1-28-2020

Enzymatically modified isoquercitrin improves endothelial function in volunteers at risk of cardiovascular disease

Nicola P. Bondonno
Edith Cowan University

Catherine P. Bondonno
Edith Cowan Unviersity

Natalie C. Ward

Richard J. Woodman

Jonathan M. Hodgson
Edith Cowan University

10.1017/S0007114519002137
This article has been published in a revised form in the British Journal of Nutrition https://doi.org/10.1017/S0007114519002137. This version is free to view and download for private research and study only. Not for re-distribution, re-sale or use in derivative works. © The Authors 2019.
Bondonno, N. P., Bondonno, C. P., Ward, N. C., Woodman, R. J., Hodgson, J. M., & Croft, K. D. (2020). Enzymatically modified isoquercitrin improves endothelial function in volunteers at risk of cardiovascular disease. *British Journal of Nutrition, 123*(2), 182-189. https://doi.org/10.1017/S0007114519002137

Enzymatically-Modified Isoquercitrin Improves Endothelial Function in Volunteers at Risk of Cardiovascular Disease

Nicola P. Bondonno[1,2*], Catherine P. Bondonno[1,2], Natalie C. Ward[3,4], Richard J. Woodman[5], Jonathan M. Hodgson[1,2], Kevin D. Croft[1].

[1] School of Biomedical Science, University of Western Australia, Royal Perth Hospital, Perth, Western Australia, Australia;

[2] School of Medical and Health Sciences, Edith Cowan University, Perth, Western Australia, Australia;

[3] School of Public Health and Curtin Health Innovation Research Institute, Curtin University, Perth, Western Australia, Australia;

[4] Medical School, University of Western Australia, Perth, Western Australia, Australia;

[5] Centre for Epidemiology and Biostatistics, School of Public Health, Flinders University of South Australia, Adelaide, South Australia, Australia.

*__Correspondance__: Nicola P Bondonno

Level 3, Medical Research Foundation

Rear 50 Murray St, Perth Western Australia, Australia WA 6000

Tel: 618 63042532

Email: n.bondonno@ecu.edu.au

Short title: EMIQ® acutely improves endothelial function

Keywords: Enzymatically modified isoquercitrin, endothelial function, blood pressure, cognitive function.

Clinical trial registry number and website: Australian New Zealand Clinical Trial Registry (ACTRN12617001202358); http://www.anzctr.org.au/

ABSTRACT

A higher intake of foods rich in flavonoids such as quercetin can reduce the risk of cardiovascular disease. Enzymatically modified isoquercitrin (EMIQ®) has a bioavailability 17-fold higher than quercetin aglycone and has shown potential cardiovascular disease moderating effects in animal studies. The present study aimed to determine if acute ingestion of EMIQ® improves endothelial function, blood pressure, and cognitive function in human volunteers at risk of cardiovascular disease. Twenty-five participants (12 males, 13 females) with at least one cardiovascular disease risk factor completed this randomized, controlled, crossover study. In a random order, participants were given EMIQ® (2 mg aglycone equivalent)/kg body weight or placebo alongside a standard breakfast meal. Endothelial function, assessed by flow mediated dilatation (FMD) of the brachial artery was measured before and 1.5 hrs after intervention. Blood pressure (BP), arterial stiffness, cognitive function, BP during cognitive stress and measures of quercetin metabolites, oxidative stress and markers of nitric oxide (NO) production were assessed post-intervention. After adjustment for pre-treatment measurements and treatment order, EMIQ® treatment resulted in a significantly higher FMD response compared to the placebo [0.60%, 95% CI: 0.03, 1.17 ($p=0.04$)]. Plasma concentrations of quercetin metabolites were significantly higher ($p<0.001$) after EMIQ® treatment compared to the placebo. No changes in blood pressure, arterial stiffness, cognitive function, or biochemical parameters were observed. In this human intervention study, the acute administration of EMIQ® significantly increased circulating quercetin metabolites and improved endothelial function. Further clinical trials are required to assess whether health benefits are associated with long-term EMIQ® consumption.

INTRODUCTION:

There is mounting evidence from both cohort studies and clinical trials that an increased intake of plant foods, specifically those rich in flavonoids, can reduce the risk of cardiovascular disease [1]. Quercetin (in its glycosylated form) is the most commonly consumed flavonoid compound within the flavonol subclass [2]. The main dietary contributors to quercetin intake include tea, apples, onions, and broccoli [3].

Evidence for the health promoting effects of quercetin comes primarily from animal and *in vitro* studies [4]. In a randomized-controlled cross-over study of men and women, apples with skin significantly reduced systolic blood pressure, improved endothelial function and increased plasma nitric oxide (NO) [5]. These apples with skin provided 184 mg of quercetin and 180 mg of (−)-epicatechin while the apple flesh only (control) provided less than 5 mg of quercetin and (−)-epicatechin. Previous studies using both pure quercetin aglycone (1095 mg) [6] and quercetin glucoside supplementation (50 – 400 mg) [7; 8] have shown no effect on endothelial function. This may be due to in part to the bioavailability of the quercetin interventions.

Quercetin in foods is usually found in its glycosylated form, with the presence and position of the sugar moiety influencing the site and extent to which it is absorbed [9]. The bioavailability of quercetin glucosides can be enhanced by enzymatic α-oligoglucosylation of this sugar moiety [10]. Enzymatically modified isoquercitrin (EMIQ®) is a water-soluble glucoside of quercetin produced by the deglycosylation and subsequent α-oligoglucosylation of rutin [10]. Plasma concentrations of quercetin metabolites have been shown to be approximately 2-3 times higher after the consumption of EMIQ® in comparison to isoquercitrin (quercetin-3-*O*-glucoside) and its bioavailability is 17-fold higher than that of quercetin [10; 11].

