The effect of grape-skin extract on oxidative status

Jette F. Young[1], Lars O. Dragsted[2], Bahram Daneshvar[2], Søren T. Lauridsen[2], Max Hansen[2] and Brittmarie Sandström[1]*

[1]*Research Department of Human Nutrition, Royal Veterinary and Agricultural University, Frederiksberg, Denmark*
[2]*Institute of Food Safety and Toxicology, Danish Veterinary and Food Administration, Søborg, Denmark*

(Received 5 July 1999 – Revised 14 December 1999 – Accepted 8 February 2000)

Epidemiological studies indicate that moderate alcohol consumption, particularly wine, reduce the risk of CHD. The present study was designed to investigate the effect of grape-skin extract on markers of oxidative status. The study was designed as a randomised crossover. A diet with a low content of flavonoids was served with strict control of intake in two consecutive 1-week intervention periods to fifteen subjects (nine women, six men) divided randomly into two groups. During one of the weeks the subjects from either group consumed 200 ml grape-skin extract in water (1 mg extract/ml) at each of three daily meals (31·3 mg total phenolics, including 9·0 mg catechin). An increased activity of glutathione reductase and a borderline increase of glutathione peroxidase activity in erythrocytes were observed after grape-skin intervention, while the intervention had no significant effect on superoxide dismutase or catalase. Likewise, no effect was found on 2-aminoadipic semialdehyde (AAS) residues, a plasma protein oxidation product, or on malondialdehyde in plasma or in LDL, which are markers of lipoprotein oxidation. A marginal effect of grape-skin intervention was observed on plasma ascorbate levels. Intake of the experimental diet significantly reduced plasma vitamin C and plasma AAS in both groups. This effect was most pronounced in the particular week with no grape-skin extract addition. We speculate that grape-skin extract may have a sparing effect on vitamin C. The effects of the experimental diet may be partly ascribed to a low content of several fruit- and vegetable-related antioxidants like flavonoids and vitamin C and a relatively high content of carrot-derived antioxidants, such as carotenes.

Grape-skin extract: Antioxidant status: Flavonoids

Epidemiological studies indicate that moderate alcohol consumption may reduce the risk of CHD (Klatsky & Armstrong, 1993; Grønbæk *et al.* 1995). However, it is uncertain whether alcohol is the sole effective component or whether other components may contribute as well. Some epidemiological studies find red wine intake in particular to be associated with reduced risk of CHD (Renaud & de Lorgeril, 1992; Grønbæk *et al.* 1995), but other results do not confirm this association (Klatsky & Armstrong, 1993). In search of a mechanism for a potential protective effect, a number of human studies have investigated the effect of red wine consumption on antioxidant status in plasma (Fuhrman *et al.* 1995; Whitehead *et al.* 1995) as a surrogate marker for the aortic intima and *in vitro* susceptibility of LDL to oxidation (Kondo *et al.* 1994; Fuhrman *et al.* 1995; de Rijke *et al.* 1996). Studies comparing red wine with other alcohol-containing beverages showed reduced LDL oxidation only after red wine consumption (Kondo *et al.* 1994; Miyagi *et al.* 1997). Miyagi *et al.* (1997) did not observe any postprandial reduction in LDL susceptibility to oxidation after intake of red grape juice. However, following 7 d intervention with concentrated red grape juice, Day *et al.* (1997) found an increased serum antioxidant capacity as well as a reduced LDL oxidation, indicating that alcohol may not be the sole component responsible for this effect.

Antioxidative properties of red wine and/or grapes are believed to be due to the content of phenolic components. An inverse correlation between phenolic content and *in vitro* oxidation of LDL has been found (Frankel *et al.* 1995; Meyer *et al.* 1997). In comparison to red wine, white wine has a much lower antioxidant capacity (Whitehead *et al.* 1995) and is less protective towards LDL oxidation *in vitro* (Frankel *et al.* 1995) possibly because of the lower phenolic content (Hertog *et al.* 1993; Frankel *et al.* 1995). Fuhrman *et al.* (1995) studied the effect of red and white wine consumption and found a reduction in LDL oxidation after 2 weeks of red wine intake but a slight increase in LDL

Abbreviations: AAS, 2-aminoadipic semialdehyde; CAT, catalase; Gpx, glutathione peroxidase; GR, glutathione reductase; MDA, malondialdehyde; SOD, superoxide dismutase.

* **Corresponding author:** Professor Brittmarie Sandström, fax +45 35 28 24 83, email bsa@kvl.dk

oxidation after white wine consumption. However, de Rijke *et al.* (1996) did not find any effect of either red or white wine intake on LDL oxidation. If the antioxidative properties of red wine can be attributed to the phenolics in red wine and/or grapes, an extract of these compounds should demonstrate a similar effect. *In vitro* the effects of extracted phenolics from red wine showed an increase in the lag phase of LDL oxidation (Carbonneau *et al.* 1997). Intake of this extract caused an increase in total antioxidant capacity of plasma, whereas *in vitro* susceptibility to oxidation of LDL was not affected (Carbonneau *et al.* 1997).

In the present study we investigated the effect of grape-skin extract intake on markers of oxidative status in the blood. Fifteen subjects consumed grape-skin extract resulting in a daily phenolic intake corresponding to that of approximately 380 ml red wine (de Rijke *et al.* 1996) for 7 d while keeping a strictly controlled experimental diet specifically designed to have a low content of flavonoids.

