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Comparative effects of enzogenol® and vitamin C supplementation versus vitamin C alone on endothelial function and biochemical markers of oxidative stress and inflammation in chronic smokers

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Abstract
Chronic smoking is associated with endothelial dysfunction and inflammation, with oxidative stress contributing to both these processes. In this study, we investigated the effect of combined antioxidant treatment with Enzogenol®, a flavonoid extract from the bark of *Pinus radiata* and vitamin C, over and above vitamin C alone, on endothelial function, plasma markers of inflammation and oxidative stress, blood pressure (BP) and anthropometrics. Forty-four chronic smokers without established cardiovascular disease were assigned randomly to receive either 480 mg Enzogenol® and 60 mg vitamin C, or 60 mg vitamin C alone daily for 12 weeks. Endothelial function in the brachial artery was assessed by flow-mediated vasodilation (FMD). FMD improved in both treatment groups ($p < 0.001$), with no significant difference between the two groups ($p = 0.84$). In the group receiving Enzogenol® and vitamin C, protein carbonyl levels were significantly reduced compared to the group taking vitamin C alone ($p = 0.03$). Enzogenol® and vitamin C resulted in a significant reduction in fibrinogen levels in heavy smokers compared with vitamin C alone ($p < 0.009$). These findings demonstrated that co-supplementation with Enzogenol® and vitamin C in smokers conferred no additional beneficial effect on macrovascular endothelial function over and above that seen in the vitamin C alone group. However, Enzogenol® did demonstrate additional favourable effects on protein oxidative damage and fibrinogen levels.

Keywords: *Flavonoids, antioxidants, pine bark, vitamin C, oxidative stress, endothelial function*

Abbreviations: *BP, blood pressure; FMD, flow-mediated dilation; CVD, cardiovascular disease; NO, nitric oxide; EID, endothelium-independent dilation; BMI, body mass index; hsCRP, high sensitivity C-reactive protein*

Introduction

Chronic cigarette smoking is a major risk factor for cardiovascular disease (CVD) and is associated with endothelial dysfunction in both coronary [1,2] and peripheral conductance [3,4] and resistance vessels [5]. These changes in endothelial function are an early step in the development of vascular disease [6], with nitric oxide (NO), a potent endothelial-derived vasodilator, having a pivotal role in the maintenance of vascular tone and inhibition of atherosclerotic and thrombotic processes [7]. It is well established that cigarette smoke contains large quantities of free radical and pro-oxidant compounds and many of the adverse effects of smoking appear to be the consequence of increased oxidative stress [8]. Although, the precise mechanisms for smoking-induced endothelial dysfunction have yet to

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be established, it is likely they are mediated, in part, by increased levels of oxygen-derived free radicals either impairing NO activity [1] or directly damaging the endothelium [9]. The higher levels of both oxidative damage [10] and inflammatory markers [11] found in smokers provides evidence for such an effect. Endothelial dysfunction is potentially reversible in smokers, with cessation of smoking shown to result in improved endothelial function [3].

Given that increased oxidative stress contributes to endothelial dysfunction, it would be anticipated that antioxidant therapies might improve vascular function. Flavonoids are a large group of naturally occurring polyphenols found in a wide range of food and drinks that have proven *in vitro* free radical scavenging potential [12]. Epidemiological studies have also shown that high flavonoid intake is associated with a significantly reduced incidence of coronary artery disease [13]. Enzogenol® is a commercially available water-soluble flavonoid extract derived from the bark of *Pinus radiata*. The extract, marketed in capsulated form, is composed of monomeric and oligomeric proanthocyandins, flavonoids, flavonoid glycosides, esters, natural organic acids, and has been shown to have *in vitro* antioxidant action [14]. The commercial product is supplemented with vitamin C, a potent antioxidant both *in vitro* and *in vivo* [15]. The majority of studies on the vascular effects of vitamin C have shown that both oral supplementation and intra-arterial infusions of the compound have beneficial effects [2,4,5,16,17]. Interestingly, a small number of studies have been unable to demonstrate any favourable modification following administration of vitamin C [18,19].

In addition to proven *in vitro* antioxidant activity [14,20], we found, in a 12-week open label pilot study in healthy, elderly subjects, that dietary supplementation with Enzogenol® (480 mg/day) and vitamin C (240 mg/day) was potentially associated with a significant improvement in basal and hyperaemic forearm blood flow in resistance vessels, and small but significant reductions in mean body weight, percentage body fat, systolic blood pressure, plasma viscosity, protein carbonyl levels and DNA damage [21,22].

