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Research Article

Effects of red grape skin and seed extract supplementation on atherosclerosis in Watanabe heritable hyperlipidemic rabbits

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Epidemiological studies have suggested an association between consumption of red wine and other polyphenolic compounds and prevention of cardiovascular diseases. In the present study, Watanabe heritable hyperlipidemic (WHHL) rabbits were used to investigate the effects of polyphenols in a red grape skin and seed extract (GSE) on the development of atherosclerosis. WHHL rabbits received either semisynthetic diet (casein based) or semisynthetic diet added GSE over a period of 15 wk. Plasma lipids and aortic cholesterol accumulation were measured. Feeding semisynthetic diet was associated with increasing hypercholesterolemia, which was developing slower in GSE group compared to the controls as recorded by significantly lower plasma cholesterol in dosage week 7 (males: $P < 0.05$, females: $P < 0.01$) and 11 (males: $P < 0.01$). Aortic atherosclerosis evaluated as the cholesterol content in aortic tissue was comparable in the control and GSE-dosed females, but it was significantly reduced in the abdominal part of GSE-dosed male compared to the controls ($P < 0.05$). In conclusion, feeding GSE extract to WHHL rabbits had no significant effects in females but was associated with transient less hypercholesterolemic response to semisynthetic diet and, furthermore, retarded the development of aortic atherosclerosis in males as demonstrated by significantly lower cholesterol content in the abdominal part.

Keywords: Atherosclerosis / Cholesterol / LC-MS/MS / Polyphenols / WHHL rabbits

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1 Introduction

Epidemiological studies have suggested an association between the consumption of red wine and prevention of cardiovascular diseases. The lower coronary heart disease mortality in France has been related to the high intake of red wine [1, 2]. Studies have indicated that the high content of polyphenolic components in red wine could be a major factor in the prevention of lifestyle-related diseases [2, 3]. However, little is known about the exact preventive effects of red wine and polyphenols, and it has been difficult to show unequivocal beneficial effects of red wine, grapes, grape extract, or polyphenols in epidemiological and human intervention studies [4–7]. For instance, in some studies cholesterol and triglyceride in plasma and in lipoproteins were unaffected by treatment with polyphenols such as proanthocyanidins, while other studies have shown beneficial effects on these markers [7].

Wine, grapes, and grape seed extracts are a major source of polyphenolic components such as the monomeric flavanols, catechin and epicatechin, and the oligomeric proanthocyanidins. Proanthocyanidins are also found in large amounts in cocoa and in some nuts, while fruits, black and green teas are rich sources of catechins. Dietary intake of polyphenols varies a lot between countries, and the total polyphenol intake has been estimated to reach 1 g/day in people who consume high amounts of polyphenol rich foods [8–10].

Several animal and human intervention studies have shown that alcohol, wine, and other alcoholic beverages have beneficial effects on early markers for cardiovascular diseases [11–13]. Other studies in animal models comparing red wine, grape juice, and grape seed extracts suggest that the beneficial effects on atherosclerosis specifically

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Abbreviations: bw, body weight; **GSE,** red grape skin and seed extract; **WHHL,** Watanabe heritable hyperlipidemic

are caused by their content of polyphenols, such as the flavonoids, catechins, and proanthocyanidins [5, 14 – 17].

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. This rabbit model has previously been used to study effects of dietary components on atherosclerosis [12, 18 – 20]. In the present study, WHHL rabbits of both sexes were used to investigate the effects of polyphenols in a red grape skin and seed extract (GSE) on the development of atherosclerosis. The study was conducted over a period of 15 wk. The control groups ($n = 5$ males and $n = 9$ females) received a semisynthetic diet, and the GSE groups ($n = 5$ males and $n = 8$ females) received a semisynthetic diet added 2.6% GSE. The biochemical parameters related to atherosclerosis development such as blood lipids and aortic cholesterol accumulation were measured.

