Anthocyanins increase low-density lipoprotein and plasma cholesterol and do not reduce atherosclerosis in Watanabe Heritable Hyperlipidemic rabbits

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Anthocyanin-rich beverages have shown beneficial effects on coronary heart disease in epidemiological and intervention studies. In the present study, we investigated the effect of black currant anthocyanins on atherosclerosis. Watanabe Heritable Hyperlipidemic rabbits (n = 61) were fed either a purified anthocyanin fraction from black currants, a black currant juice, probucol or control diet for 16 weeks. Purified anthocyanins significantly increased plasma cholesterol and low-density lipoprotein (LDL) cholesterol. Intake of black currant juice had no effect on total plasma cholesterol, but lowered very-low-density lipoprotein (VLDL) cholesterol significantly. There were no significant effects of either purified anthocyanins or black currant juice on aortic cholesterol or development of atherosclerosis after 16 weeks. Probucol had no effect on plasma cholesterol but significantly lowered VLDL-cholesterol and decreased aortic cholesterol accumulation. The erythrocyte antioxidant enzyme glutathione peroxidase was significantly increased by purified anthocyanins and superoxide dismutase was increased by both anthocyanin-containing treatments. Other markers of plasma antioxidant capacity, antioxidant enzymes, protein and lipid oxidation were not affected by any of the anthocyanin treatments. Adverse effects of purified anthocyanins were observed on plasma- and LDL-cholesterol. These effects were not observed with black currant juice, suggesting that black currants may contain components reducing the adverse effects of anthocyanins.

Keywords: Anthocyanins / Antioxidants / Atherosclerosis / Cholesterol / Flavonoids

Received: December 3, 2004; revised: January 31, 2005; accepted: February 1, 2005

1 Introduction

The inverse association between red wine consumption and risk for cardiac diseases have been consistently reported in epidemiology, whereas recent studies on alcohol, white wine, beer, or spirits have been less convincing [1 – 4]. The protective effects of red wine and other red grape products have been substantiated by experimental studies [1, 2, 5], supporting the beneficial effects of the red wine polyphenols [5, 6]. One of the main differences in the composition of the polyphenol fraction in red and white wine is the content of anthocyanins; *in vitro* studies have demonstrated a protective effect of anthocyanins on oxidation of low-density lipoprotein (LDL) and endothelial dysfunction [6]. Black currants (BCs) have a high content of anthocyanins (250 mg/100 g) [7] and may therefore protect against atherosclerosis as a nonalcoholic alternative to red wine. Watanabe Heritable Hyperlipidemic (WHHL) rabbits are LDL receptor-deficient and serve as a model of human homozygous familial hypercholesterolemia with morphology of atherosclerotic lesions similar to that in humans [8]. The hyperlipidemia in WHHL homozygotes is mainly the result of increased LDL levels similar to that in genetically predisposed humans. No previous studies have been carried out to investigate the influence of anthocyanins on plasma lipids, tissue cholesterol levels, or markers of oxidation in

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Abbreviations: AAS, 2-amino-adipic semialdehyde; **AF**, anthocyanin fraction; **BC**, black currant; **FRAP**, ferric reducing ability of plasma; **GPx**, glutathione peroxidase; **LDL**, low-density lipoprotein; **MDA**, malondialdehyde; **QR**, quinone reductase; **SOD**, superoxide dismatase; **TBARS**, thiobarbituric acid-reactive species; **TEAC**, trolox equivalent antioxidant capacity; **VLDL**, very-low-density lipoprotein

relation to the development of atherosclerosis. We therefore undertook a study in WHHL rabbits to test the hypothesis that treatment with anthocyanins in BC juice would reduce the risk of atherosclerosis.

