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Acute effects of chlorogenic acid on nitric oxide status, endothelial function and blood pressure in healthy volunteers: a randomised trial

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Running title: Chlorogenic acid lowers blood pressure
ABSTRACT

There is mounting evidence that specific dietary polyphenols can enhance vascular health by augmenting nitric oxide. Our aim was to investigate the acute effects of chlorogenic acid, an important dietary phenolic acid present in coffee (400 mg, equivalent to 2 cups of coffee), on nitric oxide status, endothelial function and blood pressure. Healthy men and women (n=23) were recruited to a randomised, double-blind, placebo-controlled, cross-over trial. Chlorogenic acid resulted in significantly higher plasma concentrations of chlorogenic acid ($P<0.001$). Relative to control, mean post-treatment systolic blood pressure (-2.41 mm Hg, 95% CI: -0.03, -4.78; $P=0.05$) and diastolic blood pressure (-1.53 mm Hg, 95% CI: -0.05, -3.01; $P=0.04$) were significantly lower with chlorogenic acid. Markers of nitric oxide status ($P>0.10$) and the measure of endothelial function ($P=0.60$) were not significantly influenced. Chlorogenic acid can lower blood pressure acutely; an effect which if sustained would benefit cardiovascular health.

Key Words: chlorogenic acid, vascular function, blood pressure, nitric oxide

Non-standard abbreviations and definitions: FMD, Flow mediated dilatation; NO, Nitric oxide; NOx, Nitric oxides comprising nitros(yl)ated species + nitrite; RXNO, S-nitrosothiols + other nitrosylated species
INTRODUCTION

Dietary guidelines recommend a diet rich in plant foods. Polyphenols are components of plant foods that are believed to contribute to human health. The main classes of dietary polyphenols are phenolic acids and flavonoids. There is increasing evidence flavonoids can enhance vascular health. Specific flavonoids, and foods and beverages rich in these flavonoids has been found to lower blood pressure, improve endothelial function and enhance NO status. Endothelial dysfunction is an early event in the pathogenesis of vascular disease, and is associated with increased risk of cardiovascular events. Nitric oxide plays a critical role in maintaining healthy endothelial function and vascular tone. The effects of phenolic acids on these outcomes are less clear.

Chlorogenic acid is one of the main phenolic acids in the diet with intakes reaching 1000 mg/d or more. It is the primary phenolic acid present in coffee, and is also present in particular fruits. Previous human trials suggest that acute intake of caffeinated coffee, but not decaffeinated coffee, causes detrimental effects on endothelial function. Regular consumption of green coffee extract, which is rich in chlorogenic acid, for 4 weeks resulted in dose-related decreases in blood pressure in mildly hypertensive subjects. An acute effect of chlorogenic acid on blood pressure and vascular function has been demonstrated in spontaneously hypertensive rats: effects which were blunted with a NO synthase inhibitor. These results suggest that chlorogenic acid may reduce blood pressure by augmenting NO status and improving endothelial function.

The acute effects of chlorogenic acid on NO status, endothelial function and blood pressure in humans have yet to be studied. Therefore, our aim was to investigate the
acute effect of 400 mg of chlorogenic acid, an intake equivalent to 2 cups of coffee, on plasma NO status, endothelial function and blood pressure in healthy men and women.
METHODS

Chemicals, materials and reagents

Food grade chlorogenic acid (3-O-caffeoylquinic acid, CA Reg number 327-97-9) was purchased from Atlantic SciTech Group (Linden, New Jersey). 1-Hydroxy-2-naphthoic acid 99% was purchased from Aldrich Chem Co. (Milwaukee, WI, USA), and phloretic acid (3-(4-Hydroxyphenyl) propionic acid) was from Lancaster (Morecambe, England). Caffeic acid (3,4-Dihydroxycinnamic acid) and ferulic acid (4-Hydroxy-3-methoxycinnamic acid) and isoferulic acid (3-Hydroxy-4-methoxycinnamic acid, predominantly trans) were purchased from Sigma Chemical Co. (St Louis, USA). β-Glucuronidase from Helix pomatia Type HP-2 pyridine ≥99% and N,O-Bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (BSTFA) and hydrocaffeic acid (3,4-Dihydroxyhydrocinnamic acid) were from from Sigma-Aldrich Pty. Ltd. (NSW, Australia).

