EFFECT OF SILYMARIN AND SILIBININ-PHOSPHATIDYLCHOLINE COMPLEX ON PLASMA AND LIPOPROTEIN CHOLESTEROL, AND OXIDATION OF LDL IN RATS FED ON HIGH CHOLESTEROL DIET SUPPLEMENTED WITH CURRANT OIL

Nina Škottová^a, Vladimír Krečman^b, Petr Váňa^a, Zdeněk Chmela^c, Jitka Ulrichová^b,
Vilím Šimánek^b

^a Institute of Pharmacology, Medical Faculty, Palacký University, Hněvotínská 3, 775 15 Olomouc, Czech Republic

^b Institute of Medical Chemistry, Medical Faculty, Palacký University, Hněvotínská 3, 775 15 Olomouc, Czech Republic

^c Institute of Pathological Physiology, Medical Faculty, Palacký University, Hněvotínská 3, 775 15 Olomouc, Czech Republic

Received October 14, 2000

Key words: Silymarin / Silibinin / Polyunsaturated fatty acids / Lipoprotein cholesterol / LDL oxidation

This work was aimed to investigate the currant oil-induced modulation of antihypercholesterolemic and LDL antioxidant effects of silymarin (SM), the extract from *Silybum marianum* L. containing silibinin as the main flavonolignan, and of better bioavailable silibinin-phosphatidylcholine complex (SPC) in rats fed on high-cholesterol (HC) high-fat diet. Feeding of rats on HC-diet supplemented with 10 % of currant oil (from *Ribes nigrum* L.) containing polyunsaturated fatty acids (PUFA, 61.1% of n-6 and 15.4 % of n-3) and lower amounts of saturated (SFA, 7.7%) and monounsaturated (MUFA, 14.3%) fatty acids caused a significant decrease in plasma cholesterol associated with a mild decrease in VLDL-C and an increase in HDL-C, when compared to rats fed on HC-diet with 10 % of lard fat containing low amounts of PUFA (7.7% of n-6 and 0.7 % of n-3) and higher amounts of SFA (42.7%) and MUFA (47.5%). However, currant oil feeding led to the increased oxidizability of LDL. Silymarin, but not SPC, was effective in prevention of development of dietary induced hypercholesterolemia in the both dietary fats with slightly better result in diet containing the currant oil. On the other hand, SPC suppressed more effectively than silymarin LDL oxidizability. The results suggest that antihypercholesterolemic effect of SM in rats fed on HC-diet is improved by dietary currant oil, but the currant oil induces an increased oxidizability of LDL. This can be suppressed by improvement of bioavailability of silibinin, as shown here for silibinin-phosphatidylcholine complex.

INTRODUCTION

Saturated fatty acids and cholesterol are two dietary constituents well-established as serum cholesterol-raising nutrients¹. In human, a 15 % higher LDL-cholesterol can increase the risk of coronary heart disease by about 15 %–45 %². Substitution of dietary saturated fats by oils containing high amounts of polyunsaturated fatty acids has beneficial effects on hypercholesterolemia¹. On the other hand, high intake of polyunsaturated fatty acids can induce formation of oxidatively modified LDL, which are proposed as risk factor involved in the so-called “oxidation hypothesis” of atherosclerosis^{3–5}. Since the type of dietary fat can inversely modulate plasma cholesterol level and sensitivity of LDL to oxidation, requirements of dietary antioxidants seem to be mandatory in the case of diets rich in PUFA⁶.

Flavonoids are large group naturally occurring antioxidant polyphenols and they have received considerable attention during the last decade⁷. In human the

flavonoids contribute greatly to the dietary sources of antioxidants and their intake decreased the risk of coronary heart disease as shown in Zutphen and other studies^{8–10}. Silymarin is the standardized extract of flavonolignans from medicinal plant *Silybum marianum* (L.) Gaertn., Asteraceae, containing as the main compound flavonolignan silibinin. Silymarin is used in supportive therapy of liver diseases and its cytoprotective activity is believed to be based on antioxidant properties¹¹. Recently, we have shown that silymarin (but not silibinin) prevents the development of dietary induced hypercholesterolemia in rats¹², and both silymarin and silibinin possess LDL antioxidant activity *in vitro*¹³. However, *in vivo* effects of silibinin are limited by its low bioavailability, which can be improved by complexation of silibinin with phosphatidylcholine¹⁴.

