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Effects of Pycnogenol on endothelial function in patients with stable coronary artery disease: a double-blind, randomized, placebo-controlled, cross-over study

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Aims Extracts from pine tree bark containing a variety of flavonoids have been used in traditional medicine. Pycnogenol is a proprietary bark extract of the French maritime pine tree (*Pinus pinaster* ssp. *atlantica*) that exerts antioxidative, anti-inflammatory, and anti-platelet effects. However, the effects of Pycnogenol on endothelial dysfunction, a precursor of atherosclerosis and cardiovascular events, remain still elusive.

Methods and results Twenty-three patients with coronary artery disease (CAD) completed this randomized, double-blind, placebo-controlled cross-over study. Patients received Pycnogenol (200 mg/day) for 8 weeks followed by placebo or vice versa on top of standard cardiovascular therapy. Between the two treatment periods, a 2-week washout period was scheduled. At baseline and after each treatment period, endothelial function, non-invasively assessed by flow-mediated dilatation (FMD) of the brachial artery using high-resolution ultrasound, biomarkers of oxidative stress and inflammation, platelet adhesion, and 24 h blood pressure monitoring were evaluated. In CAD patients, Pycnogenol treatment was associated with an improvement of FMD from 5.3 $\pm$ 2.6 to 7.0 $\pm$ 3.1 ($P < 0.0001$), while no change was observed with placebo (5.4 $\pm$ 2.4 to 4.7 $\pm$ 2.0; $P = 0.051$). This difference between study groups was significant [estimated treatment effect 2.75; 95% confidence interval (CI): 1.75, 3.75, $P < 0.0001$]. 15-F_{2t}-Isoprostane, an index of oxidative stress, significantly decreased from 0.71 $\pm$ 0.09 to 0.66 $\pm$ 0.13 after Pycnogenol treatment, while no change was observed in the placebo group (mean difference 0.06 pg/mL with an associated 95% CI (0.01, 0.11), $P = 0.012$]. Inflammation markers, platelet adhesion, and blood pressure did not change after treatment with Pycnogenol or placebo.

Conclusion This study provides the first evidence that the antioxidant Pycnogenol improves endothelial function in patients with CAD by reducing oxidative stress.

Clinical Trial Registration: ClinicalTrials.gov identifier: NCT00641758

Keywords Pycnogenol • Coronary artery disease • Clinical trial • Endothelial function • Oxidative stress

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Introduction

Extracts from pine tree bark have been used in traditional medicine. Pycnogenol is a bark extract of the French maritime pine tree (*Pinus pinaster* ssp. *atlantica*). The extract is prepared using a standardized procedure that includes an extraction of fresh pine bark with ethanol and water according to the requirements published by the United States Pharmacopoeia (USP).[1] The resulting product is a mixture of flavonoids as monomers, i.e. catechin and taxifolin, and condensed polymers (85%), as well as dodeoameric flavonols, designated as procyanidins, differing in structure and chain length. Procyanidins are biopolymers composed of catechin and epicatechin, widely distributed in the plant kingdom. Many edible fruits contain procyanidins, e.g. apples, berries, peanuts, and grapes. Additionally, the extract contains phenolic acids as gallic, caffeic, and ferulic acid as minor constituents. Pycnogenol also contains glycosylation products, i.e. sugar derivatives of phenolcarbonic acids and taxifolin.[2] These molecules have antioxidant properties and may act as modulators of metabolic enzymes and other cellular functions.[2]

In a pharmacokinetic study, healthy volunteers ingested a single dose of 300 mg and repeated doses of 200 mg Pycnogenol.[3] In blood samples, catechin, taxifolin, ferulic acid, caffeic acid, and the metabolite 5-(3′,4′-dihydroxyphenyl)-g-valerolactone (M1) were detected in concentrations up to 100 ng/mL. While the first four compounds are genuinely present in the maritime pine bark extract, the metabolite M1 is formed *in vivo* from catechin polymers by gut microbiota.[4,5] This metabolite had been previously also found in urine samples after Pycnogenol intake.[6] Additionally to these known constituents and metabolites, 10 yet unknown compounds were detected in human plasma samples.[7]

The effects of the identified compounds have been investigated *in vitro* with the metabolite M1 revealing the highest activity. On a microgram per millilitre basis, M1 was more potent than the original maritime pine bark extract in inhibiting the activity of matrix metalloproteinase (MMP)-1, -2 and -9.[3] M1 was also about twice as potent as hydrocortisone in inhibiting the release of MMP-9 from freshly isolated human monocytes after stimulation with bacterial lipopolysaccharide. Thus, it appears very likely that M1 plays a major role in mediating the biological effects of Pycnogenol.

