In Vivo Antioxidant Activity of Procyanidin-Rich Extracts from Grape Seed and Pine *(Pinus Maritima)* Bark in Rats

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Abstract: *Background: In vitro* evidence exists for the potential antioxidant benefits of procyanidin-rich extracts, but *in vivo* studies are scarce. We have evaluated the effects of selected procyanidin-rich extracts on oxidative stress in rats in condition of prolonged consumption of these compounds and also after single administration i.e. in postprandial conditions.

Methods: Rats were fed for 8 weeks with diets supplemented with either a grape seed extract (GE), a pine bark extract (PE), or a high-degree polymerized pine bark extract (HPE). An additional study was performed in order to assess the postprandial effect of these extracts on plasma antioxidant capacity. The ferric-reducing antioxidant power (FRAP) and thiobarbituric acid-reactive substances (TBARS) were determined in plasma. For lipid peroxidation study of heart tissue, homogenates were prepared and TBARS were measured after lipid peroxidation induced by $FeSO_4$-ascorbate.

Results: After 8 weeks of dietary treatment, total antioxidant capacity in plasma was significantly higher in the GE and PE groups as compared with the other two groups. Plasma TBARS concentrations and heart susceptibility to peroxidation were not significantly different between the groups. In the postprandial state, by comparing plasma antioxidant capacity 2 hours after ingestion of the different procyanidin-rich extracts (500 mg/kg body weight), we observed that FRAP values were higher in the procyanidin-rich extracts groups as compared with the control group. Moreover, plasma FRAP concentration was significantly higher in the GE group as compared with the other groups.

Conclusion: The results of the present experiment constitute positive evidence for an *in vivo* antioxidant effect at the plasma level of procyanidin-containing plant extracts.

Key words: Grape seed extract, pine (Pinus maritima) bark extract, procyanidins, antioxidant, lipids

Abbreviations: CT, cholesterol; FRAP, ferric-reducing antioxidant power; GE, grape seed extract; HPE, high-degree polymerized pine bark extract; PE, pine bark extract; TBARS, thiobarbituric acid-reactive substances.

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Procyanidins (PA) are among the most abundant polyphenolic compounds in plants and are common constituents of many foods (various fruits, legume seeds, chocolate) and beverages (red wine, beer, cider, tea) [1, 2]. A growing interest is also seen in the utilization of procyanidin-rich extracts as dietary food supplements [3, 4], at least in part, because of their strong *in vitro* antioxidant activity. PAs differ from all other natural polyphenols by their polymeric nature. They are made of flavan-3-ol units and occur with various degrees of polymerization. The phenolic and polymeric nature of PAs makes them good complexants with proteins and explains the astringent character the compounds lend to foods and beverages rich in PAs (e.g., grape skin and seeds, unripe fruits, wine or cider) [5]. The daily intake of PA has been estimated at 0.1–0.5 g [6, 7]. However, the complexity of their chemical structure and the lack of a suitable method for their estimation make these figures uncertain.

PAs have attracted increasing attention in the fields of nutrition, nutritional supplements and preventive medicine due to their potential health effects. *In vitro*, PAs have strong antioxidant activity as scavengers of reactive oxygen species [8, 9]. *In vivo*, PAs inhibit the progression of atherosclerosis [10] and prevent the increase of low-density lipoprotein (LDL)-cholesterol in high-cholesterol-fed rats [11]. Here we have tested and compared the effects of procyanidin-rich extracts on oxidative stress parameters by feeding rats for 8 weeks with diets supplemented with either a grape seed extract (GE), a pine bark extract (PE), or a high-polymerization degree procyanidin-rich extract from pine bark (HPE). The last extract was used to show the relationship between the degree of polymerization and biological effects studied. We also have assessed the postprandial effect of these extracts on plasma antioxidant capacity by giving rats water solutions of these extracts by gavage.