Bond Elut C18, 500 mg, 6 ml cartridges were from Varian (Lake Forrest, CA). Zorbax eclipse XDB C18 2.1 x 100mm / 3.5um column was purchased from Agilent Technologies (Agilent Technologies, Santa Clara, CA, USA). All solvents were of HPLC grade. Water was obtained with Arium 611 VF water purification system (Startorius stedim biotech, Aubagne, France).

Participants

Twenty three healthy men and women were recruited from the Perth general population by newspaper advertisement. All participants were screened prior to enrolment. Screening and study visits took place at the University of Western Australia, School of Medicine and Pharmacology located at Royal Perth Hospital. Screening consisted of a standard medical history questionnaire, routine laboratory analysis of a fasting blood sample, electrocardiography, height, weight, body mass index (BMI) and blood pressure
measurement. Exclusion criteria included current smoking, BMI <18 or >35 kg/m^2, systolic blood pressure (SBP) <100 or > 160 mmHg, diastolic blood pressure (DBP) <50 or > 100 mmHg, use of antihypertensive medication, history of cardiovascular or peripheral vascular disease, history of liver disease, renal disease, chronic gastrointestinal disorder and any major illness such as cancer, psychiatric illness, diagnosed diabetes, weight gain or loss >6% body weight within previous 6 months of the study, > 30g/day alcohol consumption or woman who were pregnant, lactating or wishing to become pregnant during the study. All participants were regular tea (mean±SD: 1.7±1.5 cups/d) and coffee (mean±SD: 1.7±1.6 cups/d) consumers. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the University of Western Australia Human Research Ethics Committee. Participants provided written informed consent before inclusion in the study. The trial was registered with the Australian New Zealand Clinical Trials Registry (ACTRN: 12611000467932)

**Study design**

A double blind, randomised controlled cross-over design study was performed. Participants were allocated to an intervention plan via block randomisation using computer-generated random numbers devised by a statistician. Each subject completed 2 visits with a washout period of 1 week in between testing days as far as possible. The preceding evening meal and breakfast on the morning of the study day were low polyphenol and was consistent across all study visits. A food diary was used to verify adherence to the study protocol. On arrival, a baseline blood sample was taken by venepuncture for analysis of plasma 5-nitrosothiols + other nitrosylated species (RXNO), nitrite, nitric oxides (NOx), chlorogenic acid and chlorogenic acid metabolites. Participants were then provided with the randomly allocated chlorogenic acid treatment. Treatments comprised water (control) and 400 mg chlorogenic acid (3-O-caffeoylquinic acid) dissolved in 200 mL low nitrate water (chlorogenic acid).
Flow-mediated dilatation (FMD) of the brachial artery using ultrasonography was used to assess endothelial function 120 min post treatment. A blood sample was taken by venepuncture 150 min post intervention for analysis of plasma RXNO, nitrite, NOx, chlorogenic acid and chlorogenic acid metabolites. Blood pressure measurements were taken prior to treatment and then again at 60, 90, 120 and 150 min post treatment.