However, it cannot be excluded that other yet unknown components or metabolites of Pycnogenol also contribute to its biological activity. Plasma samples of volunteers who ingested Pycnogenol-contained bioactive compounds which in addition to the inhibition of MMP-9 release statistically significantly inhibited nuclear factor kappa B (NF-κB) activation[8] as well as cyclooxygenase 1 and 2 activity.[9]

Pycnogenol has been shown to exert anti-inflammatory effects and to inhibit platelet aggregation, both risk factors for cardiovascular disease.[2] *In vitro*, Pycnogenol prolonged the ascorbate radical lifetime to the greatest extent by regenerating ascorbic acid.[10] Similarly, Pycnogenol inhibits LDL peroxidation, lipid peroxidation in phospholipid liposomes,[2] and lipid peroxidation caused by *t*-butylhydroperoxide. Furthermore, Pycnogenol has antioxidant activity.[10] Finally, in isolated blood vessels, Pycnogenol inhibits vasoconstriction caused by adrenalin via the production of nitric oxide.[11] The present study was designed to evaluate the effect of Pycnogenol on endothelial function in patients with coronary artery disease (CAD).

Methods

Study population

Patients with CAD, documented by coronary angiography, nuclear imaging, or a positive stress test, on stable cardiovascular medication for at least 1 month and ≥18 years of age, who gave written informed consent, were recruited at the Clinic of Cardiology, Cardiovascular Center, University Hospital Zurich, Switzerland.

Exclusion criteria were acute myocardial infarction, unstable angina, stroke, or coronary intervention/revascularization procedure within 3 months prior to study entry; uncontrolled symptomatic congestive heart failure (New York Heart Association functional class >II) in the last 4 weeks prior to study entry; smoking, alcohol, or illicit substance abuse; uncontrolled blood pressure despite adequate therapy (≥160/100 mmHg); symptomatic hypotension; ventricular tachyarrhythmias; any cardiomyopathy, untreated thyroid dysfunction, or adrenal insufficiency; renal failure (creatinine clearance using the MDRD formula[12] <50 mL/min); liver disease; chronic use of long-acting nitrates; oral or intravenous steroid therapy; insulin-dependent diabetes mellitus; anaemia (Hb < 10 g/dL); known hypersensitivity to Pycnogenol; systemic inflammatory diseases (e.g. rheumatoid arthritis, Crohn's disease); known human immunodeficiency virus infection or active virus—hepatitis; pregnancy or breast-feeding, women with childbearing potential without adequate contraception; malignancy (unless healed or in remission >5 years); recipient of any major organ transplant (e.g. lung, liver, heart) or renal replacement therapy and the participation in another study within the last month.

Study design and protocol

In this single-centre, prospective, randomized, double-blind, placebo-controlled study in a cross-over fashion, we analysed the impact of Pycnogenol on endothelium-dependent and -independent dilatation in patients with stable CAD receiving optimal standard therapy. Prespecified secondary endpoints were change in high-sensitive C-reactive protein and CD40 Ligand; change in total antioxidative capacity, oxidized LDL (oxLDL), and 8-isoprostanes; change in ambulatory blood pressure (ABP); and change in shear stress-dependent platelet function. The protocol was approved by the Institutional Review Board (Ethics Committee of the Canton Zurich) and the Swiss Agency for Therapeutic Products (Swissmedic, Bern, Switzerland). The study was registered at ClinicalTrials.gov (Identifier: NCT00641758).

After screening and recruiting, the patients were randomly assigned into two groups. For randomization, an unpredictable allocation sequence was provided by external institutions (InterCorNet, Zurich, Switzerland), which were responsible for the blinding and labelling of the drugs. The study drug and placebo were prepared in identical capsules to ensure uniform appearance of both formulations by the manufacturer Horphag Research (UK) Ltd, London, UK. All investigators including the ultrasonographers were unaware of the allocation procedure at any time. The patients were randomized to receive either Pycnogenol 200 mg/day or matching placebo in the first part or vice versa in the second part with a washout period of 2 weeks in-between.

At each visit (baseline and after 8, 10, and 18 weeks), endothelial function and ABP were measured, blood samples were drawn, the clinical status was assessed, and adverse events were recorded. At each visit, a safety analysis was performed including assessment of electrolytes, of liver and kidney function, as well as a white and red blood cell count. Pregnancy testing in women with childbearing potential

was performed only at the first visit. The patients were advised not to take their usual drugs in the morning of the examination day (all examinations and measurements were performed in the morning); moreover, the medical therapy was unchanged throughout the study. Blood samples and the evaluation of flow-mediated dilatation (FMD) were performed before the patients took their medications. The regular medications and the study drug were taken thereafter and before the 24 h ABP measurement was placed.

