

Antioxidant Status, Lipoprotein Profile and Liver Lipids in Rats Fed on High-Cholesterol Diet Containing Currant Oil Rich in n-3 and n-6 Polyunsaturated Fatty Acids

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Summary

Plant-based n-3 polyunsaturated fatty acids (PUFA) possess a prospective antiatherogenic potential. Currant oil from *Ribes nigrum* L. is one of the few plant oils containing PUFA-n-3 (15.3 mol%) in addition to PUFA-n-6 (60.5 mol%). This study was aimed at comparing the effects of currant oil with those of lard fat, rich in saturated (43.8 mol%) and monounsaturated (47.0 mol%) fatty acids, on antioxidant parameters, the lipoprotein profile and liver lipids in rats fed on 1 % (w/w) cholesterol diets containing either 10 % of currant oil (COD) or lard fat (LFD). After 3 weeks of feeding, the COD induced a significant decrease in blood glutathione (GSH) and an increase in Cu²⁺ induced oxidizability of serum lipids, but did not affect liver GSH and *t*-butyl hydroperoxide-induced lipoperoxidation of liver microsomes. Although the COD did not cause accumulation of liver triacylglycerols as LFD, the lipoprotein profile (VLDL, LDL, HDL) was not significantly improved after COD. The consumption of PUFA-n-3 was reflected in LDL as an increase in eicosapentaenoic and docosahexaenoic acid. These results suggest that currant oil affects positively the lipid metabolism in the liver, above all it does not cause the development of a fatty liver. However, adverse effects of currant oil on the antioxidant status in the blood still remain of concern.

Key words

Polyunsaturated fatty acids • Currant oil • Antioxidant status • Lipoprotein profile • Liver lipids

Introduction

Dietary polyunsaturated fatty acids (PUFA), and particularly PUFA of the n-3 subcategory, have been shown to positively affect atherogenic indices of vascular functions and of lipid metabolism such as cholesterol and triacylglycerol (TAG) lipoprotein profiles in the plasma

(Nestel 1998, Harper and Jacobson 2001). Epidemiological and clinical trial evidence suggests that n-3 PUFA might have a significant role in the prevention of coronary heart disease (Harper and Jacobson 2001). Most antiatherogenic effects have been demonstrated with the fish oil-derived n-3 PUFA (20:5 eicosapentaenoic acid – EPA and 22:6 docosahexaenoic acid – DHA). Lately, the

attention has also been given to delineation of the cardioprotective potential of plant-based n-3 PUFA, such as alpha-linolenic acid – ALA (Jeffery *et al.* 1996, Harper and Jacobson 2001), which can be converted to the longer chain EPA and DHA.

However, PUFA can also cause deterioration of the antioxidant status due to their liability to become oxidized (Porter *et al.* 1995, Noguchi and Niki 1999). A high intake of PUFA has been demonstrated to increase the formation of lipid radicals (Porter *et al.* 1995) and to deplete endogenous antioxidants (Pathasarathy *et al.* 1999). Thus, a potential destabilization of antioxidant status remains a concern, particularly when plant oils containing ALA are rich in PUFA of the n-6 subcategory (Harper and Jacobson 2001).

Currant oil from seeds of the plant *Ribes nigrum* L. containing a high proportion of PUFA (75 %) at a n-6/n-3 ratio of 4.0 is rich in alpha-linolenic acid (18:3n-3), linoleic acid (18:2n-6) and gamma-linolenic acid (18:3n-6). Currant oil has been shown to positively modify some risk factors related to the development of coronary disease such as hypertension (Engler 1993, Deferne and Leeds 1996), inflammatory processes (Ziboh and Fletcher 1992, Tate and Zurier 1994) or platelet adhesion (Bertomeu *et al.* 1990) and aggregation (Kockmann *et al.* 1989). An immune-enhancing effect of currant oil has been reported in healthy elderly subjects (Wu *et al.* 1999). Recently currant oil has been demonstrated to mildly attenuate lipoprotein changes in rats, when substituted in a high-cholesterol diet for lard fat (Škottová *et al.* 2000). Lard fat contains a majority of saturated (SFA)/monounsaturated (MUFA) fatty acids that are known to increase serum cholesterol (Grundy 1998) and TAG (Higashi *et al.* 1997). However, currant oil feeding has been associated with increased sensitivity of LDL to copper-induced oxidation *in vitro* (Škottová *et al.* 2000), suggesting a potentially negative effect of currant oil on antioxidant status.