EMIQ® has been shown to suppress the increase in systolic BP observed in spontaneously hypertensive rats, [12] and decrease oxidative stress in a mouse model of atherosclerosis [13]. EMIQ® is approved in Japan as a food additive and is safe for human consumption. Human trials carried out over a 12-week period indicate a significant reduction in body fat [14]. The effects of EMIQ® on vascular and cognitive function have yet to be investigated.

Therefore, the aim of the present study was to determine if acute ingestion of EMIQ® improves endothelial function, blood pressure, and cognitive function in human volunteers at risk of cardiovascular disease.

SUBJECTS AND METHODS:

Participants

Twenty-five eligible men and women, aged between 50 and 70 years, were recruited by newspaper advertisement from the general population of Perth, Western Australia (**Figure 1**). These volunteers attended a screening appointment within the University of Western Australia, School of Biomedical Sciences, located in the Medical Research Foundation building at Royal Perth Hospital. The screening appointment consisted of an electrocardiograph, a standard medical history questionnaire, and the measurement of height, weight, body mass index (BMI) and BP. Finally, a blood sample was taken for measurement of fasting serum total cholesterol, HDL-cholesterol (HDL-C), triglycerides (TG), LDL-cholesterol (LDL-C), and glucose. Volunteers were included in the study if they had at least one of the following risk factors for CVD: systolic BP between 120 mmHg and 160 mmHg, fasting plasma glucose between 5.6 mM and 6.5 mM, total cholesterol between 5 mM and 8 mM or a waist circumference >94 cm for men or >80 cm for women. Exclusion criteria included a BMI <18 or >35 kg/m^2; a systolic BP ≤100 or ≥160 mmHg; a diastolic BP ≤50 or ≥90 mmHg; diagnosed diabetes; fasting plasma glucose concentrations ≥6.5 mmol/L; use of BP lowering or cholesterol lowering medication; alcohol intake >210 g per week for women and >280 g per week for men; current or recent (within previous 6 months) significant weight loss or gain (>6% of body weight); actively trying to lose weight; current or recent (<12 months) smoking; history of cardiovascular or peripheral vascular disease; psychiatric or any other major illnesses; and women were lactating, pregnant or wishing to become pregnant during the study.

The study was carried out in accordance with the Declaration of Helsinki and was approved by the University of Western Australia Human Research Ethics Committee (Approval number RA/4/1/9260). All participants provided written informed consent before inclusion in the study. The trial was registered with the Australian New Zealand Clinical Trials Registry (ACTRN12617001202358).

Study design

This acute, randomized, controlled crossover trial was conducted between September 2017 and June 2018. Participants were required to visit the department three times (one practice visit and two study visits). The practice visit involved the completion of a selection of

cognitive function tests designed to induce cognitive stress (see description below) three times, in order to control for practice effects and to allow familiarization with procedure. The practice day data were not included in any analyses. Participants were required to fast for 12 hrs (water allowed *ad libitum*) prior to each subsequent study visit. These two visits were separated by at least 7 days to ensure that there were no carry-over treatment effects. Participants were instructed to have the same meal the night before, not to consume any alcohol for 24 hrs prior to the visit and not to take any medication or undergo any exercise on the morning of their visit. Endothelial function was assessed before and 1.5 hrs post-intervention. A blood pressure measurement was taken prior to and every 10 min for 80 min post treatment. Measurements of arterial stiffness were taken 2 hrs post-intervention. Participants completed the selection of cognitive function tests 2.5 hrs post-intervention, during which blood pressure was measured every 2 min. Finally, a blood sample was taken by venipuncture 3.5 hrs post-intervention.

Intervention

Participants were randomly assigned to one of two treatment orders generated using a pseudorandom number generator (https://www.randomizer.org/). The EMIQ® was prepared by San-Ei Gen F.F.I., Incorporated (Osaka, Japan). The active treatment consisted of 4.89 mg EMIQ® (2 mg aglycone equivalent)/kg body weight [(10)] plus ½ teaspoon of maltodextrin and 1½ tablespoons of Cottee's Raspberry flavoured cordial mixed with 250 mL water. The placebo treatment was ½ teaspoon of maltodextrin and 1½ tablespoons of cordial mixed with 250 mL water. The cordial was used to mask the taste and the colour of the EMIQ®. Both treatments were given with a standardised breakfast of 2 pieces of white bread and cheese. Participants and all researchers performing the tests and analysing the results were blinded to the treatment.

Assessment of endothelial function

In order to assess endothelial function, flow-mediated dilatation (FMD) of the brachial artery was calculated by a trained ultrasonographer, dedicated to the research protocol and blinded to the interventions used. Participants were studied in a quiet, temperature-controlled room (21 to 25 °C). Participants rested in a supine position for 20 min prior to the initiation of the FMD measurement. The right arm was extended and supported comfortably on a foam mat. For the ultrasound, a 12-MHz transducer connected to a Philips CX50 Ultrasound Machine

was clamped in position over the brachial artery, 5 to 10 cm proximal to the antecubital crease. After a baseline artery diameter recording of 1 min, a blood pressure cuff placed around the left forearm was inflated to 200 mmHg. After 5 min the cuff was released, inducing reactive hyperaemia. The brachial artery image was recorded for 4 min (240 seconds) post-cuff deflation to assess FMD. Images were downloaded for retrospective analysis. Analysis of FMD was performed using a semi-automated edge detection software (Vascular Research Tools, Medical Imaging Applications LLC, Coralville IA), which automatically calculates the brachial artery diameter, corresponding to the internal diameter. Responses were calculated as the percentage change in brachial artery diameter from baseline, at 10 second intervals, for 240 seconds after cuff deflation.

Measurement of blood pressure and arterial stiffness

Blood pressure measurements were taken using a Dinamap 1846SX/P oscillometric recorder (Critikon, Tampa, FL, USA) attached to the non-dominant arm with participants in a seated position. Following a 10 min rest, one BP measurement was taken immediately prior to treatment consumption. BP was then measured every 10 min post-treatment consumption for 80 min (9 measurements in total).

