# *Acute study of dose-dependent effects of (-)-epicatechin on vascular function in healthy male volunteers: a randomized controlled trial*

# ACUTE STUDY OF DOSE-DEPENDENT EFFECTS OF PURE (-)-EPICATECHIN ON VASCULAR FUNCTION IN HEALTHY VOLUNTEERS: A RANDOMISED CONTROLLED TRIAL

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**ABSTRACT**

*Background & aims:* There is convincing clinical evidence to suggest that flavanol-containing foods/beverages are capable of inducing improvements in human vascular function. However, whilst pure (-)-epicatechin has been tested for efficacy, a full dose-dependency has yet to be established, particularly at doses below 1 mg $kg^{-1}$ BW. The current study examined the dose-dependent effects of pure (-)-epicatechin on human vascular function with concurrent measurement of plasma (-)-epicatechin metabolites and levels of circulating nitrite and nitrate species, $NO_x$.

*Methods:* An acute, double-blind, placebo-controlled, crossover intervention trial was conducted in 20 healthy males with 4 treatment arms: water-based (-)-epicatechin (0.1, 0.5 and 1.0 mg $kg^{-1}$ BW) and a water only as control. Vascular function was assessed by flow-mediated dilatation (FMD), laser Doppler imaging with iontophoresis (LDI) and peripheral blood pressure (BP) at baseline, 1, 2, 4 and 6 hours post-intervention. Plasma analysis of epicatechin metabolites was conducted by LC-MS and circulating plasma of nitrite and nitrate species were performed using an HPLC-based system (ENO-30). The study was registered with the National Institutes of Health (NIH)-randomized trial records (NCT02292342).

*Results:* Significant increases in % FMD were found to occur at 1 and 2 h following intake of 1 mg $kg^{-1}$ BW , and at 2 h for the 0.5 mg $kg^{-1}$ BW intake. There were no significant changes in LDI or BP at any time-points or intake levels. Increases in FMD over the 6 h timeframe were closely paralleled by the appearance of total plasma (-)-epicatechin metabolites. Dose-related but non-significant changes in circulating NOx were also observed.

*Conclusions*: Our data add further evidence that (-)-epicatechin is a causal vasoactive molecule within flavanol-containing foods/beverages. In addition, we show for the first time that intake levels as low as 0.5 mg $kg^{-1}$ BW are capable of inducing acute improvements in vascular function (FMD) in healthy volunteers.

Keywords: (-)-Epicatechin, acute study, vascular function, dose effect, FMD

## 1. Introduction

Cocoa is a rich source of flavanols which has been extensively investigated for its impact on vascular health at a number of levels, including at the population level, in a growing number of human intervention trials [1-6]. Recent dietary interventions in humans have substantiated epidemiological data on an inverse relationship between flavanol cocoa intake and the risk of cardiovascular diseases. Various flavanol-mediated bioactivities after cocoa consumption have enhance the endothelial function and vascular tone of "at risk" and "healthy" individuals by means of increasing flow-mediated dilation (FMD) of the brachial artery, lowering arterial blood pressure or increasing the circulating pool of nitric oxide [7-16].

Despite these datasets, a full understanding of the causality between components of cocoa and human vasoactivity remains to be established. The reasons for these shortcomings are, at least in part, based on the fact that food matrices contain a multitude of phytochemical constituents of which an unknown number may exert physiological effects.

The main flavanols of cocoa are specially epicatechin, catechin and their oligomeric derivatives known as procyanidins. However, the chiral nature of monomeric flavanols should be noted since the bioactivity of flavanols is significantly influenced by the (+)/(-) stereochemical configuration. Among them, (-)-epicatechin is thought to be the most likely physiologically active compound responsible for the vascular health benefits associated with cocoa since the oral administration of (-)-epicatechin produced a vascular response nearly six times higher than that of (-)-catechin [17]. Therefore, subsequent human intervention trials were focused on the elucidation of the casual vascular effects after human ingestion of pure (-)-epicatechin.