Subjects and methods

Study design and diet

The study design was a randomised crossover with a 2 d run-in period and two 1-week intervention periods in succession, as shown in Fig. 1. The experimental diet was composed of common foods selected to comply with a low content of flavonoids. The diet consisted of three menus consumed each week on days 1 and 4, 2, 5 and 7 and 3 and 6 respectively (Table 1). The subjects were divided into two groups. During one of the weeks the subjects from either group consumed 200 ml grape-skin extract (for details see later) in water (1 mg/ml) at each of three daily meals, i.e. a total daily intake of 600 mg grape-skin extract. The content of macronutrients and selected micronutrients in the diet was calculated using a computer program (Dankost; Danish Catering Center, Herlev, Denmark) which is based on the Danish Veterinary and Food Administration composition database (Møller, 1989) (Table 2). Each subject's energy requirement was estimated from body weight and degree of physical activity. All meals were prepared at the Research Department of Human Nutrition, Frederiksberg, Denmark, in individual portions

Table 1. Composition of experimental diet (g/d) at an energy intake of 10 MJ

Meal	Component	Menu 1	Menu 2	Menu 3
Breakfast	White bread roll with carrot	78	78	78
	Butter	6	6	6
	Cheese	30	30	30
	Soured milk	200	200	200
	Rye-bread crumb	30	30	30
Lunch	Rye bread	100	100	100
	Butter	8	8	8
	Tuna salad	50		
	Ham	15		
	Egg salad		50	
	Turkey		15	
	Cottage cheese and ham			60
	Roast beef			15
	Carrot salad	155	136	155
Dinner	Beef stew	270		
	Mashed potatoes	257		
	Cheese sauce		270	
	Pasta		70	
	Meat sauce			275
	Rice			70
Snack	White bread roll with carrot	117	117	117
	Cream cheese	45	45	45
	Carrot cake		60	
	Sponge cake	51		51
Milk		250	250	250

Table 2. Calculated macronutrient and selected micronutrient content of the diet/d per 10 MJ*

Nutrient	Quantity
Protein (g)	88†
Fat (g)	101‡
Carbohydrate (g)	252§
Vitamin A (μg retinol equivalents)	2981
Vitamin E (mg tocopherol equivalents)	6·0
Vitamin C (mg)	23
β-Carotene (mg)	17·4

* Calculations were made using the computer program Dankost (Danish Catering Center, Kilnagleary, Carrigaline, Cork, Republic of Ireland) which is based on the Danish Veterinary and Food Administration composition database (Møller, 1989).
† 16 % of energy.
‡ 39 % of energy.
§ 45 % of energy.

Day of study	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Restricted diet*	x	x															
Group A			-	-	-	-	-	-	-	+	+	+	+	+	+	+	
Group B			+	+	+	+	+	+	+	-	-	-	-	-	-	-	
Blood sample		x	x						x	x						x	x

Fig. 1. Schematic illustration of the crossover design. *Instructions to omit all flavonoid-containing food items for 2 d before intervention; (−), experimental diet with low-flavonoid content; (+), experimental diet with low-flavonoid content and 600 mg grape-skin extract/d (Grape polyphenol powder P2157; Quest International, Kilnagleary, Carrigaline, Cork, Republic of Ireland). For details of experiment diet see Tables 1 and 2; for details of daily polyphenol intake see Table 3.

according to energy requirement. One of the daily main meals was taken at the department while remaining (cold) meals were provided daily for intake at home. Subjects were instructed to return any leftovers to the department for weighing and subtraction from planned intake. Freeze-dried coffee powder was provided for preparation of coffee. Coffee intake varied between subjects but was held constant over the 2 weeks. No other foods and drinks than those provided from the department were allowed. Fasting blood samples were collected in the morning of day 0, 1, 7, 8, 14, 15, thus reflecting the preceding 24 h, i.e. sample days 0 and 1 reflect baseline concentrations before intervention (day −1 and day 0) and the sample day 8 reflects the last 24 h of the first intervention week (day 7). The samples will be referred to in the text according to the 24 h they reflect. Body weight was determined before and after intervention to check weight stability. During the 2 d preceding the two 1-week intervention periods, the subjects were instructed to exclude flavonoid-containing foods from their diet: tea, wine, spices and all fruits and vegetables except potatoes were excluded from their diet.

Grape-skin extract

Grape polyphenol powder (P2157; Quest International, Kilnagleary, Carrigaline, Cork, Republic of Ireland) was an extract of black grapes (*Vitis vinifera*) standardised for colour strength and polyphenol content. The daily intake of some of the quantitatively most important compounds in the grape-skin extract is listed in Table 3. The polyphenol content was analysed on the same batch as used for the intervention by an independent certified laboratory on behalf of the manufacturer.

Subjects

Fifteen subjects (nine women, six men) volunteered for the study. The average age was 24 (range 21–33) years and average BMI was 22·3 (range 19·1–24·4) kg/m^2. None of the subjects were pregnant, lactating, or had any chronic illness, and all were non-smokers. Subjects received oral and written information about the study, and gave their written consent. The subjects were initially interviewed to assess that their habitual diet was an ordinary varied Danish diet before run-in, and that they were taking no medicine or supplements. No dietary records were made. The study was approved by the local Research Ethics Committee of Copenhagen and Frederiksberg (Journal number KF01-092/96).

Table 3. Daily polyphenol intake from 600 mg grape polyphenol powder*

Compound	Daily intake (mg)
Total phenolics	31·3
Catechin	9·0
Caffeic acid	1·6
Epicatechin	1·5
Rutin	1·5
Quercetin	1·2
Myricetin	1·8
Resveratrol	0·7
Unidentified phenolics	14·0

* P2157; Quest International, Kilnagleary, Carrigaline, Cork, Republic of Ireland. Analysis was made on the same batch as used in the present study by an independent certified laboratory on behalf of the manufacturer.