Therefore, the aims of this work were (i) to evaluate antioxidant status in the blood and liver, (ii) to determine the liver and plasma lipid content (cholesterol and triacylglycerols) including the plasma lipoprotein profile, and (iii) to define the lipoprotein fatty acid composition in a comparative study in rats fed high-cholesterol diets containing currant oil or lard fat.

Material and Methods

Chemicals

Currant oil from *Ribes nigrum* L. was purchased from Jan Dekker (The Netherlands). Fentanyl was from

Janssen (Belgium), medetomidin (Domitor) from Pfizer (USA), diazepam (Apaurin) from Krka (Slovenia) and cholesterol was purchased from Sigma (USA). *Tert*-butyl hydroperoxide (*t*BH) was obtained from Merck (Germany) and the Bio-La-Test Cholesterol 250 E and Bio-La-Test Triacylglycerol T 500 were from Lachema (Czech Republic). Standards for the determination of fatty acids were from Sigma. Other chemicals were of analytical grade.

Animals

All animal procedures were approved by the Ethics Committee from the Ministry of Education, Czech Republic. Male Wistar rats (body weight 220-250 g) were kept in standard laboratory conditions with free access to water. They were fed *ad libitum* on these diets: 1) STD - standard laboratory diet (KrmMo Mohelsky, Brno, Czech Republic), 2) COD – currant oil diet prepared by adding 1 % (w/w) of cholesterol and 10 % (w/w) of currant oil to the standard diet, and 3) LFD – lard fat diet prepared by adding 1 % (w/w) of cholesterol and 10 % (w/w) of lard fat to the standard diet. Each group consisted of six animals. Amounts of diets consumed were checked daily per cage of two rats, body weights of rats were measured individually at intervals as indicated in Figure 1B. All groups of rats were fed for three weeks.

After overnight fasting the rats were anesthetized by i.m. administration of fentanyl (40 µg/kg b.w.) in combination with medetomidin (200 µg/kg b.w.), followed by i.m. administration of diazepam (5 mg/kg b.w.). The blood was taken from the aortic bifurcation and divided into three aliquots. The first aliquot of blood was collected into EDTA.Na₂ (1 mg/ml) and plasma was separated by centrifugation (2500 x g, 20 min, 10 °C). The second aliquot of blood was used for isolation of serum by centrifugation (2500 x g, 20 min, 10 °C) and the third aliquot of blood was deproteinized by TCA (final concentration 0.05 g/ml) and used for determination of glutathione (GSH). The liver was removed and rinsed in ice-cold saline, weighed and divided into three portions. One piece of liver was frozen for analysis of the lipid content. For determination of GSH the liver sample was immediately homogenized (5 % w/v) in an ice-cold solution of 0.02 mol/l EDTA.Na₂ and deproteinized by TCA (final concentration 0.05 g/ml). For assay of lipid peroxidation in liver microsomes, the tissue sample was homogenized (10 % w/v) in ice-cold TRIS-HCl buffer (3 mmol/l) containing 0.25 mol/l sucrose and 0.1 mmol/l EDTA.Na₂, pH 7.4.