Endothelium-dependent and -independent vasodilatation

Flow-mediated dilatation was performed according to the current guidelines[13,14] and as described previously.[15] In brief, a B-mode high-resolution ultrasound scan of the left brachial artery was obtained by highly trained and experienced sonographers in a longitudinal section between 2 and 10 cm above the elbow, using a high-resolution 10 MHz linear array transducer and a high-resolution ultrasound system Siemens X300 (Siemens Switzerland AG, Zurich, Switzerland). The analogue video signal was acquired with a video processing system that computed the artery diameter in real-time (FMD Studio,[16] a system for real-time measurement, Institute of Clinical Physiology, Pisa, Italy). The high reproducibility of the method has been demonstrated recently.[17,18] The baseline vessel size was considered as the mean of the measures obtained during the first minute. Flow-mediated dilatation was calculated as the maximal per cent increase in diameter above the baseline. Endothelium-independent dilatation was measured after sublingual glycerol trinitrate (GTN; 0.4 mg, Nitrolingual Spray, Pohl-Boskamp, Hohenlockstedt, Germany) application by recording arterial diameter continuously for at least 6 min. The response to GTN is calculated as the maximum per cent increase in vessel size above the baseline. The reproducibility of our laboratory was published previously.[15]

Special laboratory analysis

Oxidative stress markers

8-Epi-PGF2alpha (15-F_{2t}-isoprostane) was measured in the plasma with an 8-Isoprostane Enzyme Immunoassay [8-isoprostane Express EIA Kit, Cayman Chemicals, Ann Arbor, MI, USA; intra-assay coefficient of variation (CV) 7.2%, inter-assay CV 15.5%].

High-sensitivity C-reactive protein

A high-sensitive immunoluminometric assay (Imulite 2002, DCP, Los Angeles, CA, USA) was used to measure high-sensitivity C-reactive protein levels as described by Wood *et al.*[19]

Soluble intercellular adhesion molecule-1, soluble vascular cell adhesion molecule-1, soluble CD40 Ligand, and oxidized low-density lipoprotein

EDTA plasma was drawn from each patient and immediately frozen at −70°C. Plasma samples were thawed only once for aliquotation before they were subjected to analyses. Quantitative detection of soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble CD40 Ligand (sCD40L) (all Bender Med Systems, Vienna, Austria), oxLDL (Immundiagnostik, Bernsheim, Germany), and of Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) (Uscn Life Sciences Inc., Wuhan, China) was performed using enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions. Briefly, pre-coated microwell plates absorbed with specific capture antibodies directed against the respective molecule were washed thoroughly with the washing buffers provided. Plasma samples were diluted where indicated and applied in duplicates before a 1–2 h incubation at room temperature. Detection of the target molecules was performed using a horseradish peroxidase-conjugated detection antibody specific for the respective molecule and visualized during a colour-giving reaction using the provided TMB substrate solution. Absorption was measured at 450 nm and values were normalized to the respective internal standards supplied by the manufacturer.

Total antioxidant capacity

Total antioxidant capacity of plasma was measured using the total antioxidant capacity kit (Abcam, Cambridge, UK) according to the manufacturer's instructions. Briefly, plasma was allowed to reduce Cu^{2+} for 1.5 h at room temperature. Reduced Cu^{+} was chelated with a colorimetric probe and absorbance was measured at 570 nm. Results were expressed as trolox equivalent according to a trolox standard curve.

Endothelin-1

The endothelin-1 (ET-1) sandwich ELISA system from Assay Designs (Ann Arbor, MI, USA) was used according to the manufacturer's instructions to measure ET-1 protein concentrations in EDTA plasma samples. Briefly, 500 μL of each plasma sample was extracted with 650 μL extraction solvent [acetone:1 N HCl:water (40:1:5)] and dried under nitrogen streaming. Reconstituted in 120 μL assay buffer, samples were employed for testing. The sensitivity of the assay was determined to be 0.41 pg/mL, the intra-assay CVs were 6.7% at 35.9 pg/mL, 8.9% at 2.3 pg/mL, and 8.8% at 1.1 pg/mL; cross-reactivity with human Big ET-1, ET-2, and ET-3 was low (human Big ET-1 < 0.1%, ET-2 = 21%, and ET-3 = 3.6%).

Asymmetric dimethylarginine

Asymmetric dimethylarginine (ADMA) concentration was measured from plasma samples by a competitive ELISA system including an acylation step as pre-treatment (DLD Diagnostika GmbH, Hamburg, Germany), with a standard range from 0.1 to 5.0 μmol/L. Twenty microlitres of each plasma sample were employed for testing. The detection limit of the system was 0.05 μmol/L. As described previously,[20] the intra-assay CVs were 5.7% at 0.66 μmol/L and 6.4% at 1.01 μmol/L; the inter-assay CV ranged from 8.3 to 10.3%. Cross-reactivity with arginine and other methylarginines was low [arginine <0.02%; NG-monomethyl-L-arginine 1.0%; symmetric dimethylarginine (SDMA) 1.2%]. The correlation coefficients with liquid chromatography–mass spectrometry and gas chromatography–mass spectrometry were high (0.991; 0.984), with a good linearity between 0.1 and 5.0 mol/L.

Symmetric dimethylarginine

Symmetric dimethylarginine was measured from serum samples by a competitive ELISA system including an acylation step as pre-treatment (DLD Diagnostika GmbH) with a standard range from 0.08 to 3.0 μmol/L. Twenty microlitres of each plasma sample were employed for testing. The detection limit of the system was 0.05 μmol/L. The intra-assay CVs were 5.7% at 0.52 μmol/L, 6.1% at 0.75 μmol/L, and 4.7% at 1.72 μmol/L; cross-reactivity with arginine and other methylarginines was low (arginine <0.01%; NG-monomethyl-L-arginine 0.70%; ADMA 0.44%).