Office BP, central diastolic BP, and arterial stiffness were assessed using the SphygmoCor Xcel (AtCor Medical, Sydney, Australia). Following a 5 min rest in a supine position, BP measurements were taken on three occasions at 1 min intervals, the first measurement was discarded and the second and third measurements were averaged. Arterial stiffness was determined by measuring augmentation Index (AIx) using the Sphygmocor Xcel as described previously [15] and was standardised to a heart rate of 75 bpm (AIx75).

Cognitive stress test and blood pressure

A selection of cognitive function tests designed to induce cognitive stress using the Computerised Mental Performance Assessment System (COMPASS, BPNRC, Newcastle Upon Tyne, UK) was used. This series of tests, described in detail previously [16], comprised of two computerized serial subtraction tasks (Serial Threes and Serial Sevens) and a Bakan Rapid Visual Information Processing task (RVIP), each repeated three times. Briefly, for the Serial Three and Serial Seven subtraction tasks, participants were asked to continuously subtract three or seven from a random starting number between 800 and 999. This task lasted for two min and both the number of correct responses and the total number of subtractions

completed were recorded. For the RVIP task, participants were asked to identify when three odd or three even digits appeared consecutively in series of single digit numbers presented at a rate of 100 per min. This task lasted for five min and both the number of correct responses and the average response time were recorded. Participants rested for 20 min prior to and 15 min after the cognitive testing, which took approximately 30 min. For the duration of this task (65 min total), blood pressure was measured every 2 min with a Dinamap 1846SX/P oscillometric recorder (Critikon, Tampa, FL, USA) attached to the non-dominant arm of participants in a seated position.

Plasma analyses

Plasma samples, obtained 3.5 hrs post-intervention by venepuncture, were collected into EDTA tubes with added butylated hydroxytoluene and immediately centrifuged at 5000 x g, at 4°C for 5 min. One 2 mL aliquot of plasma was kept on liquid nitrogen for immediate analysis of nitrogen oxides (NOx) and the remaining plasma was stored at -80°C until analysis.

Quercetin metabolites

Plasma quercetin metabolites were measured by liquid chromatography/tandem mass spectrometry (LCMSMS) as described previously [7]. Briefly, plasma (250 µL) collected in EDTA was incubated for 2 hrs with β-glucuronidase enzyme. As β-glucuronidase has both glucuronidase and sulfatase activity, but is unable to enzymatically cleave methyl conjugates, isorhamnetin (O-methylated quercetin) was measured. Following solid phase extraction, quercetin aglycone and isorhamnetin were measured on a Thermo Scientific TSQ Quantum Ultra Triple Quadrupole LCMS System (ThermoFisher Scientific, Waltham, MA, USA). Calibration curves for quercetin and isorhamnetin with the fisetin as the internal standard were used for quantification.

Measurement of plasma total nitrogen oxides (NOx)

The concentrations of total NOx (nitric oxide, nitros(yl)ated species and nitrite) in plasma were determined using a previously described gas phase chemiluminescence assay [17]. Blood was collected into N-ethylmaleimide (10 mmol/L) and EDTA (2 mM), mixed and centrifuged at 3000 x g (5 min, 4°C). Fresh plasma was kept on ice in the dark and analysed within 1 hr. 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 NOx was quantified by the NO signal peak area of samples against a nitrite standard (300 µL, 0.5 µM $NaNO_2^-$). Quantification of NO released by the redox reactions occurred by its chemiluminescence reaction with ozone using the Nitric Oxide Analyzer (CLD66, Eco Physics, Sweden).

NO_2 and NO_3

Nitrate (NO_3) and nitrite (NO_2) concentrations were measured using gas chromatography/mass spectrometry (GCMS) as described previously [18], with N^{15} labelled NO_3 and NO_2 internal standards.

Glucose

Plasma glucose was measured using a fully automated analyser (Architect ci8200/c16000 Analyser).

F_2-isoprostanes

Systemic oxidative stress was determined by measuring plasma F_2-isoprostane concentrations as described previously [19]. Briefly, F_2-isoprostane concentrations were calculated in pmol/L from ratio of the peak areas for the *m/z* 569 and *m/z* 573 ions, with a deuterium labelled internal standard, 8-iso-Prostaglandin $F_{2\alpha}$-d_4.

Statistics

Sample size was calculated with FMD as the primary endpoint. Assuming a within-subject correlation of r=0.25, 24 pre-intervention measures, 24 post-intervention measures, and a within group SD of 6%, a sample size of 25 subjects would provide more than 80% power at alpha=0.05 to detect a difference of 0.85% in FMD. Treatment effects for post-intervention FMD were obtained using linear mixed models with adjustment for treatment order, the pre-treatment FMD curve, and the time since cuff release with time included as a categorical variable (0 to 240 seconds at 10 second intervals). In order to allow the treatment effect to differ over time, we also included a treatment x time interaction term. The subject identifier was included as a random intercept. Assessment of post-treatment BP was similar to that for FMD, with time included as a categorical variable (0 to 80 minutes at 10-minute intervals). When assessing the effect of EMIQ® on the BP during the cognitive stress test, we

additionally adjusted for the rest-period BP. For all other outcomes, treatment effects were obtained using linear mixed models with adjustment for treatment order. Statistical analyses were performed using STATA/IC 14.2 (StataCorp LLC).

RESULTS:

Baseline data

Recruitment began on 25th September 2017 and the study concluded on 12th June 2018. Twenty-five participants (12 males, 13 females) completed the study (**Figure 1**). The baseline characteristics of the study participants are shown in **Table 1**.

Endothelial function

Data from one participant was excluded prior to statistical analysis as the clarity of the FMD image was poor. The observed mean percentages of FMD for 24 participants, between 0 and 240 seconds at 1.5 hrs post-intervention, are presented in **Figure 2**. Overall, FMD after the EMIQ® treatment was significantly higher when compared to control [adjusted mean difference=0.60%, 95% CI: 0.03, 1.17; p=0.040].