The objective of the present study was to assess whether Enzogenol® improved vascular function and a range of clinical and biochemical parameters on a background of low dose vitamin C in chronic smokers, a group known to have increased levels of markers of oxidative stress [23]. This study is an extension of our earlier work with the aim of establishing whether the changes observed in clinical and biochemical parameters were a consequence of vitamin C or combined Enzogenol® and vitamin C supplementation. Participants were administered vitamin C at 60 mg/day in the present study, as this falls within the range of the recommended daily intake for vitamin C [24,25]. The parameters measured in the study included endothelial function assessed by flow-mediated vasodilation (FMD) of the brachial artery, blood pressure, body weight, percentage body fat and plasma markers of oxidative damage and inflammation. The study also incorporated measurements of standard biochemical and haematological safety parameters.

Materials and methods

Study design and participants

The study was a double blind, comparative design in which participants received a 12 week course of either 480 mg of Enzogenol® and 60 mg of vitamin C or 480 mg of a lactose filler and 60 mg of vitamin C. The protocol was approved by the Canterbury Ethics Committee with informed, written consent being obtained from the participants prior to the study.

The number of subjects required to provide the study with sufficient statistical power was based on a report that showed flavonoids from purple grape juice increased FMD by at least 3% compared to placebo, with a standard deviation of 3% [26]. Assuming that a 3% differential effect on FMD represented the minimum clinically significant effect, we calculated 42 subjects were required to provide our study with 90% statistical power at a two tailed probability level <0.05. Allowing for a dropout rate of 10%, we enrolled 51 chronic smokers, aged 40–70 years, using either newspaper advertisements or notices displayed at Christchurch Hospital. Subjects were included in the study if they had a smoking history of 1 or more pack-years, with a pack-year defined as smoking 20 cigarettes per day for 1 year or the equivalent amount of pipe tobacco. Subjects were excluded from the study if they had either diabetes mellitus according to the diagnostic criteria defined by the WHO 1999, a sitting blood pressure greater than 140/95 mmHg, another significant clinical disease entity, or were taking hormone replacement therapy, flavonoid or antioxidant preparations or any medications that may have influenced endothelial function.

Participants attended a screening visit to check eligibility and collection of a brief medical history that included age, ethnicity, past and current medical disorders and smoking history. Participants were asked not to undergo any lifestyle changes or alter their medications for the duration of the study.

Clinical investigations

Following the collection of baseline data, participants were stratified according to the number of pack-years smoked and then allocated randomly to the two treatment groups. The capsules contained either 120 mg of Enzogenol® and 15 mg of vitamin C or 15 mg of vitamin C and 120 mg of a lactose filler. The

Enzogenol® extract had been prepared by filtration and membrane separation techniques (USA patent number 5,968,517) and had a proanthocyandidin content between 83 and 90% determined by gel permeation chromatography. Participants were requested to take 2 capsules prior to breakfast and 2 before their evening meal, providing a dosage of 480 mg of Enzogenol® and 60 mg of vitamin C or 60 mg of vitamin C per day. This was the same dosage of Enzogenol® used in our pilot study in healthy elderly subjects [21]. Data were collected at baseline, and after 6 and 12 weeks of supplementation, with compliance being checked at 6 and 12 weeks by counting the number of returned capsules. Participants were required to fast for at least 8 h prior to all the study visits and abstain from any medications and smoking on the morning of the visits.

Vascular studies

Endothelial-dependent FMD of the brachial conduit artery was measured by ultrasound after 5 min of forearm ischaemia. After this measurement, the subject was administered 800 μg of sublingual glyceryl trinitrate, followed 4 min later by assessment of endothelial-independent vasodilation (EID). Both these procedures were performed using a fully digitised ultrasound system (Logiq 700 Expert Series, GE Medical Systems) with a high-resolution, broad-spectrum transducer (6–13 MHz, LA39, GE Medical Systems). The protocol was based on that described by Celermajer et al. [27], and adhered to the guidelines of the International Brachial Reactivity Task Force [28]. Briefly, the participant stayed in a recumbent position for 15 min prior to commencement of the measurements. The non-dominant arm was then placed in a specially designed cradle and the ultrasound transducer fixed in position with a stereotactic clamp. The brachial artery was imaged in the longitudinal plane and examinations made only when well defined, double-line patterns were observed proximally and distally throughout the entire region of measurement. Image quality was given priority, with anatomical landmarks and direct measurements being used to minimise positional variations between study visits. Scans were recorded on super VHS tapes and digitised (Pinnacle DV500 Plus, Pinnacle Systems®). All recordings were by the same investigator and the frame analyses were completed prior to unblinding of the study medication allocation. Diastolic frames were identified by gating simultaneously recorded Doppler curves and then measured frame-by-frame using specially developed edge-detection software. Vessel diameter was defined as the distance between the distal and proximal luminal–intimal interface. FMD and EID were expressed as the percentage increase in vessel diameter from baseline (i.e. FMD ((maximal diameter post-ischaemia − baseline diameter)/baseline diameter) × 100, EID ((mean post sublingual glyceryl trinitrate diameter − baseline diameter)/baseline diameter) × 100). The coefficient of variation (CV) was 3% for measurement of baseline diameters, 22% for FMD and 11% for EID.