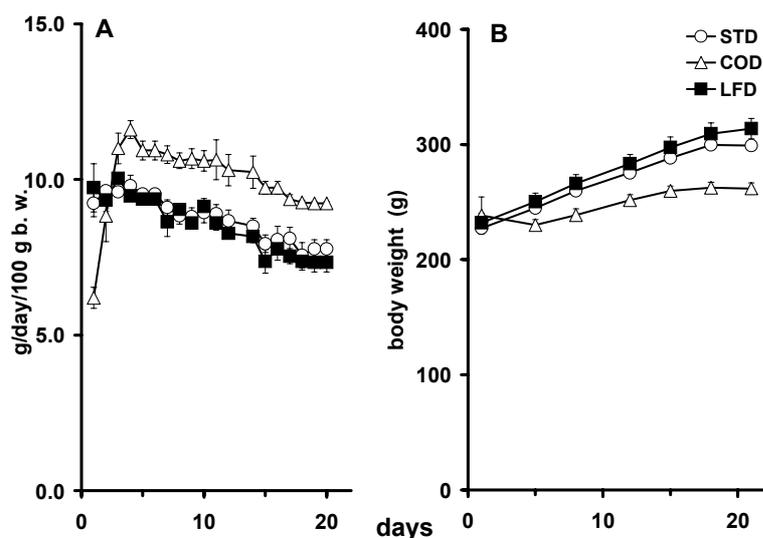


Fig. 1. Diet consumption (A) and growth curves (B) in rats fed on high-fat high-cholesterol diets. Results are expressed as means \pm S.E.M., $n=6$.

Lipid analysis

Plasma lipoproteins (VLDL, LDL and HDL) were isolated by sequential density gradient ultracentrifugation (Havel *et al.* 1955). Liver lipids were extracted according to Haug and Hostmark (1987). Cholesterol and TAG were measured enzymatically using Bio-La-Tests. Fatty acid composition of dietary fats, diets, plasma and lipoprotein fractions was analyzed by gas chromatography after transmethylations (Lin and Horning 1975, Chvojková *et al.* 2001). Individual peaks of fatty acid methyl esters were identified by comparing retention times with those of the standards. The relative content of individual fatty acids was expressed as mol%.

Glutathione and lipid peroxidation assay

GSH was determined in supernatants obtained by centrifugation (2500 \times g, 15 min, 10 °C) of TCA-deproteinized blood and liver samples (Sedlak and Lindsay 1968). Plasma TBARS (thiobarbituric acid reactive substances) were measured according to Buege and Aust (1978). Oxidation of serum lipids was induced by adding copper ions to the serum (final concentration of Cu^{2+} was 72 $\mu\text{mol/l}$ in serum diluted 1:50 in PBS buffer, pH 7.5) and monitored as conjugated diene formation at 245 nm (Schnitzer *et al.* 1998). For *t*BH-induced lipid peroxidation the liver microsomes were isolated by differential centrifugation (Haraguchi *et al.* 1995) and stored at -80 °C. Lipid peroxidation in microsomal fractions, resuspended in 1.15 % KCl and 50 mmol/l TRIS-HCl, 0.1 mmol/l EDTA.Na₂ buffer, pH 7.6 (1 mg of protein/ml), was induced by adding *t*BH (final concentration 1.0 mmol/l). TBARS were determined

(Buege and Aust 1978) in reaction mixtures after 1 h incubation at 37 °C. Microsomal proteins were estimated according to Lowry *et al.* (1951).

Statistics

The Statgraphics (version No 5.0) test was used for determination of statistical significance.

Results

Table 1 shows fatty acid composition of the low-fat commercial diet (standard diet) and two high-fat high-cholesterol diets (standard diet supplemented with 1 % of cholesterol and with either 10 % currant oil or lard fat), and fatty acid composition of both fats. Currant oil contains 13.0 mol% of alpha-linolenic acid 18:3n-3 and total amount of PUFA-n-3 is 15.3 mol%. Nevertheless, PUFA-n-6 are the main components (60.5 mol%) of currant oil due to 49.6 mol% of linoleic acid 18:2n-6. Lard fat is characterized by high contents of MUFA (47.0 mol%), due to oleic acid 18:1 (44.0 mol%), and SFA (43.8 mol%). SFA/PUFA and PUFA n-6/n-3 ratios are noticeably lower in currant oil. Fatty acid composition of currant oil and lard fat is proportionally reflected in the final composition of COD and LFD.

From the fourth day till the end of the experiment, the consumption of COD was higher than of the LFD and STD (Fig. 1A). Higher consumption of COD was associated with a lower weight gain (Fig. 1B) compared to that of rats fed on LFD or STD, but no gastrointestinal signs were evident in COD fed rats.

Table 1. Fatty acid composition of experimental diets and dietary fats (currant oil and lard fat).