**Measurement of plasma S-nitrosothiols + nitroso species (RXNO), nitrite and nitric oxides (NOx)**

Plasma was collected from blood samples taken at baseline and 150 min post treatment for immediate analysis of S-nitrosothiols + other nitrosylated species (RXNO), nitrite and NOx (nitros(yl)ated species+nitrite). The concentrations of RXNO, nitrite and total NOx in plasma were determined using a previously described gas phase chemiluminescence assay. Blood was collected into N-ethylmaleimide (10 mmol/L) and EDTA (2 mM), mixed and centrifuged at 3000 x g (5min, 4°C). Fresh plasma was kept on ice in the dark and analysed within 1 hr. For determination of nitroso species, fresh plasma (300 µl) was treated with sulphanilamide solution (30 µl, 0.5% in 0.1 M HCl) for 3 min to remove endogenous nitrite. Antifoam (200 µL) was added prior to injection into the radical purger containing potassium iodide (0.125 g) and iodine (0.05 g) in water (2.5 mL) / glacial acetic acid (7.5 mL) at room temperature. Plasma S-nitrosothiols (+ nitrosylated species) were quantified by the NO signal peak area of samples pretreated with sulphanilamide against a nitrite standard (300 µl, 0.5 µM NaNO₂). Quantification of NO released by the redox reactions occurred by its chemiluminescence reaction with ozone using the Nitric Oxide Analyzer (CLD66, Eco Physics, Sweden).

**Blood pressure**

Blood pressure was measured using a Dinamap 1846SX/P oscillometric recorder (Critikon Inc., Tampa, FL, USA) prior to treatment and then at 60, 90, 120 and 150 post treatment.
Participants were rested for 5 min in a supine position before blood pressure measurements were taken. Five blood pressure measurements were taken at 2-min intervals. The first measurement was discarded and the mean of the remaining four measurements was calculated. Blood pressure measurements for all time points (60, 90, 120 and 150) were used in the analysis.

**Flow mediated dilatation of the brachial artery**

A trained ultrasonographer dedicated to the research protocol and blinded to the treatments used performed flow mediated dilatation (FMD) of the brachial artery assessed by ultrasonography. All measurements were performed according to published protocol \(^{14}\). Briefly, subjects were rested in a supine position in a quiet, temperature controlled room (21°C to 25°C). The left arm was extended and supported comfortably on a foam mat. ECG was monitored continuously. For the ultrasound, a 12-MHz transducer connected to an Acuson Aspen 128 ultrasound device (Acuson Corporation) was fixed in position with a clamp over the brachial artery 5 to 10 cm proximal to the anti-cubital crease. After a baseline artery diameter recording of 1 min, a blood pressure cuff was placed around the left forearm and inflated to 200 mmHg. The cuff was released after 5 min inducing reactive hyperaemia. The brachial artery image was recorded for 4 min post cuff deflation to assess FMD. Images were downloaded for retrospective analysis. Analysis of FMD of the brachial artery was performed with semiautomated edge-detection software \(^{14}\) which automatically calculated the brachial artery diameter, corresponding to the internal diameter. This was gated to the R wave of ECG, with measurements taken at end diastole. Responses were calculated as the percentage change in brachial artery diameter from baseline. The FMD was measured at 30 s intervals to 4 min post cuff deflation (0, 30, 60, 90, 120, 150, 180, 210 and 240 s). Peak FMD was also assessed. The analysis was performed by an experienced observer blinded to the treatments used.
Measurement of plasma chlorogenic acid using liquid chromatography-tandem mass spectrometry