Collection of blood samples and separation of erythrocytes and LDL

Venous blood samples were taken in the morning after minimum 12 h fasting and supine resting for 10 min. Subjects were instructed to avoid heavy physical activity for 36 h prior to blood sampling. Blood samples were collected in heparin-coated tubes and centrifuged at 1500 ***g*** for 10 min at room temperature. Plasma was stored at −20°C until analysis (maximum 7 months). Erythrocytes were washed three times with three volumes NaCl (9 g/l), resuspended in one volume purified water for lysis, and then stored at −80°C until analysis. Samples for ascorbate determination were kept on ice, centrifuged at 3000 ***g*** for 15 min at 4°C and the plasma was stabilised by addition of one volume metaphosphoric acid (100 g/l) before freezing at −80°C. LDL was isolated from plasma shortly after blood sampling by a single density gradient ultracentrifugation as described by Terpstra *et al.* (1981) using 1 ml/plasma and analysed for malondialdehyde (MDA) immediately.

Antioxidant enzymes

Automated assays were performed on a Cobas Mira analyser (F. Hoffmann-La Roche Ltd, Basel, Switzerland) to determine the activity of the antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (Gpx), glutathione reductase (GR) and catalase (CAT) in red blood cell lysates. SOD, Gpx and haemoglobin were determined using commercially available kits (SD125, RS505 and HG980 respectively; Randox, Ardmore, UK). GR activity and CAT activity were determined according to methods described by Wheeler *et al.* (1990). Glutathione, FAD, purpald and potassium periodate were purchased from Sigma Chemical Co. (St Louis, MO, USA). One red blood cell sample was selected as standard and analysed on twenty different days in order to obtain an established mean and standard deviation. This sample was also analysed in duplicate with each batch of analyses. The accepted interval for each of these determinations was the established mean ±2SD for each of the four enzyme activities, otherwise the batch was reanalysed. Inter-day CV (n 4) for this sample was found to be 7·6 % (GR), 2·8 % (Gpx), 6·3 % (SOD) and 8·1 % (CAT), whereas intra-day variation was below 3 %.

Determination of malondialdehyde

Total MDA was determined in plasma or LDL by a MDA-HPLC method described previously (Lauridsen & Mortensen, 1999). Briefly, the antioxidant butylated hydroxytoluene was added to the LDL samples to give a final concentration of 1 mmol/l and an alkaline hydrolysis was performed by adding NaOH (final concentration 0·5 mol/l) and heating for 30 min at 60°C to release any

protein-bound MDA (Carbonneau *et al.* 1991). Samples were reacted with 2-thiobarbituric acid (Wallin *et al.* 1993) and after a centrifugation for 5 min at 10 000 ***g***, the supernatants were analysed by HPLC: samples (40 μl aliquots) were analysed by chromatography using a linear gradient (0–50 %, 17 min) of H_2O–acetonitrile with 0·1 % trifluoroacetic acid. The HPLC analysis was performed on a Hewlett-Packard 1100 system (Hewlett-Packard, Waldbromn, Germany) with diode array detector, using a Purospher RP-18 column (4 × 250 mm, 5 μm; Hewlett-Packard) and detection at 532 nm. Four MDA standards were included in each 2-thiobarbituric acid reaction and HPLC-run. Sample peaks with retention times and u.v.-spectra identical to the MDA–2-thiobarbituric acid standards were identified and the concentration of MDA was calculated from a standard curve and expressed as pmol MDA/mg protein. The intra-day CV for a standard was below 3·5 % (*n* 4). MDA was purchased from Aldrich Chemical Co. (Steinheim, Germany), butylated hydroxytoluene, 2-thiobarbituric acid and NADPH were purchased from Sigma Chemical Co. Acetonitrile and methanol used were HPLC grade from Rathburne (Walkerburn, Lothian, UK).

Determination of 2-aminoadipic semialdehyde in plasma proteins

These procedures were performed as previously described by Daneshvar *et al.* (1997). Briefly, 1 mg protein was dissolved in 0·25 mol *N*-(morpholino)ethanesulfonic acid/l buffer pH 6·0 containing 10 g sodium dodecyl sulfate/l and heated for 1 min at 100°C. A solution of 0·25 mol fluoresceinamine/l in 0·52 mol NaOH/l was added, followed by 0·25 mol $NaCNBH_3$/l in 0·25 mol *N*-(morpholino)ethanesulfonic acid/l pH 6·0, and the mixture was incubated for 1 h at 37°C. The mixture was applied onto a G-25 gel filtration column (Pharmacia, Uppsala, Sweden), and the column eluted with *N*-(morpholino)ethanesulfonic acid buffer. The protein fraction was collected and precipitated by addition of TCA. The precipitate was hydrolysed in 6 mol HCl/l at 110°C for 24 h, and an internal standard of acetaldehyde–fluoresceinamine adduct was added. The hydrolysate was filtered and injected into an HPLC with a Pyrospher RP-18 column (4 × 250 mm, 5 μm; Hewlett-Packard) eluted by a linear gradient of 0–50 % acetonitrile in 2 mmol formic acid/l, pH 3·2. The eluate was monitored at 454 nm, the absorption maximum of decarboxylated fluoresceinamine, and at 275 nm for determination of free L-tyrosine, a marker for the quantity of hydrolysed protein. The result was corrected for the efficiency of protein hydrolysis and for the recovery of internal standard. Each sample was analysed on two different days. The variation between the two determinations of 2-aminoadipic semialdehyde (AAS) in any plasma protein sample was not allowed to exceed 10 %, otherwise samples were reanalysed. Mean intra-day variation was below 4 %.

Determination of plasma ascorbic acid

The concentration of ascorbic acid in metaphosphoric acid-stabilised plasma was analysed before intervention (baseline) and after each week of intervention, with and without grape-skin extract respectively. The concentration of ascorbic acid in the diet was constant throughout the intervention periods (calculated ascorbic acid content of the diet/d per 10 MJ is shown in Table 2) and thus independent of the intervention. Plasma ascorbic acid was determined by HPLC according to Kall & Andersen (1999).

