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Effects of microcrystalline plant sterol suspension and a powdered plant sterol supplement on hypercholesterolemia in genetically obese Zucker rats

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Abstract

Because dietary fat appears to be an effective vehicle for dispensing plant sterols into the diet, a special plant-sterol-containing ingredient has recently been developed. This ingredient is a plant sterol suspension in oil in which the sterols are in microcrystalline form. The objective of the present study was to analyse the cholesterol-lowering effects and safety of two different plant sterol preparations, an orally administered microcrystalline plant sterol suspension (MPS) in rapeseed oil and a powdered plant sterol supplement, in obese Zucker rats. Dietary plant sterol supplements (0.5%, w/w) were given concurrently with a high cholesterol diet (HCD, 1% cholesterol and 18% fat, w/w). No significant changes in serum triglyceride, blood glucose, serum glutamate oxaloacetic transaminase and glutamic pyruvic transaminase values or body and liver weights were observed. The powdered plant sterol supplement lowered the serum cholesterol by 25% ($P < 0.05$) and the MPS diet by 35% ($P < 0.001$) compared with HCD by the end of the 12-week experiment. Interestingly, the plant sterol supplements also produced a marked reduction in serum ubiquinone levels, suggesting a possible effect on isoprene synthesis. Unlike the powdered plant sterol, both MPS and plain rapeseed oil decreased the serum baseline diene conjugation values, suggesting that they protect against oxidative stress-induced lipid peroxidation in rats. This lipid peroxidation diminishing effect is probably due to some antioxidative components in rapeseed oil. These findings indicate that an unesterified plant sterol, such as the microcrystalline suspension in oil, effectively prevents cholesterol absorption in obese Zucker rats.

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Introduction

A high serum cholesterol concentration, essential hypertension, obesity, high sodium intake, diabetes and cigarette smoking are some of the major risk factors for coronary heart disease (Jousilahti et al 1996; Miettinen et al 1996; Karppanen & Mervaala 1998; Ogden et al 2000). Several large-scale intervention trials have shown that cholesterol-lowering drug therapy in hypercholesterolaemic patients beneficially affects the mortality risk of coronary heart disease (CHD) (Gould et al 1998). The 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase inhibitors (statins) are the most effective agents currently available to treat hypercholesterolaemia (Illingworth & Tobert 1994). However, the prevention of CHD is still too large a problem to be accomplished with drugs alone. The HMG-CoA reductase inhibitors are expensive, and it is therefore important to look for alternative methods of lowering cholesterol. Lifestyle modifications and dietary intervention are significant features in the prevention of primary and secondary CHD (Blazing & O'Connor 1999).

Since the 1950s, β-sitosterol, the most common plant sterol, has been known as a potent serum cholesterol-lowering agent in experimental animals (Behar & Anthony 1955; Gould 1955; Bhattacharayya & Lopéz 1979). Dietary plant sterols have also been reported to reduce serum cholesterol concentrations in humans (Pollak 1952; Pollak & Kritchevsky 1981; Howard & Kritchevsky 1997; Law 2000). From a structural

standpoint, this inhibition is most clearly related to substitutions in position 24 on the sterol side chain. Serum plant sterol levels are also reliable indicators of the cholesterol absorption efficiency in humans (Miettinen et al 1990). Because of their higher affinity for mixed micelles compared with cholesterol, plant sterols displace cholesterol from the micelles, thereby reducing cholesterol absorption and, subsequently, serum cholesterol levels (Ikeda et al 1989; Heinemann et al 1991). Recently, a tremendous amount of research has been conducted with plant sterols and stanols to determine the best way to administer them. High dosages of plant sterols have been applied in order to lower plasma cholesterol concentrations in hypercholesterolaemic subjects (Ling & Jones 1995; Miettinen et al 1995; Hendriks et al 1999). Although β-sitosterol is detectable to some extent in the plasma, liver, aorta, adrenal and adipose tissues of rats, no harmful adverse effects have been observed (Sugano et al 1977; Malini & Vanithakumari 1990). However, the data from randomized studies indicate that plant sterols can reduce the absorption of fat-soluble vitamins, e.g. carotenes and tocopherols, which have been shown to be important in the protection of low-density lipoprotein (LDL) cholesterol from oxidation (Law 2000). No other side effects have been observed in humans, except in individuals suffering from phytosterolaemia (Lütjohann & von Bergman 1997; Plat et al 1999, Stalenhoef et al 2001).

