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Pilot Study on the Clinical Effects of Dietary Supplementation With Enzogenol®, A Flavonoid Extract of Pine Bark and Vitamin C

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Flavonoids are naturally occurring plant compounds with established *in vitro* antioxidant properties and potential cardioprotective effects. We carried out a 12-week pilot study on the effects of dietary supplementation with an extract of bioflavonoids prepared from the bark of *Pinus radiata* trees [Enzogenol®] containing added vitamin C. Data was collected from 24 healthy subjects aged between 55–75 years at baseline and at 6 and 12 weeks and included, routine biochemical and haematological indices, and anthropometric, blood pressure, forearm blood flow and haemorheological measurements. Enzogenol® supplementation at a dosage of 480 mg/day of pine bark extract and 240 mg/day vitamin C did not result in changes in any biochemical or haematological indice and was associated with a significant reduction in the means of body weight, percentage body fat, systolic blood pressure and plasma viscosity. Basal and hyperaemic blood flow in forearm resistance vessels measured by plethysmography increased significantly during the study. The findings of this pilot study indicate that dietary supplementation with Enzogenol® is safe and well tolerated and is associated with a number of beneficial effects on a range of established cardiovascular risk factors. These changes need to be validated by a placebo-controlled study but are consistent with other studies that have reported beneficial clinical effects following supplementation with bioflavonoids. Copyright © 2003 John Wiley & Sons, Ltd.

Keywords: Bioflavonoids; pine bark; antioxidants; vitamin C; clinical study; cardiovascular.

INTRODUCTION

Free radical oxidative stress has been implicated in the pathogenesis of a wide range of vascular and metabolic disorders (Baynes and Thorpe, 1999; Lum and Roebuck, 2001). As a consequence, there is increasing interest in compounds with antioxidant properties that may combine with natural defence mechanisms to inhibit free-radical damage and therefore limit disease progression. The flavonoids are a large group of naturally occurring polyphenols found in fruits, vegetables, grains, bark, tea and wine that have proven *in vitro* free-radical scavenging potential (Duthie and Crozier, 2000). While epidemiological studies have provided support for the concept that high dietary intake of flavonoids may have a protective effect against cardiovascular disease (Keli *et al*., 1996; Knekt *et al*., 1996) and cancer (DeWeese *et al*., 2001) the bioavailabilty and clinical efficacy of these compounds provided as nutritional supplements is yet to be fully established (Duthie and Crozier, 2000).

Enzogenol® is a water-soluble extract of monomeric and oligomeric proanthocyanidins, flavonoids, flavonoid glycosides, esters and natural organic acids prepared from the bark of *Pinus radiata.* The extraction process yields approximately 5% by weight of the original bark. The extract has been shown to have an *in vitro* antioxidant action as measured by inhibition of micelle oxidation and red blood cell haemolysis (Gieseg and Baird, 1998) and a nitro blue tetrazolium enzymatic method (Wood *et al*., 2001). Enzogenol® was also observed to have an apparent protective effect against tumour development in a strain of experimental mice (Duncan, 1998). The extract is currently available as a capsulated dietary supplement in several countries including New Zealand, Australia, America, United Kingdom, Germany, Japan and Singapore. This paper reports the results of an open-labelled pilot study on dietary supplementation with Enzogenol® in 26 healthy people, aged between 55–75 years, that investigated the effect of the extract on a number of cardiovascular indices including blood pressure and forearm plethysmography, anthropometric measurements and red blood cell [RBC] and plasma haemorheological properties. In addition, the effect of the extract on a range of biochemical and haematological safety parameters was investigated.

METHODS

The study protocol was approved by the Christchurch Ethics Committee. The study was an open label design of 12 weeks duration during which time participants were provided with Enzogenol® capsules. The capsules used in the study contained 120 mg of a water-soluble extract of pine bark obtained from trees of similar age.

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The extract was prepared by filtration and membrane separation techniques [USA patent number 5,968,517] with specification for proanthocyanidin content of between 83–90% using gel permeation chromatography. Each capsule also contained 60 mg of added vitamin C.