Materials and Methods

Procyanidin-rich extracts

GE-Grape seed extract (Vitaflavan®), PE-pine bark extract (Oligopin®), and HPE, a high-degree polymerization procyanidin-rich extract of pine were supplied from DRT-S.A., (Dax, France). The composition (wt %) of extracts established by gel permeation chromatography and high performance liquid chromatography (HPLC) (provided by DRT) was:

– GE: Phenolic and cinnamic acids (2% – mainly gallic acid); flavonols and flavones (catechin – 12%, epicatechin – 6%); procyanidolic oligomers (dimers – 20%, trimers – 25%, tetra-, penta-, and hexamers – 35%).
– PE: Phenolic and cinnamic acids (14% – mainly ferulate glucoside, ferulic acid, caffeic acid, protoctechic acid and p. cumaric acid); flavonols and flavones (catechin – 8%, taxifoliol – 8%); procyanidolic oligomers (dimers – 20%, trimers – 20%, tetra-, penta- and hexamers – 30%).
– HPE: Phenolic and cinnamic acids (15% – mainly glucoside forms of phenolic and cinnamic acids found in PE); flavonols, flavones, and procyanidolic oligomers (15%); high-molecular weight procyanidins (70%).

Animals and diets

Long-term effects of procyanidin supplementation

The rats were maintained and handled according to the recommendations of the INRA Ethics Committee, in accordance to decree no. 87–848. Weaning male Wistar-Han rats (IFFA-CREDO; L'Arbresle, France), 6 weeks old, weighing 190 ± 1 g (mean ± SEM) were housed in wired-bottomed cages (2 per cage) in a temperature-controlled room (22°C) with a 12-hour light/dark cycle. They were then randomly divided into experimental groups (10 per group) and fed appropriate diets for 8 weeks. Food and distilled water were provided *ad libitum*. The synthetic diets contained (in g/kg): 200 casein, 240 sucrose, 240 dextrose, 220 butter, 50 alphacel, 3 DL-methionine, 2 choline bitartrate, 35 mineral mix, 10 vitamin mix (ICN Biomedicals, Orsay, France). To restrain antioxidant protection the basal vitamin E, Cu, Se, Zn, and Mg supply were limited to one third of the AIN-76 recommended values. Procyanidin-rich extracts containing diets contained 0.5% (wt/wt) of GE, PE, or HPE, respectively. Food intake and body weight were performed once per week. One rat was removed from the experiment because of dental problems causing lower food intake.

At the time of sampling (during the post-absorptive period, namely 0900 hours), rats were weighed and anesthetized with sodium pentobarbital (40 mg/kg body weight, intraperitoneally). Blood was collected from the abdominal aorta into heparinized tubes. Plasma obtained after low-speed centrifugation (2000 × g, 15 minutes), was stored at –80°C for biochemical analysis. The heart was rapidly removed and washed in ice-cold saline solution (9 g NaCl/L), then placed in liquid N2 and stored at –80°C before analysis.

Postprandial effect of procyanidins on plasma antioxidant capacity

Weaning male Wistar-Han rats (IFFA-CREDO), 6 weeks old, weighing 190–210 g were housed in wired-bottomed cages (2 per cage) in a temperature-controlled room (22°C) with a 12-hour light/dark cycle. They were fed a standard rat chow diet and distilled water *ad libitum* be-

fore the experiment. After an overnight fast (12 hours), four groups of rats (5 to 10 per group) were randomly administered water or procyanidins by oral gavage. Blood samples were collected from the retro-orbital sinus.

Analytical procedures

Total cholesterol (TC) (Biomérieux, Charbonnières-les-bains, France) was determined in plasma by enzymatic procedure. Plasma glucose concentration was measured colorimetrically according to Bergmeyer *et al* [12].

