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Polymeric grape seed tannins prevent plasma cholesterol changes in high-cholesterol-fed rats

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The objective of this study was to determine the effect of grape seed tannins in either monomeric or polymeric form on plasma cholesterol in rats. These tannins were supplemented at a level of 2% of the diet of high-cholesterol-fed Sprague Dawley rats for 9 weeks; the diets were compared with a diet without tannins (control diet) and with a standard diet. Rats fed diets containing 1% cholesterol showed an increase in total and low-density lipoprotein plasma cholesterol and a decrease in plasma high-density lipoprotein. These changes were prevented by the addition of 2% polymeric grape tannins but not by monomeric tannins. Similarly, liver weight, liver lipids and total liver cholesterol were increased by dietary cholesterol and these increases were largely inhibited by the tannin polymers but not the monomers. The polymers significantly increased the faecal excretion of lipids and cholesterol. In conclusion, we have shown that polymeric grape seed tannins exert a hypocholesterolaemic effect in high-cholesterol-fed rats.

INTRODUCTION

The relation between high levels of plasma lipids and the incidence of atherosclerosis and cardiovascular disease has long been established. The most convincing evidence of this relation is the fact that, in some animal species, atherosclerosis may be experimentally induced by use of cholesterol-rich diets. In rats, cholesterol feeding increased low-density lipoprotein cholesterol (LDLC) and decreased high-density lipoprotein cholesterol (HDLC) (Mahley & Holcombe, 1977). It is often considered that elevated plasma cholesterol, especially LDLC, is a predisposing factor for atherosclerosis and cardiovascular disease frequently occurring in industrialised countries (Steinberg, 1983; Goldstein & Brown, 1987). On the other hand, HDLC exerts a protective effect (Mattson & Grundy, 1985).

Many vegetable foodstuffs, especially fruits and beverages, contain high amounts of tannins. Green tea tannins (condensed tannins) have been reported to exert a hypocholesterolaemic effect in cholesterol-fed rats (Muramatsu *et al.*, 1986). More recently, Yugarani *et al.* (1992) demonstrated that dietary tannic acid and morin, a flavonol related to quercetin, decreased plasma total and LDLC. The presence of condensed tannins in grape seeds is well documented (Oh & Hoff, 1979; Da Silva *et al.*, 1991). These polyphenolic procyanidins arise from flavan-3-ols and are encountered as monomers, dimers, trimers, oligomers and polymers (Swain, 1977; Da Silva *et al.*, 1991); they have been identified in grape seeds (Joslyn & Dittmar, 1962; Czochanska *et al.*, 1979; Bourzeix *et al.*, 1986; Boukharta *et al.*, 1988; Ozmiansky & Lee, 1990).

Elsewhere, in many countries, a high intake of saturated fats is strongly correlated with cardiovascular disease but not in some regions of France: polyphenolic-rich foods and marked wine consumption may be, in part, responsible for this phenomenon (Renaud & De Lorgeril, 1992). The present study was designed in order to evaluate the effects of such procyanidins, either in monomeric or in polymeric form, on plasma cholesterol level in high-cholesterol-fed rats.

MATERIALS AND METHODS

Preparation of procyanidin extracts

Grape seed tannins were purchased from DRT (Les Dérivés Résiniques et Terpéniques, Dax, France) and contained essentially monomeric (11%), dimeric (31%), trimeric (21%) and tetrameric (37%) procyanidins, according to the manufacturer. Monomers and polymers were separated according to the procedure of Boukharta *et al.* (1988). Briefly, the powdered tannins were solubilised in 70% acetone and saturated with

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sodium chloride to salt out the acetone phase. The acetone was removed under vacuum and the resulting aqueous solution was then submitted to repeated ethyl acetate extractions. The ethyl acetate extracts were combined and evaporated to dryness under vacuum; this fraction yielded monomeric procyanidins and was named 'monomers'. Polymeric procyanidins were obtained after freeze-drying the aqueous phase. Non-polymeric contaminants were discarded by subjecting the dry extract to low-pressure liquid chromatography using Sephadex LH-20 gel (Kantz & Singleton, 1990); non-polymeric phenols were eluted from the gel by 60% methanol; the absorbed polymeric procyanidins were eluted with 50% acetone. The acetone was removed under reduced pressure and the resulting aqueous phase was lyophilised. This extract was designated 'polymers'.