Ambulatory blood pressure measurement

Ambulatory blood pressure measurements were obtained over 24 h using the 'Tracker NIBP 2' (Delmar, Del Mar Reynolds Medical, Hertford, UK) before and after the active treatment phase according to recent guidelines.[21] Patients were asked to keep their arm calm while the cuff was inflating and to avoid excessive physical exertion

during monitoring. The monitors were programmed to take readings every 15 min during daytime and every 30 min during night-time.

Shear stress-dependent platelet function

Shear stress-dependent platelet function was assessed with a cone and platelet analyzer as described previously.[15,22]

Statistical analysis

The primary endpoint was the change in FMD after 8 weeks of treatment with Pycnogenol compared with placebo. Based on previous studies,[23] the estimated sample size for this study is 25 (to show a difference of 1.3% in FMD with a standard deviation of 1.5%, giving 5% two-sided significance level and 85% power when using a *t*-test). Analysis was performed using the Wilcoxon and Mann–Whitney *U*-tests (to account for possible non-normality of the endpoints), using methods discussed by Senn.[24] That is, we considered two distinct groups of patients: Group 1 who received Pycnogenol followed by placebo and Group 2 who received placebo followed by Pycnogenol. Within each group, data are summarized by examining within patients changes between Periods 1 and 2 (for Group 1, this is the change from baseline while on placebo minus the change in baseline while on Pycnogenol; for Group 2, the change from baseline while on Pycnogenol minus the change in baseline while on placebo). These unpaired change scores are then analysed using an unpaired Wilcoxon and the Mann–Whitney *U*-test, as proposed by Hill and Armitage,[25] and later discussed by Senn.[24] Statistical tests were preformed two-sided. The effect of Pycnogenol is estimated as the average of the two group-specific mean change scores. The period effect is estimated using the difference in the two group-specific mean change scores (divided by 2). The carry-over effect was excluded using an unpaired Wilcoxon test of within-patient change from baseline including only the first period of treatment. A Bonferroni–Holm[26] correction was made if necessary. For the 'responder analysis', patients were divided evenly into groups based on their FMD to Pycnogenol and placebo. Those with better responses were considered 'responders' and the others, 'non-responders'. The analyses for C-reactive protein and 15-F_{2t}-isoprostane were then performed again separately for the two groups. Results are presented as mean $\pm$ SD or SEM as described. Analysis of the primary endpoint was performed in the R programming language (R Development Core Team, 2009). The statistical software package SPSS 17 (SPSS Inc., Chicago, IL, USA) was used to evaluate difference in the clinical characteristics. Statistical significance was accepted at $P < 0.05$.

Results

Study population

A total of 28 patients were enrolled, 5 patients discontinued the study prematurely, 3 patients withdrew consent and 2 patients were excluded for adverse events: one patient in the Pycnogenol group was hospitalized for acute decompensated heart failure; one patient in the placebo group was excluded after development of an itching rash. Therefore, 23 patients (19 males and 4 females) with a mean age of 63.1 $\pm$ 7.1 years (range: 49–73 years) and a body mass index of 27.3 $\pm$ 3.3 kg/m^2 completed the study protocol and were included into the analysis. The mean left ventricular ejection fraction was 62 $\pm$ 10%. With the included 23 patients, the statistical power of 80% is sufficient to detect differences of 1.3% in FMD between the two treatments. All patients had stable CAD, 11 patients with one-vessel disease, 1 patient with two-vessel disease, and 11 patients with three-vessel disease.

Effect of Pycnogenol on endothelium-dependent and -independent vasodilatation

After 8-week treatment with Pycnogenol, FMD increased from 5.3 $\pm$ 2.6 to 7.0 $\pm$ 3.1%, while it remained unchanged in the placebo group (5.4 $\pm$ 2.4 to 4.7 $\pm$ 2.0%; *Table 1* and *Figure 1*). The estimated effect of Pycnogenol on FMD was an increase of 2.75 compared with placebo [mean difference 2.75 with an associated 95% confidence interval (CI: 1.75, 3.74), $P < 0.0001$], a statistically significant treatment effect. The period effect for this endpoint was estimated to be -0.32 [95% CI (-1.31, 0.68), $P = 0.51$] and, therefore, not statistically significant. The endothelium-independent dilatation induced by GTN remained unchanged before and after treatment with Pycnogenol (*Table 1*).

Effect of Pycnogenol on ambulatory blood pressure

Systolic and diastolic ABP remained unchanged before and after treatment with Pycnogenol (*Table 2*). The estimated effect of Pycnogenol on systolic blood pressure was an increase of 1.17 mmHg compared with placebo [mean difference 1.17 mmHg with associated 95% CI (-4.71, 7.04), $P = 0.68$], a statistically non-significant treatment effect. The estimated effect of Pycnogenol on diastolic blood pressure was an increase of 0.06 mmHg compared with placebo [mean difference 0.06 mmHg with associated 95% CI (-3.26, 3.37), $P = 0.97$], a statistically non-significant treatment effect.