From an acute study conducted with healthy male adults emanated that the oral administration of chemically pure (-)-epicatechin closely emulated acute vascular effects of flavonol rich cocoa [18]. All individuals had significantly increased the peripheral arterial tonometry index and the FMD at two hours after (-)-epicatechin ingestion at doses of 1 or 2 mg $kg^{-1}$ of body weight, potentially though induction of an NO-mediated pathway [18]. Controversially, other controlled, double-masked, crossover study in humans concluded that although (-)-epicatechin intake increased low-mediated arterial dilation, this outcome did not reach statistical significance disproving the health benefits of (-)-epicatechin [19]. Some authors disproved this statement due to the heterogeneity in vascular status of the study population, especially considering the small total number of participants of whom 22 % would be considered as hypertensive [20]. This fact should have not overlooked and unappreciated due to its impact on outcomes and final interpretations which could mask meaningful conclusions. Therefore, due to the controversy regarding to the physiological effect of (-)-epicatechin, further research including reliable clinical trials with appropriate population should be conducted to address its potential health benefits. On the other hand, the minimum effective dose of (-)-epicatechin to induce significant physiological effects is another important remaining challenge which should be addressed.

For that reasons, the current trial investigates the impact of 3 intake levels of pure (-)-epicatechin, 0.1, 0.5 and 1.0 mg $kg^{-1}$ on human vascular function over a 6 h period, primarily by assessment of flow-mediated dilatation (FMD). The study was designed to objectively elucidate the minimum effective dose and timeframe at which improvements in FMD are observed in response to pure (-)-epicatechin intake.

## 2. VOLUNTEERS AND METHODS

### *2.1.Clinical trial ethics*

The clinical study was conducted in line with the guidelines in the Declaration of Helsinki and study protocols were approved by the University of Reading Research Ethics Committee, UK (reference: 11/31) and Kantonale Ethikkommission Bern, Switzerland (reference 039/12). The trial was registered with the National Institute of Health (NIH) records on ClinicalTrials.gov website (NCT02292342).

### *2.2. Volunteers and Intervention*

A total of 20 individuals were recruited (Figure 1A) to voluntarily participate in the trial via poster advertisement at the University of Reading and surrounding area. Volunteers were assessed for health status using a standard health & lifestyle questionnaire and recruited on the basis of their compliance to the inclusion and exclusion criteria for the trial. Inclusion criteria: signed consent form, male aged 18-40 years, non-smoker, absence of metabolic (e.g. diabetes) and cardiovascular-related disorders (no pre-existing CVD and/or previous incidents), normal blood pressure (>150/90 mm Hg) and haematological parameters (liver enzymes, heamoglobin, hematocrit and leukocyte counts). Exclusion criteria: individuals who were or had administered medication (including anti-inflammatory, antibiotics or blood pressure lowering medication) or nutritional supplements (including vitamin, mineral and fish oil supplements) with 2 months prior to the trial start date. Vegetarians/vegans and individuals with an extreme exercise routine were also excluded on the basis of their regular diet and activity. Throughout the trial period (including during washout), volunteers were asked to maintain their normal diet, activity and fluid intake except for 24 h before and during the study day, where volunteers were asked to restrict their diet to low polyphenol-containing foods, including fruit, vegetables, cocoa, chocolate, tea and wine; intake of nitrate-rich