Blood pressure and arterial stiffness

Mean systolic BP and diastolic BP, measured immediately before treatment (time=0) and then every 10 min for 80 min, are shown in **Figure 3**. There was no significant difference between EMIQ® and placebo for post-treatment systolic BP (p=0.916) or diastolic BP (p=0.073). Similarly, there was no difference for MAP (p=0.188) or heart rate (p=0.290). There was no significant difference between treatments for mean systolic BP, diastolic BP, central diastolic pressure, MAP, heart rate, or AIx75, measured 2 hrs post-treatment (**Table 2**).

Cognitive stress test and blood pressure

There were no significant effects associated with the EMIQ® treatment on any of the cognitive stress test outcomes, nor on BP measurements taken during the cognitive stress tests (**Tables 3 and 4**).

Plasma analyses

Plasma concentrations of quercetin aglycone and isorhamnetin were significantly higher ($p<0.001$) after the ingestion of EMIQ® compared to the placebo (**Table 3**). There was no significant difference in plasma glucose, NOx, NO_2, NO_3, or F_2-isoprotanes between EMIQ® and placebo treatments.

DISCUSSION:

This is the first study to investigate the acute effects of an enzymatically-modified isoquercitrin compound on measures of vascular health, blood pressure and cognitive function in humans. In this randomised controlled cross-over study, the acute administration of EMIQ® significantly increased plasma quercetin metabolites and improved endothelial function. No changes in blood pressure, arterial stiffness, cognitive function, blood pressure during cognitive stress or biochemical parameters were observed.

In the present study plasma concentrations of quercetin aglycone and isorhamnetin were significantly higher after the ingestion of EMIQ® compared to the placebo. Previously, the plasma concentration of conjugated quercetin metabolites has been shown to reach a maximal level at approximately 1.5 h after intake of EMIQ® [10]. For this reason, our primary outcome, endothelial function, was assessed 1.5 hrs post-intervention.

Endothelial dysfunction, defined as an impairment in endothelium-dependent relaxation, is implicated in prehypertension, hypertension, atherosclerosis, and stroke [20; 21], and is associated with an increased risk of cardiovascular disease [22; 23]. The results of the present study suggest that EMIQ® improves postprandial endothelial function. In two previous studies, acute quercetin administration (1095 mg quercetin aglycone [6]; and doses of quercetin-3-*O*-glucoside ranging from 50 - 400 mg [7]) had no effect on FMD. In the study by Larson *et al*, FMD was measured 10 hrs post-quercetin aglycone administration, the time at which quercetin metabolites were shown to peak in the plasma [6]. The pharmacokinetics of quercetin aglycones and EMIQ are very different; in the present study FMD was measured 1.5 hrs post-intervention, at the time quercetin metabolites have been shown to peak in the plasma following EMIQ administration [10]. Quercetin-3-O-glucoside has been shown to peak in the plasma between 1.5 and 3 hrs post-intervention; in our previous study [7], FMD was

measured 1 hr post-intervention. The differences in study design and the form in which the quercetin was administered make the direct comparison to the present study difficult. Another important discrepancy in study design is that EMIQ was administered alongside a meal in the present study, likely influencing the effect of quercetin on endothelial function [24; 25]. Overall, FMD in the present study was relatively poor as we recruited participants with at least one risk factor for cardiovascular disease. Although the improvement observed was subtle (0.66%), if sustained it could have important clinical implications as a 1% increase in FMD is associated with a 13% lower risk of cardiovascular events (RR: 0.87, 95% CI: 0.83- 0.91) [23].

Evidence suggests that quercetin can improve endothelial health through NO-mediated vasorelaxant activity as well as through the prevention of oxidant induced endothelial dysfunction [4]. An acute increase in plasma NO status has been observed after both a high-flavonoid apple intervention [17] and quercetin (200 mg) intervention [26]. In the present study we did not observe any increase in plasma NOx, or the end products of NO metabolism, NO_3 and NO_2. This may have been due to the timing of our blood sample, which was 1.5 hrs after the measurement of endothelial function, and 3.5 hrs post-intervention. Changes in NO are likely to be transient, and because of the difficulty in detecting small changes in NO, the timing of the measurement is likely to be critical.

In our previous randomized clinical trial with healthy men and women, improvements in BP were also observed with the concomitant increase in nitric oxide status and FMD, after intake of flavonoid-rich apples [17]. In a meta-analysis of randomized controlled trials, significant reductions in both systolic BP (−3.04 mm Hg, 95% CI: −5.75, −0.33, p=0.028) and diastolic BP (−2.63 mm Hg, 95% CI: −3.26, −2.01, p<0.001) were seen following quercetin supplementation [27]. Furthermore, in spontaneously hypertensive rats, EMIQ® administered at a dose of 26 mg/kg/d suppressed the increase in systolic BP more effectively than quercetin aglycone [12]. In the present study we observed no effect of EMIQ® on post-treatment BP nor on BP during the cognitive stress test. This lack of effect may be due to the dose given; in the aforementioned meta-analysis there was only a significant effect on BP when quercetin was administered at doses greater than 500 mg/day [27]. Additionally, the health status of the participants may have played a role as a decrease in systolic BP following quercetin supplementation is generally seen in hypertensive individuals [28; 29; 30], not in pre-hypertensive or normotensive individuals [6; 30; 31; 32; 33; 34].

Many functions of the endothelium are affected under oxidative stress, including endothelial cell apoptosis [35] and adhesion of inflammatory cells [36], initiating the development of atherosclerosis. Quercetin may decrease oxidative stress through the stimulation of protective defences and repair systems [37]. In apolipoprotein E (apoE)–deficient atherogenic mice, EMIQ® supplementation for 14 weeks significantly suppressed aortic and aortic sinus atherosclerotic lesion areas and decreased levels of 4-hydroxy-2-nonenal, a marker of oxidative stress [13]. In the present study we observed no acute changes in levels of plasma F_2-isoprostanes, an *in vivo* biomarker of oxidative stress, following the EMIQ® intervention. It may be that long-term interventions are required to observe changes in biomarkers of oxidative stress. Evidence that quercetin may impede the development of CVD by moderating oxidative stress is stronger in animal studies than human studies [4].