2 Materials and methods

2.1 Animals and diets

Homozygous WHHL rabbits of both sexes (10 males, 17 females) with body weight (bw) of 979 ± 99 g (mean ± SD), plasma cholesterol 23.8 ± 2.6 mM, and triglyceride 6.3 ± 1.6 mM at 7 wk of age were obtained from our own breeding colony. A previous study has shown that the aortas are free from lesions at this age [21]. Animal experiments and housing procedures were performed in accordance with 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 and the Danish Animal Experimental Inspectorate approved the study and all procedures.

Before the initiation of the study, all animals received a standard diet, Altromin 2123 (Altromin International, Lage, Germany). The ingredients of standard diet, Altromin 2123 were as follows: 120 g/kg crude protein, 30 g/kg crude fat, 200 g/kg crude fibers, 90 g/kg ash, 120 g/kg moisture, 440 g/kg nitrogen-free extract, 8 g/kg calcium, 6 g/kg phosphorous, 3 g/kg magnesium, 3 g/kg sodium, and 15 g/kg potassium. At 7 wk of age, the rabbits were allocated to two groups based on plasma cholesterol and triglyceride concentrations, litter, and bw. The control groups ($n = 5$ males and $n = 9$ females, respectively) received daily 100 g of semisynthetic diet, Altromin C 2000 diet (Altromin International). The ingredients of semisynthetic diet, Altromin C 2000 were as follows: 170 g/kg crude protein, 50 g/kg crude fat, 230 g/kg crude fibers, 85 g/kg ash, 100 g/kg moisture, 180 g/kg disaccharide, 185 g/kg polysaccharide, 9 g/kg calcium, 7 g/kg phosphorous, 3 g/kg magnesium, 2.5 g/kg sodium, and 15 g/kg potassium. The second groups, two GSE groups ($n = 5$ males and $n = 8$ females) received 100 g semisynthetic diet (Altromin C 2000) added 2.64% w/w red grape polyphenols HW 75 – 10 (batch no. 2542663), a dark purple, spray dried, free-flowing extract of skins and seeds of grapes from Chr. Hansen A/S, Denmark. GSE is rich in polyphenols, and contents in the diets were determined according to methods previously described; *i. e.*, the Folin – Ciocalteau method for total phenolics, expressed as gallic acid equivalents [22], and the DMACA-HCL procedure for proanthocyanidins, expressed as catechin equivalents [23]. The semisynthetic diet contained 88 mg polyphenols *per* 100 g diet, of these 14 mg was proanthocyanidins, while the diet added GSE contained 855 mg polyphenols *per* 100 g diet, of these 265 mg was proanthocyanidins. All rabbits had free access to tap water. All animals were observed twice daily for any changes in the clinical appearance. The feed intake in all groups was recorded daily, and the bw weekly. Due to a recorded observation of lower feed intake in the GSE groups (males and females), pair feeding of the control groups was introduced after 4 wk of dosing to avoid differences in weight gain between the groups.

After 15 wk of treatment, the rabbits were sacrificed by intravenous injection of pentobarbital (100 mg/kg bw) into the marginal ear vein and sampling was performed as described elsewhere [24].

2.2 Sampling and analysis of blood and aortic atherosclerosis

Blood samples from the marginal ear vein of unanaesthetized animals fasted overnight were collected in heparin tubes. Plasma cholesterol and triglyceride were measured before the treatment and monthly until termination, where in addition, the concentration of cholesterol and triglyceride in lipoproteins was determined, by the use of kits (cholesterol, CHOD-PAP 1489232 216 and triglyceride, GPO-PAP 1488872 216 both from Roche Diagnostics, Mannheim, Germany) and an automatic analyzer (Hitachi 912; Roche Diagnostics). Plasma was isolated from blood samples by centrifugation at $2600 \times g$ for 10 min at 4°C. Plasma samples for the determination of lipoproteins were stored at –80°C for about 4 months before analyses. After thawing of the plasma samples, the lipoproteins were separated by density gradient ultracentrifugation at $100\,000 \times g$ for 18 h at 20°C, using 900 μL of plasma, according to Terpstra *et al.* [25]. The lipoproteins were separated in four fractions containing HDL, HDL/LDL, LDL, and IDL/VLDL, respectively. The fourth fraction of lipoproteins containing IDL/VLDL was added to 200 μL of 10% Triton X-100 and stored overnight at 20°C and then the suspension was homogenized by vortex. The concentrations of cholesterol and triglyceride were analyzed in all fractions by the automatic analyzer (Hitachi 912).