2 Materials and methods

2.1 Animals, housing, and clinical observations

Animal experiments and housing procedures were performed in accordance to the Danish Animal Experimentation Act on a license granted by the Ministry of Legal Affairs and the Convention ETS 123 of the Council of Europe. Sixty-one homozygous WHHL rabbits of both sexes (26 males, 35 females) with 22.1 ± 2.12 mM plasma cholesterol (mean ± SD), 6.21 ± 2.56 mM triglycerides, and 1.03 ± 0.27 kg body weight at 6 weeks of age were obtained from our own breeding colony. A previous study has shown that the aortas are free from lesions at this age [9]. The study and all procedures were approved by the Danish Animal Experimental Inspectorate.

2.2 Experimental design

The rabbits were allocated to four groups (I–IV) of 14–16 animals based on plasma cholesterol and triglyceride concentrations, litter, body weight, and sex. From the seventh week of life the control group (group I, $n = 16$) received daily 100 g standard diet (No. 2113; Altromin International, Lage, Germany). Two anthocyanin treatment groups received either 100 g standard diet added an anthocyanin fraction (AF) purified from BC (Polyphenols Laboratories AS, Sandnes, Norway) (100.3 ± 12.8 mg pure anthocyanins/100 g diet, group II, $n = 16$) or 100 g standard diet and BC juice (58 mg anthocyanins/100 mL; Valloe Saft, Koege, Denmark) *ad libitum* instead of drinking water (group III, $n = 16$) (see Table 1). A positive control group (IV, $n = 14$) received 100 g/d standard diet supplemented with 0.5 w/w% probucol (Sigma Chemicals, St. Louis, MO, USA) in the feed. The AF dose was chosen to result in similar total anthocyanin intake as in the juice group (see Table 2), based on a pilot study investigating the palatability of the juice (data not shown). The anthocyanin content in the AF was 35 g/100 g and the total phenol content was 47 g/100 g determined by Folin-Ciocalteu reagent [10]. The other phenolics contained in the AF were mainly hydroxycinnamic acid esters and it contained also other BC berry components, mainly monosaccharides (information provided by the manufacturer). All animals except for those in the juice group had free access to tap water. The feed intake in all groups and liquid intake in the control and juice group was recorded daily and body weight weekly. After 16 weeks of treatment, the animals were sacrificed by intravenous injection of pentobarbital (100 mg/kg body weight) into the marginal ear vein and sampling was performed as described elsewhere [10]. The liver and the heart were weighed and 38 livers randomly selected from all treatment groups were frozen in liquid nitrogen and stored at -80°C until analyses.

2.3 Blood sampling and analyses of blood lipid and antioxidant markers

Blood samples were collected in EDTA tubes from the marginal ear vein of unanesthetized animals fasted overnight before treatment and then monthly until termination. Plasma cholesterol and triglycerides were measured monthly until termination, where in addition the concentration of lipoproteins was determined, using an automatic analyzer (Hitachi 912; Roche Diagnostics, Mannheim, Germany) with < 3% CV. Lipoproteins were separated by single density gradient ultracentrifugation for 18 h at 21°C [11]. The following oxidation parameters were determined before treatment and at termination on a Cobas Mira S analyser (Triolab, Brøndby, Denmark): the ferric reducing ability of plasma (FRAP) [12] (CV < 2%), the trolox equivalent antioxidant capacity of plasma (TEAC) (Total Antioxidant Status kit Randox NX2332, Ardmore, UK), the activities in erythrocyte lysates of glutathione reductase (GR) (CV 5.2%), glutathione peroxidase (GPx) (CV 8.1%), and catalase (CAT) (CV 8.4%) according to Wheeler *et al.* [13], and superoxide dismutase (SOD) (CV 9.6%) and hemoglobin using commercial kits (Randox; Cat. No. SD125 and HG980). Plasma protein oxidation, determined as 2-aminoadipic semialdehyde residues (AAS) (CV 10.9%) and malondialdehyde (MDA) in lipoproteins (CV 5.4%) were determined as described previously [14, 15], and vitamin C was determined in six animals randomly chosen from each treatment group according to Kall and Andersen [16] (CV 1.2%). Thiobarbituric acid-reactive species (TBARS) in plasma [17] (CV 7%) was determined at 6 weeks and at termination.