There is evidence that flavonols, the flavonoid subclass to which quercetin belongs, can reduce the risk of type 2 diabetes (T2D). This comes from an observational study which found that each 2.5- fold increase in flavonol intake was associated with a 26% lower incidence of T2D [38], and a meta-analysis demonstrating a significant reduction in fasting plasma glucose (difference in mean = −0.18 mmol/L; 95% CI: −0.29, −0.08), following flavanol intake [39]. However, fasting plasma glucose was not significantly lower in any of the studies with a quercetin intervention. In the present study we saw no acute effect of EMIQ® on plasma glucose. Given that the study population was relatively normoglycemic, this may be better investigated in a hyperglycemic population.

Evidence that quercetin can improve cognitive function comes wholly from animal studies [40; 41; 42]. To our knowledge this is the first study to examine the acute effects of a quercetin derivative on cognitive function in humans. We saw no significant effects of EMIQ® on any measures of cognitive function in the present study. This warrants further investigation; future studies could consider a long-term intervention and a broader range of cognitive function measurements.

Strengths of the present study are that any inter-subject variability was accounted for by our cross-over study design and adjustments for baseline measurements of FMD controlled for day to day variability. There are, however, several limitations: firstly, only the primary outcome was measured before and after the intervention. Secondly, the study was only powered to detect a change in the primary outcome meaning that we may have been underpowered to detect changes in secondary outcomes. So as not to affect the measurement

of any other outcomes, the blood sample was taken 1.5 hrs after the measurement of FMD (3.5 hrs post-intervention). Additionally, this study only investigated one dose of EMIQ®, with the primary outcome measured at only one time-point.

In this human intervention study, the acute administration of EMIQ® significantly increased circulating quercetin metabolites and improved postprandial endothelial function. The addition of EMIQ® to commercially available foods and beverages may have positive effects on vascular function. The potential health benefits associated with regular consumption warrants investigation in longer term randomised controlled trials.

Acknowledgements:

The authors wish to thank the volunteers who participated in the study. JMH was supported by a National Health and Medical Research Council Senior Research Fellowship.

Financial support:

This study was funded by San-Ei Gen F.F.I., Incorporated (Osaka, Japan).

Conflicts of Interest:

The authors declare no conflict of interest. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Authorship:

NPB, CPB, JMH, NCW, and KDC were responsible for the project conception; NPB and CPB conducted the research; NPB and RJW analysed the data; NPB prepared the manuscript; CPB, JMH, NCW, RJW and KDC critically reviewed the manuscript.

REFERENCES:

1. Williamson G (2017) The role of polyphenols in modern nutrition. *Nutrition bulletin* **42**, 226-235.
2. Bondonno NP, Lewis JR, Blekkenhorst LC *et al.* (2019) Association of flavonoids and flavonoid-rich foods with all-cause mortality: The Blue Mountains Eye Study. *Clinical Nutrition*.
3. Neveu V, Perez-Jiménez J, Vos F *et al.* (2010) Phenol-Explorer: an online comprehensive database on polyphenol contents in foods. *Database* **2010**.
4. Bondonno NP, Bondonno CP, Hodgson JM *et al.* (2015) The efficacy of quercetin in cardiovascular health. *Current Nutrition Reports* **4**, 290-303.
5. Bondonno CP, Yang X, Croft KD *et al.* (2012) Flavonoid-rich apples and nitrate-rich spinach augment nitric oxide status and improve endothelial function in healthy men and women: a randomized controlled trial. *Free Radic Biol Med* **52**, 95-102.
6. Larson A, Witman MAH, Guo Y *et al.* (2012) Acute, quercetin-induced reductions in blood pressure in hypertensive individuals are not secondary to lower plasma angiotensin-converting enzyme activity or endothelin-1: nitric oxide. *Nutr Res* **32**, 557-564.
7. Bondonno NP, Bondonno CP, Rich L *et al.* (2016) Acute effects of quercetin-3-O-glucoside on endothelial function and blood pressure: a randomized dose-response study. *The American journal of clinical nutrition* **104**, 97-103.
8. Dower JI, Geleijnse JM, Gijsbers L *et al.* (2015) Effects of the pure flavonoids epicatechin and quercetin on vascular function and cardiometabolic health: a randomized, double-blind, placebo-controlled, crossover trial. *The American journal of clinical nutrition* **101**, 914-921.
9. Hollman PC (2004) Absorption, bioavailability, and metabolism of flavonoids. *Pharmaceutical biology* **42**, 74-83.
10. Murota K, Matsuda N, Kashino Y *et al.* (2010) α-Oligoglucosylation of a sugar moiety enhances the bioavailability of quercetin glucosides in humans. *Archives of biochemistry and biophysics* **501**, 91-97.
11. Makino T, Shimizu R, Kanemaru M *et al.* (2009) Enzymatically modified isoquercitrin, α-oligoglucosyl quercetin 3-O-glucoside, is absorbed more easily than other quercetin glycosides or aglycone after oral administration in rats. *Biological and Pharmaceutical Bulletin* **32**, 2034-2040.
12. Emura K, Yokomizo A, Toyoshi T *et al.* (2007) Effect of enzymatically modified isoquercitrin in spontaneously hypertensive rats. *Journal of nutritional science and vitaminology* **53**, 68-74.
13. Motoyama K, Koyama H, Moriwaki M *et al.* (2009) Atheroprotective and plaque-stabilizing effects of enzymatically modified isoquercitrin in atherogenic apoE-deficient mice. *Nutrition* **25**, 421-427.
14. Yoshimura M, Maeda A, Takehara I *et al.* (2008) Body fat reducing effect and safety of the beverage containing polyphenols derived from Japanese pagoda tree (enzymatically modified isoquercitrin) in overweight and obese subjects. Jpn. *Pharmacol Ther* **36**, 919-930.
15. Hwang M, Yoo J, Kim H *et al.* (2014) Validity and reliability of aortic pulse wave velocity and augmentation index determined by the new cuff-based SphygmoCor Xcel. *J Hum Hypertens* **28**, 475-481.
16. Scholey AB, French SJ, Morris PJ *et al.* (2010) Consumption of cocoa flavanols results in acute improvements in mood and cognitive performance during sustained mental effort. *Journal of Psychopharmacology* **24**, 1505-1514.
17. Bondonno CP, Yang X, Croft KD *et al.* (2012) Flavonoid-rich apples and nitrate-rich spinach augment nitric oxide status and improve endothelial function in healthy men and women: a randomized controlled trial. *Free Radical Biology and Medicine* **52**, 95-102.
18. Yang X, Bondonno CP, Indrawan A *et al.* (2013) An improved mass spectrometry-based measurement of NO metabolites in biological fluids.(Report). *Free Radic Biol Med* **56**, 1.
19. Mori TA, Croft KD, Puddey IB *et al.* (1999) An Improved Method for the Measurement of Urinary and Plasma F_2-Isoprostanes Using Gas Chromatography–Mass Spectrometry. *Anal Biochem* **268**, 117-125.