In this study the rats were fed on high cholesterol diet and effects of silymarin and silibinin: phosphatidylcholine complex on changes of plasma lipoprotein levels and oxidizability of LDL induced by dietary PUFA-rich currant oil were investigated.

MATERIAL AND METHODS

Chemicals. Silymarin (Galena Opava, Czech Republic) was extracted from *Silybum marianum*, var. *Silyb* (L.) Gaertner. (Asteraceae) and contained (HPLC analysis) 44.5 % of silibinin (silybin), 1.4 % of silydianin, 10.9 % of silychristine and 2.5 % of taxifolin. Silibinin:phosphatidylcholine complex (Silipide) was generous gift from Dr. G. P. Forni (Indena Milano, Italy) and currant oil from Dr. K. Vrkoč (Academy of Sciences, Czech Republic). Bio-La-Test Oxochrom Cholesterol 250E was from Lachema (Czech Republic). Other chemicals were of analytical grade.

Animals. Female Wistar rats (160–170 g b.w.) were kept in standard laboratory conditions with free access to water and high cholesterol diet containing 1 % of cholesterol and 10 % of lard fat or currant oil (from the seeds of *Ribes nigrum*, L.) for 3 weeks. Silymarin (1% w/w) or silibinin:phosphatidylcholine complex (0.1 % w/w) were administered as dietary supplements in high cholesterol diets. Neither amounts of consumed diet nor body weight were affected due to the dietary interventions. After overnight fasting the blood was taken from aortic bifurcation under ether anesthesia. The blood was collected into Na₂EDTA (mg.ml⁻¹) and NaN₃ (0.1 mg.ml⁻¹).

Analysis. Plasma was separated by centrifugation for 20 min at 2500 x g. Plasma lipoproteins (VLDL, LDL, HDL₂ and HDL₃) were isolated by sequential ultracentrifugation in density gradient¹⁵, cholesterol was measured enzymatically using Bio-La-Test. LDL oxidation was evaluated as Cu²⁺-induced formation of malondialdehyde measured by thiobarbituric acid reactive substances assay¹⁶. Composition of fatty acids in lard fat and currant oil was analyzed by gas chromatography¹⁷ as previously described¹⁸ and relative content of individual fatty acids was expressed as a percentage of individual peak areas of total sum peak areas. Statistical analysis was performed using Student's t-test.

RESULTS

Data presented in the Table 1 show that supplementation of high cholesterol diet with 10 % of currant oil leads to development of lower level of plasma cholesterol than supplementation of the diet with lard fat. This effect of currant oil is associated with a mild decrease in VLDL cholesterol and an increase in HDL cholesterol due to the HDL₂ cholesterol. In currant oil supplemented diet silymarin administration resulted in slightly lower level of plasma and VLDL cholesterol than in lard fat diet. Administration of silibinin:phosphatidylcholine complex did not additionally decrease the levels of plasma cholesterol in any dietary fat, but in currant oil supplemented diet mild positive changes in cholesterol distribution among lipoproteins appeared – a decrease in VLDL and LDL, and an increase in HDL due to HDL₂. Currant oil induced an increase in Cu²⁺-oxidation of LDL. Silymarin slightly and silibinin:

phosphatidylcholine complex significantly reduced LDL oxidizability in the both high fat diets.