Statistics

Biomarker analyses were performed on duplicate blood samples (taken on two successive days) during run-in, after 1 week and after 2 weeks. The means of these duplicates were used in the statistical analyses. The effect of period and the presence of carry-over (period × effect interactions) were determined by *t* tests according to Pocock (1998). Due to the risk of period effects when no wash-out period was used, the effects of intervention was determined for all biomarkers by the paired *t* test as suggested by Pocock (1998) to reduce such effects. In the case of significant carry-over, intervention effects were determined by comparing groups A and B by *t* test in the first period only. ANOVA (GLM procedure, SAS 6·12; Statistical Analysis Systems Institute Inc., Cary, NC, USA) with a repeated statement for the sampling days was used to test for intra-individual variation during the whole 15 d experimental period for each of the biomarkers. Further analyses of these variations were performed by comparing intra-individual differences in biomarker responses between sampling times by paired *t* tests. Due to the many tests performed, a P value $<0·01$ was considered statistically significant.

Results

Subjects

The subjects were weight stable, i.e. body weight changes were less than 1 kg, except in one individual who lost 1·3 kg over the 2-week intervention period.

Biomarkers of antioxidant status

There were no significant differences in biomarkers between males and females or between the two groups in the run-in period but group A had somewhat lower levels of MDA in LDL than group B before run-in ($P = 0·03$). There was a significant period effect in the case of ascorbate ($P < 0·001$) and significant carry-over (period × effect interaction) in the case of GR ($P = 0·001$), whereas carry-over for Gpx was borderline ($P = 0·04$). The effects of the cross-over intervention with and without grape-skin extract on the biomarkers for antioxidative status are shown in Table 4. None of the biomarkers MDA, AAS, CAT or SOD were significantly changed after grape-skin extract addition to the experimental diet. Plasma ascorbate showed a borderline increase during the grape-skin intervention after correction for period effects ($P = 0·02$). Due to carry-over, the effects of GR and Gpx could only be assessed by comparing the two groups after the first week of

Table 4. Concentration of malondialdehyde in LDL, plasma 2-aminoadipic semialdehyde residues and activities of superoxide dismutase, glutathione peroxidase, glutathione reductase and catalase in red blood cells after 1 week's intake of the experimental diet† with (+) and without (−) 600 mg grape-skin extract/d‡

(Mean values and 99 % CI for fifteen subjects)

	−Grape skin		+Grape skin		Paired difference	
Biomarker	Mean	99 % CI	Mean	99 % CI	Mean	99 % CI
MDA in LDL (pmol/mg protein)	706	595, 818	712	593, 831	5·9	−152, 164
MDA in plasma (pmol/mg protein)	50·5	46·3, 54·8	53·6	47·7, 59·4	3·0	−3·0, 9·0
Plasma AAS (pmol/mg protein)	18·5	16·9, 20·1	19·0	17·4, 20·6	0·47	−1·91, 2·85
Ascorbic acid (μmol/l)	35·7	29·2, 42·2	38·3	33·1, 43·5	2·5	−2·5, 8·9
RBC SOD (U/g Hb)	1038	997, 1079	1043	1001, 1085	5·5	−31·5, 42·6
RBC Gpx (U/g Hb)	43·1	37·0, 49·2	50·0	43·8, 56·27	6·9§	
RBC GR (U/g Hb)	6·79	6·22, 7·36	8·09*	7·52, 8·66	1·30§	
RBC CAT (U/g Hb)	14·0	13·0, 15·0	13·7	12·6, 14·8	−0·28	−0·75, 0·20

MDA, malondialdehyde; AAS, 2-aminoadipic semialdehyde; RBC, red blood cell; SOD, superoxide dismutase; Hb, haemoglobin; Gpx, glutathione peroxidase; GR, glutathione reductase; CAT, catalase.
Mean value was significantly different from that of subjects without grape-skin intervention: *$P < 0·05$.
† For details of experimental diet see Tables 1 and 2.
‡ Grape polyphenol powder P2157; Quest International, Kilnagleary, Carrigaline, Cork, Republic of Ireland. For details of daily polyphenol intake see Table 3.
§ Due to significant carry-over for GR and Gpx, the effect of intervention was tested in period 1 between the groups, and the differences are therefore not paired.

intervention. In period 1 there was a significant difference between the groups with respect to GR ($P = 0·001$) and a borderline effect with respect to Gpx ($P = 0·06$).

A significant effect of the overall dietary change to the experimental diet was observed for AAS (decrease, $P < 0·0001$) and ascorbate (decrease, $P < 0·0001$) whereas it was borderline for MDA (increase, $P = 0·03$) by repeated samples ANOVA (Table 5). In order to look further into the cause of these changes we analysed sequentially the changes from the run-in period to period 1 and from period 1 to period 2 in each group (Fig. 2). In group A, which had no grape-skin extract in period 1 we observed a significant decrease for AAS ($P < 0·001$) and ascorbate ($P = 0·002$) and a significant increase with respect to MDA in LDL ($P < 0·01$), confirming the observed overall effects. In group B we observed similar but weaker trends for AAS ($P = 0·02$) and ascorbate ($P = 0·06$). In the second period we observed no further significant changes in group A whereas in group B the further decline was significant for ascorbate ($P < 0·01$) and borderline for AAS ($P = 0·05$). The activities of red blood cell antioxidant enzymes SOD, GR and CAT did not change with the experimental diet as determined by repeated samples ANOVA, but Gpx increased non-significantly ($P = 0·07$). Weak decreases in GR ($P = 0·07$) and CAT ($P = 0·04$) in group A from run-in to the end of period 1 as determined by paired *t* tests were also observed.