Formulation problems have limited the widespread use of phytosterols. Their physical properties, i.e. low solubility in fats and insolubility in water, limit the use of plant sterols in dietary products. To achieve a maximum decrease in cholesterol absorption, plant sterols should be simultaneously present with cholesterol in the intestinal lumen (Mattson et al 1982). Therefore, clinically relevant reductions in cholesterol have in the past been achieved by the consumption of large quantities of plant sterols as capsule or powder formulations, which is usually impractical, and similar results have been achieved by smaller doses of plant sterols dispersed in food (Mensink & Plat 1998). Esterification of plant sterols or stanols with fatty acids increases their solubility in fats and oil, and thus facilitates their incorporation into fat-containing foods (Jandacek et al 1977).

The main objective of the present study was to investigate the cholesterol-lowering effects of an orally administered microcrystalline plant sterol suspension (MPS) and a powdered plant sterol supplement in genetically obese Zucker rats. In addition, the possible difference in effect between these two formulations and several parameters describing the safety of these formulations were assessed. The advantage of using a suspension is the high effective surface area of plant sterol crystals due to their small particle size compared to powdered sterols.

In the animal model of type II diabetes with obesity, a mutation of the leptin-receptor gene leads to hyperphagia, hyperlipidaemia and obesity (Ionescu et al 1985; Kasiske et al 1992; Phillips et al 1996). Other metabolic changes such as peripheral insulin resistance, hyperinsulinaemia and impaired glucose tolerance are observed, and hyperglycaemia also occurs (Kasiske et al 1985; McCaleb & Sredy 1992; Phillips et al 1996). Recent studies have shown that a cholesterol-rich diet significantly increases serum cholesterol in obese Zucker rats (Higueruelo et al 1998; Vaskonen et al 1999, 2001). In the present study, β-sitosterol supplements were administered concurrently with a high cholesterol/high fat diet. The effect on body and liver weight and tissue biochemical parameters [blood glucose and cholesterol, serum glutamate oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT), serum ubiquinone (Q9), serum triglycerides, serum baseline diene conjugation (BDC) and total peroxyl radical trapping antioxidant potential (TRAP)] were also measured in order to elucidate the cholesterol lowering effects and possible adverse effects of the investigated dietary supplements.

Materials and Methods

The animals

A total of 40 genetically obese Zucker female rats (strain: Hsd0la: Zucker-fa), purchased from Harlan (Harlan UK Ltd, UK), were used in the study. The animals were housed in groups of four per cage, and were allowed to adapt for 5 days under standard conditions of a 12-h (06.00–18.00) light–dark cycle and free access to food and tap water. At the beginning of the study the serum cholesterol values were measured, and the 7- to 9-week-old rats were divided into five subgroups ($n = 8$ in each group). The study was approved by the Animal Experimentation Committee of the Biocenter of the University of Helsinki in accordance with internationally accepted principles.

Experimental design and diets

All the groups of rats were fed with different experimental ingredients for 12 weeks. The serum cholesterol values and body weights were analysed at the beginning of the experiment and after 2, 4, 8 and 12 weeks. The other analyses described in the Biochemical determinations section were measured at the end of the study.