The cholesterol content in the parts of intima-inner media was determined as described previously and expressed as micromole total cholesterol *per* milligram wet weight of aortic tissue [24].

2.3 Urine collection and determination of catechins by LC-MS/MS

Urine from four control rabbits and nine rabbits from the GSE group was collected during 24 h in dose week 12. Collection was initiated when the diets were administrated and continued for 6 h using collection trays containing 100 mL 0.5 M citric acid for stabilization. The collection trays were removed and new urine collections were initiated 6–24 h after the feed administration. The two portions of urine from each rabbit were pooled and stored at −20°C until analyses.

Before analyses, the urine samples were thawed, aliquots of each sample were centrifuged for 10 min at $10\,000 \times g$ and the pH value in 1 mL urine supernatant was adjusted to pH 5 (4.9–5.1) by the addition of approximately 800 μL of 4 M sodium acetate buffer containing 10 mg/mL ascorbic acid. The urine samples were enzymatically hydrolyzed by the addition of 1 μL of β-glucuronidase and 5 μL of arylsulfatase to 994 μL of diluted urine. β-Glucuronidase (*Escherichia coli*) and arylsulfatase type VI (*Aerobacter aerogenes*) were obtained from Roche, Switzerland and Sigma, St. Louis, MO, respectively. The samples were purged with argon and incubated in sealed vials for 1 h at 37°C under continuous shaking.

Samples for calibration curves were prepared in triplicate by spiking the blank urine from control rabbits with (+)-catechin and (−)-epicatechin (obtained from Sigma-Aldrich Chemie, Germany). The blank urine was treated similarly to the samples. Aliquots of 970 μL blank urine were added to a mixture of catechin and epicatechin standards dissolved in 24 μL of 25% ACN, 0.5% formic acid in concentrations of 5.2, 10.4, 20.8, 41.7, or 83.3 μg/mL resulting in final concentrations of 0.125, 0.25, 0.5, 1.0, or 2.0 μg/mL, respectively. The calibration samples were then enzymatically hydrolyzed, as described for the samples prior to analyses.

Urine samples and standards for calibration curves were analyzed immediately after hydrolysis by LC-MS/MS. For analyses of the GSE, a solution of 10 μg/mL GSE in 25% ACN containing 0.5% formic acid was filtrated and analyzed by LC-MS/MS.

LC-MS/MS analyses were performed on an Agilent 1100 series Capillary LC system (Agilent Technologies, Wallbronn, Germany) coupled to a Bruker Daltonics esquire HCT mass spectrometer (Bruker Daltonics, Bremen, Germany). The LC system was equipped with a high-pressure flow cell assembly and photodiode array detector. Injection volume was 10 μL and the compounds were separated on a Zorbax SB-C18, 5 μm, 150 × 0.5 mm column (DE-45B 01561, Agilent Technologies). The primary flow rate was 0.5–0.8 mL/min with a microsplit column flow rate of 50 μL/min. The oven temperature was 40°C. Solvents were A: 1% formic acid and B: ACN (HPLC grade ACN and formic acid (distilled before use) were obtained from Romil, Cambridge, UK). Solvent gradient was: 0–3 min, 1% B v/v; 8 min, 25% B v/v; 15 min, 28% B v/v; 16–18 min, 100% B; and 19–20 min, 1% B v/v.

The MS/MS system was equipped with an electrospray interface and operating in a negative ion mode (ESI-NEG), with a capillary voltage of 3270 V; nebulizer pressure 12.0 psi; drying gas flow 6.0 L/min, and drying temperature at 300°C. The ESI interface and mass spectrometer parameters were optimized to obtain the maximum sensitivity. Trap settings: smart target 50 000; maximum accumulation time 50.00 ms; scanning from 100–350 *m/z* with an average of 2. MS data were acquired from 8–13.5 min with the entire tune parameter settings especially optimized for the catechins. The $[M-H]^-$ ions were isolated as the precursor ion, *m/z* 289 for catechin and epicatechin with an isolation width of 2.0 *m/z*. The fragmentation cutoff for the compounds was set to 33% of the precursor mass. Finally, the specific fragment ion of catechin and epicatechin *m/z* 245 was isolated with the isolation width of 2.0 *m/z*. For data analyses, the program Quant analyses were used (Bruker Daltonics). All peaks were manually integrated. A model for the regression of peak area ratio of calibration standards was used to calculate a linear equation; used to calculate the concentration of catechin and epicatechin in the urine samples.