Discussion

The effect of a grape-skin extract on biomarkers of oxidative status was studied in order to demonstrate any antioxidative *in vivo* characteristics of polyphenolic compounds in red wine. In order to facilitate a comparison between grape-skin extract and wine, the dose was selected to match realistic intakes of polyphenolics from red wine. The daily intake of grape-skin extract in this study corresponded to approximately 330 ml red wine when based on catechin content and approximately 430 ml wine when compared with the quercetin content of the wine used in an intervention study by de Rijke *et al.* (1996). In the present study the subjects were maintained on a low-flavonoid diet with strict control of intake in order to increase the possibility of detecting any effect of grape-skin

Table 5. Concentration of malondialdehyde in LDL, plasma 2-aminoadipic semialdehyde residues and activities of superoxide dismutase, glutathione peroxidase, glutathione reductase and catalase in red blood cells at baseline and after 1 and 2 weeks on the experimental diet†

(Mean values and 99 % CI in fifteen subjects)

	Baseline		1 week		2 weeks	
Biomarker	Mean	99 % CI	Mean	99 % CI	Mean	99 % CI
MDA in LDL (pmol/mg protein)	542	404, 679	735	609, 860	684	583, 785
MDA in plasma (pmol/mg protein)	51·8	47·6, 56·1	51·4	46·4, 56·3	52·2	47·3, 58·1
Plasma AAS (pmol/mg protein)	24·0	21·8, 26·2	19·8	18·2, 21·5	17·6*	16·5, 18·7
Ascorbic acid (μmol/l)	59·7	48·0, 71·4	40·7	34·4, 47·0	33·5*	29·0, 38·0
RBC SOD (U/g Hb)	1017	969, 1065	1030	996, 1063	1051	1004, 1098
RBC Gpx (U/g Hb)	44·0	39·2, 48·8	46·3	41·5, 51·2	46·8	41·9, 51·6
RBC GR (U/g Hb)	7·45	6·92, 7·98	7·39	6·80, 7·99	7·39	6·83, 7·95
RBC CAT (U/g Hb)	14·3	12·8, 15·7	13·8	12·8, 14·8	12·6	12·5, 16·1

MDA, malondialdehyde; AAS, 2-aminoadipic semialdehyde; RBC, red blood cell; SOD, superoxide dismutase; Hb, haemoglobin; Gpx, glutathione peroxidase; GR, glutathione reductase; CAT, catalase.
Significant intra-individual decreasing trend with time on flavonoid-free diet by repeated samples ANOVA (*$P < 0·01$).
† For details of experimental diet see Tables 1 and 2.