Animals and diets

Male Sprague Dawley rats (Iffa Credo, France) weighing 190.8±2.4 g were housed in individual stainless-steel metabolic cages in a room maintained at 25±1°C with a 12 h light–dark cycle. They were divided into four equal groups of six animals according to the average body weight and received the diets according to a pair-feeding schedule. Water was provided *ad libitum*. The experiment was carried out over 63 days; food consumption and body weight were controlled daily.

The composition of the four experimental diets is reported in Table 1. The standard diet contained no added cholesterol and no tannins; the control diet contained no tannins and was hypercholesterolaemic; by addition of lard to this diet, saturated fatty acids were enhanced and polyunsaturated fatty acids reduced in order to promote the hypercholesterolaemic effect. Two other dietary groups were prepared by adding either 2% monomeric tannins or 2% polymeric tannins to the control diet. Cholesterol and tannins were incorporated at the expense of corn starch. The fatty acid composition of the diets is shown in Table 2.

Analytical procedure

At the end of the experimental period, the animals were fasted for 16 h and anaesthetised with sodium pentobarbital (Pentobarbital 6%, 60 mg/kg body weight). Blood was withdrawn by cardiac puncture with heparin-moistened syringes and collected into ice-cold tubes. Plasma was prepared by centrifugation and assayed enzymically for total, free, LDL and HDL cholesterol using Boehringer Mannheim kits (Boehringer Mannheim, Meylan, France). Cholesterol ester levels were calculated as the difference between free and total cholesterol values. Livers were excised, blotted dry, weighed and stored frozen (–80°C); livers and the faeces collected during the last 5 days were extracted for lipids with chloroform/methanol (2:1) according to the procedure of Folch *et al.* (1957); then, total liver and faecal cholesterol were determined as described by Carlson and Goldfarb (1977) using kits supplied by Boehringer Mannheim. The fatty acid composition of the diets was analysed by gas chromatography using methyl esters of fatty acids.

Table 1. Composition of experimental diets[a]

Ingredient	Standard	Control	Tannin supplemented
Casein[b] (14·44% N)	21·7	21·7	21·7
DL-Methionine[c]	0·4	0·4	0·4
Corn starch	47·9	42·9	40·9
Sucrose	5	5	5
Cellulose powder	5	0	0
Vegetable oil[d]	8	2	2
Lard	0	15	15
Vitamin mix[e]	1	1	1
Mineral mix[f]	11	11	11
Cholesterol	0	1	1
Tannins[g]	0	0	2

[a] Expressed on a dry matter basis.
[b] N × 6·38.
[c] Casein was supplemented with 2% DL-methionine.
[d] Maize oil/sunflower oil (1:1).
[e] Composition expressed in international units or g per kg of vitamin mix: retinyl acetate, 1 980 000 IU; cholecalciferol., 600 000 IU; DL-tocopheryl acetate, 17·0; menadione, 4·0; thiamin-HCl, 2·0; riboflavin, 1·5; calcium pantothenate, 7·0; pyridoxin–HCl, 1·0; inositol, 15·0; cyanocobalamin, 5×10^{-3}; ascorbic acid, 80·0; nicotinic acid, 10·0; choline–HCl, 136·0; folic acid, 0·5; *p*-aminobenzoic acid, 5·0; D-biotin, 3×10^{-2}.
[f] Salt mixture consisted of (g kg^{-2}): calcium dihydrogen phosphate, 430·0; potassium chloride, 100·0; sodium chloride, 100·0; magnesium chloride, 50·0; magnesium sulphate, 50·0; ferric oxide, 30·0; manganese sulphate, 2·5; zinc sulphate, 2·0; cupric sulphate, 0·5; cobalt sulphate, 4×10^{-3}; potassium iodide, 8×10^{-3}.
[g] Either monomers or polymers in accordance with dietary group.