Analysis of fatty acid composition of dietary fats – lard fat and currant oil (Table 2) demonstrates that the contents of myristic, palmitic, stearic and oleic acid are lower, and of linoleic, linolenic and octadecatetraenoic acid are higher in currant oil than in lard fat. Table 3 summarizes types of fatty acids in lard fat and currant oil showing majority of polyunsaturated fatty acids in currant oil and majority of saturated and monounsaturated fatty acids in lard fat. Ratio PUFA/SFA in currant oil is higher (almost 10 times) and ratio PUFA n-6/n-3 lower than in lard fat.

DISCUSSION

Feeding of rats for 3 weeks with high cholesterol diet containing 10 % of lard fat induced the development of higher hypercholesterolemia than the diet containing 10 % of currant oil. This can be related to the qualitative differences in fatty acid composition between the lard fat (majority of SFA and MUFA) and currant oil (majority of PUFA). An important role in prevention of degree of diet-induced hypercholesterolemia can be mainly ascribed to the higher content of n-6 PUFA in currant oil. It has been shown that n-6 PUFA lower plasma and LDL-cholesterol level when substituted for saturated fatty acids in the diet¹. Antihypercholesterolemic effect of currant oil was associated with a mild decrease in VLDL and an increase in HDL, both changes accepted as positive trends in distribution of cholesterol among lipoproteins.

In contrast to cholesterol lowering effect of currant oil there is an increased sensitivity of LDL to copper-induced oxidation, probably due to the higher content of n-6 and n-3 polyunsaturated fatty acids, which are prone to oxidative processes¹⁹. It has been suggested that high intake of PUFA may be disadvantageous, because the oxidative degradation of PUFA may lead to the formation of modified atherogenic LDL³. At present there is accumulating evidence that PUFA-rich diets increase the lipoprotein susceptibility to undergo oxidative modifications, while oleate-rich diets (and generally MUFA-rich diets) produce lipoproteins more resistant to *in vitro* oxidation^{6, 19–21}. Thus the high content of monounsaturated oleic acid (44.2 %) in lard fat could contribute to the relative resistance of LDL to oxidation after lard fat feeding.

In agreement with our previous results¹² silymarin administered as a 1 % dietary supplement in high-cholesterol, high-fat diets inhibited the development of diet-induced hypercholesterolemia in rats and positively affected cholesterol distribution in lipoprotein fractions (decrease in VLDL and LDL, increase in HDL). A combination of silymarin with currant oil in high cholesterol diet led to the slightly lower level of plasma cholesterol than the combination of silymarin with lard fat suggesting the trend of adding effect of both cholesterol

Table 1. Effects of silymarin and silibinin: phosphatidylcholine complex on plasma and lipoprotein cholesterol, and Cu²⁺-mediated oxidation of LDL in rats fed on high-fat (lard fat or currant oil), high-cholesterol diet

Cholesterol (mmol.l ⁻¹)	Controls		Silymarin (1%)		Silibinin:phosphatidylcholine complex (0.1%)	
	lard fat (n = 8–9)	currant oil (n = 10)	lard fat (n = 5)	currant oil (n = 5)	lard fat (n = 4–5)	currant oil (n = 5)
Plasma	3.01±0.23	2.40±0.16 ^{(a)*}	1.55±0.19 ^{(a)****}	1.36±0.10 ^{(b)****}	3.35±0.42	2.38±0.49
VLDL	2.26±0.21	1.81±0.18	0.67±0.14 ^{(a)****}	0.53±0.09 ^{(b)****}	2.22±0.26	1.29±0.32
LDL	0.21±0.02	0.18±0.02	0.09±0.02 ^{(a)****}	0.13±0.02	0.15±0.03	0.12±0.01 ^{(b)**}
HDL	0.26±0.03	0.33±0.05	0.37±0.04 ^{(a)*}	0.34±0.05	0.23±0.03	0.36±0.05
HDL ₂	0.20±0.02	0.27±0.04	0.33±0.04 ^{(a)**}	0.31±0.04	0.20±0.02	0.33±0.04
HDL ₃	0.06±0.009	0.06±0.013	0.04±0.005	0.04±0.008	0.03±0.005 ^{(a)**}	0.03±0.003 ^{(b)*}
Cu ²⁺ oxidation (nmol MDA/ μmol LDL-C)	18.44±1.37	23.51±1.76 ^{(a)*}	14.95±2.98	19.48±1.03	8.41±1.37 ^{(a)****}	17.12±1.91 ^{(b)*}