For the analysis of chlorogenic acid, plasma samples were defrosted and were extracted using solid phase extraction (SPE) method. Bond Elut C18 500 mg cartridges were first conditioned with 2 ml of methanol followed by 2 ml water. Aliquot of 500 µl plasma sample was spiked with 10 µl internal standard (IS) (1-Hydroxy-2-naphthoic acid) working solution (1ng/ml). Then plasma was acidified by adding 2 ml of buffer (0.1M sodium acetate pH 3) and then vortexed. After acidification, samples are applied to SPE cartridges and washed with water and hexane to remove any interfering substances. Compounds are eluted with methanol and collected in a glass tube. The methanol was evaporated under a stream of nitrogen and the residue was then reconstituted with mobile phase (100 µl). Standard stock solutions were prepared by dissolving a required amount of chlorogenic acid and IS in methanol, which was then further diluted appropriately with methanol to desired concentration. The concentration of IS working solution was 100 pg/µl. All solutions were stored at -20°C until use. Calibration curve of standards used to quantify chlorogenic acid in plasma were drawn from the analysis of plasma spiked with 10 µl of IS working solution (1ng/ml) and different concentrations of chlorogenic acid standards ranging from 10 to 1000 ng/ml. Coefficients of linearity for the calibration curve was typically $R^2 > 0.99$. Chlorogenic acid and IS were analyzed on a Thermo Scientific TSQ Quantum Ultra Triple Quadrupole LC-MS System (ThermoFisher Scientific) equipped with electrospray ionization source (ESI) operated in negative- ion mode using a Zorbax eclipse XDB C18 2.1 x 100mm / 3.5um column. Injection volume was 25 µl. The mobile phases were: A: 2 mM ammonium acetate pH=3.6 and B, methanol at a flow rate of 400ul/min. Chromatography is achieved using solvent A (100%) from 0 to 4min, then ramped to B (100%) from 4 to 12 min, then A (100%) from 13 to 15min. Multiple reaction monitoring (MRM) was used to monitor precursor to product ion transitions of m/z 354.1 [M-
to a strong product ion at m/z 191 for chlorogenic acid (Retention time is 6.91 min). IS includes m/z 188 [M-1] and a strong product ion at m/z 144 (Retention time is 10.03 min). The operating conditions for MS analysis were ion spray voltage 3500 V; capillary temperature and voltage, 350 °C and 35 V, respectively; sheath gas (nitrogen) and auxiliary gas pressure, 60 and 50 psi, respectively. The mass spectrometer was employed in MS/MS mode using argon as collision gas (1.2 mTorr). Collision energy (CE) was optimized at 19eV for chlorogenic acid and 20eV for IS.

**Measurement of plasma chlorogenic metabolites using gas chromatographic mass spectrophotometer**

For the analysis of chlorogenic acid metabolites, plasma samples were defrosted and were extracted using liquid-liquid extraction method. Plasma samples (750 µl) was first spiked with 50 µl of IS 1ng/µl. The mixture was then acidified with 0.2M hydrochloric acid to pH 5. Then, 30 µl of β-glucuronidase was added and incubated at 37°C overnight. The sample was then acidified to pH 3.5 with 2M HCl and extracted with ethyl acetate. The mixture was then centrifuged for 5 min, and the ethyl acetate layer was then recovered and re-extracted with 5% K₂CO₃. Ethyl acetate phase was then removed to waste and the carbonate phase was immediately reacidified to pH 1-2 with 6M HCl. This layer was re-extracted with 1 ml of ethyl acetate. Ethyl acetate layer was recovered and evaporated under a stream of nitrogen. The residue was derivatized with pyridine and BSTFA and heated at 40°C for 60 minutes prior to GC-MS analysis.

Calibration curve of standards used to quantify chlorogenic acid metabolites in plasma were prepared from the analysis of mixture 50 µl of IS working solution (1ng/µl) and different concentrations of individual metabolite compound ranging from 10 to 500 ng/µl. Coefficients of linearity for the calibration curve was typically $R^2 > 0.99$. 

A Hewlett-Pakard (HP) 6890 Series Gas Chromatograph System coupled with Agilent 5973 Network mass selective detector was used for the analysis. Samples were separated on a 30m, 0.25µm, 0.25 mm, high resolution gas chromatography column (HP-1MS J&W Scientific, Folsom, CA). The maximum temperature of column was 330 °C and the total run time was 14.17 min. Injection volume was 1.0 µl in the splitless mode. The interface temperature was held at 280 °C. Selected ion monitoring was used after initial scan of standard compounds, the following ions were monitored for detection of chlorogenic acid metabolites and the Internal Standard: ions m/e 338.0 and 308.0 for ferulic acid and iso-ferulic acid; ions m/e 179.0 and 310.0 for phloretic acid; 396.0 and 219.0 for caffeic acid; ions 209.0 and 340.0 for hydrocaffeic acid and ion 313.0 for IS. Electron impact ionization energy was 70 eV. All compounds were identified by characteristic ions and retention time compared to authentic standards.

