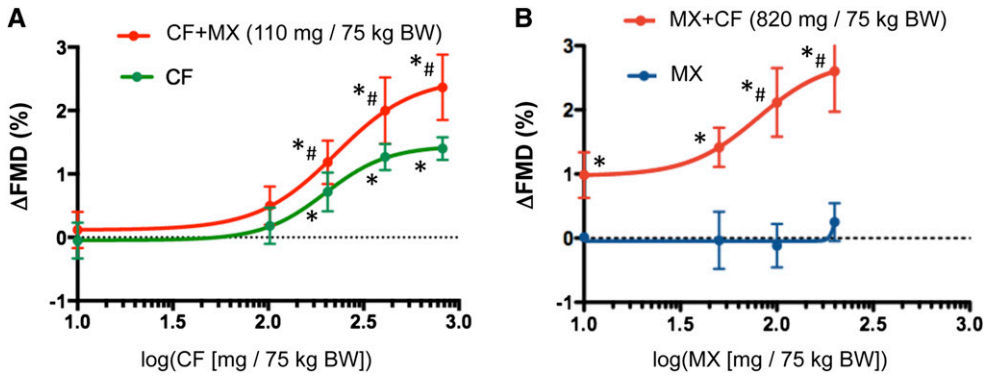

FIGURE 3 Intake-dependent interactions between CFs and MXs. (A) Potentiation of FMD response at 2 h after the ingestion of increasing amounts of CFs alone (102, 205, 410, and 820 mg/kg BW; green line) and co-administered with a fixed amount of MXs (122 mg/kg BW; red curve). (B) The blue line shows that FMD values remained unchanged at 2 h after the ingestion of increasing amounts of MXs (0, 61, 122, and 244 mg/kg BW); the red line shows FMD values when a fixed amount of CFs (820 mg/kg BW) was co-administered. Symbols are mean values and error bars are SDs (n = 24; 2-factor mixed-model ANOVA; $P < 0.01$ for time-by-treatment interaction for all parameters). $^{*}P < 0.05$ compared with respective 0-mg amount. $^{\#}P < 0.05$ compared with the value of the other group. BW, body weight; CF, cocoa flavanol; FMD, flow-mediated vasodilation; MX, methylxanthine.

R^2 = 0.84) (Figure 3A). In this context, ΔFMD_{max} in the group that consumed CFs and methylxanthines together was significantly greater than the ΔFMD_{max} attained after the intake of a same amount of CFs without methylxanthines. However, no significant differences were observed between the ED_{50} of CFs when consumed with or without methylxanthines.

The next test was to determine whether the intake of methylxanthines alone can improve FMD. The results showed that no changes in FMD in the group that consumed methylxanthines only (n = 6) in amounts ≤244 mg (Figure 3B). However, when the subjects consumed 820 mg CFs in combination with increasing amounts of methylxanthine, this resulted in a methylxanthine intake–dependent increase in FMD, with a ΔFMD_{max} of 2.8% (95% CI: 1.7%, 3.8%) after 244 mg methylxanthines and an ED_{50} of 80 mg (95% CI: 40, 160 mg; R^2 = 0.67) (Figure 3B). The baseline FMD values at 0 h were not significantly different between the groups.

Methylxanthine intake increases CF-related FMD improvements concomitant with increased plasma concentrations of SREMs (study 3)

The consumption of CFs with and without methylxanthines (study 3) significantly increased the FMD response, with a maximal FMD response 2 h after intake in both groups (**Figure 4**A). In keeping with the results of the previous sections, the FMD response in study protocol 3 was significantly greater when CFs were consumed with methylxanthines. In addition to the improvements in FMD, the ingestion of the test drink containing both CFs and methylxanthines resulted in a significantly higher C_{max} of SREMs than after the ingestion of the test drink containing only CFs (Figure 4B). The $AUC_{0–5\ h}$ of plasma concentration over time after the intake of the test drink containing CFs and methylxanthines was 37% ± 17% higher than the analog $AUC_{0–5\ h}$ after the intake of the drink containing CFs only (7.2 ± 1.9 compared with 10.9 ± 1.2 μmol/h; P = 0.007). No differences in the amount of SREMs excreted in the 0- to 24-h urine samples were observed after the intake of either drink (8.6 ± 1.4 compared with 8.2 ± 2.7 μmol; $P > 0.05$ for the test drinks that contained CFs and methylxanthines compared with the test drink that contained CFs alone).