146 foods/beverages such as leafy green vegetables, beetroot, processed meat and tap water (rich
147 source of nitrate in UK). They were also asked to restrict vigorous exercise to > 20 min per
148 day and the consumption of alcohol to > 168 g alcohol per week (14 arbitrary units in UK).
149
150 *2.3. Interventions: Pure (-)-epicatechin test drinks*
151 To investigate the dose-dependent activity of pure (-)-epicatechin relative to vascular
152 function in healthy males at and below 1 mg $kg^{-1}$ BW, pure, food-grade (-)-epicatechin (EC)
153 (Yancui Import & Export Corporation Limited, Shanghai, China). Analysis of EC was
154 performed at NRC (Lausanne, Switzerland) to ascertain its purity and safety for use in clinical
155 human intervention trials. (-)-Epicatechin was stored at -80 º till use and test interventions
156 were prepared at 3 doses of pure EC (1.0, 0.5, and 0.1 mg $kg^{-1}$ EC) by dissolution in low-
157 nitrate drinking water at room temperature (3 mL $kg^{-1}$ of BW) [18]. Volunteers were
158 randomly allocated to each intake level of pure EC or the control treatment (water only) via a
159 block randomisation method (A-D).
160
161 *2.4. Study design*
162 The trial was a randomised, double-blind, placebo-controlled, crossover intervention
163 with 4 treatment arms in which volunteers consumed a water-based test drink containing 0.1,
164 0.5 and 1.0 mg/kg BW of (-)-epicatechin or a control drink containing water only. All
165 volunteers on the study were recruited under the supervision of a qualified research nurse,
166 alongside trained researchers. All responsible parties involved in conduction of the trial and
167 assessment of the study outcomes were blinded to treatment allocation, as well as the
168 participating volunteers. In compliance with the study protocol, volunteers were required to
169 attend the Hugh Sinclair Unit of Human Nutrition on 4 separate occasions to assess each
170 treatment during a 6 h time course where they were randomised to a treatment schedule via a

block randomisation system (Figure 1B). 24 h prior to a study day visit, volunteers were asked to follow a low-polyphenol/nitrate diet in which consumption of specific foods and beverages were restricted and were instructed not to consume alcohol or partake in strenuous physical activity during this restrictive period. A low-fat, low-polyphenol meal was also provided to all volunteers the evening prior to each visit and were asked to consume this before 20:00 in order to allow for a 12 h overnight fast.

On arrival to the Nutrition Unit the weight of each volunteer was recorded to determine the precise amount (mg) of (-)-epicatechin they will receive relative to the arm of the trial they were currently assigned to. Volunteers were cannulated (left arm) by a qualified research nurse and blood and urine samples were collected in the fasted state. Following a 30 min period of inactivity under temperature-controlled conditions 21ºC, baseline FMD measurements of the brachial artery (primary outcome), peripheral blood pressure (systolic and diastolic BP), laser Doppler imaging with iontophoresis to measure cutaneous perfusion of acetylcholine and sodium nitroprusside (LDI) and plasma nitrite/nitrate and epicatechin metabolites analyses were carried out. Following baseline measurements, volunteers were orally administered test or control interventions (30 sec max). Additional vascular measures including FMD and BP were conducted at 1, 2, 4 and 6 hours post intake and LDI at 2, 4 and 6 hours after (-)-epicatechin ingestion. Blood samples were collected at 1, 2, 4 and 6 hours after consumption and urine at 2 and 6 h during the visit and for a total of 24 h. Volunteers received a small low-fat, low-flavonoid containing lunch 4.5 h post intervention. All volunteers followed a 14-day washout period between study days, where they were asked to adhere to their normal diet and exercise regime.

*2.5. Vascular measurements*

196 *2.5.1. Flow-mediated dilatation*

197 FMD of the brachial artery was the primary end point measure of the study measured
198 following standard guidelines [21] using an ALT Ultrasound HDI5000 system (ATL
199 Ultrasound, UK), with a semi-automated computerized analysis system (Brachial Analyzer,
200 Medical Imaging Applications-llc, IL, US). After 30 min supine rest in a quiet, air-
201 conditioned room, the brachial artery was imaged longitudinally at 2-10 cm proximal to the
202 antecubital fossa. Baseline images recorded for 60 s, after which a blood pressure cuff placed
203 around the forearm was inflated to 220 mm Hg. After 2 min of occlusion, the pressure was
204 rapidly released to allow reactive hyperemia, with image collection continuing for 5 min post
205 release. A single, fully trained researcher, who was blinded to the intervention details,
206 analyzed all image files and peak diameter was defined as the maximum diameter obtained
207 after the occlusion was released. FMD response was calculated as relative diameter change
208 from baseline as compared to peak diameter during hyperemia and presented as percentage
209 change.