Fatty acid (mol%)	Low-fat diet	High-fat high-cholesterol diet		Dietary fat	
	STD	COD	LFD	Currant oil	Lard fat
12:0	0.2	0.1	0.1	–	0.1
14:0	1.0	0.4	1.1	0.1	1.1
16:0	23.4	9.7	24.5	5.6	24.5
17:0	0.3	0.2	0.4	0.1	0.3
18:0	5.2	2.6	16.5	1.6	17.4
20:0	0.4	0.3	0.3	0.4	0.4
22:0	0.6	0.2	0.2	–	–
16:1	2.5	0.8	2.3	0.2	2.2
18:1	27.6	20.3	40.9	14.8	44.0
20:1	1.0	1.0	0.9	1.0	0.8
18:3 (n-3)	3.0	8.7	0.7	13.0	0.4
18:4 (n-3)	–	1.2	–	2.3	–
20:3 (n-3)	–	–	0.1	–	0.1
18:2 (n-6)	30.9	46.8	10.8	49.6	6.8
18:3 (n-6)	–	6.9	–	10.9	–
20:2 (n-6)	–	0.2	0.3	0.3	0.4
20:3 (n-6)	–	–	0.1	–	0.1
20:4 (n-6)	–	–	0.2	–	0.2
Unidentified peaks	3.9	0.6	0.6	0.1	1.2
Amounts of FA (mg/g)	18.6	106.3	115.6	–	–
SFA	31.1	13.5	43.1	7.8	43.8
MUFA	31.1	22.1	44.1	16.0	47.0
PUFA (n-3)	3.0	9.9	0.8	15.3	0.5
PUFA (n-6)	30.9	53.7	11.1	60.5	7.1
SFA/MUFA	1.00	0.61	0.98	0.49	0.93
SFA/PUFA	0.92	0.21	3.53	0.10	5.48
PUFA n-6/n-3	10.30	5.42	13.88	3.95	14.20

Figure 2 shows the antioxidant parameters of the blood and liver of rats fed STD, COD and LFD. COD, compared to LFD, decreased the blood GSH concentration and insignificantly increased the plasma TBARS and *t*BH-induced lipoperoxidation of liver microsomes. On the other hand, the LFD decreased the content of GSH in the liver. Liver weight did not differ between the COD and LFD groups (9.89 ± 0.30 g vs 10.17 ± 0.52 g, N.S.), but was higher after both experimental diets in comparison to STD (8.09 ± 0.30 g, $p < 0.01$). Influence of experimental diets on the kinetics of serum oxidation induced by Cu^{2+} ions is shown in Figure 3. Feeding of rats by COD resulted in reduction of the lag time (62.16 ± 2.87 min) in comparison to the

LFD (215.74 ± 18.76 min) as well as the STD groups (86.81 ± 5.36 min).

Categories of fatty acids, grouped according to the degree of saturation and to the n-3 or n-6 subcategory, found in the plasma, VLDL, LDL and HDL of rats fed on experimental diets are shown in Figure 4. Administration of COD, rich in PUFA, resulted in higher PUFAn-6 in HDL (18:3, 20:2, 20:4, 22:4, not shown) and PUFAn-3 in LDL (see also Fig. 5). Ratios of n-6/n-3 in LDL was 0.91 after COD and 2.52 after LFD, and in HDL 3.19 after COD and 1.76 after LFD. SFA and MUFA in LDL were lower in the COD group compared to the LFD group.

Categories of fatty acids (SFA, MUFA, PUFA-n-6 and n-3) in total plasma and VLDL were the same after COD and LFD feeding. Figure 5 demonstrates the spectrum of individual fatty acids in LDL. In the COD fed group saturated fatty acids 16:0 and 18:0 were lower as well as the monounsaturated 20:1, while PUFA-n-3 20:5, 22:5 and 22:6 (but not 18:3) were higher in comparison to

LFD. However, the 22:6 in HDL was substantially lower in the COD group (not shown). Although the total PUFA-n-6 in LDL did not differ between COD and LFD groups (see Fig. 4), there were differences in individual PUFA-n-6 (18:2, 20:4 and 22:4 were lower, whereas 18:3 and 20:2 were higher in COD than in the LFD fed group).