2.4 Statistics

All data are expressed as means ± SD. Data were tested for normal distribution and for homogeneity of variance by standardized residuals' plot. When necessary, logarithmic transformations were performed. Normally distributed data were analyzed by *t*-test and data not normally distributed were analyzed by nonparametric Mann-Whitney *U* test. The effects with p-values <0.05 were considered statistically significant. All statistical analyses were performed using SPSS (SPSS for Windows, version 14.0, SPSS Chicago, IL, USA).

3 Results

3.1 Animal welfare

Feeding a semisynthetic diet with or without GSE and pair feeding had no effect on the clinical appearance in any of the rabbits. The content of GSE, total polyphenols, and proanthocyanidins in feed, feed intake, initial, and terminal bws are shown in Table 1. Initial and terminal bws and feed intake were similar in all groups.

3.2 Intake and excretion of catechins

To control the bioavailability of GSE polyphenols from the feed, catechin and epicatechin were used as compliance markers and were measured in urine from the rabbits by

Table 1. Body weight, feed intake, and dose of GSE

	Control group		GSE group	
	Male (n = 5)	Female (n = 9)	Male (n = 5)	Female (n = 8)
Initial bw (kg)	0.92 ± 0.04	0.98 ± 0.14	1.01 ± 0.08	0.99 ± 0.09
bw at termination (kg)	2.44 ± 0.13	2.49 ± 0.27	2.27 ± 0.20	2.23 ± 0.24
Relative feed intake (g/kg bw/day)	33.0 ± 1.6	32.9 ± 1.9	31.8 ± 3.4	35.5 ± 3.3
Relative dose of GSE (mg/kg bw/day)	–	–	839.3 ± 88.6	925.3 ± 88.1
Relative dose of total polyphenols (mg/kg bw/day)	29.0 ± 1.4	29.0 ± 1.7	271.8 ± 28.9	299.7 ± 28.5
Relative dose of proanthocyanidins (mg/kg bw/day)	4.61 ± 0.22	4.61 ± 0.27	84.3 ± 8.9	92.9 ± 8.8

All data are mean ± SD.

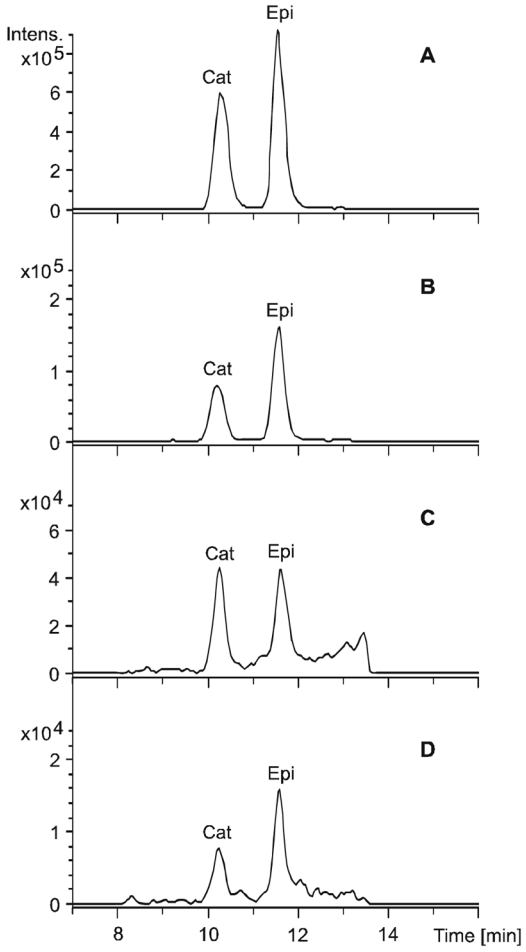

Figure 1. MS/MS ion chromatograms showing the time segment 8–13.5 min for precursor ion *m/z* 289 fragmented to *m/z* 245. (A) Catechin/epicatechin standard (1 μg/mL). (B) GSE (10 μg/mL). (C) Blank urine spiked with catechin/epicatechin standard (1 μg/mL), and (D) Urine from rabbit fed with GSE. Ten microliters of all samples were injected.