All the diets were based on the standard rodent chow in milled form (R-36, Lactamin, Sweden, Table 1). In all the groups the average daily consumption of chow was 25 g, and each group was allowed water ad libitum. The dietary regimens for each group of female rats are presented in

Table 1 Nutritional value and electrolytes of the experimental diet (standard rodent fodder R-36, Lactamin, Sweden).

Ingredients	%	Electrolytes	%
Protein	18.5	Ca	1.0
Fat	4.0	P	0.8
of which linolic acid	1.0	Na	0.3
Carbohydrates	55.7	Cl	0.4
Fibre	3.5	Mg	0.2
Ash	6.3	K	0.6
Water	< 12.0		
Energy content (kJ $100\,g^{-1}$)	1260.0		

Table 2. All the added dietary supplements were mixed with the normal rat chow before administrating the chow to the animals. The high cholesterol diet (HCD, group 2) was produced by adding 1% cholesterol (5-cholesten-3β-ol, Sigma Chemical Co., St Louis, MO) and 18% unsalted butter (Valio Oy, Seinäjoki, Finland) to the standard rodent chow. According to gas chromatography-mass spectrometry (GC-MS) analysis the cholesterol was 99% pure.

The diet with powdered plant sterols was prepared by adding 0.5% β-sitosterol (E. Merck, Darmstadt, Germany), 1% cholesterol and 18% unsalted butter to the chow (group 3, Table 2). According to GC-MS analysis β-sitosterol composed of 76.8% β-sitosterol, 13.2% β-sitostanol, 8.8% campesterol and 1.2% campestanol. The composition of the sterol raw materials and cholesterol in the plasma were determined by GC-MS on samples prepared by modifying the method described by Phillips et al (1999).

Because dietary fat appears to be an effective vehicle for dispersing plant sterols in the diet, a special plant-sterol-containing ingredient, MPS, was developed. MPS was prepared by using a recently developed method for reducing the particle size of plant sterols by recrystallizing plant sterols in oil in the presence of water. The preparation method of MPS is published and described in detail elsewhere (Christiansen et al 2002). MPS consists of plant sterols (17%), rapeseed oil (70%) and water (13%). The plant sterols are initially dissolved in oil by heating the mixture to 100 °C. While cooling, water was added to achieve rapid crystallization and conversion of the liquid into a semi-solid mass. Rapid solidification prevents crystal growth of the plant sterols, and the resulting fat is smooth. The MPS diet (group 4, Table 2) contained the same amount of plant sterols as the diet for group 3 (Table 2). The advantage of using a suspension is the high effective surface area of plant sterol crystals due to their small particle size compared to powdered sterols. Because plant sterols are finely dispersed in rapeseed oil in MPS, the effect of plain rapeseed oil supplement without plant sterols was also studied to separate the possible cholesterol-lowering effect of rapeseed oil from that of microcrystalline plant sterols (group 5).

Table 2 Dietary regimens for each group of rats.

Group	Fodder
1	Standard rat chow, water ad libitum
2	Standard rat chow + HCD[1], water ad libitum
3	Standard rat chow + HCD[1] + 0.5% β-sitosterol in powder form, water ad libitum
4	Standard rat chow + HCD[1] + MPS 3% (corresponding to 0.5% β-sitosterol, 2.1% rapeseed oil and 0.4% water), water ad libitum
5	Standard rat chow + HCD[1] + rapeseed oil 2.1%, water ad libitum

Dietary supplements, i.e. high cholesterol diet, plant sterol and oil supplements, were mixed with the standard rat chow before administration to the animals. [1]1% cholesterol and 18% unsalted butter.

Biochemical determinations

At the end of the 12-week experimental period, the animals were anaesthetized with carbon dioxide, decapitated and about 4 mL of blood was collected in test-tubes. After decapitation, the liver was dissected out, inspected macroscopically and weighed. Blood cholesterol, glucose, GOT and GPT were measured on a Reflotron IV instrument (Boehringer Mannheim GmbH, Mannheim, Germany). Fresh venous blood was used for performing the strip test for cholesterol. Blood collected in test-tubes containing sodium EDTA as an anticoagulant was used for the other strip tests at the end of the experiment. The test strips were purchased from Boehringer Mannheim GmbH (Germany).