Other biochemical analyses

Routine biochemical analyses on fasting blood samples taken at the screening visit were carried out in the Path West laboratory at Royal Perth Hospital, Western Australia. A routine enzymatic colorimetric test with a fully automated analyser (Roche Hitachi 917, Roche Diagnostics Australia Pty. Ltd., Castle Hill, New South Wales, Australia) was used to measure serum total cholesterol, HDL cholesterol and triglycerides. LDL cholesterol concentrations were calculated using the Friedewald formula. Serum glucose was measured using an automated analyser (Roche Hitachi 917).

Statistics

Sample size was calculated on the primary endpoints of plasma RXNO, FMD and blood pressure. With α=0.05, 20 participants provided >80% power to detect a 30% difference in plasma RXNO, a 20% difference in FMD (a 1.5% absolute difference), and a 3.0 mm Hg
difference in systolic blood pressure. Statistical analyses were performed using SPSS 15.0 (SPSS Inc, Chicago, IL) and SAS 9.2 (SAS institute Inc., Cary, NC, USA). Non-normally distributed data were log-transformed prior to analysis. Participant characteristics are presented as mean±SD. Results in the text and tables are presented as mean (95% CIs) or geometric mean (95% CIs) for non-normally distributed variables. Results in figures are presented as mean±SEM or geometric mean (95% CIs) for non-normally distributed variables. Outcome variables were analysed with mixed models in SAS using the PROC MIXED command. Subject was included as a random factor in all models. Models for plasma RXNO, plasma nitrite, plasma NOx, peak FMD, plasma chlorogenic acid and chlorogenic acid metabolites included fixed effects for group (control or chlorogenic acid) and order. Models for blood pressure also included fixed effects for baseline value and time (60, 90, 120 and 150 min).
RESULTS

Baseline data

Recruitment began December 8, 2010 and the study ended June 6, 2011. Twenty-three participants (4 men, 19 women) completed the study (Figure 1). The characteristics of the study participants are shown in Table 1.

Blood pressure, endothelial function and biomarkers of nitric oxide status

Mean post-treatment systolic blood pressure (-2.41 mm Hg, 95% CI: -0.03, -4.78; P=0.05) and diastolic blood pressure (-1.53 mm Hg, 95% CI: -0.05, -3.01; P=0.04) between 60 and 150 min were significantly lower for chlorogenic acid relative to placebo (Figure 2). There was no significant difference in peak FMD (0.41 %, 95% CI: -1.29, 2.10; P=0.62) at 120 min (Figure 3). Relative to placebo, chlorogenic acid did not significantly influence plasma RXNO (13.73 nmol/L, 95% CI: 12.23, 15.42; P=0.11), plasma nitrite (2.71 nmol/L, 95% CI: 2.49, 2.91; P=0.79) and plasma NOx (11.53 nmol/L, 95% CI: 11.04, 12.03; P=0.14) at 150 min (Figure 4).

Plasma chlorogenic acid and its metabolites

Intact chlorogenic acid was measured in plasma at 150 min. Typical chromatogram traces from chlorogenic acid treated participants (pre- and post-treatment) are shown in Figure 5. The concentration of chlorogenic acid in plasma was significantly higher following 400 mg chlorogenic acid compared to placebo (P< 0.001; Figure 6A), however the concentration of chlorogenic acid metabolites, isoferulic acid, ferulic acid, phloretic acid, caffeic acid and hydrocaffeic acid were not significantly different according to treatment (Figure 6B).
DISCUSSION

The aim of this study was to assess whether the consumption of pure chlorogenic acid can acutely augment NO status, improve endothelial function and lower blood pressure in healthy men and women. Enhanced NO status and improved endothelial function may explain previously reported benefits on blood pressure \(^{16}\). While we did observe a significant decrease in systolic and diastolic blood pressure, and a concomitant increase in chlorogenic acid in the plasma, the effects on NO status and FMD did not reach significance.