The effects of theobromine and caffeine on plasma SREM concentrations after the intake of (−)-epicatechin (study 4)

Results similar to study 3 were obtained when applying study protocol 4, in the context of which CFs and methylxanthines were provided in a cocoa-free formulation that emulated the composition of naturally occurring cocoa with respect to its content of (−)-epicatechin, theobromine, and caffeine. The consumption of the test drinks containing either (−)-epicatechin alone and a combination of (−)-epicatechin and theobromine and caffeine significantly increased the SREM C_{max}, with maximal values 2 h after intake (Figure 4C). Consistent with data from study protocol 3, a higher SREM C_{max} was detected after the consumption of the test drink containing theobromine and caffeine and (−)-epicatechin than with the C_{max} attained after the intake of the test drink containing only (−)-epicatechin ($P < 0.005$). The plasma AUCs of SREM concentrations compared with the time for ≤4 h ($AUC_{0–4\ h}$) after the intake of the test drink containing (−)-epicatechin and theobromine and caffeine was 22% ± 5% higher than the $AUC_{0–4\ h}$ after the intake of the drink containing (−)-epicatechin only (1.43 ± 0.22 compared with 1.76 ± 0.40 μmol/h; P = 0.014). No differences in the amount of SREMs excreted in the 0- to 24-h urine samples were observed after the intake of either drink [10.8 ± 2.1 compared with 11.1 ± 2 0.1 μmol; $P > 0.05$ for the test drink containing (−)-epicatechin and theobromine and caffeine compared with (−)-epicatechin only].

DISCUSSION

The intake of test drinks containing CFs either with or without methylxanthines resulted in acute improvements of established functional cardiovascular biomarkers in healthy adults, namely FMD, CAC, PWV, and diastolic BP. These findings are in agreement with previous studies that have reported beneficial vascular effects after the intake of CFs or foods containing flavanols (7, 8). In contrast, the intake of methylxanthines alone did not mediate acute changes in the biomarkers assessed herein. Although the consumption of methylxanthines has been reported to modulate certain vascular parameters (15, 22), it is plausible that the amounts of methylxanthines administered herein or the

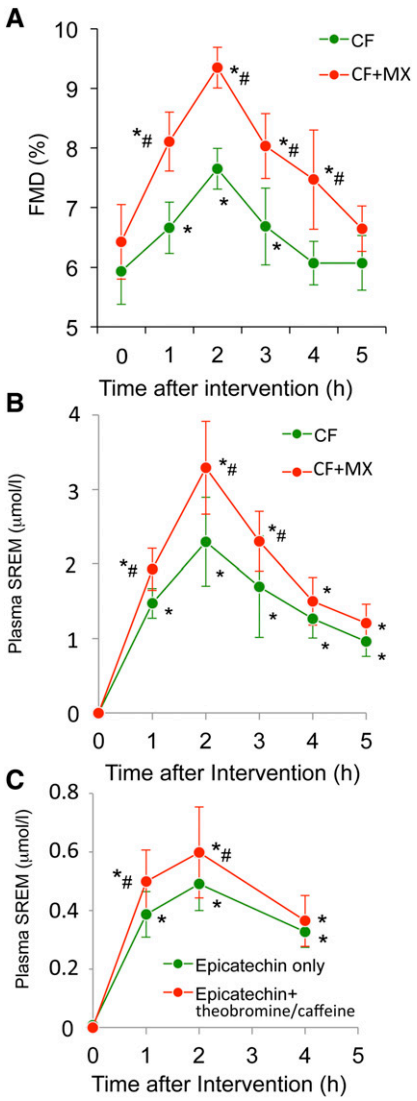

FIGURE 4 Time course of endothelial function (FMD) (A) and SREMs (B) in plasma after the ingestion of CFs (green lines) alone or co-ingested with MXs (red lines). (C) Time course of SREMs in plasma after the intake of (−)-epicatechin alone (green line) or (−)-epicatechin and theobromine and caffeine (red line). Symbols are mean values and error bars are SDs [n = 5 for study 3 and n = 6 for study 4; 2-factor repeated-measures ANOVA; P = 0.0004 (A), P = 0.0052 (B), and P = 0.004 (C) for time-by-treatment interactions]. *$P < 0.05$ compared with respective baseline at 0 h. $^{\#}P < 0.05$ compared with respective CF or (−)-epicatechin administration. CF, cocoa flavanol; FMD, flow-mediated vasodilation; MX, methylxanthine; SREM, structurally related (−)-epicatechin metabolite.

matrix in which they were consumed were not conducive for mediating the effects on the observed parameters. When CFs and methylxanthines were ingested simultaneously, as occurs normally in the context of cocoa that contains CFs, changes over the baseline in FMD, CACs, and PWV of 47%, 42%, and 57%, respectively, were observed, indicating the existence of a synergistic mode of action. The results of study 2 confirmed these findings and further proved that this synergistic effect depended on the amount of CFs and methylxanthines consumed. An important consequence of this synergism is that it may explain to a certain degree the variability in the magnitude of effects observed in different studies with test materials containing varying amounts of CFs and methylxanthines (23). In this context, we and others have shown that the intake of pure (−)-epicatechin can acutely improve endothelial function after the consumption of 1–2 mg/kg BW (5, 10). It is important to note that the effect size of (−)-epicatechin intake observed herein was smaller than an earlier study (5) in which the test drink contained both CFs and methylxanthines.