LC-MS/MS. The LC conditions resulted in a good separation of these compounds and the specific MS/MS conditions resulted in the isolation of molecular ions $[M-H]^-$ at *m/z* 289 with a characteristic fragment ion at *m/z* 245. The identification of the two catechins in the GSE was confirmed by comparison with authentic standards. The content of catechin and epicatechin in the GSE was calculated to 1.46 and 1.96%, respectively. At the time of urine collection, the intake of catechin and epicatechin was calculated to be about 32 and 42 mg/day, respectively, and the 24 h excretion of catechin and epicatechin in urine was found to be about 35–36 and 27–45 μg, respectively (Table 2). There was no urinary excretion or trace of catechin or epicatechin in the urine samples from the control rabbits. Figure 1 shows MS/MS ion chromatograms of the molecular ions $[M-H]^-$ at *m/z* 289 fragmented to *m/z* 245 of catechin and epicatechin in a standard sample, in the GSE product, in a blank urine sample spiked with catechin and epicatechin, and in a urine sample from a rabbit fed with GSE.

Table 2. Dose and excretion of catechin and epicatechin

	GSE group	
	Male (n = 3)	Female (n = 6)
Feed intake (g/day)[a]	72.8 ± 23.4	85.8 ± 11.0
Dose of GSE (g/day)[a]	1.92 ± 0.62	2.27 ± 0.29
Dose of catechin (mg/day)[a]	28.1 ± 9.0	33.1 ± 4.3
Dose of epicatechin (mg/day)[a]	37.6 ± 12.1	44.3 ± 6.0
Excreted catechin in urine (μg/day)[b]	36.3 ± 18.9	35.0 ± 24.3
Excreted epicatechin in urine (μg/day)[b]	45.0 ± 38.0	27.5 ± 24.0
Content of catechin in GSE (%)[b]	1.46	
Content of epicatechin in GSE (%)[b]	1.96	

All data are mean ± SD.

a) Feed intake, dose of GSE, catechin, and epicatechin are calculated from the data of feed intake the first 2 days of doses in week 12.

b) The content of catechin and epicatechin in GSE and the excretion of catechin and epicatechin are based on LC-MS/MS measurements.

3.3 Cholesterol and triglyceride in plasma, lipoproteins, and aorta

During feeding with a semisynthetic diet, plasma cholesterol increased in both groups. However, hypercholesterole-

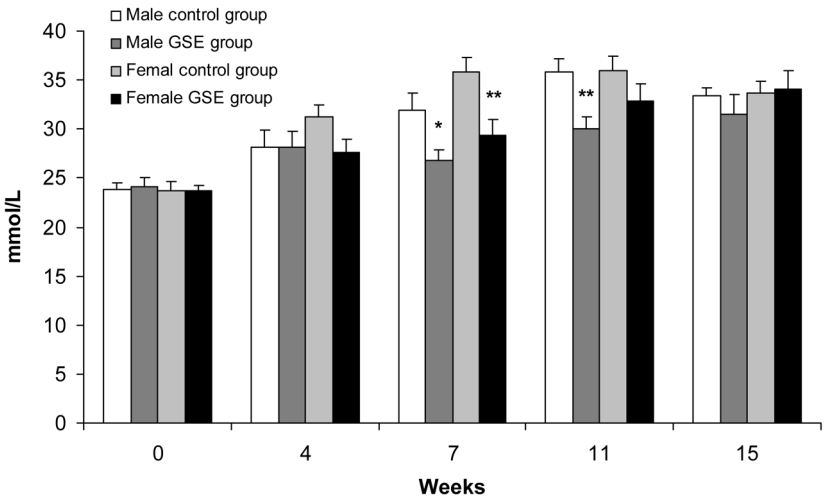

Figure 2. Plasma cholesterol concentration during 15 wk of treatment. Values are mean ± SD of male control group ($n = 5$), male GSE group ($n = 5$), female control group ($n = 9$), and female GSE group ($n = 8$). * $P < 0.05$ and ** $P < 0.01$ relative to the control groups at each time point (Asymptomatically significant 2 tailed. Mann-Whitney *U* test).