An acute intake of 400 mg of chlorogenic acid resulted in significantly lower systolic and diastolic blood pressure. In a previous study, 4 weeks of regular (chronic) consumption of green coffee extract, which is rich in chlorogenic acid, led to lower blood pressure in mildly hypertensive men \(^{16}\). A dose-related reduction in systolic and diastolic blood pressure was observed, with the highest dose of 100 mg/d of chlorogenic acid resulting in lower systolic and diastolic blood pressures of approximately 3.5 mm Hg and 3.0 mm Hg, respectively \(^{16}\). The blood pressure measurements were performed in the morning, in the fasting state, and 24 hours after the last dose of chlorogenic acid, and are therefore not due to acute effects of chlorogenic acid \(^{16}\). We have now demonstrated that chlorogenic acid can acutely reduce blood pressure. It is not known whether these acute effects would be found over and above any chronic effects.

Coffee consumption has an adverse effect on blood pressure \(^{17}\). However, trials with caffeinated coffee reveal a weaker effect on blood pressure than those with pure caffeine \(^{18}\), suggesting that other coffee components counteract the caffeine effect. It is possible that chlorogenic acid contributes to blunting of the blood pressure raising effect of caffeine in coffee. Plums and other fruit such as berries are also rich in chlorogenic acid \(^{19}\). The blood
pressure lowering effect of chlorogenic acid may contribute to the growing evidence supporting the protective effect of fruit against cardiovascular disease and hypertension\textsuperscript{1, 20, 21}.

Lower blood pressure was observed without any concomitant significant difference in NO status or FMD of the brachial artery. FMD of the brachial artery after a period of ischemia is primarily mediated by NO\textsuperscript{22}. Plasma S-nitrosothiols and nitrite are considered to be reliable measures of endogenous NO production\textsuperscript{23, 24}. These molecules also form part of a circulating NO pool in that they have the potential to be converted back to NO. A lower FMD is associated with a significantly higher risk of subsequent cardiovascular events\textsuperscript{6}. Thus, augmented NO status and increased FMD would be consistent with benefits on cardiovascular risk. In our study, FMD was measured at 120 min post chlorogenic acid intake and NO status was measured 150 min post chlorogenic acid intake. We observed a non-significant trend for both FMD and RXNO to be higher after the chlorogenic acid in comparison to placebo. These results are consistent with previous studies showing that FMD did not significantly improve 1-2 h after decaffeinated coffee intake\textsuperscript{9, 10}. Although we found that plasma concentrations of chlorogenic acid were significantly elevated at 150 min, peak plasma concentrations of chlorogenic acid may be closer to 30-60 min post ingestion\textsuperscript{25}. Furthermore, the observed blood pressure difference appeared to be largest between 60 to 90 min, and smaller beyond 90 min. These results raise the possibility that acute effects on NO, FMD and blood pressure coincide with peak plasma chlorogenic acid concentrations.

This idea is not supported by the results of Suzuki et al.\textsuperscript{12}, who demonstrated an acute reduction in blood pressure in spontaneously hypertensive rats 9 hours after a single dose of chlorogenic acid. In addition, this effect was inhibited by use of a NO synthase inhibitor\textsuperscript{12}. An improvement in acetylcholine-induced endothelium-dependent vasodilation in the aorta was also observed\textsuperscript{12}. These apparent discordant results could be due to the use of a very high dose of chlorogenic acid, which may result in a more sustained elevation in circulating
chlorogenic acid and metabolites. The dose of chlorogenic acid used in our study was equivalent to that obtained from drinking 2 cups of coffee, an easily achievable amount. Additional studies are needed to investigate the time course of acute vascular effects.