MDA – malondialdehyde, C – cholesterol, (a) – vs lard fat control, (b) – vs currant oil control,

*p< 0.05, **p< 0.02, ***p< 0.01, ****p< 0.001

Table 2. Fatty acid composition of dietary fats

Fatty acid (%)	Lard fat	Currant oil
14:0	1.2	0.1
16:0	25.2	5.5
16:1	2.1	0.2
18:0	15.7	1.6
18:1	44.2	13.2
18:2 (n-6)	7.4	48.7
18:3 (n-6)	-	12.4
18:3 (n-3)	0.6	12.9
18:4 (n-3)	-	2.5
20:0	0.3	0.3
20:1	1.2	0.9
20:2	0.6	0.3
20:3 (n-6)	0.1	-
20:3 (n-3)	0.1	-
20:4 (n-6)	0.2	-
22:0	-	0.1

Table 3. Saturated and unsaturated fatty acids of dietary fats

Fatty acids (%)	Lard fat	Currant oil
Saturated (SFA)	42.7	7.7
Monounsaturated (MUFA)	47.5	14.3
(n-6) polyunsaturated (PUFA)	7.7	61.1
(n-3) polyunsaturated (PUFA)	0.7	15.4
PUFA/SFA ratio	0.2	9.9
(n-6)/(n-3) PUFA ratio	11.0	4.0

lowering compounds. The finding that the better bioavailable silibinin-phosphatidylcholine complex¹⁴ is not effective in prevention of diet-induced hypercholesterolemia supports our previous suggestion¹² that other constituent(s) of silymarin than silibinin may be responsible for antihypercholesterolemic activity of the extract.

However, the supplementation of the both high-cholesterol, high-fat diets with silibinin-phosphatidylcholine complex generated LDL more resistant to oxidation than 10 times higher concentration of silymarin in the diets. It suggests that LDL antioxidant activity of silibinin found *in vitro*¹³ is determined *in vivo* by silibinin bioavailability. Therefore an improvement of bioavailability of silibinin as a component of silymarin seems to be necessary to enable the LDL antioxidant activity in addition to antihypercholesterolemic effect of silymarin + currant oil combination.

ACKNOWLEDGEMENTS

The authors thank to the Ministry of Education (project MSM 151100003), Medical Faculty Olomouc (grant 12101 101) and Internal Grant Agency, Ministry of Health (grant NL 5267-3/1999), Czech Republic, for their financial supports.