After clotting in an ice bath for 10 min, the blood was centrifuged with a Sorvall Instruments RC-5C ultracentrifuge (DuPont Company, Newton, CT) with fixed-angle rotors at +4 °C (3000 rpm for 10 min) in order to separate the serum. Serum samples for triglyceride determinations were stored at −20 °C until assayed. For determining the BDC and TRAP, the serum was kept at −80 °C until analyzed. For the determination of BDC, the lipids were extracted from serum samples (100 μL) by chloroform/methanol 2:1. The chloroform was separated, and the samples were dried under nitrogen and then redissolved in cyclohexane before being analyzed spectrophotometrically (at 234 nm) as described by Corongiu & Milia (1983). The spectrophotometric analyses were performed on a Perkin-Elmer Lambda 2 spectrometer (Perkin Elmer, CT). The capacity of the individual rat serum samples to trap peroxyl radicals was estimated by chemiluminescence-based methodology, previously described in detail by Ahotupa et al (1997). Chemiluminescence measurements were performed on a Bio-Orbit 1251 Luminometer connected to a personal computer with special programs for the assays (Bio-Orbit, Turku, Finland).

For Q9 determinations, blood samples were centrifuged on an Eppendorf 5417C centrifuge (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) for 30 min (+4 °C, 14 000 rpm). The quantitative determination of Q9 was performed by a standard HPLC test using electrochemical detection (Okamoto et al 1985; Edlund 1988). The following equipment was used: Kontron HPLC system 600 (Kontron Instruments, Rotkreuz, Switzerland), Pharmacia LKB HPLC Pump 2248 (Pharmacia, Sweden) and Hypersil ODS (10 μm) precolumn connected to Beckman Ultrasphere ODS (5 μm), electrochemical detection unit Coulchem II, conditioning cell, model 5021, and analytical cell, model 5011, spectrophotometer Uvikon 722 LC and BBC Goerz Metrawatt recorder.

Serum triglyceride concentrations were measured using commercial enzymatic kits manufactured by Konelab (Konelab, Espoo, Finland). All the solvents used were of analytical grade.

Statistical analysis

Statistical analysis was carried out by one-way analysis of variance (ANOVA), followed by the Fischer's least

significant difference. P values of less than 0.05 were considered to indicate a significant difference. The data were analysed using SYSTAT statistical software (SYSTAT Inc., Evanston, IL). The results are expressed as means ± s.e.m.

Results

The weight development of the rats was monitored at 2-week intervals for one month, and then at 4-week intervals up until 12 weeks. The weight of the rats increased almost linearly in all the groups. There were no statistically significant differences in weight gain among the groups (Table 3). No statistically significant differences were found between the groups in the liver weights of the rats receiving different diets at the end of the experiment. Macroscopically, the rat livers in the groups receiving a standard diet, diet enriched with powdered plant sterol, and MPS containing plant sterol appeared normal. Liver lesions were clearly seen in the rats receiving HCD (group 2) and HCD with rapeseed oil (group 5).

HCD provoked an elevation of serum cholesterol level at week 2 of the experimental period (Table 4). The hypercholesterolaemic effect persisted up until the end of the study. Powdered plant sterol (group 3) and plant sterol containing MPS (group 4) antagonized the elevation of cholesterol throughout the study. No statistical differences were found between these groups although rats receiving the MPS diet had a tendency to have lower cholesterol values at week 2 and week 12.