A dosage of 480 mg/day of flavonoid extract was selected for the study on the basis that this represented an approximately equivalent dose of 5 mg/kg body weight found to be associated with increased survival in a strain of experimental mice. [Dr K. Duncan, personal communication]. The dosage was taken as two 120 mg capsules, each containing 60 mg vitamin C, prior to breakfast and the evening meal. Compliance in the study was checked by counting the number of capsules returned at the study visits.

As the study was a pilot investigation, the number of participants required to provide sufficient statistical power was estimated from results of a similar investigation that measured changes in flow-mediated dilatation in subjects with coronary artery disease following ingestion for 14 days of grape juice containing flavonoid compounds (Stein *et al*., 1999). Assuming Enzogenol® induced a vascular response of similar magnitude [mean increase in flow mediated dilatation 4.2 ± 4.4%], it was calculated using GraphPad Instat™ that a total of 25 patients would provide the study with a power of 95% at a significance level of 0.05.

Twenty-six people aged between 55–75 years and free from any significant clinical disease entities were invited to participate in the study by advertising in a local newspaper. The subjects' suitability for the study was checked by a registered nurse who obtained a brief medical history that included age, ethnicity, past and current medical disorders and smoking history. Exclusion criteria included, diabetes mellitus and subjects who were currently smoking or taking any other medications including flavonoid or antioxidant preparations prior to the study.

The following data were collected at baseline and at 6 and 12 weeks at the same time of the day [approximately 9.00am] with the participants being asked to fast for 12 h prior to each of these appointments. Height, weight and waist circumference were recorded and body mass index [BMI] calculated. The percentage body fat was determined by a bioelectrical impedance method using a body fat analyser [Tanita Corp., Tokyo, Japan]. This measurement had a coefficient of variation of 0.8%. Systolic and diastolic blood pressure were then measured in triplicate by the same operator using a sphygmomanometer. These recordings were taken after 5 min rest in the supine position and at 2 min intervals with the mean of the 2nd and 3rd readings being used in the data analysis. Radial pulse rate was also recorded. Venous blood samples were then obtained with minimal stasis followed by the collection of a random urine sample. The urine sample was centrifuged at 1000 g for 10 minutes prior to being analysed. Urine albumin concentration was determined using an immunoenzymometric assay (Townsend, 1986) and the results expressed relative to the urine creatinine concentration measured by the alkaline picrate method. The plasma concentration of the following parameters were measured in an Aeroset analyser Model LN [Abbott Laboratories, Illinois, IL, USA]; glucose, creatinine, bilirubin, alkaline phosphatase, aspartate amino transferase [AST], alanine amino transferase [ALT], γglutamyl transferase [GGT], total cholesterol, HDL-cholesterol, LDL-cholesterol, triglyceride and the cholesterol HDL-cholesterol ratio. Apolipoprotein B concentration was also determined by nephelometry in a Array® 360 system [Beckman Coulter Inc, USA] Haematological indices were measured using an automatic cell counter and analyser [Coulter Electronics, Luton, UK].

The following cardiovascular parameters were investigated on two occasions during the study [i.e. at baseline and at 12 weeks]. Blood flow was measured in the left and right [control] forearm using a venous occlusion plethysmograph [Hokanson Inc., Washington, USA] according to a modification of the method of Sax *et al.* (1987) following the guidelines described by Playford and Watts (1998). After 10 min rest in the supine position, forearm basal blood flow was measured for 3 min. Hyperaemic flow was then measured in the left forearm immediately following deflation of a brachial cuff that had been inflated to supra-systolic levels for 5 min. Basal blood flow was measured simultaneously in the right forearm as a control for the procedure. The average of the flow curve gradients was calculated for baseline blood flow in both forearms independently. The hyperaemic response was defined as the gradient of the first blood flow curve after brachial cuff deflation. The results were expressed as ml/min/dL. We have determined previously that the coefficient of variation for this procedure is <15%. Haemorheological profiles of EDTA anti-coagulated blood samples were obtained and consisted of haematocrit, whole blood viscosity at high shear rate [200 s^{-1}] measured in duplicate at 37 °C in a cone-plate rheometer [Brookfield Engineering, Massachusetts, USA] and plasma viscosity measured in triplicate at 25 °C in a capillary viscometer [Coulter Electronics, Luton, UK] (Harkness, 1971). The blood and plasma viscosity measurements had a coefficient of variation of 6% and 3% respectively. RBC rheology was assessed by calculating an index of RBC deformability, obtained by standardising the high shear rate blood viscosity data to a haematocrit of 0.45 (Matrai *et al*., 1987) and also by measuring RBC osmotic fragility measured in duplicate according to the method of Dacie and Lewis (1995). Plasma protein profiles were also determined and consisted of total protein and albumin concentration [Abbot Aeroset analyser], total globulin concentration measured using a modified Hopkins-Cole reaction (Goldenberg and Drewes, 1971) and plasma fibrinogen concentration analysed by rocket immuno-electrophoresis.