2.4 Determination of phase 2 enzymes in plasma, red blood cells (RBCs) and liver

At termination, the activity of quinone reductase (QR) and glutathione-*S*-transferase (GST) was determined in duplicate liver cytosol samples (diluted 300 times) according to Ernster [18] and Habig *et al.* [19], respectively, adapted to a Cobas Mira analyzer (CV < 7%).

2.5 Urine collection, excretion of anthocyanins, and determination of 2,3-dinor-TxB_2

Urine samples for anthocyanin analyses were obtained from eight animals in each group before start of treatment, and at 1, 2, 3, 5, 7, 9, 13, and 16 weeks of treatment. Collection

was initiated when the test diets were administered and continued for 4 h using collection trays containing 80 mL aqueous citric acid (0.5 M) for stabilization with 0.7 mg/L cyanidin-3,5-diglucoside (Cy-3,5-diglc) as an internal standard. A longer collection time was impossible due to rapid degradation of the anthocyanins in the collection trays. Urine samples containing spilled juice were easily recognized based on colorization and omitted. Samples were collected, treated, and analyzed according to Nielsen *et al.* [20] (CV% < 10). An indicator of platelet activation, urinary 2,3-dinor-TxB_2, was analyzed from 16-h urine samples (collected after 16 weeks of treatment) of five animals per group. Samples were purified [21], diluted 1 : 200, and analyzed with ELISA (#519051; Cayman Chemicals, Ann Arbor, MI, USA) (CV% 9). Urinary creatinine was measured by the Jaffe method (Konelab 20, Espoo, Finland).

2.6 Biochemical and microscopic evaluation of aortic atherosclerosis

The cholesterol content in the parts of intima-inner media was determined as previously described [10] and expressed as µmol of total cholesterol per square centimeter of aorta and as nmol total cholesterol per mg wet weight of aortic tissue. Microscopic quantification of atherosclerotic lesions was performed by point counting at the level of first intercostal arteries as described previously [10] and was expressed as the ratio of intima to media and as the area of intima in square millimeters.

2.7 Anthocyanin and vitamin C content in the experimental diets

The concentration and batch-to-batch content of anthocyanins in the anthocyanin treatments were monitored every two weeks throughout the study, essentially as described previously [22], see Table 1. The vitamin C content in the juice and the diet was monitored every two weeks throughout the study according to Kall and Andersen [16] with the modification that the feed was extracted with 2% w/v metaphosphoric acid/0.1% w/v aqueous oxalic acid (Table 1).

Table 1. Content of vitamin C and anthocyanins in test diets and BC juice (mg/100 g)

	I. Control	II. AF	III. Juice	IV. Probucol
Vitamin C	3 ± 2	3 ± 2	34.5 ± 0.5	4 ± 3
Dp-3-glc	n.d.	8.6 ± 1.6	4.1 ± 0.1	n.d.
Dp-3-rut	n.d.	47.2 ± 5.9	31.0 ± 0.8	n.d.
Cy-3-glc	n.d.	5.7 ± 1.0	1.9 ± 0.1	n.d.
Cy-3-rut	n.d.	38.7 ± 5.4	21.6 ± 0.6	n.d.
Total anthocyanins	n.d.	100.3 ± 12.8	58.5 ± 1.5	n.d.