Liver function tests were assessed by determining serum GOT and GPT. Blood glucose was measured. Dietary supplements in the different groups produced no statistically significant changes in blood glucose concentrations (data not shown). The serum GPT values in the rat group receiving powdered plant sterol were higher ($P < 0.05$) compared with the rats receiving the standard diet (group 1). No other intergroup differences in GPT values were observed. Because no difference was observed between group 3 and the group receiving HCD (group 2), and no difference was observed between the group receiving the other plant sterol supplement (group 4) with the other groups, this deviation is not considered significant. Thus, no clear explanation for the significant deviation between group 3 and 1 could be found. No statistically significant changes were observed between the groups in the serum GOT values at the end of the experiment.

Lipid oxidation was estimated by the baseline level of diene conjugation in the serum lipid fraction. HCD raised the BDC to ca. 4-fold compared with the group receiving the standard diet (Figure 1). Statistically significant differences were also found between groups 4 and 5 compared with the values obtained for group 2 (HCD). When estimating the antioxidant potency, no statistically significant differences were found between the groups in the capacity of the individual rat serum samples to trap peroxyl radicals.

Changes in serum Q9 levels in groups 3 and 4 were statistically significant and very significant, respectively, compared with the values in rat group 2 (HCD, Figure 2). These significant differences disappeared when the Q9 levels were adjusted for the levels of cholesterol.

No statistically significant differences in serum triglyceride values were observed between the groups (data not shown).

Discussion

Some food products containing plant sterols are already on the market, and are used to lower or control cholesterol levels in mildly hypercholestrolaemic individuals (Miettinen et al 1995; Hendriks et al 1999). However, the commercial products are not based on free plant sterols and esterification or other chemical reaction is therefore needed to incorporate sterols into dietary products.

The low solubility of sterols in foods can be improved with a new method described by Christiansen et al (2002). The physical state of this plant-sterol-containing ingredient is a microcrystalline suspension in oil. In the present study, plant sterol was administered simultaneously either as a powder (group 3, Table 2) or in microcrystalline form, i.e. MPS (group 4, Table 2). Both of the dietary plant sterol formulations reduced serum cholesterol levels in the genetically obese Zucker rats used in studies measuring the effects of a cholesterol-rich diet (Higueruelo et al 1998; Vaskonen et al 1999, 2001). The cholesterol-lowering effect of the studied plant sterol preparations are in accordance with earlier studies on different animal models (Gould 1955; Bhattacharayya & Lopéz 1979) as well as in

Table 3 Mean ± s.e.m. (n = 8) body weight in genetically obese Zucker rats.

Week	Group 1	Group 2	Group 3	Group 4	Group 5
0	270.13 ± 7.16	271.50 ± 11.35	277.00 ± 11.23	278.63 ± 12.14	269.38 ± 17.62
2	338.50 ± 4.04	358.63 ± 14.78	354.13 ± 9.01	367.13 ± 11.94	360.00 ± 16.29
4	406.5 ± 19.06	390.88 ± 2.75	409.88 ± 8.62	425.13 ± 12.27	417.00 ± 0.32
8	464.38 ± 4.37	479.14 ± 36.16	464.00 ± 20.91	503.88 ± 20.26	506.75 ± 12.23
12	505.25 ± 5.72	524.86 ± 46.42	511.25 ± 32.63	553.63 ± 23.47	561.88 ± 14.92

Explanations for groups 1–5 are given in Table 2.

Table 4 Mean ± s.e.m. (n = 8) cholesterol values in genetically obese Zucker rats.

Week	Group 1	Group 2	Group 3	Group 4	Group 5
0	2.88 ± 0.07	2.96 ± 0.06	2.95 ± 0.11	2.96 ± 0.03	2.95 ± 0.07
2	2.94 ± 0.06***	4.49 ± 0.11	3.82 ± 0.11**	3.33 ± 0.12***	4.13 ± 0.31
4	3.28 ± 0.26**	4.99 ± 0.39	4.09 ± 0.18	3.79 ± 0.16*	5.11 ± 0.57
8	3.10 ± 0.12***	6.16 ± 0.65	4.42 ± 0.43**	4.40 ± 0.27**	6.23 ± 0.52
12	3.23 ± 0.14***	7.16 ± 0.87	5.34 ± 0.32*	4.64 ± 0.27***	6.67 ± 0.52

*Comparison of serum cholesterol levels with the serum cholesterol levels of rats receiving the HCD was by Fischer's least significant difference test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Explanations for groups 1–5 are given in Table 2.