A recent study on the effects of (−)-epicatechin intake (24) did not detect statistically significant long-term changes in FMD. It may be that investigations that use a purified dietary constituent do not fully mimic the effects or effect sizes observed when the same constituent is consumed in its native food or food matrix. Our data demonstrate that the effect of (−)-epicatechin intake–related changes in vascular function is greater when the flavanol is consumed with methylxanthine, similar to the consumption of (−)-epicatechin as part of certain teas or cocoas.

These data indicate that methylxanthines, when consumed together with CFs, lead to significantly higher concentrations of plasma SREMs without affecting excretion. These findings confirm previous results in rats that suggested that theobromine intake increases the absorption of (−)-epicatechin (12, 25). A similar pattern is observed when aspirin is consumed together with caffeine (18). Potential mechanisms by which methylxanthines may increase (−)-epicatechin (or aspirin) absorption are unknown but may include specific effects on gastrointestinal physiology (e.g., motility and/or secretion, blood flow, gastric pH) or even the modulation of transporters that regulate the efflux of SREMs in the gut. The fact that cocoa methylxanthines and theobromine and caffeine increased the concentration of SREMs in plasma compared with the concentration that would be reached when CFs or pure (−)-epicatechin were consumed alone may explain how cocoa methylxanthines may enhance the vascular effects of CFs. However, methylxanthines are compounds that could also interact with CF effects at a biochemical level via the modulation of biological effector processes. Methylxanthines have been reported to act on adenosine receptors and are phosphodiesterase inhibitors (26, 27). These molecular actions may also provide a feasible explanation for a synergistic effect of CFs and methylxanthines at the level of CACs, diastolic BP, FMD, and PWV. However, because the mechanisms of action of CFs have not yet not been fully elucidated, it is difficult to ascertain whether the effect of methylxanthines on adenosine receptors and phosphodiesterase activity are related to the CF-methylxanthine interactions that modulate the vascular effects observed herein or whether there are still other molecular targets yet to be identified. Thus, future studies are needed to fully understand CF-methylxanthine interactions and the potential molecular events that mediate the observed synergies on the level of vascular function.

The methylxanthines in cocoa are composed predominantly of theobromine, with only small amounts of caffeine (110 mg theobromine and 10 mg caffeine). However, dietary flavanols are also found in other food sources in which the predominant methylxanthine is caffeine, as is the case for tea, which represents the largest contributor of flavanols in the European diet (1). In this context, it is pertinent to ask whether the findings presented in this study can be extrapolated to circumstances in which flavanols are co-ingested with other types of methylxanthines, particularly caffeine. Previous studies have suggested that theobromine and caffeine may have different potencies when mediating

a given effect (12, 28). With regard to BP, BP-lowering and BP-increasing effects have been reported for theobromine and caffeine, respectively. In healthy volunteers, the intake of 700 mg theobromine decreased BP acutely, whereas 120 mg caffeine increased BP (12). Other studies have suggested that caffeine may adversely affect arterial stiffness (14, 29), whereas the consumption of chocolate containing theobromine did not show any effect on arterial stiffness (30, 31). Thus, the extrapolation of our findings to foods other than cocoa, including green and black teas, requires further investigation.

We investigated the vascular effects after the intake of CFs and methylxanthines under conditions in which these substances were either ingested together or individually and in amounts that ranged between 0 and 820 mg for CFs and 0 and 220 mg for methylxanthines. The intake amount ranges investigated in this study encompass the daily intake amounts observed in population-based assessments (1) and are thus relevant in the context of nutrition. The key findings were as follows: *1*) CF-mediated vascular effects were enhanced when CFs were co-ingested with methylxanthines, whereas the ingestion of cocoa methylxanthines alone did not result in significant effects on the assessed biomarkers of vascular function; *2*) the modulation of CF-related vascular effects by the co-ingestion of methylxanthines depended on the relative amount of CFs and methylxanthines consumed; and *3*) the co-ingestion of CFs and methylxanthines resulted in a significantly higher SREM C_{max} and AUC of the plasma concentration over time than CF intake alone. Taken together, our results demonstrate a significant interaction between cocoa methylxanthines and CFs in which methylxanthines, likely by affecting CF absorption, enhance the vascular effects commonly ascribed to CF intake with cocoa.