20. Cosentino F, Volpe M (2005) Hypertension, stroke, and endothelium. *Curr Hypertens Rep* **7**, 68-71.
21. Yang Z, Ming X-F (2006) Recent advances in understanding endothelial dysfunction in atherosclerosis. *J Clin Med Res* **4**, 53-65.
22. Halcox JP, Schenke WH, Zalos G *et al.* (2002) Prognostic value of coronary vascular endothelial dysfunction. *Circ J* **106**, 653-658.
23. Inaba Y, Chen JA, Bergmann SR (2010) Prediction of future cardiovascular outcomes by flow-mediated vasodilatation of brachial artery: a meta-analysis. *The international journal of cardiovascular imaging* **26**, 631-640.
24. de Koning EJ, Rabelink TJ (2002) Endothelial function in the post-prandial state. *Atherosclerosis Supplements* **3**, 11-16.
25. Grassi D, Draijer R, Schalkwijk C *et al.* (2016) Black tea increases circulating endothelial progenitor cells and improves flow mediated dilatation counteracting deleterious effects from a fat load in hypertensive patients: a randomized controlled study. *Nutrients* **8**, 727.
26. Loke WM, Hodgson JM, Proudfoot JM *et al.* (2008) Pure dietary flavonoids quercetin and (-)-epicatechin augment nitric oxide products and reduce endothelin-1 acutely in healthy men. *Am J Clin Nutr* **88**, 1018-1025.
27. Serban MC, Sahebkar A, Zanchetti A *et al.* (2016) Effects of quercetin on blood pressure: a systematic review and meta-analysis of randomized controlled trials. *Journal of the American Heart Association* **5**, e002713.
28. Larson A, Bruno R, Guo Y *et al.* (2009) Acute quercetin Supplementation does not lower blood pressure or Ace Activity in Normotensive Males. *Journal of the American Dietetic Association* **109**, A16.
29. Egert S, Bosy-Westphal A, Seiberl J *et al.* (2009) Quercetin reduces systolic blood pressure and plasma oxidised low-density lipoprotein concentrations in overweight subjects with a high-cardiovascular disease risk phenotype: a double-blinded, placebo-controlled cross-over study. *Br J Nutr* **102**, 1065-1074.
30. Edwards RL, Lyon T, Litwin SE *et al.* (2007) Quercetin reduces blood pressure in hypertensive subjects. *J Nutr* **137**, 2405-2411.
31. Dower JI, Geleijnse JM, Gijsbers L *et al.* (2015) 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* **101**, 914-921.
32. Perez A, Gonzalez-Manzano S, Jimenez R *et al.* (2014) The flavonoid quercetin induces acute vasodilator effects in healthy volunteers: Correlation with beta-glucuronidase activity. *Pharmacol res* **89**, 11-18.
33. Conquer J, Maiani G, Azzini E *et al.* (1998) Supplementation with quercetin markedly increases plasma quercetin concentration without effect on selected risk factors for heart disease in healthy subjects. *J Nutr* **128**, 593-597.
34. Nakayama H, Tsuge N, Sawada H *et al.* (2013) Chronic intake of onion extract containing quercetin improved postprandial endothelial dysfunction in healthy men. *J Am Coll Nutr* **32**, 160-164.
35. Touyz R, Schiffrin E (2004) Reactive oxygen species in vascular biology: implications in hypertension. *Histochem Cell Biol* **122**, 339-352.
36. Taniyama Y, Griendling KK (2003) Reactive oxygen species in the vasculature molecular and cellular mechanisms. *Hypertension* **42**, 1075-1081.
37. Forman HJ, Davies KJ, Ursini F (2014) How do nutritional antioxidants really work: Nucleophilic tone and para-hormesis versus free radical scavenging< i> in vivo</i>. *Free Rad Biol Med* **66**, 24-35.
38. Jacques PF, Cassidy A, Rogers G *et al.* (2013) Higher dietary flavonol intake is associated with lower incidence of type 2 diabetes. *The Journal of nutrition* **143**, 1474-1480.
39. Menezes R, Rodriguez-Mateos A, Kaltsatou A *et al.* (2017) Impact of flavonols on cardiometabolic biomarkers: A meta-analysis of randomized controlled human trials to explore the role of inter-individual variability. *Nutrients* **9**, 117.