The ferric-reducing antioxidant power (FRAP) was determined in plasma using the method of Benzie and Strain [13], which measures the reduction of ferric iron to ferrous form in the presence of antioxidants components. The colorimetric measurement was performed at 593 nm and the reaction was monitored for up to 8 minutes. Results were calculated from a standard scale of $FeSO_4$. Plasma thiobarbituric acid-reactive substances (TBARS) levels were determined by spectrofluorometry (LS 5, Perkin Elmer, Norwalk, CT). A modified method of Ohkawa *et al* [14] was used as previously described [15]. For lipid peroxidation study of heart tissue, homogenates were prepared on ice using a ratio of 1g wet tissue to 9 mL 150 mmol/L KCl using a Polytron homogenizer. Thiobarbituric acid-reactive substances (TBARS) were measured, using a spectrophotometer (Uvikon 941 plus series, Kontron Instruments, St. Quentin en Yvelines, France), in tissue homogenates after lipid peroxidation induced by $FeSO_4$ (2 µmol/L)-ascorbate (50 µmol/L) for 30 minutes in a 37°C water bath in an oxygen-free medium, using a standard of 1,1,3,3-tetraethoxypropane as previously described [15].

Statistical analysis

Statistical analyses were performed using "GraphPad" InStat (GraphPad Software Inc, San Diego, CA) software package. Results were expressed as means ± SEM. All data were subjected to one-way analysis of variance (ANOVA) to compare multiple group means, followed by the Student-Newman-Keuls test to determine statistical significance among the dietary groups. Differences were considered significant when $p < 0.05$.

Results

Long-term effects of procyanidin supplementation

Food intake and body weight were not significantly different between studied groups (Table I). Plasma cholesterol (CT) and glucose concentrations were not significantly different between different dietary groups (Table II).

Total antioxidant capacity of plasma, as measured by the FRAP assay, was significantly higher in the GE and PE groups as compared with the other two groups (Table II). Plasma TBARS concentration and heart lipid susceptibility to peroxidation were not significantly different between the groups (Table II). However, when analyzing the results in comparison with the control group, heart TBARS was significantly lower in the GE group ($p < 0.05$; Student's-t-test).

Postprandial effect of procyanidins on plasma antioxidant capacity

We first looked for the optimal conditions in which procyanidin-rich extracts antioxidant functions could be studied as a function of time and dose after gavage of procyanidin-rich extracts solutions. In this first assay, we used GE as this extract showed the greater antioxidant function *in vivo* in our conditions after 8 weeks of experiment. The evolution of plasma antioxidant capacity, as measured by FRAP (measured at 0.5, 1, 2, 4 hours), with increasing concentrations of GE as a function of time shows that FRAP values were significantly higher with doses of GE higher than 500 mg/kg body weight (kinetic not shown). For the dosage of 500 mg/kg body weight, the higher values were observed 2 hours after GE administration. Figure 1 shows results 2 hours after gavage with various dosages of GE. By comparing plasma antioxidant capacity 2 hours after ingestion of the different procyanidin-rich extracts (500 mg/kg body weight), we observed that FRAP values were higher in the procyanidin-rich extracts receiving groups as compared with the control group (Table III). Moreover, plasma FRAP concentration was significantly higher in the GE group as compared with the oth-

Table 1: Food intake and body weight in rats consuming control or supplemented diet with grape seed extract (GE), pine bark extract (PE), or the high-polymerization degree procyanidin-rich extract (HPE) for 8 weeks[1]

	Control (10)	GE (10)	PE (9)	HPE (10)	ANOVA[2]
Body weight, g	379 ± 11	413 ± 11	389 ± 9	397 ± 7	NS
Food intake, g/day	17.4 ± 0.5	18.1 ± 0.5	15.8 ± 0.4	17.3 ± 0.8	NS

[1] (n) animals per group; values are means ± SEM.
[2] one-way ANOVA; NS $p > .05$.