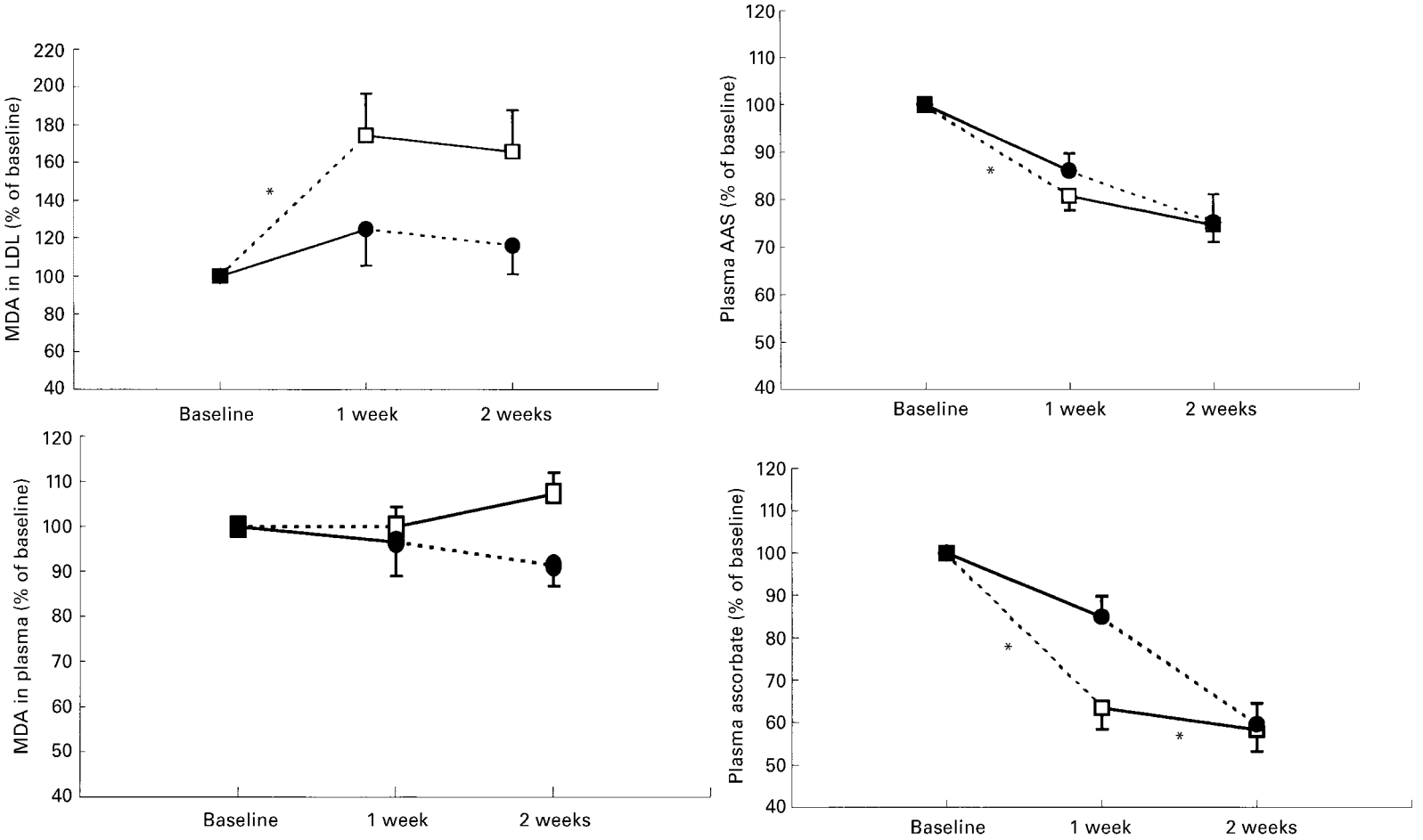

Fig. 2. Concentration of malondialdehyde (MDA) in LDL and plasma, plasma 2-aminoadipic semialdehyde (AAS) residues and plasma ascorbate at baseline and after 1 and 2 weeks on the experimental diet in eight subjects from group A (□) and seven subjects from group B (●) with (−) or without (----) 600 mg grape-skin extract/d (Grape polyphenol powder P2157; Quest International, Kilnagleary, Carrigaline, Cork, Republic of Ireland). For details of experimental diet see Tables 1 and 2; for details of daily polyphenol intake see Table 3. Values are means with standard errors of the means represented by vertical bars. Mean values were significantly different from values at previous time point: $^{*}P < 0{\cdot}05$.

extract intervention. An increased activity of GR and borderline increases of Gpx activity in red blood cells and of plasma ascorbate concentrations were observed in the period of grape-skin intervention whereas other indices of oxidative status were not affected. It is unlikely that the small increase in Gpx during the total trial period was due to an improved Se status. The experimental diet did not contain any rich source of Se and a 2-week intervention period would not be sufficient to achieve major changes in red blood cell Gpx (Thomson *et al.* 1988). It has furthermore been demonstrated that ascorbic acid improves the bioavailability of Se (Mutanen & Mykkänen, 1985) and thus if anything the Se status would be impaired as a consequence of the low ascorbic acid intake.

An intervention period of 1 week may seem inadequate for affecting red blood cell enzyme activities, but in previous studies we have observed an increase in Gpx activity after 1 week of apple–blackcurrant juice intake (Young *et al.* 1999), increased GR after 3 weeks of spinach intervention (Castenmiller *et al.* 1999) and increased GR and SOD after 1 week of parsley intake (Nielsen *et al.* 1999). In the present study, the Gpx and GR activities were higher in period 1 in group B who had grape-skin extract than in group A. Since group B had somewhat higher activity in these enzymes than group A throughout the trial periods, this may have been chance findings in the present study. Intake of grape-skin extract did not seem to affect plasma lipid oxidation.

We used a direct assay to assess LDL and plasma lipid oxidation *in vivo* by determining the actual concentration of the lipid oxidation product (MDA) in LDL and plasma by HPLC. The MDA and the Cu^{2+}-induced lipoprotein oxidation lag time assays have been found to give inversely proportional results in experimental animals (S Lauridsen, A Mortensen, P Olsen, B Mayer and LO Dragsted, unpublished results). We observed a non-significant overall increase in the content of MDA in LDL during the 2-week trial due to a significant increase of MDA in LDL in group A during period 1 with no grape-skin (Fig. 2). We speculate that the grape-skin intervention in group B may have prevented this effect on MDA in LDL, and that the lack of a similar effect in period 2 might be explained by carry-over. For several other biomarkers, including ascorbate, AAS, CAT and GR we also observed a more marked change in group A than in group B during period 1 (Fig. 2). This might indicate an effect of the grape-skin extract on these markers in the opposite direction to that of the experimental diet. The dose of absorbed phenolics may have been too small or the intervention period too short to observe a significant protective effect of grape-skin intake on most of these biomarkers. Most likely, the strong effects of the experimental diet caused period effects and the lack of a wash-out period caused carry-over of any effects of the grape-skin extract due to our experimental design.

Other studies of 2-weeks duration have shown decreased *in vitro* oxidation of LDL in fasting blood samples, after ingestion of 400 ml red wine/d with habitual diet (Fuhrman *et al.* 1995) and a similar effect was observed after ingestion of approximately 450 ml (0·8 g ethanol/kg) red wine/d with a controlled diet low in phenolic substances (Kondo *et al.* 1994). In contrast, de Rijke *et al.* (1996) did not observe any effect on *in vitro* LDL oxidation after 4 weeks intake of 550 ml red wine/d with a self-selected low-flavonoid diet. Thus, the background diet may have affected the different responses to wine and grape-skin extract. It is also possible that the processing of the grape skin including the removal of alcohol had changed the antioxidative properties of the phenolics or their bioavailability (Duthie *et al.* 1998).

As opposed to a possible increase in lipid oxidation with the experimental diet we observed a decrease in protein oxidation. In other studies we have observed a direct association between plasma ascorbate and AAS, indicating that ascorbate or other plant-derived antioxidants may be involved in Fenton-type oxidation of albumin (Castenmiller *et al.* 1999; Young *et al.* 1999; B Daneshwar, unpublished results). In the present study, the basic experimental diet was made to comply with a low-flavonoid intake. These compounds are predominantly present in fruits and vegetables, which are also the main sources of ascorbic acid. Decreases in plasma ascorbate can therefore be considered a marker of decreases in several non-grape-skin antioxidants resulting from the experimental diet. The experimental diet contained no fruits and only carrots as vegetables and had a calculated average ascorbic acid content of 23 mg/d per 10 MJ, which is approximately 25 % of the average intake for this age-group in Denmark (91 mg/d per 10 MJ) (Haraldsdóttir *et al.* 1991). According to Biesalski *et al.* (1997) the optimal plasma concentration of ascorbic acid for preventing increased risk of CHD is >50 μmol/l. After 1 week of intervention the mean plasma ascorbic acid concentration was at 40·7 μmol/l decreasing further to 33·5 μmol/l after the second week of intervention. The decrease was only significant for group A during period 1 and for group B during period 2 (Fig. 2), i.e. in the periods when no grape skin was supplied. We speculate that this might be due to a weak sparing effect of grape-skin extract on vitamin C.