Effect of Pycnogenol on oxidative stress

15-F_{2t}-Isoprostane was reduced significantly after 8-week treatment with Pycnogenol compared with baseline and placebo (*Table 2* and *Figure 2*). The estimated effect of Pycnogenol on 15-F_{2t}-Isoprostane was a decrease of 0.06 pg/mL compared with placebo [mean difference 0.06 pg/mL with an associated 95% CI (0.01, 0.11), $P = 0.012$], a statistically significant treatment effect. This effect was even more pronounced, when the patients, 'responders' and 'non-responders', were analysed. The estimated effect of Pycnogenol on 15-F_{2t}-Isoprostane (responders) was a decrease of 0.1 compared with placebo [mean difference 0.1 with associated 95% CI (0.00, 0.20), $P = 0.045$], a statistically significant treatment effect, while no change was observed in the 'non-responders'. The estimated effect of Pycnogenol on 15-F_{2t}-isoprostane (non-responders) was an increase of 0.04 compared with placebo [mean difference 0.04 with associated 95% CI (-0.02, 0.10), $P = 0.15$], which was not statistically significant.

Effect of Pycnogenol on markers of inflammation

The estimated effect of Pycnogenol on C-reactive protein was an increase of 2.18 mg/L compared with placebo [mean difference 2.18 mg/L with associated 95% CI (-3.35, 7.71), $P = 0.42$], a statistically non-significant treatment effect. Moreover, all of the

Table 1 Clinical measures before and after 8-week treatment with Pycnogenol or placebo and concomitant medication

	Baseline for treatment period	Pycnogenol	Baseline for control period	Placebo
Clinical parameters				
FMD (%)	5.3 ± 2.6	7.0 ± 3.1*,#	5.4 ± 2.4	4.7 ± 2.0
GTN (%)	18.4 ± 5.4	19.4 ± 4.8	19.5 ± 5.4	19.7 ± 5.7
24 h SBP (mmHg)	125.8 ± 8.7	125.5 ± 8.1	124.8 ± 9.7	125.7 ± 8.1
24 h DBP (mmHg)	75.0 ± 7.0	74.3 ± 7.3	73.9 ± 7.5	74.4 ± 6.6
Office SBP (mmHg)	129.9 ± 12.5	131.8 ± 14.5	130.1 ± 12.1	129.8 ± 11.7
Office DBP (mmHg)	77.4 ± 6.8	78.9 ± 7.7	78.9 ± 7.5	79.7 ± 8.0
Office HR (b.p.m.)	58.3 ± 8.3	59.4 ± 9.0	60.3 ± 12.3	57.6 ± 9.9
Concomitant medication				
Aspirin	23/23 (100%)			
Statin	20/23 (87%)			
ACE-inhibitor/ARB	18/23 (78%)			
β-Blocker	17/23 (74%)			
Diuretics	8/23 (35%)			
Calcium-antagonist	4/23 (17%)			
Clopidogrel	4/23 (17%)			
Ezetimibe	4/23 (17%)			
Oral anti-diabetics	4/23 (17%)			
Marcoumar	1/23 (4%)			
α-Antagonist	1/23 (4%)			

*$P < 0.05$ vs. baseline.
#$P < 0.05$ vs. placebo.

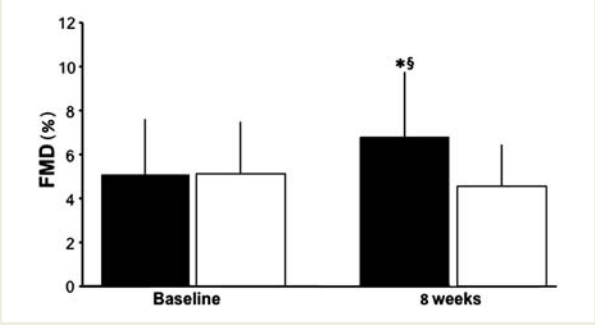

Figure 1 Flow-mediated dilatation (FMD, %) of the brachial artery in patients receiving Pycnogenol (black bar) or placebo (white bar). *$P < 0.05$ vs. baseline and §$P < 0.05$ vs. placebo.

following parameters remained unchanged before and after treatment with Pycnogenol or placebo (*Table 2*): total antioxidant capacity [mean difference −0.19 μM with associated 95% CI (−2.20, 1.83), $P = 0.85$], sVCAM [mean difference −20.44 ng/mL with associated 95% CI (−128.88, 88.00), $P = 0.7$], sICAM [mean difference −67.38 ng/mL with associated 95% CI (−141.83, 7.07), $P = 0.073$], sCD40L [mean difference 0.02 ng/mL with associated 95% CI (−0.21, 0.25), $P = 0.85$], and oxLDL [mean difference −2.56 ng/mL with associated 95% CI (−18.59, 13.47), $P = 0.74$], and Lp-PLA_2 [mean difference 74.83 ng/mL with associated 95% CI (−46.10, 195.76), $P = 0.21$].