Mean ± SD of feed analyses performed every 2nd week is given (n = 8). n.d., not detectable; Dp, delphinidin; cy, cyanidin; glc, glucoside; rut, rutinoside

2.8 Statistics

Data were tested for normal distribution by Shapiro-Wilks test and for homogeneity of variance by standardized residuals plots or by Levenes test. When necessary, logarithmic transformations were performed. From groups I–III data were analyzed by analysis of variance followed by Duncan's test. Data from group IV (positive control) were compared separately with data from controls by Student's *t*-test. Data, which could not be analyzed by analysis of variance (ANOVA) or *t*-test due to lack of normal distribution or homogeneity of variance, were analyzed by the Wilcoxon rank scores test. Time trends in blood lipids by group, sex, and litter were analyzed by repeated samples analysis of covariance using the GLM procedure with the Greenhouse-Geisser test since the sphericity criterion was not met. Significant interactions between time and group were further analyzed by least squares means. The number of animals per group having atherosclerotic lesions was analyzed by Fisher's exact test. Correlation analyses were performed as Pearson correlations. $P < 0.05$ was considered significant throughout. Statistical Analysis System (SAS) software was used for all analyses (release 8.1; SAS Institute, Cary, NC, USA).

3 Results

3.1 Animal welfare and compliance to treatments

No effect of the treatment on clinical appearance was observed in any of the rabbits. The content of anthocyanins in the feed and juice is shown in Table 1. The weight gain was significantly higher ($P < 0.05$) in the juice and probucol groups than in the control group (Table 2). A significant decrease in the relative fluid intake ($P < 0.005$) was detected in the juice group in comparison to the control. Urinary excretion of anthocyanins was only observed in the groups dosed with anthocyanins, and was significantly higher in the juice group than in the AF group (Table 3). Also a higher plasma vitamin C level ($P < 0.0005$) was observed in the juice group than in the other groups, whereas a significantly lower ($P < 0.05$) vitamin C plasma level was detected in the groups dosed with AF and probucol in comparison to the control (Table 3).

3.2 Plasma and lipoprotein cholesterol, triglycerides, and protein

There was a significant increase in plasma cholesterol with time in all groups and a significant time*group interaction ($P < 0.01$). The AF group had, however, a significantly higher and increasing level of plasma cholesterol compared to all other groups at time 4, 12, and 16 weeks (Fig. 1). Also

Table 2. Body weight, feed, and fluid intake of WHHL rabbits and dose of test compounds

	No. of animals	Body weight		Relative feed intake	Fluid intake	Dose of test compounds
		Before	Gain			
		(kg)		(g/kg bw/day)	(g/kg bw/day)	(mg/kg bw/day)[a)]
I. Control	15	1.23 ± 0.21	1.43 ± 0.20	48.6 ± 3.6	115.1 ± 35.6	
II. AF	16	1.25 ± 0.15	1.51 ± 0.18	47.0 ± 3.1		47.0 ± 3.1[b)]
III. Juice	16	1.22 ± 0.18	1.66 ± 0.17*	45.2 ± 3.8	84.7 ± 11.4**	48.0 ± 6.5[b)]
IV. Probucol	14	1.26 ± 0.19	1.65 ± 0.25*	45.6 ± 4.0		226.8 ± 17.8

All data are mean ± SD. bw, body weight
a) Calculation based on relative feed and juice intake and results in Table 1
b) Total anthocyanins
* $P < 0.05$ to group I, ** $P < 0.005$

Table 3. Concentration of vitamin C in plasma and urinary excretion of anthocyanins

	I. Control	II. AF	III. Juice	IV. Probucol
Vitamin C plasma[a)] (μg/mL)	7.85 ± 1.93	5.10 ± 1.45**	11.71 ± 1.81***	4.94 ± 1.56**
Anthocyanin excr.[b)] (μg/4 h)	n.d.	3.9 ± 7.0*	8.8 ± 6.8**	n.d.