40. Sabogal-Guáqueta AM, Munoz-Manco JI, Ramírez-Pineda JR *et al.* (2015) The flavonoid quercetin ameliorates Alzheimer's disease pathology and protects cognitive and emotional function in aged triple transgenic Alzheimer's disease model mice. *Neuropharmacology* **93**, 134-145.
41. Wang D-M, Li S-Q, Wu W-L *et al.* (2014) Effects of long-term treatment with quercetin on cognition and mitochondrial function in a mouse model of Alzheimer's disease. *Neurochemical research* **39**, 1533-1543.
42. Priprem A, Watanatorn J, Sutthiparinyanont S *et al.* (2008) Anxiety and cognitive effects of quercetin liposomes in rats. *Nanomedicine: Nanotechnology, Biology and Medicine* **4**, 70-78.

TABLES

Table 1. Baseline characteristics of study participants

Characteristic	Mean ± SD
Age (years)	64.1 ± 6.3
Weight (kg)	77.6 ± 14.9
BMI (kg/m^2)	27.0 ± 3.7
Systolic BP (mmHg)	128.6 ± 15.3
Diastolic BP (mmHg)	73.6 ± 8.4
Total cholesterol (mM)	5.6 ± 1.0
Triglyceride (mM)	1.0 ± 0.3
Low- density lipoprotein cholesterol (mM)	3.7 ± 0.8
High-density lipoprotein cholesterol (mM)	1.5 ± 0.4
Fasting plasma glucose (mM)	5.2 ± 0.5
Creatinine (mM)	67.9 ± 10.3
eGFR (mL/min/1.73 m^2)	86 ± 7.2

n=25; males=12, females=13
BP, blood pressure.

Table 2. Pulse wave analysis

	EMIQ®	Control	p-value
Systolic BP (mmHg)	129.3 ± 3.2	130.9 ± 3.2	0.489
Diastolic BP (mmHg)	73.8 ± 1.8	74.1 ± 1.8	0.702
Central DP (mmHg)	74.3 ± 1.8	75.0 ± 1.8	0.518
MAP (mmHg)	88.9 ± 2.1	89.8 ± 2.1	0.455
HR (bpm)	55.9 ± 1.7	57.3 ± 1.7	0.281
AIx75 (%)	13.9 ± 2.0	13.2 ± 2.0	0.635

Results are presented as means ± sd (n=25). P-values for treatment effect were obtained using linear mixed models with adjustment for treatment order. BP, blood pressure; MAP, mean arterial pressure; PR, pulse rate.

Table 3. Cognitive function measurements

	EMIQ®	Control	p-value

Serial 3 subtractions total	25.1 ± 9.0	25.2 ± 9.7	0.710
Serial 3 subtractions correct	27.0 ± 8.7	26.8 ± 9.4	0.701
Serial 7 subtractions total	19.1 ± 7.1	18.6 ± 7.9	0.569
Serial 7 subtractions correct	16.3 ± 7.7	16.4 ± 7.7	0.595
RVIP correct	54.1 ± 22.5	53.5 ± 22.4	0.299
RVIP response time (msec)	559.8 ± 57.8	551.8 ± 56.9	0.210

Cognitive test measurements, 2.5 hours post consumption of EMIQ® or placebo treatment. Results are presented as means ± sd (n=23). P-values for treatment effect were obtained using linear mixed models with adjustment for treatment order and time.

Table 4. Blood pressure during cognitive stress testing

	EMIQ®	Control	p-value
Systolic BP (mmHg)	129.8 ± 18.0	125.2 ± 19.9	0.468
Diastolic BP (mmHg)	72.1 ± 10.1	73.3 ± 14.4	0.510
MAP (mmHg)	94.8 ± 12.4	93.4 ± 15.0	0.466
PR (bpm)	63.3 ± 10.1	65.8 ± 15.6	0.728

Results are presented as means ± sd (n=25). Blood pressure was measured every 2 minutes for 34 minutes (17 measures total). P-values for treatment effect were obtained using linear mixed models with adjustment for treatment order, time and blood pressure during pre-test rest period. BP, blood pressure; MAP, mean arterial pressure; PR, heart rate.

Table 5. Plasma analyses

	EMIQ®	Control	p-value

Glucose (mmol/L)	4.7 ± 0.1	4.7 ± 0.1	0.994
NOx (nM NO_2)	36.1 ± 6.8	36.3 ± 7.1	0.979
NO_3 (μM)	29.9 ± 1.8	30.9 ± 1.8	0.587
NO_2 (μM)	2.5 ± 0.2	2.8 ± 0.2	0.263
F_2-Isoprostanes (pmol/L)	540.7 ± 33.5	517.2 ± 33.5	0.510
Quercetin aglycone (nM)	144.9 ± 12.3	12.6 ± 12.3	<0.001
Isorhamnetin (nM)	245.5 ± 16.5	41.7 ± 16.5	<0.001

Measurements of plasma glucose, NOx, nitrate (NO_3), nitrite (NO_2), F_2-isoprostanes, and quercetin metabolites, 3 hours post consumption of EMIQ® or placebo treatment. Results are presented as means ± sd (n=25). P-values for treatment effect were obtained using linear mixed models with adjustment for treatment order.

FIGURES

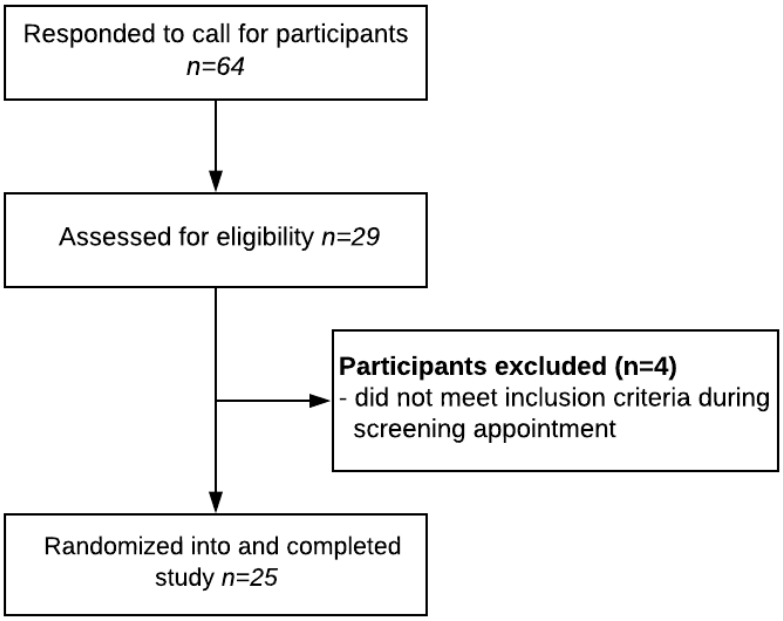

Figure 1. Participant flow diagram.