Effect of Pycnogenol on symmetric dimethylarginine, asymmetric dimethylarginine, and endothelin-1

The estimated effect of Pycnogenol on SDMA was a decrease of 0.06 μM compared with placebo [mean difference −0.06 μM with associated 95% CI (−0.16, 0.04), $P = 0.23$], a statistically non-significant treatment effect. The estimated effect of Pycnogenol on ADMA was a decrease of 0.05 μM compared with placebo [mean difference −0.05 with associated 95% CI (−0.12, 0.03), $P = 0.19$]. Though 57% of the patients receiving Pycnogenol and only 30% of the patients taking placebo revealed lower ADMA concentrations after the respective treatment period, the effect was not statistically significantly different (*Table 2*).

The mean ET-1 concentrations of the participants were throughout at the lower limit of the reported physiological range between 1.10–2.70 ng/L[27] and no statistically significant treatment effect was observed (*Table 2*).

Effect of Pycnogenol on shear stress-dependent platelet function

Shear stress-dependent platelet function did not change after treatment with Pycnogenol (*Table 2*). The estimated effect of Pycnogenol on area fraction was a decrease of 0.22 compared with placebo [mean difference −0.22 with associated 95% CI (−1.85, 1.42), $P = 0.78$], a statistically non-significant treatment effect.

Table 2 Laboratory values before and after treatment with Pycnogenol or placebo

	Baseline before treatment period	Pycnogenol	Baseline before control period	Placebo
Hb (g/L)	14.5 ± 1.2	14.5 ± 1.2	14.6 ± 1.2	14.7 ± 1.2
Hk (%)	41.4 ± 3.2	41.3 ± 3.8	41.1 ± 3.4	41.6 ± 3.3
Sodium (mmol/L)	141.1 ± 2.3	141.6 ± 2.2	141.4 ± 2.1	141.9 ± 2.1
Potassium (mmol/L)	3.9 ± 0.3	3.8 ± 0.4	3.9 ± 0.4	3.8 ± 0.4
Creatinine (μmol/L)	91.7 ± 13.9	90.6 ± 25	91.6 ± 16.7	93.4 ± 14.1
Glucose (mmol/L)	5.7 ± 1.4	5.6 ± 1.3	5.7 ± 1.1	5.7 ± 1.3
TC (mmol/L)	4.6 ± 0.6	4.6 ± 0.7	4.7 ± 0.7	4.8 ± 0.8
HDL-C (mmol/L)	1.4 ± 0.4	1.4 ± 0.4	1.4 ± 0.4	1.4 ± 0.4
LDL-C (mmol/L)	2.5 ± 0.5	2.5 ± 0.6	2.6 ± 0.6	2.7 ± 0.7
TG (mmol/L)	1.4 ± 0.7	1.5 ± 0.7	1.5 ± 0.9	1.5 ± 0.9
ALT (U/L)	27.7 ± 4.9	30.0 ± 7.0	29.4 ± 6.3	29.2 ± 5.4
AST (U/L)	29.5 ± 8.2	31.6 ± 9.3	92.6 ± 9.7	32.9 ± 9.9
High-sensitivity C-reactive protein (mg/L)	1.52 ± 1.37	1.52. ± 1.33	1.63 ± 1.71	1.91 ± 3.4
Lp-PLA_2 (ng/mL)	315.89 ± 23.5	321 ± 27.7	279.4 ± 20.4	368.6 ± 24.8
15-F_{2t}-Isoprostane (pg/mL)	0.71 ± 0.09	0.66 ± 0.13*,#	0.72 ± 0.09	0.75 ± 0.10
ADMA (μM)	0.47 ± 0.11	0.49 ± 0.11	0.45 ± 0.12	0.50 ± 0.12
SDMA (μM)	0.47 ± 0.13	0.47 ± 0.13	0.45 ± 0.12	0.51 ± 0.13
Endothelin-1 (ng/L)	1.20 ± 0.36	1.27 ± 0.30	1.23 ± 0.27	1.21 ± 0.30
Platelet adhesion (%)	3.84 ± 2.0	3.49 ± 1.45	3.2 ± 1.6	3.2 ± 2.0
Total antioxidant capacity (μM)	10.12 ± 1.65	10.1 ± 1.28	9.65 ± 2.72	9.78 ± 2.70
sVCAM (ng/mL)	869 ± 232	791 ± 226	824 ± 265	783 ± 244
sICAM (ng/mL)	284 ± 90	232 ± 77	277 ± 91	274 ± 130
sCD40L (ng/mL)	0.54 ± 1.11	0.50 ± 1.0	0.54 ± 1.10	0.46 ± 1.01
OxLDL (ng/mL)	77.9 ± 119.0	78.9 ± 123.5	85.8 ± 155.4	88.5 ± 154.0

*$P < 0.05$ vs. baseline.
#$P < 0.05$ vs. placebo.