Systolic and diastolic blood pressure and heart rate were measured in triplicate using an automated sphygmomanometer (Omron 705CP, Matsusaka Co., Ltd., Japan). These recordings were taken at 2 min intervals, 5 min after the patient had rested in the supine position, with the mean of the second and third recordings being used in the data analysis.

Anthropometric measurements

Height, weight and waist circumference were measured and body mass index (BMI) calculated. The percentage body fat was determined by a bioimpedance method using a body fat analyser (Tanita Corp., Tokyo, Japan).

Laboratory investigations

An ELISA assay adapted from the method of Buss et al. [29] was used to determine protein carbonyl concentration. This assay had a CV of less than 10% (Zentec, Dunedin, New Zealand). Plasma oxidised LDL levels were determined using an ELISA assay (Mercodia AB, Sweden) with a CV of $< 7.5\%$. Plasma inflammatory markers measured included plasma fibrinogen concentration analysed by rocket immuno-electrophoresis, high sensitivity C-reactive protein (hsCRP) concentration determined by rate nephelometry, and plasma viscosity measured in duplicate at 25°C in a capillary viscometer (Coulter Electronics, Luton, UK). Plasma concentrations of glucose, creatinine, albumin, bilirubin, alkaline phosphatase (ALP), aspartate amino transferase (AST), alanine amino transferase (ALT) and glutamyl transferase (GGT), total cholesterol, triglyceride and HDL cholesterol were determined using an Aeroset analyser Model LN (Abbott Laboratories, IL, USA). LDL cholesterol was calculated by the Friedewald equation. Haematological indices were measured using an automatic cell counter and analyser (Coulter Electronics).

Statistical analysis

Statistical analysis was performed using SPSS Base version 10.0 (SPSS, Inc., Chicago, IL). Comparisons of changes in the variables from baseline between the two treatment groups were tested using ANOVA with repeated measures. Smoking history was used as a between subject factor in all the analyses. Correlations between selected variables were examined using Pearson's correlation coefficient. Variables that were

not normally distributed were $\log_e$ transformed prior to analysis and expressed as geometric means. Statistical significance was inferred when $p < 0.05$.

Results

Baseline characteristics and compliance

Of the 51 subjects recruited, 7 were withdrawn from the study, 4 for non-compliance, 1 had a prolapsed disc, 1 had a displaced fracture, and 1 due to psychiatric illness. Data from the remaining 44 subjects were included in the analysis, with 22 subjects in each treatment group. There were no differences in clinical characteristics between the two groups at baseline as shown in Tables I and II. Mean pack years smoked at baseline in the Enzogenol® and vitamin C group was 34.2 ± 4.2 compared to 33.0 ± 3.1 in the vitamin C only group ($p = 0.82$). The mean age of both groups was 49 ± 2 years. Mean compliance with treatment was 88% and was similar in the two treatment groups.

Participants were stratified according to smoking history as either <33 pack-years (lighter smoking history, $n = 24$) or >33 pack-years (heavier smoking history, $n = 20$). Mean pack years smoked was significantly greater in those with a heavier smoking history compared to those with a lighter smoking history (48 ± 3 (34–80) versus 22 ± 2 (8–32) pack-years respectively, $p < 0.001$). In subjects with a lighter smoking history, mean pack years smoked in the vitamin C alone group was 24 ± 2 (11–32) pack-years compared to 19 ± 3 (8–30) pack-years ($p = 0.10$) in the Enzogenol® and vitamin C group. In subjects with a heavier smoking history, mean pack years smoked was 46 ± 5 (34–75) pack-years in the vitamin C alone group versus 49 ± 5 (34–80) pack-years in the Enzogenol® and vitamin C group ($p = 0.61$).

As anticipated there was a significant difference in mean age based on smoking history; 46 ± 1 (40–57) years in the lighter smoking group compared to 54 ± 2 (42–67) years in heavier smoking group ($p < 0.001$), but within each group the mean age was comparable between the two treatment regimes. Mean age of subjects with a lighter smoking history was 46 ± 2 (40–57) years in the vitamin C group versus 46 ± 2 (40–56) years in the Enzogenol® plus vitamin C group ($p = 0.92$). In subjects with a heavier smoking history the mean age was 55 ± 3 (46–67) years in the vitamin C group compared to 53 ± 5 (42–61) years in the Enzogenol® and vitamin C group ($p = 0.43$).