REFERENCES

1. Grundy, S. M. (1998) Nutrient composition and the metabolic syndrome. In: *Atherosclerosis XI*. (Eds. Jacotot B., Mathé D. and Fruchart J. C.), 519–527, Elsevier Science Pte Ltd., Singapore.
2. Lamarche, B., Tchernof, A., Moorjani, S., Cantin, B., Dagenais, G. R., Lupien, P. J., Despres, J. P. (1996) Small, dense low-density lipoprotein phenotype as a predictor of the risk of ischemic heart disease in men: prospective results from the Quebec Cardiovascular Study. *Circulation* 94, 69–75.
3. Steinberg, D., Pathasarathy, S., Carew, T. E., Khoo, J. C., Witztum, J. L. (1989) Modifications of low-density lipoprotein that increase atherogenicity. *N. Engl. J. Med.* 320, 915–924.
4. Witztum, J. L. (1994) The oxidation hypothesis of atherosclerosis. *Lancet* 344, 793–795.
5. Aviram, M. (1996) Interaction of oxidized low density lipoprotein with macrophages in atherosclerosis, and the antiatherogenicity of antioxidants. *Eur. J. Clin. Chem. Clin. Biochem.* 34, 599–608.
6. Carmena, R., Ordovas, J. M., Ascaso, J. F., Camejo, G., Hurt-Camejo, E., Martinez-Valls, J. (1998) Dietary composition and low-density lipoprotein (LDL) modifications. In: *Atherosclerosis XI*. (Eds. Jacotot B., Mathé D. and Fruchart J. C.), 507–517, Elsevier Science Pte Ltd., Singapore.
7. Lairon, D., Amiot, M. J. (1999) Flavonoids in food and natural antioxidants in wine. *Curr. Opin. Lipidol.* 10, 23–28.
8. Hertog, M. G. L., Feskens, E. J. M., Hollman, P. C. H., Katan, M. B., Kromhout, D. (1993) Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen elderly study. *Lancet* 342, 1007–1011.
9. Keli, S. O., Hertog, M. G. L., Feskens, E. J. M., Kromhout, D. (1996) Dietary flavonoids, antioxidant vitamins, and incidence of stroke: the Zutphen study. *Arch. Intern. Med.* 156, 637–642.
10. Knekt, P., Jarvinen, R., Reunanen, A., Maatela, J. (1996) Flavonoid intake and coronary mortality in Finland: a cohort study. *Br. Med. J.* 312, 478–481.
11. Valenzuela, A., Garrido, A. (1994) Biochemical bases of the pharmacological action of the flavonoid silymarin and its structural isomer silibinin. *Biol. Res.* 27, 105–112.
12. Krečman, V., Škottová, N., Walterová, D., Ulrichová, J., Šimánek, V. (1998) Silymarin inhibits the development of diet-induced hypercholesterolemia in rats. *Planta Med.* 64, 138–142.
13. Škottová, N., Krečman, V., Šimánek, V. (1999) Activities of silymarin and its flavonolignans upon low density lipoprotein oxidizability *in vitro*. *Phytother. Res.* 13, 535–537.
14. Morazzoni, P., Montalbetti, A., Malandrino, S., Pifferi, G. (1993) Comparative pharmacokinetics of silipide and silymarin in rats. *Eur. J. Drug Metab. Pharmacokinet.* 18, 289–297.
15. Havel, R. J., Eder, H. A., Bragdon, J. H. (1955) The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* 34, 1345–1353.
16. Fernandes, A. C., Filipe, P. M., Freitas, J. P., Manso, C. F. (1996) Different effects of thiol and nonthiol ACE inhibitors on copper-induced lipid and protein oxidative modification. *Free Radic. Biol. Med.* 20, 507–514.
17. Shen-Nan-Lin, Horning, E. C. (1975) Analysis of long-chain acids of human plasma phosphatidylcholines (lecithins) and cholesteryl esters by glass open tubular capillary column gas chromatography for stroke patients and for normal subjects. *J. Chrom.* 112, 483–497.
18. Večeřa, R., Chmela, Z., Hřebíček, J., Škottová, N. (1994) Unsaturated fatty acids incorporated in HDL in hypo- and hyperalphalipoproteinemia – relation to the HDL-cholesterol level. *Acta Univ. Palacki. Olomuc.* 137, 35–38.
19. Aviram, M. (1998) Macrophages, LDL oxidation and atherosclerosis. In: *Atherosclerosis XI*. (Eds. Jacotot B., Mathé D. and Fruchart J. C.), 507–517, Elsevier Science Pte Ltd., Singapore.
20. Frémont, L., Gozzelino, M. T., Franchi, M. P., Linard, A. (1998) Dietary flavonoids reduce lipid peroxidation in rats fed polyunsaturated or monosaturated fat diets. *J. Nutr.* 128, 1495–1502.
21. Baroni, S. S., Amelio, M., Sangiorgi, Z., Gaddi, A., Battino, M. (1999) Solid monounsaturated diet lowers LDL unsaturation trait and oxidisability in hypercholesterolemic (type IIb) patients. *Free Radic. Res.* 30, 275–285.