Table 2: Plasma and heart biochemical parameters in rats consuming control or supplemented diet with grape seed extract (GE), pine bark extract (PE), or the high-polymerization degree procyanidin-rich extract (HPE) for 8 weeks[1]

	Control (10)	GE (10)	PE (9)	HPE (10)	ANOVA (p-value) [2]
Total cholesterol, mM	1.73 ± 0.06	2.06 ± 0.12	1.84 ± 0.12	1.78 ± 0.06	NS
Glucose, mM	9.40 ± 0.10	10.02 ± 0.30	9.46 ± 0.18	9.63 ± 0.24	NS
Plasma FRAP, µmol/L	184 ± 4a	222 ± 11b	229 ± 10b	210 ± 11ab	0.010
Plasma TBARS[4], nmol/mL	3.0 ± 0.1	3.2 ± 0.1	3.2 ± 0.1	3.1 ± 0.1	NS
Heart TBARS[4], nmol/wet wt. g	269 ± 14	224 ± 16	234 ± 11	277 ± 24	NS

[1] (n) animals per group; values are means ± SEM. Values in a row with superscripts not sharing a letter are different, $p < 0.05$;
[2] p-value, one-way ANOVA; NS $p > 0.05$
[3] FRAP, ferric-reducing antioxidant power
[4] TBARS, thiobarbituric acid reactive substances

er two groups supplemented with procyanidin-rich extracts.

Discussion

The present work was designed to assess the effects of long- and short-term administration of procyanidin-rich extracts on selected oxidative stress parameters. A high-polymerization degree procyanidin-rich extract was used to show the relationship between the degree of polymerization and biological effects studied. The results show that long-term GE and PE consumption leads to higher plasma antioxidant capacity. This increased antioxidant capacity of plasma was also observed after measuring GE, PE, and HPE in short-term postprandial conditions. Of particular interest was the comparison of the action of these extracts during long-term supplementation and in postprandial conditions. In fact, it could be argued that the long-term effect could result mainly from the effect on various metabolic systems implicated in free radical production and antioxidant protection, but postprandial effects could result from the presence of significant concentrations of absorbed molecules or their metabolites.

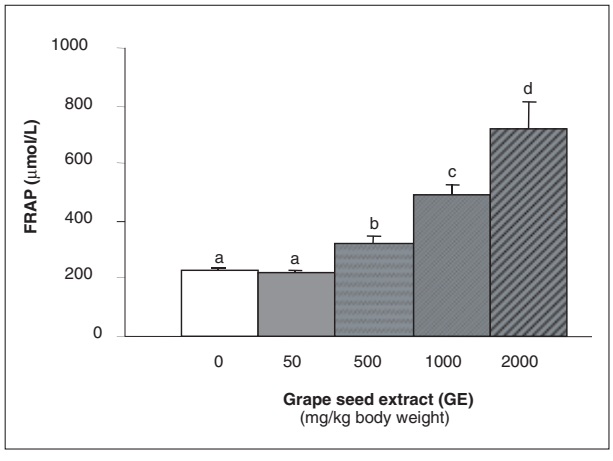

Figure 1: Dose effect of grape seed extract (GE) on plasma antioxidant capacity (FRAP; ferric-reducing antioxidant power) 2 hours after gavage. Values are means ± SEM, 5 animals per group; one-way ANOVA; $p < 0.05$; bar graphs not sharing a letter are different, $p < 0.05$