Table 3. Cholesterol and triglyceride in lipoproteins at the termination

	Control group		GSE group	
	Male ($n = 5$)	Female ($n = 9$)	Male ($n = 5$)	Female ($n = 8$)
	(mmol/L)			
Total cholesterol	28.3 ± 3.45	29.8 ± 4.75	30.1 ± 3.67	31.1 ± 2.84
HDL cholesterol	0.46 ± 0.15	0.60 ± 0.20	0.43 ± 0.22	0.55 ± 0.10
HDL/LDL cholesterol	0.75 ± 0.31	0.64 ± 0.39	0.66 ± 0.28	0.85 ± 0.38
LDL cholesterol	8.75 ± 2.94	6.95 ± 3.60	9.62 ± 1.34	8.64 ± 3.02
IDL/VLDL cholesterol	19.9 ± 4.99	22.7 ± 4.91	18.2 ± 2.43	22.6 ± 2.51[a)]
Total triglyceride	2.03 ± 0.77	1.25 ± 0.58	1.25 ± 0.38	1.09 ± 0.34
HDL triglyceride	0.07 ± 0.028	0.049 ± 0.015	0.074 ± 0.024	0.091 ± 0.016[b)]
HDL/LDL triglyceride	0.070 ± 0.047	0.028 ± 0.020	0.056 ± 0.029	0.056 ± 0.019[c)]
LDL triglyceride	0.45 ± 0.13	0.23 ± 0.14[d)]	0.32 ± 0.07	0.26 ± 0.08
IDL/VLDL triglyceride	1.46 ± 0.73	1.02 ± 0.53	0.84 ± 0.27	0.83 ± 0.31

All data are mean ± SD. The data were not normally distributed and were therefore analyzed by asymptomatically significant 2 tailed Mann-Whitney *U* test.

a) Significantly different from the male GSE group value, $P < 0.01$.
b) Significantly different from the female control group value, $P < 0.01$.
c) Significantly different from the female control group value, $P < 0.05$.
d) Significantly different from the male control group value, $P < 0.05$.

mic response in males and females from the GSE groups was lower than that of the controls (Fig. 2). The difference between the male groups reached statistical significance in weeks 7 and 11 ($P < 0.05$ and $P < 0.01$, respectively) and in females in week 7 ($P < 0.01$). A similar tendency was seen at dosage weeks 4 and 11 of the females, but not at the end of the study, where the concentration of cholesterol in the plasma in the control groups was decreased to the same level as in the GSE groups.

There was no significant difference in the triglyceride level between the groups during the study period. The level of triglyceride in all groups decreased from about 6.2–1.5 mmol/L within the first 4 wk of treatment. From dosage week 4 to termination of the study, the concentration of triglyceride in plasma in all groups was stabilized at similar levels (data not shown) and at termination the plasma triglyceride concentrations in the control groups and GSE groups were 1.67 ± 0.78 and 1.16 ± 0.36, respectively.

The concentration of cholesterol and triglyceride in lipoproteins at week 15 of the treatment was similar between the groups with a few exceptions (Table 3). IDL/VLDL cholesterol was significantly higher in the female GSE group ($P < 0.01$) than in the male GSE group, HDL triglyceride was significantly higher in the female GSE group ($P < 0.01$) than in the female control group, HDL/LDL triglyceride was significantly higher in the female GSE group ($P < 0.05$) than in the female control group, and LDL triglyceride was significantly lower in the female control group ($P < 0.05$) than in the male control group.