Statistical analysis

Significance of differences between mean values (±SEM) were determined by one-way analysis of variance for repeated values (Winer, 1971); *a priori* contrasts of the groups were tested with Fisher's protected least signifi-

Table 2. Fatty acid composition of experimental diets

Fatty acid	Standard diet (g/100 g fatty acids)	Control and experimental diets (g/100 fatty acids)
14:0	—	1·25
16:0	10·55	23·80
16:1ω7	—	2·20
18:0	2·50	12·65
18:1ω9	44·25	42·30
18:2ω6	40·20	16·60
20:0	1·00	0·12
20:1ω9	—	0·90
22:0	1·50	0·18
Total saturated	15·55	38·00
Total monounsaturated	44·25	45·40
Total polyunsaturated	40·20	16·50

Table 3. Effect of diets on weight gain, food intake and plasma and liver cholesterol in rats[a]

	Standard	Control	Monomers	Polymers
Weight gain (g/day)	159 ± 11ab	187 ± 16c	177 ± 17ac	152 ± 12b
Food intake (g/day)	12·4 ± 0·6a	12·7 ± 0·1a	12·5 ± 0·1a	12·5 ± 0·5a
Plasma cholesterol (mg/100 ml)				
Total	78·1 ± 7·5a	121·1 ± 6·7b	112·2 ± 9·9b	79·1 ± 4·5a
Free	15·5 ± 1·0a	24·6 ± 2·1b	23·6 ± 2·1b	16·2 ± 0·5a
Esterified[b]	62·5 ± 6·5a	96·4 ± 5·34	89·3 ± 5·2	62·8 ± 4·2a
HDL	57·5 ± 5·78a	13·9 ± 2·3	24·6 ± 2·4	51·6 ± 6·1a
LDL	12·4 ± 1·2a	69·3 ± 1·2b	60·4 ± 4·1b	13·3 ± 3·5a
Liver				
Weight (% body wt)	3·01 ± 0·06a	3·88 ± 0·26b	3·60 ± 0·33bc	3·27 ± 0·22ac
Total lipids (mg/liver)	55·3 ± 2·5	171·4 ± 2·1	146·0 ± 9·0	82·4 ± 1·7
Total cholesterol (mg/g liver)	2·4 ± 0·4	42·7 ± 4·8	28·2 ± 1·2	12·4 ± 1·4

[a] Mean ± SEM; $n = 6$. Values without common following letters are significantly different at $P < 0·05$.
[b] Esterified cholesterol was calculated as (total cholesterol–free cholesterol).

cant differences at the probability level of 95%, by using a Stat View 512⁺ micro-computer program (Brain Power, Calabassa, CA, USA).

RESULTS

Control rats had increased body weight gain whereas animals from polymer and standard groups showed an identical growth; rats fed monomers had greater weight gain than rats fed polymers (Table 3). Food intake did not discriminate between the dietary groups by the pair-feeding technique.

The plasma total, free and LDL cholesterol levels were the highest in the control diet and were significantly decreased in polymer diet and reached the basic level of the standard diet; monomer diet led to intermediate results. The esterified cholesterol level changed according to an identical pattern, except that the addition of monomers in the diet led to a slight decrease compared to controls. In contrast, HDLC level was highest in standard and polymer groups which were not significantly different, lower in rats receiving the monomeric tannins and lowest in the control group (Table 3).

Liver weight, expressed as % body weight, was the highest in control rats and decreased following tannin intake and the difference was significant in rats fed polymers. In this last group of rats, liver weight did not significantly differ from that seen in standard rats. Identically, liver total lipids and cholesterol were high in the control group, significantly decreased after feeding monomers and even more in polymer-fed rats; these parameters failed to reach the basic levels exhibited by the standard rats (Table 3).