210

211 *2.5.2. Laser Doppler Imaging*

212 Laser Doppler flowmetry/imaging to measure cutaneous perfusion accompanied by
213 iontophoresis of acetylcholine and sodium nitroprusside was carried out as previously
214 described [22] as secondary outcome. Measurements were taken after 30 min of
215 acclimatization in a supine position in a quiet, temperature controlled room (22 - 24 ºC). The
216 incremental area under the flux versus time curve for 20 scans was used as a measure of micro
217 vascular response to acetylcholine (Ach; endothelium dependent vasodilation) and sodium
218 nitroprusside (SNP; endothelium independent vasodilation).

219

220 2.5.3. Systolic and diastolic blood pressure

Systolic and diastolic blood pressure measures were performed automatically using an Omron, MX2 digital upper-arm blood pressure monitor (Omron Electronics Ltd, Milton Keynes, UK). Volunteers were previously rested in the supine position for approximately 30 min, during FMD measurement, and required to remain in this position or become semi-recumbent during repeated measures. Blood pressure readings were taken every 2 min until 3 successful readings were obtained, according to consistency. The average of these readings were calculated in excel and reported as mean and SEM.

*2.6. Biochemical analyses*

Blood samples were drawn from volunteers (Figure 1) via an *in situ* cannula and blood vacutainer system into EDTA (flavanol metabolite analysis) and sodium heparin (nitrite/nitrate analysis). Samples were collected on ice and immediately centrifuged (1700 x g; 15 min at 4°) and plasma aliquots of 1 ml were frozen at -80° until analysis. Plasma stored for metabolite analysis and nitrate/nitrite analysis were treated with ascorbic acid (200 mg/ml; 5 % v/v) and nitrite preservation solution (100 μM N-ethylmaleimide, NEM), respectively.

*2.6.1. Plasma metabolite analysis*

Analysis of total plasma (-)-epicatechin metabolites TPEM was as previously described [23]. Plasma samples (200 μl) were thawed on ice and spiked with internal standards. After protein precipitation, samples were filtered, washed with 200 μl methanol and dried at room temperature under a flow of nitrogen. Finally, the residue was dissolved in 100 μl of 8 % acetonitrile in acidified water. Half of the volume was directly injected into the ultraperformance liquid chromatography UPLC-ESI-MS/MS system for quantification of total (-)-epicatechin metabolites which were separated by reversed-phase UPLC using a C18-column Acquity UPLC HSS (2.1 mm x 100 mm, 1.8 μm), (Waters AG, Baden-Dättwil,

Switzerland) following the chromatographic method described by Actis-Goretta et al. [23]. Data were collected and processed using Analyst software (AB Sciex Switzerland GmbH, C/o Applied Biosystems Europe BV, Zug, Switzerland). Each participants' samples were analyzed within a single assay batch in random sequence. The samples were analyzed blind.

*2.6.2. Plasma nitrite and nitrate analysis:*

Plasma levels of circulating nitrite and nitrate were individually assessed using an Eicom NOx Analyzer ENO-30 (San Diego, USA), a dedicated high-performance liquid chromatography (HPLC) system developed by Eicom Corporation (Japan). This system uses a post-column diazo coupling reaction (Greiss reaction) combined with HPLC using a NO-PAK separation column. The followed method was that proposed by Ishibashi et al. [24].