Aortic atherosclerosis evaluated by biochemical measurement of aortic cholesterol content demonstrated significantly less atherosclerotic lesions in the abdominal aorta of

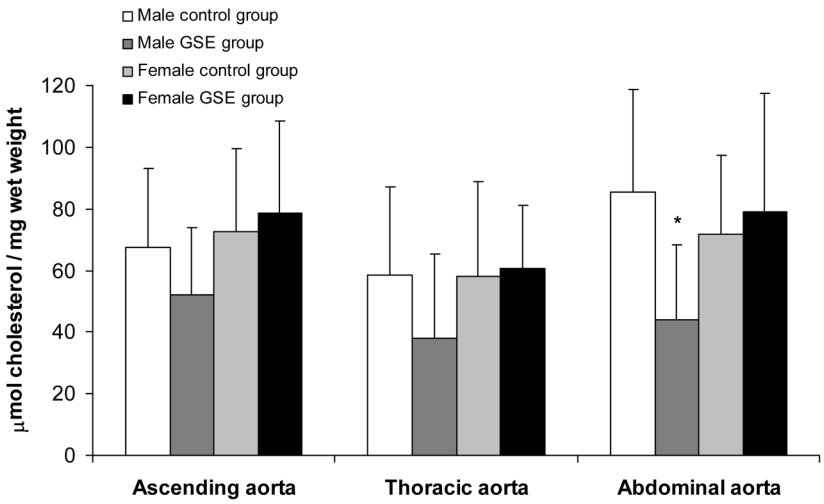

Figure 3. Accumulation of cholesterol in the aorta. Values are mean ± SD of male control group ($n = 5$), male GSE group ($n = 5$), female control group ($n = 9$), and female GSE group ($n = 8$). * Significantly different from the male control group, $P < 0.05$ (Asymptomatically significant 2 tailed. Mann-Whitney *U* test).

male GSE group ($P < 0.05$) compared to the controls (Fig. 3). Furthermore, a decreasing trend was found in the cholesterol content in the ascending and the thoracic parts of the aorta of male GSE compared to the controls. No difference in aortic cholesterol was found between the groups of females.

4 Discussion

Several animal and epidemiological studies have been conducted to show the beneficial effects of red wine, grape skin, and seed polyphenols and proanthocyanidins on the development of cardiovascular disease [4, 6, 11, 17, 26, 27]. WHHL rabbits are a well-known model of human hypercholesterolemia and have been used to study the effects of dietary components on the development of atherosclerosis. This model has also been used to study the effects of red wine and pure alcohol on atherosclerosis [12]. Our previous study of the effects of anthocyanins on atherosclerosis demonstrated an increase in the total and LDL cholesterol of WHHL rabbits fed a diet added with pure anthocyanins, but not of those, which received a black currant juice containing anthocyanins instead of drinking water [20]. This observation led us to the suggestion that the black currant juice might contain other components, for instance other polyphenolic compounds which might reduce atherosclerosis. Therefore, we decided to investigate the effects of a polyphenolic rich red grape extract on the development of atherosclerosis in WHHL rabbits.

Before the present study was designed, we conducted a pilot study with WHHL rabbits fed a normal rabbit standard diet based on crude plant components (Altromin 2123, Altromin International) supplemented with the same amount of the GSE as in the present study. Two groups of WHHL rabbits; a control group ($n = 9$) and a GSE group ($n = 9$), received 100 g feed daily during a period of 16 wk. However, the pilot study showed no significant differences between the two groups. The feed intake, growth rates, and concentration of cholesterol and triglyceride in plasma were comparable in both groups during the whole period of treatment. At termination (week 16), the concentration of cholesterol and triglyceride in lipoproteins and aortic atherosclerosis evaluated biochemically were also similar in the two groups (data not shown). The standard diets based on plant components (hereunder Altomin 2123) contain polyphenolic compounds [28, 29] and are not atherogenic compared to semisynthetic casein-based diets [30 – 33]. Therefore, it cannot be excluded that the polyphenols naturally present in plant component-based standard diets could mask the effect of GSE supplementation. Thus the lack of effects in the pilot study, when using Altromin 2123 as a control diet, prompted us to perform the present study. By using a semisynthetic diet, we ensured that the only polyphenols consumed by the WHHL rabbits were those from GSE.