Faecal lipid excretion was significantly increased by feeding tannins, especially in polymeric form. Cholesterol excretion was greatly enhanced by monomers as well as by polymers (Table 4).

DISCUSSION

Feeding experiments with rats have generally demonstrated that diets, in which the fat component comprises predominantly saturated fatty acids, increase plasma cholesterol concentrations that are significantly higher than those resulting from diets in which polyunsaturated fatty acids (PUFA) predominate (McNamara, 1987; Grundy & Denke, 1990). Dietary cholesterol also exerts specific effects on plasma cholesterol (McNamara, 1987; Grundy & Denke, 1990). This was confirmed herein when comparing standard and control diets. The major finding of the present experiments was that the addition of grape seed tannins to a hypercholesterolaemic diet, and particularly the polymeric fraction, reproduced the same effects as a standard diet, on plasma cholesterol concentrations. This is clearly shown, for the first time, by this study, which emphasised differential effects between monomeric and poly-

Table 4. Effect of diets on total lipid and cholesterol excretion during the last 5 days of the experiment[a]

	Standard	Control	Monomers	Polymers
Faecal dry weight (g)	6·90 ± 0·50a	5·05 ± 1·04	6·45 ± 0·55a	6·80 ± 0·60a
Total lipid intake (g)	1·96 ± 0·06	4·71 ± 0·14a	4·61 ± 0·08a	4·69 ± 0·12a
Total lipid excretion (mg)	300 ± 25	541 ± 56	701 ± 51	875 ± 64
Excreted/ingested ratio (%)	15·3 ± 1·1a	11·5 ± 1·0	15·2 ± 0·9a	18·7 ± 1·2
Cholesterol intake (mg)	ND[b]	247 ± 8a	244 ± 4a	246 ± 7a
Cholesterol excretion (mg)	ND	122 ± 10	225 ± 15a	241 ± 9a
Excreted/ingested ratio (%)	ND	50·0 ± 5·2	93·1 ± 5·3a	98·5 ± 4·2a

[a] Mean ± SEM; $n = 6$. Values without common following letters are significantly different at $P < 0·05$.
[b] ND, not determined.

meric grape tannins . This effect, however, was observed by Würsch (1979) in rats fed tannin-rich carob pod fibre. Muramatsu *et al.* (1986) reported that dietary green tea catechins have a hypocholesterolaemic effect in experimental animals. Grape tannins have been largely neglected. The present results showed that cholesterol feeding triggered off an increase in LDLC in the control rats compared with standards; dietary polymeric tannins acted conversely. The mechanisms and consequences of the reverse cholesterol transport, that is the movement of cholesterol from the extra-hepatic tissues to the liver, and the role of plasma HDL as a physiological acceptor of tissue cholesterol have been reviewed (Millner, 1990). Moreover, the ratios of HDLC to total cholesterol of standard rats and polymer-fed ones are closely related (0.73 and 0.65, respectively). The data reported recently by Badimon *et al.* (1992) supported the protective effect of HDL while LDL was a risk factor (Goldstein & Brown, 1987). Thus, the authors suggest that polymeric grape seed tannins may be protective against atherosclerosis and cardiovascular disease, particularly because they also decreased plasma LDLC level. The study shows that monomeric grape seed tannins were not as effective in lowering plasma cholesterol.

The mechanism underlying these effects is not clear; the increased cholesterol excretion in rats fed tannins suggested that they decreased intestinal cholesterol absorption. This was supported by the work of Ikeda *et al.* (1992) using tea catechins. Nevertheless, the high cholesterol excretion by rats fed monomers remained difficult to explain. Elsewhere, an increase in the excretion of bile acids in faeces was observed in antibiotic-treated rats fed a diet containing condensed tannins (Horigome *et al.*, 1988). Since Kuyvenhoven *et al.* (1989) have reported that an increased excretion of faecal bile acids would depress body cholesterol resulting in lower blood cholesterol, an increased excretion of bile acids may be another mechanism by which polymeric grape seed tannins act. Further work providing a better understanding of the system involved is under investigation.

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