Statistical analysis of the data was carried out using Graphpad Prism™. Data distribution was assessed using the normality test. All indices were found to have a Gaussian distribution. Descriptive statistics included, range, mean, standard deviation and standard error. Changes in the parameters from baseline values were assessed using two-tailed paired *t*-tests. The relationship between selected variables was assessed using Pearson's correlation analysis. The level of significance for all the statistical analyses was set at the 0.05 level.

RESULTS

Two subjects were withdrawn from the study, one person for non-compliance, while the other person

Table 1. Clinical, vascular and haemorheology data. Mean [SD]

Index	Baseline	Week 6	Week 12
Anthropometrics			
BMI [kg/m^2]	26.1 [4.6]	25.8 [4.6] **	25.7 [4.7] ***
Waist circumference [cm]	90.4 [13.9]	88.9 [15.0] *	89.3 [15.1]
Body fat [%]	30.9 [13.4]	30.3 [13.2] *	29.3 [13.5] ***
Blood pressure			
Systolic [mmHg]	130 [18]	123 [15] **	123 [15] **
Diastolic [mmHg]	78 [11]	77 [11]	76 [10]
Heart rate [beats/min]	65 [8]	65 [10]	66 [11]
Plethysmography			
Basal flow [ml/min/dL]			
Left arm	1.98 [0.82]	–	2.32 [0.75] *
Right arm ¥	2.08 [1.02]	–	2.49 [0.96] **
Reactive hyperaemic flow [ml/min/dL]			
Left arm	22.9 [7.5]	–	27.0 [7.2] **
Right arm – control basal flow	2.04 [1.13]	–	2.51 [1.07] *
Haemorheology profile			
Haematocrit	0.43 [0.03]	–	0.42 [0.03]
High shear rate blood viscosity [mPa.s]	3.98 [0.44]	–	3.90 [0.38]
RBC deformability Index [mPa.s]	4.22 [0.28]	–	4.27 [0.30]
RBC osmotic fragility			
Lysis at 0.475% NaCl [%]	‘13.5 [7.3]	–	13.7 [11.4]
Median RBC fragility [%NaCl]	0.438 [0.007]	–	0.440 [0.011]
Plasma viscosity [mPa.s]	1.64 [0.06]	–	1.58 [0.06] ***
Plasma total protein [g/L]	71.8 [3.1]	–	70.4 [3.3]**
Plasma albumin [g/L]	42.0 [1.8]	–	41.2 [1.7] *
Plasma globulins [g/L]	28.0 [3.7]	–	27.1 [3.4]
Plasma fibrinogen [g/L]	3.90 [0.63]	–	3.95 [0.73]
Albumin:fibrinogen ratio	11.1 [2.1]	–	10.6 [3.3]

p value of comparison with baseline. * ≤0.05 ** ≤0.01 *** ≤0.001
¥ Combined data of basal flow measurements and control measurements for hyperaemic flow.

developed a serious condition, Kingella endocarditis during the course of the study. The remaining 24 subjects [14 males, 10 females; mean age 64 years, range 56–75 years] completed the study and are included in the data analysis. At baseline, one-third of the study participants had dyslipidaemia, defined as either a plasma LDL-cholesterol concentration >3.5 mmol/L or plasma triglyceride concentration >2.0 mmol/L. One-quarter of the subjects reported a history of osteoarthritis while one person had been investigated previously for ischaemic heart disease. Eleven people [46%] had a history of smoking cigarettes although it was confirmed that no person was currently smoking. Compliance during the study was satisfactory with a mean of 93% of the Enzogenol® capsules being taken during the study.