In general our results are concordant with *in vitro* and animal supplementation studies (8, 9, 10, 16, 17, 18) that have previously described antioxidant potential of procyanidin-rich extracts. Hence, Blaszo *et al* [19] observed that the anti-inflammatory action of pine bark extract in mice was correlated with its antioxidant action. In addition, procyanidins from grape seed extracts (71 mg/kg) leads to significantly decreased tissue peroxidability and to enhanced antioxidant status [20]. In another experimental model, dietary-induced hypercholesterolemic rabbits, Yamakoshi *et al* [10] observed that the reduced aortic malondialdehyde (MDA) level found in animals supplemented with procyanidins was correlated with cholesterol concentration in this tissue. Moreover, rabbits fed procyanidin-supplemented diets showed lower plasma cholesteryl ester hydroperoxyl (ChE-OOH) formation after AAPH induction. In recent work [21] it has been shown that after the meals, catechin and epicatechin were present in conjugated forms in both plasma and urine. In contrast, no procyanidins or conjugates were detected in the plasma or urine of rats. Procyanidins were not cleaved into bioavailable monomers and had no significant effects on the plasma levels or urinary excretion of the monomers when supplied together in the grapeseed extract. It was concluded that the nutritional effects of dietary procyanidins are unlikely to be due to procyanidins themselves or monomeric metabolites with the intact flavonoid ring structure, as they do not exist at detectable concentrations *in vivo*. Thus, the observed increase in antioxidant capacity 2 hours after administration of studied extracts is probably related to the absorption of compounds of lower molecular weight. The antioxidant action of procyanidin-rich extracts was also observed after long-term feeding. This

Table III: Plasma antioxidant capacity measured by FRAP in rats 2 hours after a gavage of water solutions (500 mg/kg body weight) of grape seed extract (GE), pine bark extract (PE), or the high-polymerization degree procyanidin-rich extract (HPE)[1]

	Control (10)	GE (10)	PE (10)	HPE (10)	ANOVA(p-value) [2]
FRAP[3], µmol/L	220 ± 6a	335 ± 10b	292 ± 13c	259 ± 17c	0.0001

[1] (n) animals per group; values are means ± SEM. Values in a row with superscripts not sharing a letter are different, $p < 0.05$
[2] p-value, one-way ANOVA; NS $p>0.05$
[3] FRAP, ferric-reducing antioxidant power

is in agreement with the recently published observation that 6 weeks of oral consumption of pine bark extracts (150 mg/day) significantly increased plasma antioxidant capacity in humans [16]. Especially, after long-term administration, procyanidins may have biological effects even though they are not absorbed into the systemic circulation. As chelating agents they may interact with minerals such as Fe(III) and influence their bioavailability [1]. Furthermore, biological effects of procyanidins in inner tissues could be mediated by some metabolites formed in the colon and then absorbed through the colon barrier. Studies have identified mono- and dihydroxylated phenylpropionic, phenylacetic, and hippuric acid as well as various phenylvalerolactones as flavanol metabolites in plasma [22, 23]. It has been recently observed that feeding rats wine polyphenol extracts for 8 days resulted in excretion of aromatic acid metabolites bearing a phenolic group that has reducing and antioxidant capacity [24]. Results of this later study suggest that aromatic acids may thus make an important contribution to the protection against oxidative stress and explain some of the biological effects reported for procyanidins and other high-molecular weight polyphenols in animals and human studies [1].

In the present study we also evaluated plasma cholesterol concentrations after prolonged administration of procyanidin-rich extracts. We have not observed significant changes in cholesterolemia. Previously published work by Tebib *et al* [11] has shown a beneficial effect of grape seed extracts on plasma cholesterol concentrations. This latter result was obtained in rats that were fed 2% tannins, fat-rich (17%), and cholesterol-supplemented (1%) diets. This discrepancy between these results and our observation probably results from the different contents, of both cholesterol and procyanidin, used. In other work, the hypolipemic effect was not observed in rabbits that were fed a supplemented-cholesterol (1%) and grape seed extract (1%) diet [10], despite the fact that procyanidin leads to a reduction of aortic cholesterol depot in these animals. This previous observation suggests a potential beneficial effect of procyanidins despite the fact that no effect was observed on cholesterolemia.

In conclusion, the results of the present experiment state for an *in vivo* antioxidant effect of procyanidin-containing extracts. The observed results could be related to the metabolic effects of procyanidins (mainly observed during long-term studies) and the presence of some antioxidant molecules or their metabolites (mainly revealed in the postprandial conditions).

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