Chlorogenic acid and its metabolites were measured in plasma at baseline and 150 min post-treatment. A significant increase was seen in the plasma level of chlorogenic acid. Although some studies have failed to detect chlorogenic acid in the plasma of rats and human subjects after ingestion of chlorogenic acid as the pure compound or in coffee, other studies have detected chlorogenic acid in its unchanged form in urine suggesting absorption of the compound without structural modification. The undetectable chlorogenic acid in previous studies may have resulted from the limit of detection of the assay being used or due to degradation of chlorogenic acid during sample treatment.

After absorption, chlorogenic acid is metabolised to caffeic acid, quinic acid and ferulic acid. Phenolic groups may then be further metabolized by methylation or conjugation with glucuronic acid or sulfatase in the liver. Previous studies in rats suggest that ferulic acid can reduce blood pressure and improve vascular function by scavenging superoxide and increasing NO bioavailability or by acting directly on the vascular endothelium. In this study, no increase in ferulic acid or the other metabolites was detected at 150 min post-ingestion of chlorogenic acid. This does not rule out the possibility that ferulic acid, as a metabolite of chlorogenic acid ingested, could have an effect on NO, FMD and blood pressure at a later time point. According to Stalmach et al., metabolites such as dihydroferulic acid had peak plasma concentration (C\text{max}) values after 4 hours of coffee ingestion, indicating absorption in the large intestine with possible catabolism by the colonic microflora. Similarly Renouf et al. reported that dihydrocaffeic acid and dihydroferulic acid appeared in plasma 6 to 8 hours after coffee ingestion. It is possible that these metabolites could contribute to a more sustained effect on blood pressure.
The bioactivity of polyphenols rely largely on their bioavailability. A study evaluating pharmacokinetic profile and apparent bioavailability of chlorogenic acid in plasma and urine of 10 healthy adults after consumption of a decaffeinated green coffee extract showed that there is a large variation in chlorogenic acid absorption, metabolism and kinetics among different individuals. A better understanding of the consumption and bioavailability of dietary polyphenols will lead to the better evaluation of their role in disease prevention.

The exact molecular mechanisms by which chlorogenic acid or its metabolites could lower blood pressure and improve vascular function are yet to be elucidated. However, a number of potential pathways have been proposed. These include direct scavenging of free radicals; inhibition of NADPH oxidase, an enzyme producing reactive oxygen species; increased NO production and/or inhibition of angiotensin-converting enzyme. The importance of these and other potential mechanisms in vivo is not clear.

The main limitation of our study may be the timing of blood samples for NO measurement and the timing of FMD measurement. In addition we used a representative chlorogenic acid, which was the 3-caffeoylquinic acid. Major dietary sources such as coffee predominately contain 5-caffeoylquinic acid. At this stage, we do not know if different chlorogenic acid isomers have the same biological activity. However, this study has been able to demonstrate acute intake of pure chlorogenic acid in a relevant achievable dietary dose can significantly lower blood pressure in healthy subjects.

In conclusion, we found an acute blood pressure lowering effect of chlorogenic acid. The significant increase of plasma chlorogenic acid with no difference seen in plasma level of chlorogenic acid metabolites suggests that intact chlorogenic acid absorbed in the small intestine, rather than its metabolites could be responsible. Future studies should investigate both the dose response and time course of these effects.
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Figure legend

**Figure 1.** Participant flow from recruitment through screening and randomisation to trial completion.

**Figure 2.** The effect of chlorogenic acid on: (A) systolic blood pressure from 60 min to 150 min post treatment; (B) mean baseline-adjusted systolic blood pressure post treatment; (C) diastolic blood pressure from 60 min to 150 min post treatment; and (D) mean baseline-adjusted diastolic blood pressure post treatment. Results are expressed as geometric mean (95% CIs). A mixed random-effects linear model was used to compare groups (n=23).

**Figure 3** The effect of chlorogenic acid on flow mediated dilatation 120 min post treatment. Results are expressed as mean (SEM). A mixed random-effects linear model was used to compare groups (n=23).