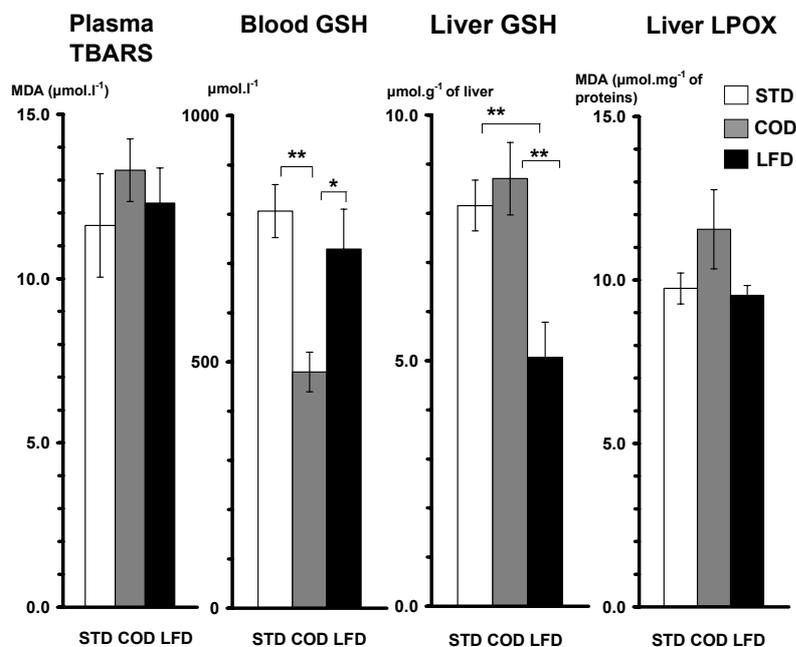


Fig. 2. Oxidation parameters in blood, plasma and liver in rats fed on high-fat high-cholesterol diets. Results are expressed as means \pm S.E.M., $n=6$, $*p<0.05$, $**p<0.01$.

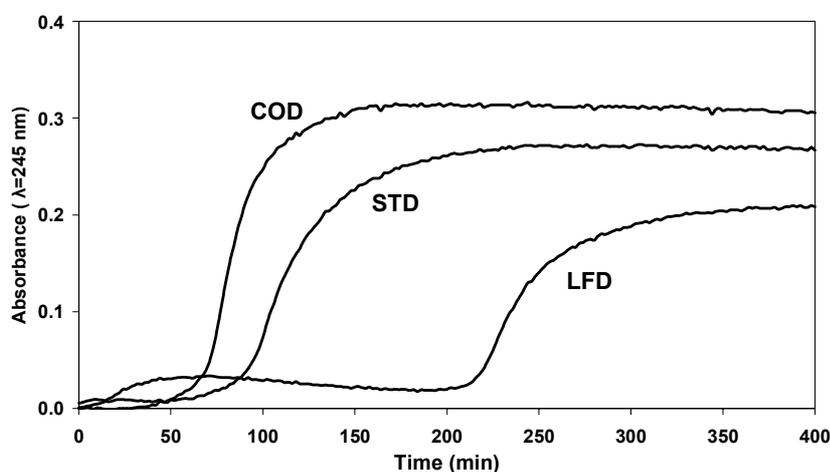


Fig. 3. Kinetics of lipid oxidation induced by Cu^{2+} ions in serum of rats fed on high-fat high-cholesterol diets. The record of time courses is representative for one analytical run.

Figure 6 demonstrates that both high-cholesterol high-fat diets caused moderate increases in total plasma cholesterol and TAG. Plasma lipids as well as lipoprotein profiles were not significantly distinguishable in the COD group in comparison with the LFD group. Both high-cholesterol diets induced accumulation of cholesterol in the liver. However, in contrast to the LFD, the COD did not induce any significant accumulation of TAG in the liver.

Discussion

In the present report, feeding rats a high-cholesterol diet containing currant oil instead of lard fat was found to induce pro-oxidant changes in markers of oxidative stress in the blood. These changes were manifested as a significant depletion of blood concentrations of reduced glutathione, non-significant increases in plasma TBARS, and a reduction in lag time

for diene production induced in serum lipids by copper ions *in vitro*, indicating an increased susceptibility of serum lipids to oxidation (Schnitzer *et al.* 1995, 1998).