Data are given as mean ± SD. n.d., not detectable
a) Determined at 16 weeks of treatment
b) Determined at 1, 2, 3, 5, 7, 9, 13, and 16 weeks of treatment
* $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$ relative to controls

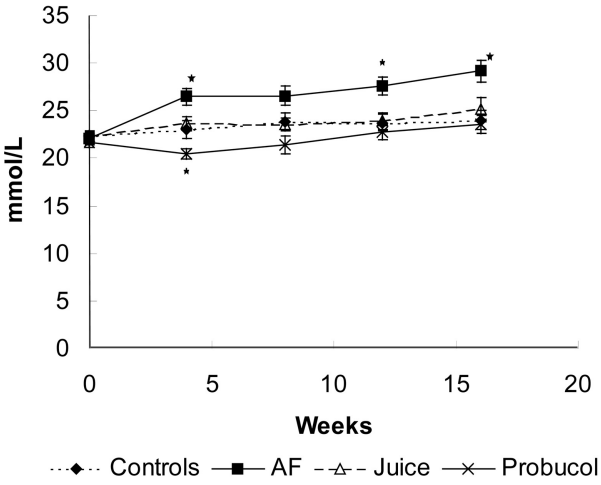

Figure 1. Plasma cholesterol concentration during the 16 weeks of treatment. *$P < 0.05$ relative to the control at each timepoint.

LDL cholesterol was significantly increased and triglycerides in LDL borderline increased after 16 weeks ($P < 0.05$ and $P = 0.056$, respectively) in the AF group in comparison to the control, whereas BC juice and probucol lowered very-low-density lipoprotein (VLDL) cholesterol (Fig. 2). Plasma levels of triglycerides were not different between the groups (Table 4). In the probucol group only a transitory decrease in plasma cholesterol ($P < 0.05$) was observed at 4 weeks and a borderline lower level at 8 weeks ($P = 0.06$) (Fig. 1).

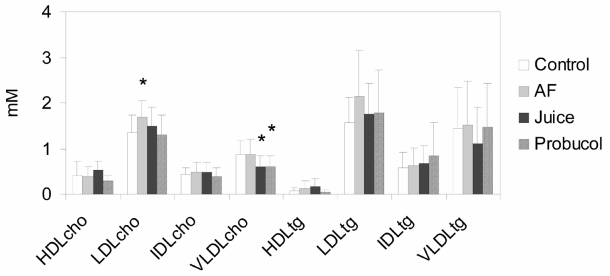

Figure 2. Cholesterol (cho) and triglycerides (tg) in lipoproteins after 16 weeks of treatment. LDL-, IDL-, and VLDL-cholesterol values are divided by 10.
* $P < 0.05$ relative to the control.

3.3 Markers of oxidation and activated platelets

Both MDA in LDL and VLDL as well as FRAP were significantly increased in the probucol group, whereas plasma TEAC, AAS, and TBARS were unaffected in all groups (Table 4). The antioxidant enzyme SOD was significantly increased in both the BC juice group and in the AF group, and GPx was increased by the AF treatment. The other antioxidant enzymes were not affected by any of the treatments. Furthermore, liver GST, QR, and urinary 2,3-dinor-TxB_2 were unchanged in all treatment groups.

3.4 Aortic atherosclerosis

The cholesterol content in the ascending, the thoracic and the abdominal aorta in all treatments was comparable

Table 4. Plasma triglycerides, biomarkers of oxidative stress, activity of phase 2 enzymes, and urine marker of activated platelets