**Figure 4.** The effect of chlorogenic acid on plasma concentrations of (A) S-nitrosothiols+other nitroso species (RXNO) and (B) nitrite 150 min post treatment. Results are expressed as geometric mean (95% CIs). A mixed random-effects linear model was used to compare groups (n=23).
**Figure 5.** Typical LC/MS/MS chromatogram trace from the analysis of chlorogenic acid concentrations at baseline and 150 min post treatment in chlorogenic acid treated participants. Relative abundance of chlorogenic acid peak (retention time: 6.9 min) increased in post-treatment plasma compared to pre-treatment plasma.

**Figure 6.** Plasma concentration of intact chlorogenic acid (A) and chlorogenic acid metabolites (B) for control and chlorogenic acid treatments at 150 min post-treatment. Increased concentration was found in intact chlorogenic acid in the chlorogenic acid treated group compared to control ($P<0.001$). Levels of chlorogenic acid metabolites were not significantly different between the interventions ($P=0.09$, $P=0.16$, $P=0.66$, $P=0.77$, $P=0.72$ for isofurulic acid, ferulic acid, phloretic acid, caffeic acid, hydrocaffeic acid respectively; n=10). Results are expressed as mean ± 95% CI. A mixed random-effects linear model was used to compare groups (n=23).
Table 1. Baseline characteristics of study subjects (n=23; males= 4; females= 19)

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<th>Characteristic</th>
<th>Mean ± SD</th>
<th>Range</th>
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<tr>
<td>Age (years)</td>
<td>52.3 ± 10.6</td>
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<td>Weight (kg)</td>
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<td>Body Mass Index (kg/m²)</td>
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<td>Systolic blood pressure (mmHg)</td>
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</tr>
<tr>
<td>Pulse pressure (mmHg)</td>
<td>60.9 ± 7.3</td>
<td>37 - 69</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.9 ± 0.9</td>
<td>3.1 – 5.6</td>
</tr>
<tr>
<td>Triglyceride (mmol/L)</td>
<td>0.9 ± 0.4</td>
<td>0.4 – 1.7</td>
</tr>
<tr>
<td>High density lipoprotein (mmol/L)</td>
<td>1.4 ± 0.3</td>
<td>0.8 – 2.2</td>
</tr>
<tr>
<td>Low density lipoprotein (mmol/L)</td>
<td>3.1 ± 0.7</td>
<td>1.8 – 4.5</td>
</tr>
<tr>
<td>Fasting plasma glucose (mmol/L)</td>
<td>5.1 ± 0.4</td>
<td>4.1 – 5.8</td>
</tr>
</tbody>
</table>
Responded to newspaper advert or recruitment letter  \( n = 73 \)

Screened  \( n = 35 \)

Excluded  \( n = 8 \)
- did not meet inclusion criteria during telephone screening  \( n = 6 \)
- did not meet inclusion criteria during screening appointment  \( n = 2 \)

Eligible to participate  \( n = 28 \)

Decided not to participate  \( n = 5 \)

Randomised and completed all treatments  \( n = 23 \)
Figure 2

(A) Graph showing systolic blood pressure (mm Hg) over time (min) for control and chlorogenic acid groups. 
(B) Bar chart showing systolic blood pressure (mm Hg) comparison between control and chlorogenic acid groups, with P = 0.05. 
(C) Graph showing diastolic blood pressure (mm Hg) over time (min) for control and chlorogenic acid groups. 
(D) Bar chart showing diastolic blood pressure (mm Hg) comparison between control and chlorogenic acid groups, with P = 0.04.
Figure 4

![Figure 4](image-url)
Figure 5:
Figure 6

A

P < 0.0001

Chlorogenic acid (ng/ml)

Control Chlorogenic acid

B

Control Chlorogenic acid

Concentration (ng/µL)

Isoferulic acid Ferulic acid Phloretic acid Caffeic acid Hydrocaffeic acid