Vascular and clinical investigations

The data on endothelial function is summarised in Table I and shows there were no differences in ultrasound parameters between the two treatment groups at baseline. Endothelial-dependent FMD of the brachial artery improved markedly after 6 ($p = 0.009$) and 12 ($p = 0.001$) weeks of supplementation in both the Enzogenol® and vitamin C group and vitamin C alone group. Figure 1 shows the

Table I. Changes in vascular parameters and anthropometry and plasma markers of inflammation and oxidative stress during the study

	Vitamin C (n=22)			Enzogenol® and Vitamin C (n=22)		
	Baseline	6 weeks	12 weeks	Baseline	6 weeks	12 weeks
Endothelial Function						
Vessel size (mm)	2.87 ± 0.13	2.86 ± 0.12	2.89 ± 0.14	2.96 ± 0.12	2.85 ± 0.14	2.88 ± 0.13
FMD (%)	5.88 ± 1.57	7.12 ± 1.59*	7.37 ± 1.84**	6.65 ± 1.60	7.60 ± 1.63*	8.40 ± 1.88**
EID (%)	30.87 ± 4.91	29.91 ± 4.60	32.63 ± 5.15	28.90 ± 4.06	31.61 ± 3.80	30.70 ± 4.26
Blood pressure						
Systolic (mmHg)	120 ± 3	121 ± 3	123 ± 3	119 ± 3	122 ± 3	117 ± 3
Diastolic (mmHg)	76 ± 2	74 ± 2	77 ± 2	74 ± 2	77 ± 2	74 ± 2
MAP (mmHg)	91 ± 2	90 ± 2	93 ± 2	89 ± 2	92 ± 2	89 ± 2
Heart rate (beats/min)	67 ± 2	63 ± 2	69 ± 2	64 ± 2	67 ± 2	64 ± 2
Anthropometrics						
Weight (kg)	79.4 ± 3.5	77.4 ± 3.9	79.4 ± 3.3	77.7 ± 3.9	79.6 ± 3.4	78.2 ± 4.0
BMI (kg/m^2)	27.0 ± 0.9	25.9 ± 1.0	27.0 ± 0.8	26.0 ± 1.1	27.1 ± 0.9	26.2 ± 1.1
Waist (cm)	92.0 ± 3.1	86.9 ± 2.6	92.2 ± 3.1	87.1 ± 2.6	92.0 ± 3.1	87.9 ± 2.7
Body fat (%)	34.5 ± 2.8	30.0 ± 3.7	34.9 ± 2.7	30.7 ± 3.7	34.7 ± 2.7	31.0 ± 3.6
Oxidative Stress Marker						
Protein carbonyls (pmol/mg)	53 ± 12	52 ± 10	54 ± 12	65 ± 14	54 ± 13	43 ± 17*+
Oxidised LDL in ApoB (U/l)	76.3 ± 5.8	74.4 ± 5.4	71.7 ± 5.2	72.5 ± 5.8	72.0 ± 5.4	73.1 ± 5.2
Inflammatory Markers						
Plasma fibrinogen (g/l))	3.3 ± 0.1	3.2 ± 0.1	3.1 ± 0.1	3.6 ± 0.1	3.3 ± 0.1	3.3 ± 0.1
hs CRP (mg/l)	1.41 ± 0.23	1.65 ± 0.24	1.69 ± 0.25	1.42 ± 0.23	1.44 ± 0.24	1.31 ± 0.25
Plasma viscosity (mPa.s)	1.62 ± 0.02	1.64 ± 0.02	1.62 ± 0.02	1.62 ± 0.02	1.61 ± 0.02	1.61 ± 0.02

* $P = 0.009$, ** $P = 0.001$ for comparison from baseline within treatment groups. $^{+}P = 0.03$ for comparison of changes from baseline between treatment groups. Data expressed as the mean ± SEM.

Table II. Changes in biochemical and haematological safety parameters during the study