Low plasma ascorbic acid may also specifically have led to the observed increase of MDA in LDL in group A in the first period of the present study (Fig. 2). Repletion of ascorbic acid in plasma from 13·5 μmol/l to 51·7 μmol/l, by citrus fruit supplementation, significantly increased the lag time of *in vitro* LDL oxidation (Harats *et al.* 1998), indicating that ascorbic acid had a protective effect despite its absence from the LDL particles. The experimental diet had a relatively high calculated content of β-carotene (Table 2). Early *in vitro* studies of LDL oxidation suggested a possible role of β-carotene in delaying the onset of LDL oxidation (Esterbauer *et al.* 1989); in addition, a supplementation study using a β-carotene concentration ten times higher (180 mg/d) than that of the experimental diet in the present study showed a decrease in *in vitro* oxidation of LDL (Levy *et al.* 1996). However, other supplementation studies have not shown any consistent effect on the oxidative susceptibility of LDL particles (Tribble, 1999). Exogenous antioxidants such as flavonoids or other polyphenols have not been investigated as plasma antioxidants in a similar way, so their overall contribution cannot be compared. The decrease in ascorbate *per se* may also have resulted in the decreased AAS. However, other dietary factors co-existing with ascorbate

are more likely to have caused such effects since ascorbate does not seem otherwise to have pro-oxidant effects in the blood (Frei *et al.* 1988). Alternatively, the experimental diet may have contained other antioxidants (e.g. carotenes, simple phenolic acids) capable of specifically protecting lysine residues in plasma proteins, thereby causing the observed decrease in AAS. However, recent results from a spinach intervention study does not support a role for β-carotene or lycopene in protection of lysine residues (Castenmiller *et al.* 1999).

In conclusion, with the exception of a small effect of GR activity, grape-skin extract in an amount corresponding to the phenolic content of 300–400 ml red wine did not influence markers of antioxidative status. The experimental diet *per se* led to non-significantly increased MDA concentration in LDL, and to reduced plasma protein oxidation and reduced plasma ascorbate. This demonstrates that changes in markers of oxidative stress and of antioxidative defence can occur within normal variations in dietary intake of antioxidant-rich foods.

Acknowledgements

This study has been carried out with financial support from the Commission of the European Communities, Agriculture and Fisheries (FAIR) specific RTD program, CT 95-0158 'Natural Antioxidants in Foods'. It does not necessarily reflect its views and in no way anticipates the Commission's future policy in this area. The authors wish to thank the laboratory technicians Anni Schou, Vibeke Kegel, and Ella Jessen for excellent assistance and Carina Andersen and Morten Kall, Institute of Food Chemistry and Nutrition, Danish Veterinary and Food Administration, Copenhagen, Denmark for the plasma ascorbic acid analyses.

References

Biesalski HK, Böhles H, Esterbauer H, Fürst P, Gey F, Hundsdörfer G, Kasper H, Sies H & Weisburger J (1997) Antioxidant vitamins in prevention. *Clinical Nutrition* **16**, 151–155.

Carbonneau M-A, Léger CL, Monnier L, Bonnet C, Michel F, Fouret G, Dedieu F & Descomps B (1997) Supplementation with wine phenolic compounds increases the antioxidant capacity of plasma and vitamin E of low-density lipoprotein without changing the lipoprotein Cu^{2+}-oxidizability: Possible explanation by phenolic location. *European Journal of Clinical Nutrition* **51**, 682–690.

Carbonneau M-A, Peuchant E, Sess D, Canioni P & Clerc M (1991) Free and bound malondialdehyde measured as thiobarbituric acid adduct by HPLC in serum and plasma. *Clinical Chemistry* **37**, 1423–1429.

Castenmiller JJM, Lauridsen ST, Dragsted LO, van het Hof KH, Linssen JPH & West CE (1999) Beta-carotene does not change markers of enzymatic and non-enzymatic antioxidant activity in human blood. *Journal of Nutrition* **129**, 2162–2169.

Daneshvar B, Frandsen H, Autrup H & Dragsted LO (1997) Gamma-glutamyl semialdehyde and 2-amino-adipic semialdehyde: biomarkers of oxidative damage to proteins. *Biomarkers* **2**, 117–123.

Day AP, Kemp HJ, Bolton C, Hartog M & Stansbie D (1997) Effect of concentrated red grape juice consumption on serum antioxidant capacity and low-density lipoprotein oxidation. *Annals of Nutrition & Metabolism* **41**, 353–357.

de Rijke YB, Demacker PNM, Assen NA, Sloots LM, Katan MB & Stalenfoef AFH (1996) Red wine consumption does not affect oxidizability of low-density lipoproteins in volunteers. *American Journal of Clinical Nutrition* **63**, 29–34.

Duthie GG, Pedersen MW, Gardner PT, Morrice PC, Jenkinson AM, McPhail DB & Steele GM (1998) The effect of whiskey and wine consumption on total phenol content and antioxidant capacity of plasma from healthy volunteers. *European Journal of Clinical Nutrition* **52**, 733–736.

Esterbauer H, Striegl G, Puhl J & Rothender M (1989) Continuous monitoring of *in vitro* oxidation of human low density lipoprotein. *Free Radical Research Communications* **6**, 67–75.

Frankel EN, Waterhouse AL & Teissedre P-L (1995) Principal phenolic phytochemicals in selected California wines and their antioxidant activity in inhibiting oxidation of human low-density lipoproteins. *Journal of Agricultural and Food Chemistry* **43**, 890–894.