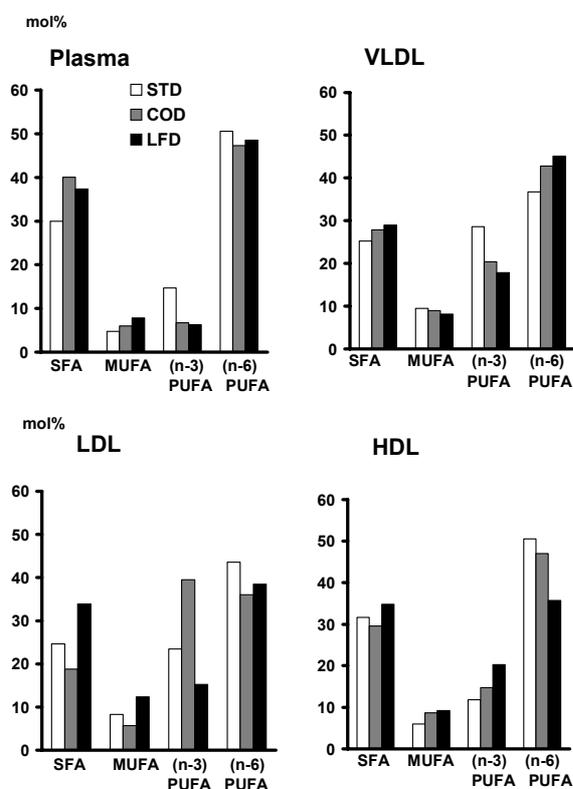


Fig. 4. Categories of fatty acids in plasma, VLDL, LDL and HDL in rats fed on high-fat high-cholesterol diets. The data represent means from two samples of pooled plasma or lipoprotein fraction (each obtained from 3 rats).

The amounts of diets consumed and the fatty acid composition of the diets suggest that the currant oil diet ensured at least a ten times higher supply of PUFA, both n-3 (mainly alpha-linolenic acid) and n-6 (mainly linoleic acid and linolenic acid) as compared to the lard fat diet which was characterized by a higher content of saturated (mainly palmitic acid and stearic acid) and monosaturated fatty acids (mainly oleic acid). However, this was not reflected in the distribution of fatty acid categories in the plasma. Although a majority of studies has demonstrated that plasma fatty acid composition can reflect dietary fatty acids (Manku *et al.* 1983, Dougherty *et al.* 1987, Judd *et al.* 1989, Lopez *et al.* 1991), this marker did not appear to be as sensitive as plasma phospholipids in short-term (weeks to months) dietary fat intake (Holman 1986, Riboli *et al.* 1987, Kwon *et al.* 1991). In fact, even plasma phospholipids may not always reflect the dietary fatty acid composition (Sinclair

et al. 1994). The inadequate response of plasma lipid fatty acid composition to dietary fatty acids could be a result of the action of many factors modulating absorption, distribution and the metabolic fate of specific fatty acids. It can involve, among others, the chemical structure of lipid sources (Carvajal *et al.* 2000, Amate *et al.* 2001), the degree of fatty acid unsaturation (Nestel 1998) as well as a high cholesterol content of the diet (Sakono *et al.* 1996).

Although total n-3 and n-6 PUFA in the plasma were not increased after COD feeding, the easier oxidizability of serum lipids was accompanied by total PUFA enrichment (due to PUFAn-3) in LDL, rendering these lipoproteins more sensitive to oxidation (Whitman *et al.* 1994). PUFAn-3 enrichment in LDL involved an increase in EPA and DHA, but not in alpha-linolenic acid, indicating elongation and desaturation processes in alpha-linolenic acid metabolism. On the other hand, PUFAn-3 were lower in HDL after COD (due to DHA). As n-6/n-3 ratio shows, the opposite trends are attributable to PUFAn-6 distribution between LDL and HDL after the COD, which is rich in linoleic acid. This fatty acid was lower in LDL similarly as arachidonic acid (20:4n-6) and docosatetraenoic acid (22:4n-6). The concentration of these PUFA are controlled primarily by the desaturase system. On the other hand, eicosadienoic acid (20:2n-6), an exclusive product of elongation, was higher. Accordingly, administration of PUFA-rich COD (relatively enriched in PUFAn-3 as shown by a lower n-6/n-3 ratio compared to that of a LFD) can lead not only to the preferential metabolism of PUFAn-3 (Gerster 1998), but also to the events channelling them into LDL. Among others, an increased EPA incorporation into liver lipids, observed after currant oil feeding in guinea pigs (Crozier *et al.* 1989), could play a role, since LDL are known to transport cholesteryl esters formed in liver. Thus specific enrichment of LDL with PUFA in COD fed rats may facilitate the oxidation of serum lipids (Schnitzer *et al.* 1998).