	Vitamin C (n=22)		Enzogenol® and Vitamin C (n=22)	
	Baseline	12 weeks	Baseline	12 weeks
Glycaemic control				
Plasma glucose (mM/l)	5.0 ± 0.1	5.0 ± 0.1	4.8 ± 0.1	5.1 ± 0.1
Renal function				
Plasma creatinine (mM/l)	0.08 ± 0.003	0.08 ± 0.003	0.08 ± 0.002	0.08 ± 0.003
Liver function				
Total protein (g/l)	72.1 ± 0.8	71.9 ± 0.6	71.1 ± 1.0	71.6 ± 0.9
Plasma albumin (mM/l)	43.2 ± 0.5	42.6 ± 0.4*	43.2 ± 0.5	42.7 ± 0.4*
Plasma bilirubin (mM/l)	8.1 ± 0.8	7.8 ± 0.7	7.4 ± 0.5	7.3 ± 0.6
Plasma ALP (mM/l)	76.0 ± 4.5	78.7 ± 3.9	73.2 ± 5.6	73.5 ± 3.0
Plasma AST (mM/l)	20.8 ± 1.6	20.9 ± 3.9	18.5 ± 1.0	18.0 ± 0.9
Plasma ALT (mM/l)	21.6 ± 2.0	21.1 ± 1.9	19.0 ± 3.0	18.7 ± 1.4
Plasma GGT (mM/l)	34.7 ± 5.0	34.7 ± 5.0	26.0 ± 5.7	26.1 ± 4.9
Lipids				
Plasma cholesterol (mM/l)	5.5 ± 0.2	5.9 ± 0.2*	5.4 ± 0.2	6.0 ± 0.2*
Plasma HDL-cholesterol (mM/l)	1.36 ± 0.09	1.38 ± 0.09	1.35 ± 0.07	1.35 ± 0.09
Plasma LDL-cholesterol (mM/l)	3.5 ± 0.2	3.8 ± 0.2*	3.4 ± 0.2	4.0 ± 0.2*+
Cholesterol:HDL-cholesterol	4.3 ± 0.3	4.5 ± 0.3	4.0 ± 0.2	4.6 ± 0.3
Plasma triglyceride (mM/l)	1.5 ± 0.2	1.6 ± 0.2*	1.4 ± 0.2	1.6 ± 0.2*
Haematology				
Haemaglobin (g/l)	143 ± 3	141 ± 3*	145 ± 3	143 ± 3*
Haematocrit	0.42 ± 0.007	0.41 ± 0.007*	0.43 ± 0.008	0.42 ± 0.008*
RBC count ($\times 10^{12}$/l)	4.56 ± 0.08	4.46 ± 0.09*	4.63 ± 0.08	4.56 ± 0.09*
RBC mean cell volume (fL)	92.3 ± 1.0	92.3 ± 1.0	92.1 ± 1.0	92.4 ± 1.0
WBC count ($\times 10^{9}$/l)	7.6 ± 0.4	7.4 ± 0.3	7.4 ± 0.5	7.4 ± 0.4
Platelet count ($\times 10^{9}$/l)	258 ± 12	265 ± 14*	271 ± 12	273 ± 13*
Platelet mean cell volume (fL)	8.4 ± 0.2	8.3 ± 0.2*	8.1 ± 0.2	8.0 ± 0.2*

*$P < 0.05$ for comparison from baseline within treatment groups. $^{+}P < 0.05$ for comparison from baseline between treatment groups. Data expressed as the mean ± SEM.

improvement in FMD from baseline in the two treatment groups after 12 weeks of supplementation (Mean change = 1.8 ± 0.73% in the Enzogenol® and vitamin C group versus 1.5 ± 0.53% in the vitamin C group). The improvement in FMD in smokers receiving Enzogenol® and vitamin C was however, not significantly greater from that in smokers supplemented with vitamin C alone ($p = 0.84$). Smoking history did not influence the effect of treatment on FMD ($p = 0.25$). There was no significant difference in EID in either treatment group during the study ($p = 0.36$). Baseline and vessel diameters and reactive hyperaemic blood flow remained unchanged in either treatment group (data not shown). Other factors that can influence endothelial function such as smoking quantity, alcohol intake and physical activity did not alter throughout the study in either treatment group (data not shown).

The results of the blood pressure and anthropometric measurements are summarized in Table I. Systolic and diastolic blood pressure, heart rate and anthropometry remained unchanged throughout the study in both treatment groups.

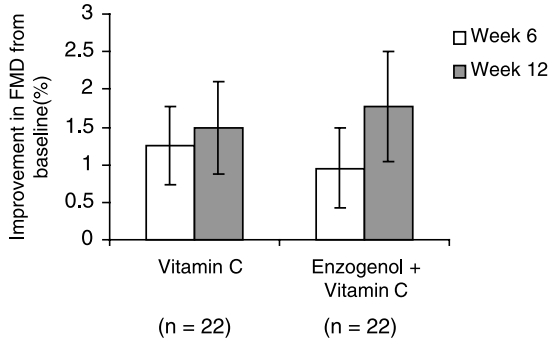

Figure 1. Improvement in baseline FMD of the brachial artery after 6 and 12 weeks of supplementation with Enzogenol® and vitamin C or vitamin C alone. $P = 0.009$ versus baseline at 6 weeks and $P = 0.001$ at 12 weeks for both treatment groups. Data are expressed as means ± SEM.