Semisynthetic (casein based) diets are sometimes poorly accepted by the rabbits, which is manifested by reduced feed intake and lower bw gain [33 – 35]. Also in the present study feeding a semisynthetic diet was associated with a decreased feed intake, which was especially pronounced in the GSE group. The possible explanation of this can be a lower palatability of GSE-added diet, as it had a bitter taste, a dark color, and a harder consistence of pellets. However, introduction of pair feeding insured a comparable relative feed intake in both groups, which was also comparable to that in a previous study in WHHL rabbits, fed the same semisynthetic diet [24].

To confirm the absorption of polyphenols from the GSE feed we measured catechin and epicatechin in urine. The urinary excretion was about 1000-fold lower than the intake of catechin and epicatechin. This was in accordance with a previous study, where a similar low excretion of catechin and epicatechin in rats was found after an oral administra-

tion with a water solution of a grape seed polyphenol compound. In urine, only about 0.3% of catechin, epicatechin, and their respective conjugates was excreted in 49 h [36].

In the present study, the hypercholesterolemic response to semisynthetic diet, which is recognized as atherogenic to rabbits [30–33], was transitory but significantly less in males and females from GSE group compared to the controls. No difference in hypercholesterolemia between the GSE and the control groups at terminations was related to a decrease in plasma cholesterol in the control animals, the reason for which remains obscure. However, an adaptation to the atherogenic diet should be considered. Several animal studies report the plasma cholesterol lowering effect of grape skin and seed extract [14, 15, 17, 26]. A lower concentration of plasma cholesterol after the consumption of red wine, dealcoholized red wine, and grape seed juice has also been shown in cholesterol fed hamsters, but not after the consumption of pure ethanol given in concentration similar to the red wine [15]. Thus the observed (though transient) less hypercholesterolemic response to feeding semisynthetic diet added GSE in the present study may be due to the intake of polyphenols from GSE, especially that the content of total phenols was about ten-fold higher in the GSE supplemented diet than in the semisynthetic control diet.

In the male rabbits, the aortic atherosclerosis evaluated as cholesterol content in the aortic tissue was significantly lower in the abdominal aorta in the male GSE group than that of the control group. In addition, the cholesterol content in ascending and thoracic parts of aorta of the male GSE group tended to be lower than that of the control group. These findings indicate a time difference in the development of atherosclerotic lesions in the aortic wall between the control and the GSE-treated male WHHL rabbits. This time difference may be crucial for the evaluation of the effect of the treatment in models with genetically conditioned spontaneous hypercholesterolemia and atherosclerosis, because progression of atherosclerosis, once started, cannot be prevented but may be retarded. Thus our findings may indicate that the ingestion of GSE in the diet retarded the development of atherosclerosis in male WHHL rabbits. This finding of a beneficial effect on atherosclerosis in WHHL male rabbits is in accordance with reports in other models. In Kurosawa- and Kusanagi-hypercholesterolemic rabbits of both sexes treated with cocoa powder containing 7.8% total polyphenols, the aortic cholesterol content was lower compared with the cholesterol content in the aorta of the control group [37]. Cholesterol fed male Golden Syrian hamsters showed a significantly lower aortic fatty streak area after the consumption of different kinds of grape seed products [26]. Another study has also shown that grape polyphenols decrease plasma triglyceride and cholesterol accumulation in the aorta of ovariectomized guinea pigs [17]. The authors suggest that polyphenols exert their protective effects in the absence of estrogen. In fact, most of the studies of wine extracts, grape seed, or polyphenolic compounds showing significant beneficial effects on atherosclerosis are conducted with male animals [14–16, 38]. Thus, further studies are warranted to clarify the impact of the hormonal differences between male and female animals on the possible preventive effects of GSE on atherosclerosis.

In conclusion, feeding GSE extract to WHHL rabbits had no significant effect on hypercholesterolemia and aortic atherosclerosis in females, but was associated with a transient less hypercholesterolemic response to semisynthetic diet and furthermore retarded the development of aortic atherosclerosis in males, as demonstrated by significantly lower cholesterol content in the abdominal part.

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