The authors' responsibilities were as follows—JIO, MWM, HS, and CH: designed the research; RS, JIO, AR-M, YH, and DN: conducted the research; HS: provided the test products; RS, JIO, and JPS: analyzed the data and performed the statistical analysis; JIO, AC, MK, HS, and CH: wrote the manuscript; RS, AC, and CH: had primary responsibility for the final content; and all authors: read and approved the final manuscript. JIO and HS are employed by Mars Inc., a company engaged in flavanol research and flavanol-related commercial activities. JPS, AC, MK, and CH have received unrestricted research grants from Mars Inc. None of the other authors reported a conflict of interest related to the study.

REFERENCES

1. Vogiatzoglou A, Mulligan AA, Luben RN, Lentjes MA, Heiss C, Kelm M, Merx MW, Spencer JP, Schroeter H, Kuhnle GG. Assessment of the dietary intake of total flavan-3-ols, monomeric flavan-3-ols, proanthocyanidins and theaflavins in the European Union. Br J Nutr 2014;111:1463–73.
2. Vogiatzoglou A, Mulligan AA, Bhaniani A, Lentjes MA, McTaggart A, Luben RN, Heiss C, Kelm M, Merx MW, Spencer JP, et al. Associations between flavan-3-ol intake and CVD risk in the Norfolk cohort of the European Prospective Investigation into Cancer (EPIC-Norfolk). Free Radic Biol Med 2015;84:1–10.
3. Heiss C, Keen CL, Kelm M. Flavanols and cardiovascular disease prevention. Eur Heart J 2010;31:2583–92.
4. Schroeter H, Heiss C, Spencer JP, Keen CL, Lupton JR, Schmitz HH. Recommending flavanols and procyanidins for cardiovascular health: current knowledge and future needs. Mol Aspects Med 2010;31:546–57.
5. Schroeter H, Heiss C, Balzer J, Kleinbongard P, Keen CL, Hollenberg NK, Sies H, Kwik-Uribe C, Schmitz HH, Kelm M. (–)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. Proc Natl Acad Sci USA 2006;103:1024–9.
6. Heiss C, Dejam A, Kleinbongard P, Schewe T, Sies H, Kelm M. Vascular effects of cocoa rich in flavan-3-ols. JAMA 2003;290:1030–1.
7. Heiss C, Jahn S, Taylor M, Real WM, Angeli FS, Wong ML, Amabile N, Prasad M, Rassaf T, Ottaviani JI, et al. Improvement of endothelial function with dietary flavanols is associated with mobilization of circulating angiogenic cells in patients with coronary artery disease. J Am Coll Cardiol 2010;56:218–24.
8. Heiss C, Sansone R, Karimi H, Krabbe M, Schuler D, Rodriguez-Mateos A, Kraemer T, Cortese-Krott MM, Kuhnle GG, Spencer JP, et al. Impact of cocoa flavanol intake on age-dependent vascular stiffness in healthy men: a randomized, controlled, double-masked trial. Age (Dordr) 2015;37:9794–9.
9. Werner N, Kosiol S, Schiegl T, Ahlers P, Walenta K, Link A, Bohm M, Nickenig G. Circulating endothelial progenitor cells and cardiovascular outcomes. N Engl J Med 2005;353:999–1007.
10. Loke WM, Hodgson JM, Proudfoot JM, McKinley AJ, Puddey IB, Croft KD. Pure dietary flavonoids quercetin and (–)-epicatechin augment nitric oxide products and reduce endothelin-1 acutely in healthy men. Am J Clin Nutr 2008;88:1018–25.
11. Langer S, Marshall LJ, Day AJ, Morgan MR. Flavanols and methylxanthines in commercially available dark chocolate: a study of the correlation with nonfat cocoa solids. J Agric Food Chem 2011;59:8435–41.
12. Mitchell ES, Slettenaar M, vd Meer N, Transler C, Jans L, Quadt F, Berry M. Differential contributions of theobromine and caffeine on mood, psychomotor performance and blood pressure. Physiol Behav 2011;104:816–22.
13. Salhiyyah K, Senanayake E, Abdel-Hadi M, Booth A, Michaels JA. Pentoxifylline for intermittent claudication. Cochrane Database Syst Rev 2012;1:CD005262.
14. Karatzis E, Papaioannou TG, Aznaouridis K, Karatzi K, Stamatelopoulos K, Zampelas A, Papamichael C, Lekakis J, Mavrikakis M. Acute effects of caffeine on blood pressure and wave reflections in healthy subjects: should we consider monitoring central blood pressure? Int J Cardiol 2005;98:425–30.
15. Papamichael CM, Aznaouridis KA, Karatzis EN, Karatzi KN, Stamatelopoulos KS, Vamvakou G, Lekakis JP, Mavrikakis ME. Effect of coffee on endothelial function in healthy subjects: the role of caffeine. Clin Sci 2005;109:55–60.
16. Papaioannou TG, Karatzi K, Karatzis E, Papamichael C, Lekakis JP. Acute effects of caffeine on arterial stiffness, wave reflections, and central aortic pressures. Am J Hypertens 2005;18:129–36.
17. Thithapandha A. Effect of caffeine on the bioavailability and pharmacokinetics of aspirin. J Med Assoc Thai 1989;72:562–6.
18. Yoovathaworn KC, Sriwatanakul K, Thithapandha A. Influence of caffeine on aspirin pharmacokinetics. Eur J Drug Metab Pharmacokinet 1986;11:71–6.
19. Ottaviani JI, Momma TY, Kuhnle GK, Keen CL, Schroeter H. Structurally related (–)-epicatechin metabolites in humans: assessment using de novo chemically synthesized authentic standards. Free Radic Biol Med 2012;52:1403–12.
20. Heiss C, Amabile N, Lee AC, Real WM, Schick SF, Lao D, Wong ML, Jahn S, Angeli FS, Minasi P, et al. Brief secondhand smoke exposure depresses endothelial progenitor cells activity and endothelial function: sustained vascular injury and blunted nitric oxide production. J Am Coll Cardiol 2008;51:1760–71.
21. Rodriguez-Mateos A, Cifuentes-Gomez T, Gonzalez-Salvador I, Ottaviani JI, Schroeter H, Kelm M, Heiss C, Spencer JP. Influence of age on the absorption, metabolism, and excretion of cocoa flavanols in healthy subjects. Mol Nutr Food Res 2015;59:1504–12.
22. van den Bogaard B, Draijer R, Westerhof BE, van den Meiracker AH, van Montfrans GA, van den Born B-JH. Effects on peripheral and central blood pressure of cocoa with natural or high-dose theobromine: a randomized, double-blind crossover trial. Hypertension 2010;56:839–46.
23. Hooper L, Kay C, Abdelhamid A, Kroon PA, Cohn JS, Rimm EB, Cassidy A. Effects of chocolate, cocoa, and flavan-3-ols on cardiovascular health: a systematic review and meta-analysis of randomized trials. Am J Clin Nutr 2012;95:740–51.
24. Dower JI, Geleijnse JM, Gijsbers L, Zock PL, Kromhout D, Hollman P. Effects of the pure flavonoids epicatechin and quercetin on vascular function and cardiometabolic health: a randomized, double-blind, placebo-controlled, crossover trial. Am J Clin Nutr 2015;101:914–21.