Oxidative stress markers

Table I shows the effects of the two supplement regimes on plasma levels of protein carbonyls, a marker of protein oxidative damage. At baseline there were no differences between treatment groups, whereas after 12 weeks of treatment, there was a significant reduction in protein carbonyl concentrations in subjects supplemented with Enzogenol® and vitamin C compared to vitamin C alone ($p = 0.03$). Figure 2 shows the percentage reduction in protein carbonyl levels from baseline after 6 and 12 weeks of treatment. Smoking history did not influence the effect of treatment on protein carbonyl levels

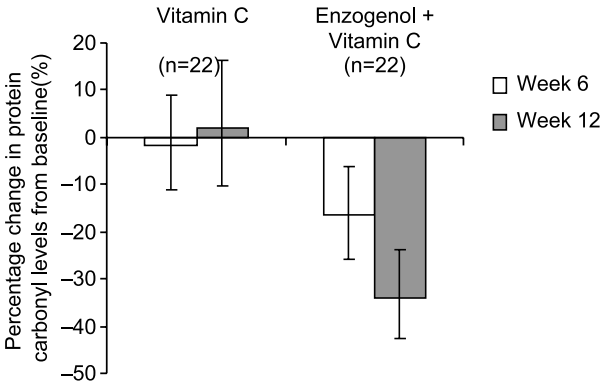

Figure 2. Percentage reduction in protein carbonyl concentration from baseline after 6 and 12 weeks of supplementation Enzogenol® + vitamin C or with vitamin C alone. $P = 0.03$ versus changes at 12 weeks from baseline between treatment groups. Data are expressed as means ± SEM.

($p = 0.85$). While our study showed a significant reduction in protein oxidative damage in the form of carbonyl groups in serum proteins, we were unable to detect any changes in levels of oxidised low-density lipoproteins in either treatment group.

Inflammatory markers

Changes in plasma inflammatory markers are summarized in Table I. During the study a small, insignificant reduction in mean plasma fibrinogen levels occurred in both treatment groups (0.1 ± 0.09 g/l in the Enzogenol® and vitamin C group versus 0.3 ± 0.09 g/l in the vitamin C group, $p = 0.06$). When the subjects were stratified according to smoking history, mean baseline fibrinogen levels for subjects with a lighter smoking history were 3.3 ± 0.08 g/l compared to 3.6 ± g/l in subjects with a heavier smoking history ($p = 0.11$). The mean reduction in fibrinogen levels in the heavier smokers was 0.32 versus 0.13 g/l in the lighter smokers ($p = 0.01$). Supplementation with both Enzogenol® and vitamin C resulted in a significantly greater reduction in plasma fibrinogen levels in subjects with a heavier smoking history (>33 pack-years) (mean reduction = 0.56 ± 0.28 g/l). In contrast, vitamin C alone was associated with a markedly smaller reduction in fibrinogen levels (0.04 ± 0.16 g/l), $p = 0.009$ in this subgroup of subjects (Figure 3). This treatment effect was however not observed in subjects with a lighter smoking history (<33 pack-years) ($p = 0.72$). Plasma viscosity and hsCRP levels remained unchanged in both treatment groups irrespective of smoking history and mean age.

Correlation analyses

Analysis of the baseline data showed no relationship between smoking history and either endothelial

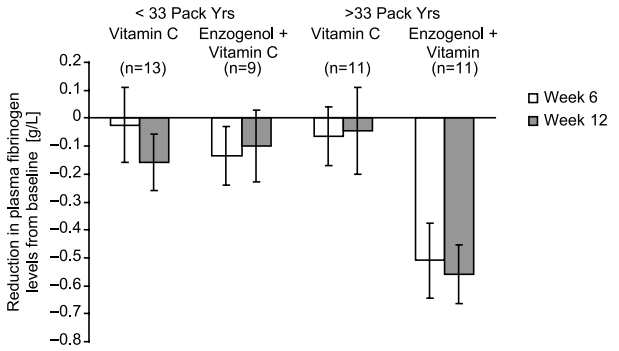

Figure 3. Reduction in plasma fibrinogen level from baseline after 6 and 12 weeks of supplementation with Enzogenol® and vitamin C or vitamin C alone stratified according to smoking history. After 12 weeks there was a significant decrease in fibrinogen levels in the Enzogenol® and vitamin C treatment group in comparison to vitamin C alone in subjects with a heavier smoking history (>33 pack years) ($p = 0.009$). There was no treatment effect in subjects with a history of lighter smoking (<33 pack-years) ($p = 0.72$). Data are expressed as means ± SEM.

function, plasma markers of oxidative damage or inflammation, blood pressure or anthropometric indices. There were positive correlations in both treatment groups between the changes in plasma fibrinogen and hsCRP ($r = 0.80$, $p < 0.001$), hsCRP and plasma viscosity ($r = 0.57$, $p = 0.03$), fibrinogen and plasma viscosity ($r = 0.57$, $p = 0.04$), and systolic blood pressure and plasma viscosity ($r = 0.58$, $p = 0.03$). No significant associations were present between changes in FMD, and plasma protein carbonyls or fibrinogen levels.