GSH plays a pivotal defensive role against oxidative insults as an endogenous scavenger of free radicals (Cooper and Kristal 1997). Its level in the blood is a sensitive indicator of antioxidant status in circulation (Piemonte *et al.* 2001). The finding of lower GSH in the blood in COD fed rats corresponds to the PUFAn-3 induced increase of oxidized glutathione in human blood (Jenkinson *et al.* 1999). Plasma TBARS levels, evaluated as a nonspecific plasma index of lipoperoxidation (Jenkinson *et al.* 1999), was not significantly increased after COD.

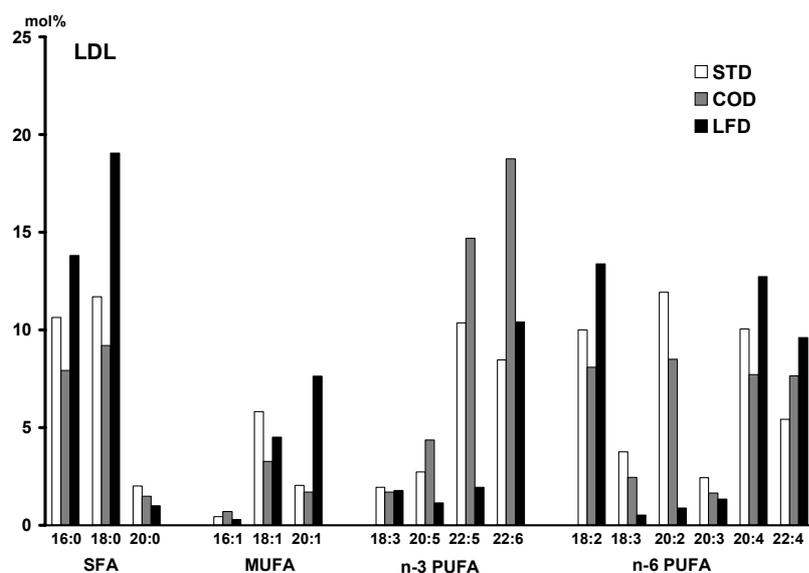


Fig. 5. Fatty acid composition in LDL in rats fed on high-fat high-cholesterol diets. The data represent means from two samples of pooled LDL (each obtained from 3 rats).