Nitrite and nitrate standard stock solutions were prepared in PBS buffer using sodium nitrite and sodium nitrate salts, respectively. On each day of analysis, a nitrite/nitrate working standard solution was prepared using the premade stock solutions and methanol. Eight calibration standards were prepared by series dilution containing nitrite and nitrate in the following concentrations: 1.6, 0.8, 0.4, 0.2, 0.1, 0.075, 0.05 and 0.025 μM and 100, 50, 25, 12.5, 6.25, 3.125 and 1.5625 μM, respectively. After deproteinisation of plasma samples with methanol, 10 μl was injected into the HPLC-system via a programmed autosampler (Waters, UK). The binary system phases were solvent A (carrier) and solvent B (reactor) with a flow rate of 330 μl $min^{-1}$ and 100 μl $min^{-1}$, respectively. The nitrite present is able to react with the Greiss reagent generating a red diazo compound, and absorbance is quantitatively measured by spectrophotometric detection at 540 nm. Furthermore, nitrate passing through the reduction column was reduced to nitrite prior to undergoing the same diazo coupling reaction. Calibration standards previously prepared were used for comparison with the peak areas of

absorption produced by the test plasma samples in order to quantify the nitrite and nitrate present.

*2.7. Power calculation and Statistical analysis*

A power calculation was performed for the primary endpoint measure of change in FMD response based on statistical limitations associated with this measure in order to determine an accurate minimum number of volunteers required to power the trial. The minimal statistically significant measurable improvement in FMD was set at an absolute change of 1.5% whilst considering a baseline vasodilation (FMD response) of 6.0 %, based on previous clinical assumptions that the minimal statistically significant improvement detectably following drug/nutrient interventions is an absolute change in FMD of 1.5-2% [21] The sample size was calculated based on a variance of repeated measures of 1.8%, deduced from the inter-individual variability analysis of data collected in a FMD pilot reproducibility study. This is consistent with previous, similar clinical trials that have used a standard deviation of 2.3% [9, 18]. Using a standard deviation of 1.8%, a significance level of 0.05 and a power of 80 % a total of 18 volunteers were required to observe significant within-subject differences between treatments of at least 1.5%. Therefore, assuming a dropout rate of 10% we aimed to recruit 20 volunteers in total.

All results were expressed as means ± standard error of the mean (SEM), and further statistically analysed using GraphPad Prism version 5 software (Graphpad Software Inc. San Diego, CA, USA). Two-factor repeated measures ANOVA was utilized to assess time course data for study endpoint outcomes in order to estimate intra-individual treatment effects, with pairwise comparisons corrected using the Bonferroni test during post-hoc analysis. Statistical significance was assumed if a null hypothesis could be rejected at $p = 0.05$ values represented

295 in figures are as follows: *$p$ = 0.01-0.05, **$p$ = 0.001-0.01, ***$p$ < 0.0001. LDI results were
296 expressed as area under the cure (AUC) and incremental AUC (iAUC) calculated using the
297 trapezoidal method. A correlation analysis was performed using Pearson's correlation
298 coefficient to assess relationship between FMD and total plasma (-)-epicatechin metabolites
299 (TPEM) during the timecourse.
300
301 **3. Results**
302 *3.1. Baseline characteristics of sample population*
303 The characteristics of the study population are detailed in Table 1. Baseline
304 characteristics were calculated and expressed as means ± SEMs and mean values for
305 parameters were all within the normal range for healthy individuals. The pure (-)-epicatechin
306 test drinks were well tolerated and no adverse advents were reported in the context of the
307 study.
308
309 *3.2. (-)-Epicatechin induced dose-dependent increases in vascular function*
310 Significant increases in % FMD were observed after ingestion of 0.5 mg $kg^{-1}$ BW at 2
311 h ($p$ < 0.01) and similarly at 1 h ($p$ < 0.01) and 2 h ($p$ < 0.001) following ingestion of the
312 highest dose, 1.0 mg $kg^{-1}$ BW. Significant differences in FMD were also identified between
313 the highest (-)-epicatechin dose (1.0 mg $kg^{-1}$ BW) and the lowest (-)-epicatechin dose (0.1 mg
314 $kg^{-1}$ BW) at 1 h ($p$ < 0.05) and 2 h ($p$ < 0.01) after consumption of the test drinks (Figure 2A).
315 Peak vasodilation occurred at 2 h after treatments (0.5 and 1.0 mg $kg^{-1}$ BW), with subsequent
316 declines in FMD response towards baseline at 4 and 6 h. FMD response (endothelium-
317 dependent brachial artery vasodilation) increased after ingestion of all 3 pure (-)-epicatechin
318 test drinks administered to volunteers in a dose-dependent manner between 1 and 2 h (Figure
319 2B). The magnitude of increase from baseline (0 h) to 1 h was 1.6 ± 0.3 % following (-)-