Marker[a)]	I. Control	II. AF	III. Juice	IV. Probucol
Triglycerides (mmol/L)	3.76 ± 1.37	4.42 ± 2.46	3.79 ± 2.15	4.20 ± 2.47
TEAC[b)] (mmol/L)	0.857 ± 0.149	0.963 ± 0.401	0.947 ± 0.234	1.13 ± 0.22
FRAP[b)] (mmol/L)	591 ± 88	651 ± 183	606 ± 122	791 ± 190**
AAS[c)] (nmol/g protein)	191 ± 10	183 ± 28	176 ± 30	195 ± 17
TBARS[d)] (µmol/L)	1.54 ± 0.32	1.71 ± 0.37	2.07 ± 1.21	1.80 ± 0.26
MDA[d)] HDL (nmol/g protein)	153 ± 58	148 ± 41	159 ± 41	165 ± 57
MDA[d)] LDL (nmol/g protein)	480 ± 181	441 ± 201	380 ± 178	1207 ± 399***
MDA[d)] VLDL (nmol/g protein)	1194 ± 350	852 ± 495	762 ± 413	2177 ± 1786*
GR[e)] (U/g hemoglobin)	8.74 ± 0.99	8.63 ± 1.08	8.98 ± 0.94	8.11 ± 0.67
GPx[e)] (U/g hemoglobin)	163 ± 26	217 ± 43***	167 ± 34	179 ± 54
SOD[e)] (U/g hemoglobin)	1388 ± 108	1401 ± 162	1490 ± 148**	1290 ± 152
CAT[e)] (U/g hemoglobin)	9.21 ± 1.54	9.97 ± 1.04	9.17 ± 1.97	8.34 ± 1.99
GST[f)] liver (U/mg protein)	961.9 ± 90.2	959.3 ± 167.1	942.6 ± 238.5	883.4 ± 206.3
QR[f)] liver (U/mg protein)	17.42 ± 4.85	17.49 ± 4.99	17.06 ± 6.04	17.04 ± 3.24
TxB_2[g)] urine (nmol/mol creatinin)	4952 ± 4581	4539 ± 3820	7405 ± 5335	4551 ± 1992

a) All biomarkers are determined after 16 weeks of treatment
b) Antioxidant capacity of plasma. TEAC, trolox equivalent antioxidant capacity; FRAP, ferric reducing ability of plasma
c) Oxidation of plasma proteins. AAS, 2-amino-adipic semialdehyde residues
d) Oxidation of plasma lipids. TBARS, thiobarbituric acid reactive species; MDA, malondialdehyde
e) Antioxidant enzymes. GR, glutathione reductase; GPx, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase
f) Phase 2 enzymes. GST, glutathione *S*-transferase; QR, quinone reductase
g) Urine marker of activated platelets. TxB_2, 2,3-dinor-thromboxane B2
* $P < 0.05$, **$P < 0.05$, ***$P < 0.0005$ to control

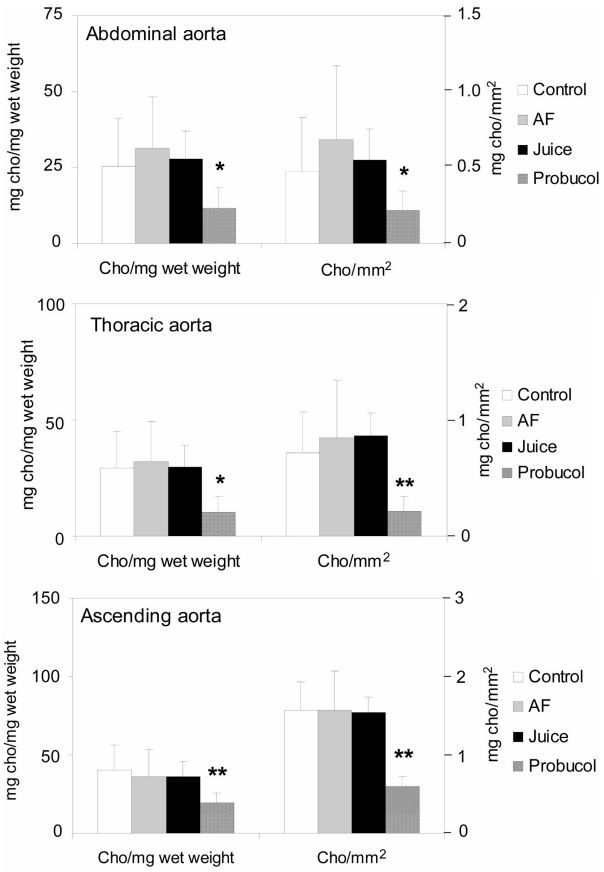

Figure 3. Accumulation of cholesterol in the aorta. * $P < 0.05$ and ** $P < 0.005$ relative to the control.