Frei B, Stocker R & Ames BN (1988) Antioxidant defenses and lipid peroxidation in human blood plasma. *Proceedings of the National Academy of Sciences, USA* **85**, 9748–9752.

Fuhrman B, Lavy A & Aviram M (1995) Consumption of red wine with meals reduces the susceptibility of human plasma and low-density lipoprotein to lipid peroxidation. *American Journal of Clinical Nutrition* **61**, 549–554.

Grønbæk M, Deis A, Sørensen TIA, Becker U, Schnohr P & Jensen G (1995) Mortality associated with moderate intakes of wine, beer, or spirits. *British Medical Journal* **310**, 1165–1169.

Haraldsdóttir J, Holm L, Jensen JH & Møller A (1991) *Danskernes kostvaner 1985 1. Hovedresultater*. Copenhagen: Danish Veterinary and Food Administration.

Harats D, Chevion S, Nahir M, Norman Y, Sagee O & Berry EM (1998) Citrus fruit supplementation reduces lipoprotein oxidation in young men ingesting a diet high in saturated fat: presumptive evidence for an interaction between vitamins C and E *in vivo*. *American Journal of Clinical Nutrition* **67**, 240–245.

Hertog MGL, Hollman PCH & van de Putte B (1993) Content of potentially anticarcinogenic flavonoids of tea infusions, wines, and fruit juices. *Journal of Agricultural and Food Chemistry* **41**, 1242–1246.

Kall M & Andersen C (1999) Improved method for simultaneous determination of ascorbic acid and dehydroascorbic acid, isoascorbic acid and dehydroisoascorbic acid in food and biological samples. *Journal of Chromatography – Biomedical Applications* **730**, 101–111.

Klatsky AL & Armstrong MA (1993) Alcoholic beverage choice and risk of coronary artery disease mortality: Do red wine drinkers fare best? *American Journal of Cardiology* **71**, 467–469.

Kondo K, Matsumoto A, Kurata H, Tanahashi H, Koda H, Amachi T & Itakura H (1994) Inhibition of oxidation of low-density lipoprotein with red wine. *Lancet* **344**, 1152.

Lauridsen ST & Mortensen A (1999) Probucol selectively increases oxidation of atherogenic lipoproteins in cholesterol-fed mice and in Watanabe heritable hyperlipidemic rabbits. *Atherosclerosis* **142**, 169–178.

Levy Y, Kaplan M, Benamotz A & Aviram M (1996) Effect of dietary supplementation of beta-carotene on human monocyte-macrophage-mediated oxidation of low density lipoprotein. *Israel Journal of Medical Sciences* **32**, 473–478.

Meyer AS, Yi O-S, Pearson DA, Waterhouse AL & Frankel EN (1997) Inhibition of human low-density lipoprotein oxidation in relation to composition of phenolic antioxidants in grapes (*Vitis vinifera*). *Journal of Agricultural and Food Chemistry* **45**, 1638–1643.

Miyagi Y, Miwa K & Inoue H (1997) Inhibition of human low-density lipoprotein oxidation by flavonids in red wine and grape juice. *American Journal of Cardiology* **80**, 1627–1631.

Mutanen M & Mykkänen HM (1985) Effect of ascorbic acid supplementation on selenium bioavailability in humans. *Human Nutrition: Clinical Nutrition* **39**C, 221–226.

Møller A (1989) *Food Composition Tables*. Copenhagen: National Food Agency.

Nielsen SE, Young JF, Daneshvar B, Lauridsen ST, Knuthsen P, Sandström B & Dragsted LO (1999) Effect of parsley (*Petroselium crispum*) intake on urinary apigenin excretion, blood antioxidant enzymes and biomarkers for oxidative stress in human subjects. *British Journal of Nutrition* **81**, 447–455.

Pocock SJ (1998) *Clinical trials. A Practical Approach*. Chichester: Wiley & Sons.

Renaud S & de Lorgeril M (1992) Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* **339**, 1523–1526.

Terpstra AHM, Woodward CJH & Sanchez-Muniz FJ (1981) Improved techniques for the separation of serum lipoproteins by density gradient ultracentrifugation: Visualization by prestaining and rapid separation of serum lipoproteins from small volumes of serum. *Analytical Biochemistry* **111**, 149–157.

Thomson CD, Steven SM, van Rij AM, Wade CR & Robinson MF (1988) Selenium and vitamin E supplementation: activities of glutathione peroxidase in human tissues. *American Journal of Clinical Nutrition* **48**, 316–323.

Tribble DL (1999) Antioxidant consumption and risk of coronary heart disease: Emphasis on vitamin C, vitamin E and β-carotene. A statement for healthcare professionals from the American Heart Association. *Circulation* **99**, 591–595.

Wallin B, Rosengren B, Shertzer HG & Camejo G (1993) Lipoprotein oxidation and measurement of thiobarbituric acid reacting substances formation in a single microtiter plate: Its use for evaluation of antioxidants. *Analytical Biochemistry* **208**, 10–15.

Wheeler CR, Salzman JA, Elsayed NM, Omaye ST & Korte DW Jr (1990) Automated assays for superoxide dismutase, catalase, glutathion peroxidase, and glutathion reductase. *Analytical Biochemistry* **184**, 193–199.

Whitehead TP, Robinson D, Allaway S, Syms J & Hale A (1995) Effect of red wine ingestion on the antioxidant capacity of serum. *Clinical Chemistry* **41**, 32–35.

Young JF, Nielsen SE, Haraldsdóttir J, Daneshvar B, Lauridsen ST, Knuthsen P, Crozier A, Sandström B & Dragsted LO (1999) Effect of fruit juice intake on urinary quercetin excretion and biomarkers of antioxidative status. *American Journal of Clinical Nutrition* **69**, 87–94.