The results of the anthropometric, blood pressure, plethysmography and haemorheology measurements are contained in Table 1. During the study there was a significant and sustained decrease in both mean BMI and mean percentage body fat. A significant reduction in mean systolic blood pressure was also observed after 6 and 12 weeks of treatment [mean decrease = 7 mmHg]. Diastolic blood pressure remained unchanged throughout the study.

After 12 weeks of treatment, there was a significant increase in basal blood flow with this change being similar in both forearms (17.2% in the left forearm [$p < 0.05$] and 19.7% in the right forearm [$p < 0.01$]). The hyperaemic response measured in the left forearm increased by 17.9% [$p < 0.01$] while basal blood flow measured in the right forearm did not change during the time the hyperaemic recordings were being taken.

A decrease in mean plasma viscosity of 0.6 mPa.s was measured after 12 weeks supplementation with Enzogenol® with this reduction being reflected in a statistically insignificant reduction in blood viscosity. Mean plasma total protein and albumin concentrations decreased significantly during the study in conjunction with a trend of decreasing mean total globulin concentration. Mean plasma fibrinogen concentration and the albumin:fibrinogen ratio did not change. No changes in RBC osmotic fragility or deformability index, were observed during the study.

Correlation matrices of the changes in plasma rheology expressed as percentage changes, demonstrated a significant relationship between the decrease in total protein concentration and the reduction in total globulin concentration [$r = 0.39$ $p = 0.05$] but not to the reduction in albumin levels [$r = 0.18$ $p = 0.40$]. No significant correlation was observed between the decrease in plasma viscosity level and changes in plasma total protein [$r = 0.34$ $p = 0.10$] or any of the individual protein fractions [albumin $r = 0.08$ $p = 0.71$; total globulins $r = 0.08$ $p = 0.72$; fibrinogen $r = 0.36$ $p = 0.11$].

The data of the biochemical and haematological safety indices are summarised in Table 2. Dietary supplementation with Enzogenol® was not associated with any changes in glycaemic control or renal and liver function. A small decrease [$p < 0.05$] in mean plasma bilirubin concentration was observed at week 12 [mean

Table 2. Biochemical and haematological safety parameters. Mean [SD]

Index	Baseline	Week 6	Week 12
Glycaemic control			
Plasma glucose [mmol/L]	5.2 [0.6]	5.1 [0.8]	5.0 [0.6]
Renal function			
Plasma creatinine [mmol/L]	0.07 [0.01]	0.07 [0.01]	0.07 [0.01]
Urine albumin creatinine ratio	1.2 [1.4]	1.0 [0.7]	1.1 [0.8]
Liver function			
Plasma bilirubin [mmol/L]	13.7 [5.3]	12.6 [4.1]	11.4 [3.8] *
Plasma alkaline phosphatase [mmol/L]	81.4 [20.5]	83.0 [22.8]	78.7 [21.7]
Plasma AST [mmol/L]	21.4 [6.6]	21.6 [5.8]	21.8 [5.8]
Plasma ALT [mmol/L]	22.5 [10.6]	21.3 [10.9]	21.3 [9.6]
Plasma GGT [mmol/L]	27.3 [16.1]	28.6 [19.8]	28.9 [21.4]
Lipid profile			
Plasma total cholesterol [mmol/L]	5.7 [1.0]	5.8 [1.0]	5.6 [1.2]
Plasma HDL-cholesterol [mmol/L]	1.60 [0.51]	1.62 [0.53]	1.65 [0.47]
Plasma LDL-cholesterol [mmol/L]	3.4 [0.9]	3.5 [0.8]	3.3 [0.8]
Plasma triglyceride [mmol/L]	1.57 [0.74]	1.47 [0.72]	1.43 [1.12]
Total cholesterol : HDL-cholesterol ratio	3.9 [1.3]	3.8 [1.2]	3.6 [1.2]
Plasma apolipoprotein B [mmol/L]	1.09 [0.25]	1.03 [0.26]	1.09 [0.30]
Haematology			
Haemoglobin [g/L]	139 [11]	139 [9]	137 [10]
RBC count [$\times 10^{12}$/L]	4.5 [0.4]	4.5 [0.4]	4.4 [0.4]
RBC mean cell volume [fL]	90.5 [4.0]	90.9 [4.0]	91.0 [4.0]
MCHC [g/L]	342 [4]	340 [5]	340 [8]
WBC count [$\times 10^{9}$/L]	5.3 [1.3]	5.5 [1.8]	5.6 [1.6]
Platelet count [$\times 10^{9}$/L]	235 [43]	233 [47]	234 [50]
Platelet mean cell volume [fL]	7.9 [0.7]	8.0 [0.7]	7.9 [0.6]

p value of comparison with baseline. * ≤0.05

decrease –2.3 mmol/L]. The mean plasma concentration of the various lipid fractions remained unchanged throughout the study. No change was observed in any of the haematological parameters measured.