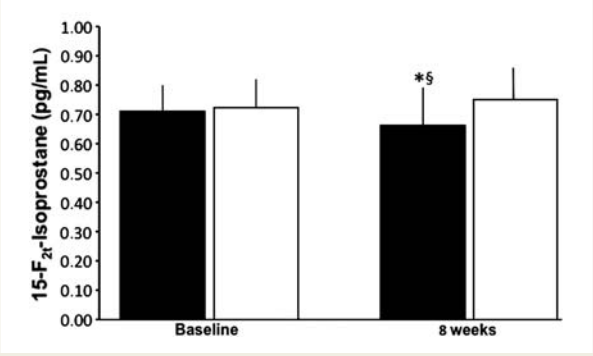

Figure 2 15-F_{2t}-Isoprostane before and after Pycnogenol (black bar) or placebo (white bar); *$P < 0.05$ vs. baseline and §$P < 0.05$ vs. placebo.

Discussion

This study demonstrates an improvement of endothelial function after 8-week treatment with the flavonoid extract Pycnogenol at a dose of 200 mg q.d. when compared with placebo in patients with stable CAD.

The balance between endothelial function and dysfunction plays an important role in the initiation and progression of atherosclerosis and in the transition from a stable to an unstable disease state.[28] As such, the evaluation of endothelial function has emerged as an important endpoint in cardiovascular research. A recent meta-analysis suggests that an impairment of brachial FMD is strongly associated with the occurrence of future cardiovascular events.[29] Therefore, preservation or recovery of endothelial function is an important therapeutic aim in the prevention of arteriosclerosis and its clinical complications such as myocardial infarction and stroke. The improvement of endothelial function in our study was associated with a reduction in 8-isoprostanes, a maker for oxidative stress, but not with components of the L-arginine metabolism or markers of inflammation, indicating that antioxidant properties of the compound appear to be involved.

An improvement of endothelial function has been demonstrated by Nishioka *et al.*[30] in a randomized, double-blind, placebo-controlled study, including 16 healthy young men who were randomized to receive either Pycnogenol (180 mg/day) or placebo for 2 weeks. The investigators measured forearm blood flow (FBF) using strain-gauge plethysmography. They found an augmented FBF response to acetylcholine (ACh) in the Pycnogenol group, while no augmentation was seen in the placebo group. However,

sodium-nitroprusside response was similar in both treatment groups and the administration of NG-monomethyl-L-arginine, a known nitric oxide synthase (NOS) inhibitor, abolished the Pycnogenol-induced augmentation to FBF response to ACh, suggesting an endothelium-dependent vasodilatation by increased NO production.

Several lines of evidence suggest that oxidative stress may promote endothelial dysfunction through different mechanisms.[31] First, diverse reactive oxygen species (ROS) are produced at increased levels within the vessel wall and they individually or in combination interfere with the function of endothelial and vascular smooth muscle cells.[32] The family of ROS includes highly bioactive, short-living molecules that are derived from a reduction in molecular oxygen. Multiple enzyme systems use different substrates as sources of electrons to produce a variety of ROS, the most important of which are NO, superoxide (O_2^-), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$). Many enzyme systems, including NAD(P)H oxidase, xanthine oxidase, and uncoupled NOS among others, contribute to production as well as to degradation of ROS.[33] $ONOO^-$ is an important mediator of lipid peroxidation and protein nitration, including oxidation of LDL, which has potent proatherogenic effects.[32] NO is a crucial mediator of endothelium-dependent vasodilatation and also plays a role in platelet aggregation and in maintaining the balance between smooth muscle cell growth and differentiation.[34] NO is rapidly inactivated by O_2^- to form $ONOO^-$, a known mechanism of endothelial dysfunction.

The short half-life of ROS makes them ideal signalling molecules, but it also confounds their measurement in complex biological systems, particularly in humans *in vivo*.[32] On the other hand, F2-isoprostanes are associated with oxidative stress and are reliable markers of *in vivo* lipid peroxidation.[35] Isoprostanes (iPs) are a family of prostaglandin (PG)-like compounds formed non-enzymatically through free radical catalysed attack on esterified arachidonate followed by enzymatic release from cellular or lipoprotein phospholipids.[32] The measurement of F2-isoprostanes (F_2-iPs), containing F-type ring analogous to $PGF_{2\alpha}$, provides a reliable tool for identifying populations with enhanced rates of lipid peroxidation.[32] In addition to their potential usefulness as indexes of oxidative stress *in vivo*, 8-iso-$PGF_{2\alpha}$ is a vasoconstrictor and modulates platelet activation in response to other agonists.[32] Interestingly, in our study, 8-iso-$PGF_{2\alpha}$ plasma levels decreased significantly by $6.43 \pm 16.3\%$ after therapy with Pycnogenol ($P = 0.012$) when compared with placebo (increase of $4.54 \pm 11.1\%$). Moreover, the pronounced improvement of endothelial function after 8 weeks of treatment with Pycnogenol in the 'responder' group was paralleled by a significant decrease in 15-F_{2t}-isoprostane, while in patients in the 'non-responder' group, no significant change was detected. These findings are in line with a study performed by Ryan *et al.*,[36] who examined the effects of Pycnogenol on different cognitive and biochemical parameters in a healthy elderly population (60–85 years old). In this double-blind, placebo-controlled, matched-pair study, 101 participants received Pycnogenol (150 mg/day) for 3 months. The participants in the Pycnogenol group showed an improved working memory and decreased levels of F2-isoprostanes compared with the control group.