320 epicatechin (1.0 mg $kg^{-1}$ BW), and at 2 h FMD increased by 1.2 ± 0.3 % and 2.9 ± 0.3 % after
321 ingestion of 0.5 mg $kg^{-1}$ and 1.0 mg $kg^{-1}$ BW (-)-epicatechin, respectively. No significant
322 changes in FMD were observed at baseline (0 h), 4 h and 6 h and no significant changes in
323 FMD were observed at any time-point following intake of 0.1 mg $kg^{-1}$ BW (-)-epicatechin and
324 the control. Both LDI (Figure 3) and BP (Figure 4) measures were not significantly altered
325 after consumption of any of the (-)-epicatechin dose intakes compared with baseline or the
326 control drink.
327
328 *3.3. Relationship of FMD to plasma (-)-epicatechin metabolites and $NO_x$*
329 Mean plasma concentrations of epicatechin metabolites ranged from 0.19 to 9.93 μmol $L^{-1}$
330 following consumption of pure (-)-epicatechin treatments (excluding control) up to 6 h. The
331 time course for the appearance of flavanol metabolites in the circulation paralleled FMD data,
332 although in all cases peak metabolite concentrations preceded peak FMD (Fig 2A) at 1 h.
333 Analysis using a 2-factor repeated measures ANOVA indicated significant increases in
334 plasma flavanol metabolites following ingestion of 0.5 and 1.0 mg $kg^{-1}$ BW pure (-)-
335 epicatechin at 1 h ($p < 0.001$), 2 h ($p < 0.001$) and 4 h ($p < 0.01$ and $p < 0.001$, respectively)
336 and additionally at 6 h ($p < 0.01$) for 1.0 mg $kg^{-1}$ BW (-)-epicatechin (Figure 5). Total
337 plasma concentrations of (-)-epicatechin metabolites were at or below the limit of detection
338 (0.01 μmol $L^{-1}$) for the control at all time points and for the lowest intake at 4 and 6 h.
339 Analysis using Pearson's correlation coefficient found positive associations with intake
340 amount between % FMD response and TPEM at 1 h and 2 h. At 1 h, coefficients between %
341 FMD and TPEM were $R^2 = 0.010$, $R^2 = 0.013$ and $R^2 = 0.174$, for 0.1, 0.5 and 1.0 mg $kg^{-1}$ BW
342 (-)-epicatechin, respectively. At 2 h correlation coefficients with TPEM were $R^2 = 0.07$ and
343 $R^2 = 0.206$ for the 0.5 and 1.0 mg $kg^{-1}$ BW (-)-epicatechin, respectively (Figure 5). A 2-factor
344 repeated measures ANOVA found no significant increases in plasma nitrite, nitrate or NOx at

any timepoint during the crossover intervention comparative to baseline and control measures ($p > 0.05$) (Figure 6).

## 4. Discussion

Previous works focused on the beneficial vascular effects of cocoa has predominantly centered on the physiological actions of the total flavanol content [3-10, 19]. However, although (-)-epicatechin seems to be the main responsible compound for vascular effect, a direct cause and effect relationships between intake and efficacy are difficult to establish following the consumption of complex food matrices such as cocoa. Therefore, a proof of concept study with pure (-)-epicatechin was performed, providing insight, for the first time, into the dose-dependent effect of its vasoactive benefits post intake and insights into the circulating metabolites and nitric oxide pool that might mediate such effects.