25. Yamamoto T, Takahashi H, Suzuki K, Hirano A, Kamei M, Goto T, Takahashi N, Kawada T. Theobromine enhances absorption of cacao polyphenol in rats. Biosci Biotechnol Biochem 2014;78:2059–63.
26. Francis SH, Sekhar KR, Ke H, Corbin JD. Inhibition of cyclic nucleotide phosphodiesterases by methylxanthines and related compounds. Handb Exp Pharmacol 2011;200:93–133.
27. Chen JF, Chern Y. Impacts of methylxanthines and adenosine receptors on neurodegeneration: human and experimental studies. Handb Exp Pharmacol 2011;200:267–310.
28. Usmani OS, Belvisi MG, Patel HJ, Crispino N, Birrell MA, Korbonits M, Korbonits D, Barnes PJ. Theobromine inhibits sensory nerve activation and cough. FASEB J 2005;19:231–3.
29. Vlachopoulos C, Hirata K, O'Rourke MF. Effect of caffeine on aortic elastic properties and wave reflection. J Hypertens 2003;21:563–70.
30. Vlachopoulos C, Aznaouridis K, Alexopoulos N, Economou E, Andreadou I, Stefanadis C. Effect of dark chocolate on arterial function in healthy individuals. Am J Hypertens 2005;18:785–91.
31. Vlachopoulos CV, Alexopoulos NA, Aznaouridis KA, Ioakeimidis NC, Dima IA, Dagre A, Vasiliadou C, Stefanadi EC, Stefanadis CI. Relation of habitual cocoa consumption to aortic stiffness and wave reflections, and to central hemodynamics in healthy individuals. Am J Cardiol 2007;99:1473–5.