Indeed, Pycnogenol is able to scavenge both hydroxyl radicals and superoxide anions[37] and increase the activity of superoxide dismutase, glutathione peroxidase, and catalase.[38] These *in vitro* effects have been confirmed in healthy subjects, treated with Pycnogenol 150 mg q.d.[39] In that study oxygen radical absorption capacity increased by 40% during treatment and returned to baseline after washout. However, there was no significant change in plasma lipid peroxidation or in LDL-cholesterol oxidation.[39] These data are in line with our study, showing no changes in plasma lipids including oxLDL or in total antioxidative capacity.

Endothelial dysfunction has been linked to elevated blood levels of ADMA, which is an endogenous inhibitor of NO synthesis.[40,41] Even small changes of ADMA concentration alter vascular NO production. Symmetric dimethylarginine correlates with renal function and indicates an increased cardiovascular risk.[42,43] It has been shown to increase production of ROS and is associated with atrial fibrillation and endothelial and platelet activation. Although ADMA plasma levels tended to be lower after Pycnogenol treatment, neither the effect on ADMA nor SDMA concentrations reached statistical significance. Although the L-arginine/NO metabolism was not studied in particular, our finding strongly suggests that alterations in the L-arginine/NO metabolisms are unlikely involved in the endothelial protective effects of Pycnogenol.

Endothelin-1 is a potent vasoconstrictor modulating the release and action of NO in the vessel wall.[44] Furthermore, previous studies suggested[45] that Pycnogenol may lower the plasma levels of the peptide. However, in the current study, no statistically significant effect of the flavonoid extract could be observed. This might be due to the fact that all participants, although CAD patients, exhibited low ET-1 plasma levels at the lower limit of the physiological reference range. Thus, it cannot be excluded that Pycnogenol might affect vascular ET-1 tissue levels.

The anti-inflammatory properties of Pycnogenol have been demonstrated in a large variety of studies, both *in vitro* and *in vivo*.[46] Pycnogenol is able to inhibit tumour necrosis factor-α-induced NF-κB activation and adhesion molecule expression in human umbilical vein endothelial cells.[47] In two double-blind, randomized, placebo-controlled trials in patients with asthma, the investigators found improved asthma symptom scores as well as improved pulmonary function, which was paralleled by a reduction in plasma, as well as in urine leucotriene concentrations after Pycnogenol treatment.[48,49] While we could demonstrate a marked improvement in endothelial function in stable CAD patients treated with Pycnogenol for 8 weeks, which was paralleled by a decrease in 15-F_{2t}-isoprostane, we could not demonstrate an improvement in inflammatory parameters (high-sensitivity C-reactive protein, sVCAM, sICAM, and sCD40L) or total antioxidative capacity. This lack of effect may be due to the sample size, which of course was calculated for the primary endpoint.

The effects of Pycnogenol on platelet function were investigated by Araghi-Niknam *et al.*[50] in 60 patients with stable CAD, using the PFA-100 test with adenosine diphosphate- or arachidonic acid-induced platelet aggregation. After 4 weeks of treatment with the flavonoid extract at a dose of 450 mg/day, a significant reduction in platelet aggregation was observed compared with placebo or baseline values. However, in our study using Pycnogenol

200 mg q.d., we did not found any difference in shear stress-dependent platelet function, which suggests that higher dosages of Pycnogenol are needed to inhibit platelet function.

Recent studies suggested a blood pressure-lowering effect of Pycnogenol. In a double-blind, placebo-controlled cross-over study involving 11 patients, supplementation with Pycnogenol 200 mg q.d. significantly reduced systolic blood pressure of patients with mild-to-moderate hypertension from 139.9 ± 3.3 to 132.7 ± 4.18 mmHg ($P < 0.05$) after 8 weeks of therapy, while diastolic blood pressure remained stable (93.8 ± 1.23 vs. 92 ± 1.7 mmHg, $P = \text{NS}$).[48] In another double-blind, placebo-controlled trial randomizing 58 hypertensive subjects, intake of 100 mg/day Pycnogenol allowed to reduce the dosage of the calcium channel blocker nifedipine required to maintain normal blood pressure levels.[45] However, we did not find any change in systolic or diastolic blood pressure in our study. This may be explained, at least in part by the fact, that the blood pressure in our study was well controlled at study entry.

Conclusion

This study demonstrates for the first time an improvement of endothelial function after 8-week treatment with Pycnogenol at a dose of 200 mg q.d. in patients with stable CAD. Pycnogenol significantly reduced oxidative stress as assessed by plasma levels of 8-isoprostanes, but left ADMA and SDMA as well as plasma ET-1 levels unaffected. The clinical implications of these antioxidative properties of the flavonoid extract need to be confirmed in large-scale clinical outcome trials.

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