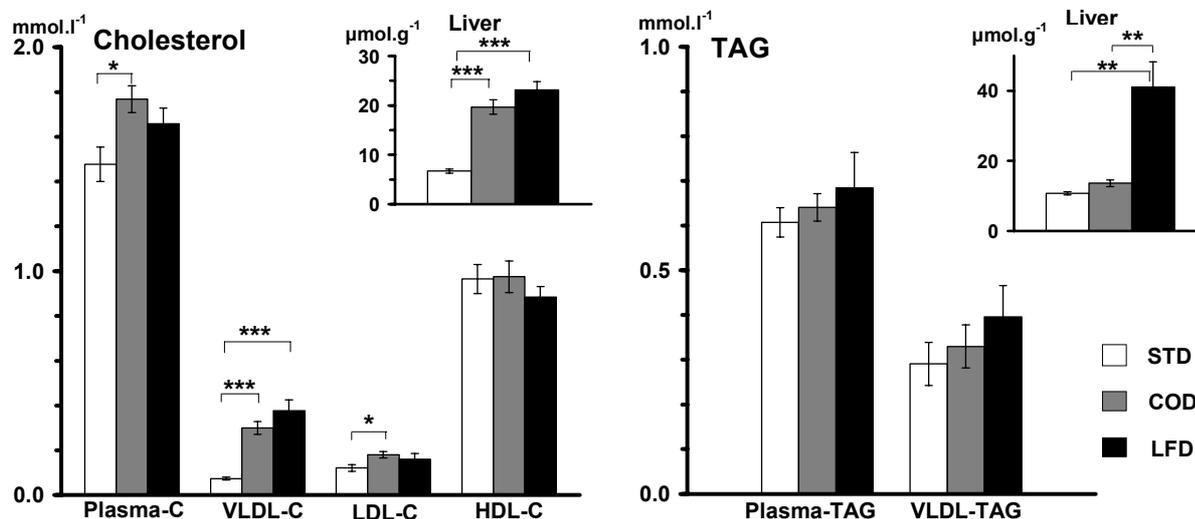


Fig. 6. Plasma lipoprotein profiles and liver lipids (cholesterol and triacylglycerols) in rats fed on high-fat high-cholesterol diets. Results are expressed as means \pm S.E.M., $n=6$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

In the liver, COD feeding did not significantly affect the lipoperoxidation of microsomes induced *in vitro* by *t*-butyl hydroperoxide. This suggests that the susceptibility of membrane fatty acids to form free radicals is not noticeably influenced. Although an increased EPA content has been observed in red blood cell membranes after currant oil intake (Diboune *et al.* 1992), the unchanged PUFA-n-3 and n-6 composition has been shown in rat cell membranes from the heart and liver (Baracca *et al.* 1994). In addition, the *in vivo* intensity of lipoperoxidation processes in the liver can be stabilized because COD did not decrease the content of

liver GSH. A maintenance of liver GSH under conditions of increased lipoperoxidation has been suggested as a supportive and compensatory mechanism (Cooper and Kristal 1997, Spolarics and Meyenhofer 2000) reflecting higher „capacity“ of liver compared to erythrocytes (Valencia *et al.* 2001). A decrease in liver GSH is often related to hepatic fatty infiltration in different experimental models (Soltys *et al.* 2001, Vendemiale *et al.* 2001). In our experiments, a lower content of liver GSH was observed after LFD, which caused an accumulation of both liver lipids – cholesterol and TAG, while feeding COD induced exclusively an accumulation

of liver cholesterol. However, in spite of this difference in lipid composition of the liver between COD and LFD groups, plasma lipids as well as lipoprotein profiles were not significantly different. This suggests that cholesterol supplementation of the diet containing the currant oil could reduce known positive effects of PUFA on plasma lipid indices (Grundy 1998, Stangl 2000, Fukushima *et al.* 2001, Zheng *et al.* 2001).

Differences in TAG content in the liver found after COD and LFD feeding can be attributed to the effects of dietary fatty acids according to their degree of saturation, since dietary cholesterol *per se* appears to contribute to the accumulation of liver triacylglycerols by stimulation of hepatic TAG biosynthesis and a decrease in oxidation of fatty acids in the rat (Fungwe *et al.* 1993). Monounsaturated oleic acid, which is present in a high proportion in LFD, can stimulate secretion of chylomicrons enriched in TAG and increase the magnitude of postprandial chylomicron remnants (Higashi *et al.* 1997). On the other hand, PUFA-rich diets reduce fatty acid availability for TAG synthesis in the liver (Nestel 1990, Brown *et al.* 1999). This is

particularly caused by the inhibition of fatty acid synthesis *de novo* and by the stimulation of fatty acid oxidation (Nestel 1998). In addition, the effects of PUFA on TAG synthesis have been related to antiadiposity (Bremer 2001). In our experiments, a higher COD consumption was associated with a smaller body weight increment. This is in agreement with the findings that feeding of rats on PUFA diets, such as sunflower oil rich in linoleic acid (Stangl 2000) and linseed oil containing a high proportion of alfa-linolenic acid (Jefferery *et al.* 1996), results in lower body fat and lower body weight.

In conclusion, this study indicates that currant oil, when substituted for lard fat in a high-cholesterol diet, partly exerts a positive effect on lipid metabolism in the liver, but has partly adverse effects on antioxidant status, especially in the blood.

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