Biochemical and haematological safety parameters

Table II summarizes the biochemical and haematological safety parameters. These parameters were comparable between the treatment groups at baseline and neither supplement was associated with any changes in glucose, renal and liver function, white count or electronic differential during the study. Minor changes were noted in plasma albumin and some haematological indices over the course of the study, but none of these changes were of a magnitude to be clinically significant. Increases in total cholesterol, LDL-cholesterol, and triglycerides were observed in both treatment groups over the 12 weeks, although the increases were not significantly different between the two groups, with the exception of LDL-cholesterol.

Discussion

In this study, we found a significant improvement in endothelium-dependent vasodilation of the brachial artery in chronic smokers with no established CVD following 12-weeks supplementation with either Enzogenol® and vitamin C, or vitamin C alone. We were, however, unable to distinguish a significant

improvement in the Enzogenol® and vitamin C group on endothelium-dependent vasodilation over and above that seen in the vitamin C alone group. Neither supplement had an effect on nitroglycerin-induced endothelium independent vasodilation, indicating that the response of the vascular smooth muscle to NO was unaltered by either antioxidant treatment. Taken together these observations imply that the increase in vasodilation was related to changes in endothelial activity.

It is possible the beneficial vasodilatory effects on the endothelium may have been attributable to vitamin C supplementation. However, without a placebo arm in the study it remains unclear whether the improvement in vasodilation was related to time effects over the 12 weeks or a treatment effect of vitamin C. To date, studies measuring the effect of oral or intravenous vitamin C on endothelial function have provided contradictory findings. The majority of investigations in smokers using vitamin C have shown beneficial effects on micro- and macro-vascular function [2,4,5,16,17], however, some have demonstrated no effect [18,19]. In our previous study, we found a significant improvement in endothelial function of the forearm resistance vessels following Enzogenol® and vitamin C supplementation in healthy elderly subjects, implying favourable effects on microvascular function [21]. This finding of improved microvascular function after supplementation of Enzogenol® and vitamin C in healthy elderly subjects lead us to investigate whether this supplementation regime also had similar effects in smokers, a group known to have increased levels of oxidative damage. We used a relatively low dose of vitamin C (60 mg/day) in the study as this was within the RDI range for vitamin C [24,25]. This was a considerably lower dose than that reported in other studies in both smokers and non-smokers, in which the oral dosage ranged from 500 to 2000 mg/day [30]. Despite the low dose vitamin C used in our study, we were able to show vitamin C had a potential beneficial effect on endothelial function. It has been suggested that vitamin C probably acts by preserving tetrahydrobiopterin, with subsequent improvement in endothelial NO synthase activity, thereby increasing NO bioactivity [31]. Interestingly, a recent, 6-week, placebo-controlled study in hypertensive patients showed no changes in endothelium-dependent vasodilation following combined treatment with grapeseed polyphenols and 500 mg vitamin C or with either treatment alone [32].

With regards to flavonoid supplementation, several studies have shown beneficial effects in both normal controls [33], and patients with CVD [26], while the one other study in smokers of which we are aware demonstrated no effect [34]. The present study was unable to distinguish any significant contribution of Enzogenol® to the improvement observed in endothelium dependent vasodilatation. As a consequence it remains unknown whether Enzogenol® has a beneficial effect on endothelial function if administered without vitamin C. In order to determine the vasodilatory potential of Enzogenol® a placebo-controlled study would be required in which the placebo would not contain any antioxidant compounds. Nonetheless, the primary objective of this study was to determine whether the commercial product, Enzogenol® and vitamin C conferred any additional benefit over and above that of vitamin C on macrovascular function. Another limitation of our investigation was the lack of measurement of vitamin C and polyphenol levels during the study.

While neither combined supplementation with Enzogenol® and vitamin C or vitamin C alone was associated with significant changes in mean systolic or diastolic blood pressure or heart rate, we observed a trend of decreasing systolic blood pressure levels in the group receiving Enzogenol® and vitamin C. This finding was consistent with our pilot study in which we measured a significant reduction in mean systolic blood pressure, but no change in diastolic blood pressure or heart rate [21]. There is some evidence from animal models that flavonoids have an antihypertensive effect, [35,36] with one study demonstrating that short-term administration of red wine flavonoids lowered blood pressure in normotensive rats [35], while other investigations have reported that flavonoids extracted from the plant *Spergularia purpurea* lowered blood pressure levels in both normal and spontaneously hypertensive rats [36]. Clinical studies in humans examining the effect of polyphenols on blood pressure have however, provided conflicting results. Several trials have shown a reduction in blood pressure following treatment with polyphenol-rich dark chocolate compared with polyphenol-free white chocolate in healthy individuals [37,38], whereas other studies have been inconclusive [39]. To our knowledge only one other study has investigated the combined effects of vitamin C and polyphenols on blood pressure [32]. This placebo-controlled trial demonstrated a clinically significant increase in systolic blood pressure in treated hypertensive patients following co-supplementation with grapeseed polyphenols and vitamin C [32]. Treatment with vitamin C alone was associated with a small but significant reduction in systolic blood but the reduction with polyphenols alone was not significant [32]. These findings were in contrast to the present study, and suggest a potential repressor effect of combined polyphenol and vitamin C treatment on systolic blood pressure. With respect to these findings, and given that our study did not have a placebo arm, further controlled studies are necessary to support the use of such combination treatment regimes, particularly in hypertensive patients. In addition the mechanisms underlying the effects of polyphenols alone or in combination with vitamin C on blood pressure require elucidation.