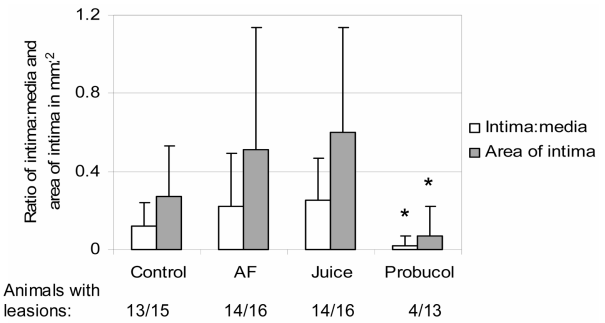

Figure 4. Microscopic quantification of atherosclerosis expressed as the ratio intima : media and the area of the intima. The number of animals with lesions per group is given below the graph.
* Significantly different with Wilkoxon's test.

between the BC groups and the control, while a significant decrease was recorded in the probucol group (Fig. 3). The microscopic quantitative evaluation of atherosclerosis confirmed the protective effect of probucol (Fig. 4). Also the number of animals with atherosclerotic lesions was significantly reduced for this group ($P = 0.0056$).

4 Discussion

The present study does not suggest a protective effect of BC anthocyanins on atherosclerosis in WHHL rabbits. On the contrary, our findings suggest an aggravating effect of

anthocyanins. The AF was found to increase plasma and LDL cholesterol significantly but did not increase aortic atherosclerosis. The increase in plasma cholesterol by AF but not by BC juice indicates that the adverse effect of the anthocyanins is counteracted by other components in the juice, not contained in the employed AF isolated from BCs.

We have recently shown, that the mechanisms for absorption and excretion of anthocyanins in WHHL rabbits are very similar in humans [20]. The significantly higher urinary excretion of anthocyanins from juice-treated than from the AF-fed rabbits (Table 3) are probably explained by the 24 h free access to the juice, whereas the feed was provided at initiation of the 4 h urine collection after about 18 h of fasting. However, the possibility of a lower absorption from a solid than from a liquid source cannot be excluded. In addition, we previously observed a food matrix effect resulting in increased absorption of anthocyanins from BC juice compared to pure anthocyanins dissolved in a citric acid solution indicating that BC juice contains compounds which increase anthocyanin absorption [20]. The significantly lower fluid intake in the juice group could be due to a low palatability of the juice to the rabbits. The animals in this group did, however, receive the same amount of anthocyanins as the AF group. The anthocyanin dose corresponds to about 3 g anthocyanin per day in a 70-kg man. If the difference in metabolic rate between rabbit and human is taken into account, the daily dose corresponds to only about 1/3 in humans, thus only 1 g pure anthocyanin equivalent to about 400 g BCs [7]. A shortage of antioxidants in the diet has been proposed to promote coronary heart disease through accumulation of oxidized LDL in macrophages [23]. Even though anthocyanins are generally regarded as very potent antioxidants, little is known about their actual function as antioxidants *in vivo*. In the present study, we observed no antioxidative effects of any of the anthocyanin treatments determined by TEAC, FRAP, AAS, TBARS, or MDA in lipoproteins. The juice and AF treatment did, however, affect the endogenous antioxidant defense by increasing the activity of SOD and GPx, respectively. The effect on GPx is in line with our previous observations where an increase was observed in humans after treatment with red grape skin extract [24]. This increase in activity of the antioxidant enzymes does, however, not seem to be related with a protection against development of atherosclerosis in WHHL rabbits.

Activated platelets are known to increase the formation of atherosclerotic plaques [25]. Anthocyanins have been found to reduce platelet aggregation in cholesterol-fed rabbits and in humans [1, 26]. In the present study, however, no significant difference between the treatments was detected in the urinary marker of platelet activation, 2,3-dinor-TxB_2.