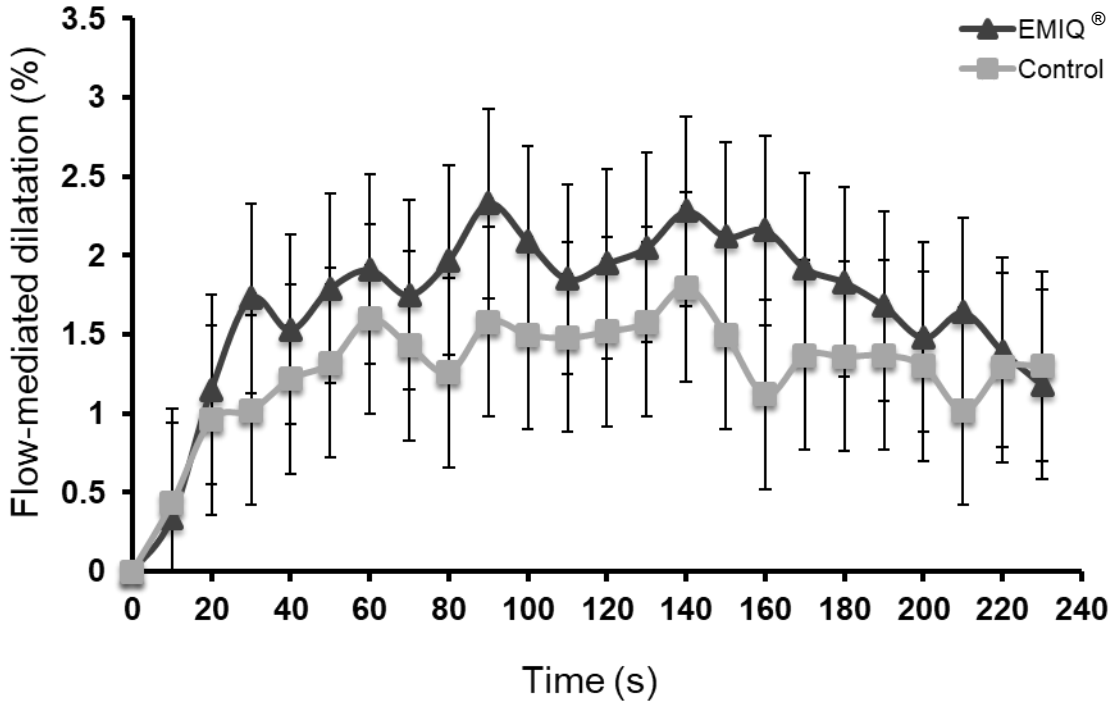

Figure 2. Acute changes in flow-mediated dilatation (FMD) over 240 s measured 1.5 h post-intervention. Results are presented as means (n=24). P-values for treatment effect were obtained using linear mixed models with adjustment for treatment order, time, and baseline (pre-treatment) FMD. Over the time course there was a significant difference between interventions (p=0.040). EMIQ®, Enzymatically-Modified Isoquercitrin.

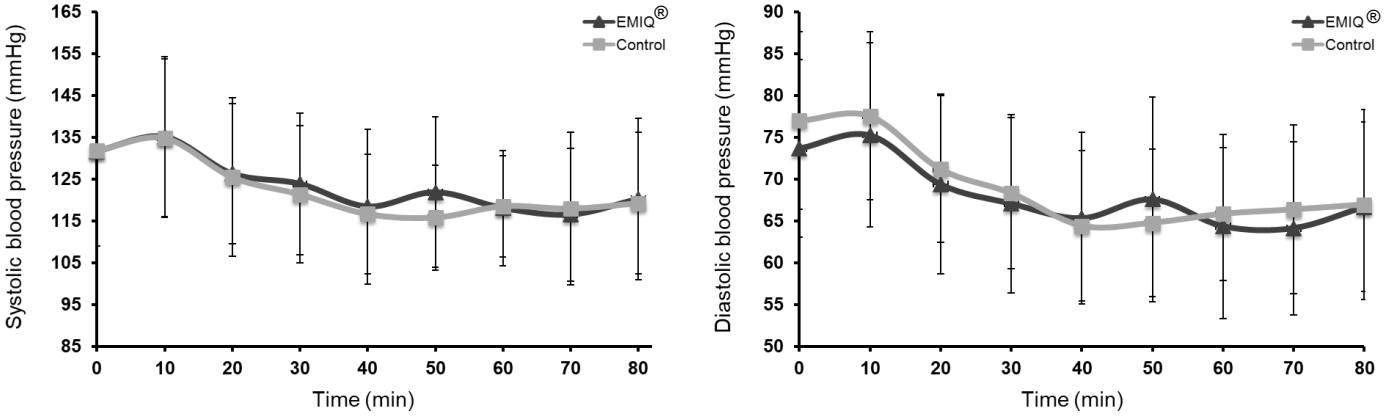

Figure 3. Acute changes in systolic and diastolic blood pressure over 80 minutes immediately post-intervention. Treatment was given immediately after the first measurement at time=0. Results are presented as means ± sd error bars (n=25). P-values for treatment effect were obtained using linear mixed models with adjustment for treatment order and time. Over the time course there was no significant difference between interventions (systolic, p=0.916; diastolic, p=0.073). EMIQ®, Enzymatically Modified Isoquercitrin.