DISCUSSION

This pilot study found that short-term dietary supplementation with a water soluble extract of *Pinus radiata* bark containing bioflavonoids [Enzogenol®] and added vitamin C had no adverse effects on routine laboratory indices of glycaemic control, renal and liver function, plasma lipid profile and haematology. Our data also indicated that the extract was associated with beneficial effects on a range of clinical parameters. These changes included a 1.5% decrease in mean body mass index, a 5.2% reduction in mean percentage body fat, a decrease in mean systolic blood pressure of 7 mmHg, an improvement in forearm blood flow characteristics and a significant decrease in plasma viscosity associated with minor changes in plasma protein profile. The dose of Enzogenol® associated with these effects was approximately equivalent to the dose of the extract that was found to improve overall condition and increase survival in an placebo-controlled study in a strain of experimental mice [Dr K. Duncan, personal communication].

It is imperative these findings are interpreted as data from an open label, uncontrolled investigation of a bioflavonoid extract that also contained the established antioxidant, vitamin C. A placebo-controlled study is therefore required in order to validate these results as it is well recognised that enrolment in a clinical study may alter participants' expectations and habits, such as diet, thereby possibly influencing anthropometric measurements (Swartzman and Burkell, 1998). Similarly, there is evidence that baseline blood pressure measurements in clinical trials are often falsely high and that the subsequent reduction in levels may represent a return to the normal mean blood pressure of the study group (Medical Research Council Working Party, 1977).

Notwithstanding the limitations of the present study, our findings substantiate the increasing volume of literature that has reported beneficial clinical changes in cardiovascular risk factors following increased consumption of bioflavonoids (Caccetta *et al*., 2001; DiCarlo *et al.*, 1999; Gulati, 1999; Wang and Ng, 1999). Flavonoids have been shown to promote relaxation of vascular smooth muscle (Fitzpatrick *et al*., 1993; Stein *et al*., 1999) and our finding of increased basal and hyperaemic forearm blood flow at the end of the study is consistent with such an affect. The hyperaemic response primarily provides an assessment of vasodilator reserve of peripheral resistance vessels, and secondly is a relatively non-specific measure of endothelial-dependent vasodilatation (Sax *et al*., 1987). The mechanism of this change in vascular reactivity is unknown but may be the result of a reduction in blood pressure restoring nitric oxide availability (Drexler, 1997) or alternatively to a decrease in free-radical inhibition of nitric oxide activity (De Caterina, 2000, Park *et al*., 2000).

The significant reduction in mean plasma viscosity level in combination with small changes in plasma protein determinants of viscosity were interesting findings in the study and are in accordance with several other

rheological investigations that observed flavonoids have an inhibitory effect on RBC aggregation (Lacombe *et al.*, 1988; Schmid-Schönbein *et al.*, 1975). The magnitude of the reduction in mean plasma viscosity with Enzogenol® [i.e. 0.06mPa.s] is of physiological significance (Harkness, 1971) and is consistent with other studies that have suggested flavonoids may be naturally occurring anti-inflammatory agents by their action of modifying eicosanoid biosynthesis or inhibiting the expression of endothelial cell adhesion proteins (Middleton and Kandaswami, 1992; Read, 1995).

In conclusion, the findings of this pilot study demonstrated that dietary supplementation with Enzogenol® in healthy people aged between 55 and 75 years was associated with beneficial changes in anthropometric, vascular and plasma rheological indices. Enzogenol® did not change standard biochemical or haematological indices and appeared to be a safe and well tolerated dietary supplement. On the basis of these findings we suggest a placebo-controlled clinical study of Enzogenol® is warranted in order to validate these potentially important clinical effects.

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