In response, this acute crossover intervention trial investigating the vascular effects of pure (-)-epicatechin provided further evidence for the increase vascular function of this compound, and in particular the impact of FMD % response of the brachial artery. In agreement with our data, the oral administration of pure (-)-epicatechin in a human intervention causally linked to the increase of FMD % [18], contrary to other studies which put to rest the idea that (-)-epicatechin represents a compound with cardiovascular health benefits [19]. Data shows clear dose-dependent increases in FMD following pure (-)-epicatechin interventions, and specifically at 1 h and 2 h after ingestion of the middle dose (0.5 mg $kg^{-1}$ BW) and highest dose (1.0 mg $kg^{-1}$ BW), where increasing magnitude and statistical significance was observed, respectively. However, after conducting post-hoc power calculations for the lower dose treatment, 0.1 mg $kg^{-1}$ BW, no significant variations were observed in comparison with those FMD % values for control treatment. Until now, the minimum dose of (-)-epicatechin proven to be vascular active was 1 mg $kg^{-1}$ BW [18]. Our

results evidence that the ingestion of lower doses, 0.5 mg $kg^{-1}$ BW, is enough to induce a physiologically increase in FMD %. Currently, evidence of a significant increase in FMD with an intervention dose as low as 0.5 mg $kg^{-1}$ pure (-)-epicatechin BW has not been directly reported, and therefore provides much needed dose-related insight regarding active and minimum-active doses capable of inducing acute improvements in FMD, within a healthy population.

From baseline mean FMD % reach the maximal change two hours after treatment ingestion at 0.5 and 1.0 mg $kg^{-1}$ BW doses which is consistent with the timecourse of total plasma (-)-epicatechin metabolites (TPEM). This fact suggests that there is an optimal level of circulating (-)-epicatechin metabolites for driving physiological effect on the endothelium after two hours of treatment ingestion. Other studies also reported intake-dependent biphasic improvements in FMD paralleled with appearance of individual phenolic plasma metabolites following an intervention of flavanol-rich food such as blueberry, cocoa or coffee, showing the higest level after 2 hours ingestion [18, 25, 26]. (−)-Epicatechin-3′-β-D-glucuronide, (−)-epicatechin-3′-sulfate, and 3′-O-methyl-(−)-epicatechin-5/7-sulfate have been reported as the major *in vivo* metabolites present in the bloodstream at 1−3 h after ingestion of cocoa, chocolate products, or pure compounds [23, 27, 28].

Potential mechanisms by which such circulating metabolites mediate their vascular effects have been postulated, and include their potential to inhibit NADPH oxidase, thus affecting superoxide production and subsequent NO bioavailability at the vascular epithelium [25, 29, 30]. Because of the fact that FMD is mediated by NO, increases in NOS activity are paralleled by increases in plasma nitroso species. Particularly, our data do not reveal significant enhancements in plasma nitrite, nitrate or NOx at any timepoint during the crossover

intervention. However, results emanated from other studies establish a causal link among parallel measurements of FMD and circulating NO species in conjunction with NOS inhibition studies after oral ingestión of flavanol which represents a mechanistically strong experiment framework [9, 18, 31, 32].

In conclusion our findings support the notion that (-)-epicatechin is an important mediator of the cardiovascular effects of flavanols rich food. A clearer picture of dose-dependency of (-)-epicatechin is drawn since our data adds new evidence demonstrating significant activity from as low as 0.5 mg kg-1 BW (-)-epicatechin, with increasing magnitude and significance to the highest dose intervened in the trial. Although, data from this acute study cannot be used to determine the long term effects of repeated consumption of (-)-epicatechin on vascular function and the study population is limited to healthy young males, findings in the current paper may provide new avenues to dietary or therapeutic interventions aimed at improving and maintaining cardiovascular health

**State of authorship**

JPES was the PI on the study, MEA was a postdoctoral researcher, MSC was pre-doctoral student assigned to the project and GS was a PhD student collaborator. JPES, MEA and MSC designed the study. MEA, MSC and GS conducted the epicatechin study. JPES, MEA and MSC collaborated on the manuscript preparation. All authors read and approved the final manuscript.

**Conflict of interest**

No conflicts of interest are stated by the authors.

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