We also found that Enzogenol® and vitamin C reduced the formation of carbonyl groups on amino acid residues, whereas levels remained unaltered with vitamin C alone. A previous study demonstrated higher protein carbonyl levels in smokers in comparison to non-smokers as a result of free radical initiated reactions [10], while in the present study baseline protein carbonyls were comparable to documented non-smoker levels of 60 pmol/mg [29]. The reduction in protein carbonyls in the Enzogenol® and vitamin C group in this study, is consistent with our pilot study on Enzogenol® [22], and confirms an earlier report that showed grape juice flavonoids decreased protein carbonyl levels by 20% in healthy adults [40]. In addition, black tea flavonoids have been associated with a reduction in protein carbonyl levels in rats [41].

A further interesting observation in our study was that Enzogenol® and vitamin C supplementation reduced fibrinogen levels in subjects with a heavier smoking history (>33 pack-years) in comparison to vitamin C alone. We confirmed the effect of treatment on fibrinogen levels in the heavy smokers was not due to higher mean age in the Enzogenol® and vitamin C supplemented group. The overall reduction in fibrinogen levels in the heavier smokers does reflect a return to the lighter smokers levels, although the reduction is more profound in the Enzogenol® and vitamin C group. This reduction in fibrinogen levels is in contrast to the findings of a previous study in smokers that showed no change in fibrinogen levels following flavonoid administration [34]. As the reference range for fibrinogen levels is 1.5–4 g/l, the smokers in our study had high normal fibrinogen levels comparable to the fibrinogen levels observed in smokers in previous trials [42–44]. Fibrinogen is an important factor in platelet aggregation, haemorheology, and endothelial cell injury, all of which have major roles in thrombogenesis. As heavy smokers are known to have a higher risk of thrombotic events [45], reductions in plasma fibrinogen of the magnitude seen with Enzogenol® and vitamin C may have significant clinical effects. The changes in plasma fibrinogen levels we observed were however, not of sufficient magnitude to cause a significant change in mean plasma viscosity levels and were also not associated with any consistent change in hsCRP levels.

Neither treatment group had changes in anthropometric measurements during the study. This finding is in contrast to our pilot study in which small but significant reductions in weight, body mass index (BMI) and percentage body fat were measured following Enzogenol® and vitamin C supplementation [21]. As the pilot study was an open-label and uncontrolled investigation, it is possible the improved anthropometry was a consequence of lifestyle changes arising from participation in the study.

Our study also provided further evidence that short-term dietary supplementation with combined Enzogenol® and vitamin C or vitamin C alone was not associated with any adverse changes in laboratory markers of renal and liver function, glycaemic control and haematology. The decreases we observed in mean plasma albumin, haemoglobin, and haemocrit and increases in mean cell volume and platelet count, while statistically significant were very small and were not considered to be clinically relevant.

We also found clinically significant increases in total cholesterol, and LDL-cholesterol and statistically significant triglyceride levels during the study in both treatment groups that have the potential to cause significant adverse effects on vasomotor function. These increases in lipoprotein levels were surprising, since they were not seen in our pilot study [21] and have not been reported in other studies on flavonoid or vitamin C supplementation [46]. Although, we cannot offer any explanation for these adverse lipid changes, it is conceivable they may reflect dietary changes in the participants rather than a treatment effect. Nevertheless, such observations warrant further investigation into the effects of flavonoid preparations on plasma lipid profiles.

Conclusion

In conclusion, this study in chronic smokers demonstrated that dietary supplementation in the Enzogenol® and vitamin C group conferred no additional improvement in endothelium dependent vasodilation over and above that observed in the vitamin C group. The improvement in endothelial function may be attributable to vitamin C; consistent with the increasing volume of evidence that vitamin C alone improves endothelial vasodilatory responses. Combined Enzogenol® and vitamin C supplementation was however associated with reductions in protein carbonyl levels and a reduction in fibrinogen levels in subjects with a heavier smoking history. These observations indicate a role for Enzogenol® to decrease oxidative damage of proteins and potentially lower the risk of thrombotic events in heavy smokers.

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