The entry and accumulation of LDL cholesterol in the aortic wall is an essential step in the atherogenesis and an increase in plasma LDL cholesterol is thus thought to be associated with an increased risk of atherosclerosis. AF increased plasma- and LDL-cholesterol in the present study. However, we observed no significant increases in either biochemical or microscopic markers of atherosclerosis. The BC juice had no significant effects on plasma- or LDL-cholesterol but lowered VLDL-cholesterol. This effect was apparently also unrelated to the development of atherosclerosis. This suggests, that the BC juice contains components, which are able to counteract the adverse effects on plasma- and LDL-cholesterol induced by the anthocyanins. Other studies in cholesterol-fed rabbits or hamsters have shown that red wine, dealcoholized red wine, or red grape juice, but not whiskey, beer, ethanol, or white wine reduce the area of aorta covered with plaques [1, 2] and reduce plasma cholesterol [5]. However, Bentzon *et al.* [27] found no effect on aortic atherosclerosis of red wine in apolipoprotein E-deficient mice. In these previous studies, the anthocyanin treatments used all contained large quantities of other berry or wine components. Pure anthocyanins were recently shown to elevate plasma levels of homocystein in rats, a recognized risk factor of vascular disease [28]. Furthermore, recent studies on red wine polyphenols suggest that it may rather be the procyanidins contained in red wine, that exert the anti-atherogenic properties [29].

Since the AF employed in the present study also contained other components from BCs than anthocyanins it cannot be definitively concluded, that the observed adverse effects on plasma- and LDL-cholesterol was due to the anthocyanin content. The impurities in the product were mainly sugars and other minor phenolics co-existing with the anthocyanins in BCs and they would thus also be present in the BC juice. Further studies using pure anthocyanins are nevertheless needed to confirm our observations, but considering the high content of anthocyanins in the purified AF it seems highly unlikely that the anthocyanins would have a protective effect against atherosclerosis.

Since rabbits have *de novo* synthesthis of vitamin C, we did not suspect the vitamin C in the juice treatment to affect the plasma vitamin C level in the rabbits. However, the juice actually resulted in elevated plasma vitamin C concentrations. We have recently dosed WHHL rabbits with vitamin C and observed no protective effects of this treatment on plasma or lipid cholesterol or on atherosclerosis development (unpublished data). The reduced plasma vitamin C level in the AF and probucol groups may result from the redox capacity of those treatments either leading to a negative feedback on the production of vitamin C or in a prooxidant reaction thus consuming plasma vitamin C. A plasma vitamin C sparing effect of grape skin extracts have

previously been suggested in humans [24] supporting the former explanation.

Probucol had only transient influence on plasma cholesterol. This is in line with the literature [15]. The theory of probucol reducing the risk of atherosclerosis by removing cholesterol from plasma by oxidation [15] is consequently not supported by the present study, although we did observe an increased level of MDA in LDL and a concomitant decrease in atherosclerosis in the probucol-fed rabbits.

In conclusion, our findings do not support the hypothesis of an anti-atherogenic effect of anthocyanins. On the contrary, an anthocyanin-rich preparation from BCs was found to increase plasma- and LDL-cholesterol, which are known risk markers for atherosclerosis. No significant increase in the development of arterial plaques and risk of atherosclerosis was observed after 16 weeks of treatment with anthocyanins. More studies on anthocyanins are warranted to confirm these results and to investigate the potential adverse effect of purified anthocyanin preparations in humans.

The authors want to thank Kobra Fahimeh Hansen, Anni Schou, Magareta Bertram, Joan Frandsen, Morten Andreasen, Rikke Johnsen, and Vibeke Kegel for excellent technical assistance. The anthocyanin fraction was kindly provided by Polyphenols Laboratories, Sandnes, Norway, and analyzed for total phenol content by Alan Mortensen, Chr. Hansen A/S, Denmark. This research was supported by the European Unions 5th framework as part of project QLK1-1999-00124. This paper does not necessarily reflect the EUs views and do not anticipates the Commissions future policy in this area. Support to this research project was also provided by the Danish Research Agency, FELFO grant No. 2056-03-0042.

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