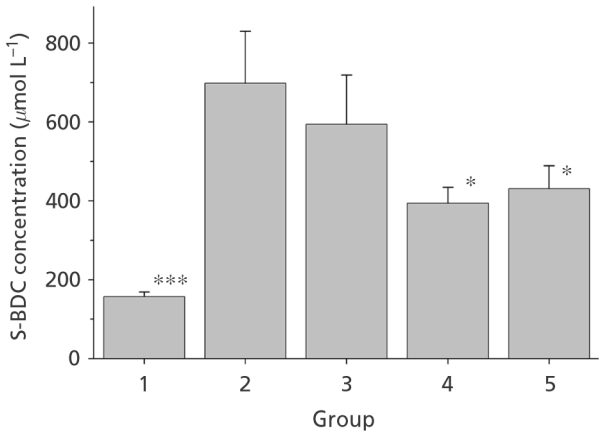

Figure 1 Changes in serum BDC levels (S-BDC, μmol L^{-1}) in Zucker rats at the end of the 12-week experiment. Comparison to the changes in the rat group receiving HCD (group 2) was by Fischer's least significant difference test. Changes in serum BDC levels are expressed as mean ± s.e.m., n = 8 (*$P < 0.05$, ***$P < 0.001$).

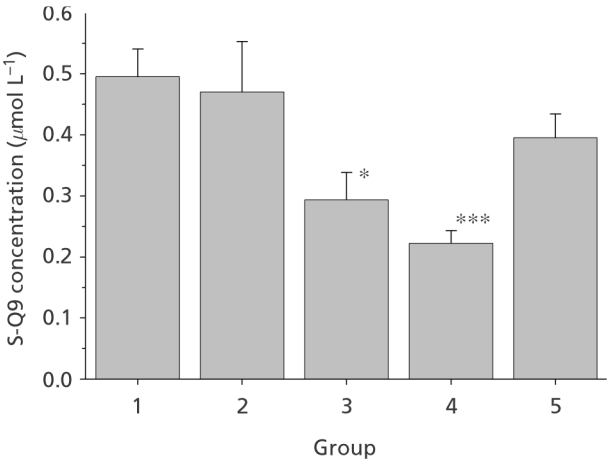

Figure 2 Changes in serum Q9 levels (S-Q9, μmol L^{-1}) in Zucker rats at the end of the 12-week experiment. Comparison to the changes in the rat group receiving HCD (group 2) was by Fischer's least significant difference test. Changes in serum Q9 levels are expressed as mean ± s.e.m., n = 8 (*$P < 0.05$, ***$P < 0.001$).

humans (Pollak 1952; Pollak & Kritchevsky 1981; Howard & Kritchevsky 1997). The MPS diet lowered the serum cholesterol by 35% ($P < 0.001$) and the powdered plant sterol enriched supplement lowered the serum cholesterol by only 25% ($P < 0.05$) compared with the control values in rats with HCD at the end of the 12-week period. Although there were no statistically significant differences between groups receiving MPS and powder form plant sterol supplements, the results indicate that different manufacturing techniques for producing microcrystalline plant sterol, combined with a suitable matrix, may increase the efficacy of the plant sterol. However, more studies are needed to monitor the differences in the potency between different plant sterol preparations.

As the liver is physiologically related in respect to excretory, metabolic and vasoregulatory functions, it has a common susceptibility to injurious factors. Serum enzymes, especially GOT and GPT, are often considered as reliable indicators of adverse drug reactions or of the presence of disease (Malini & Vanithakumari 1990). The absence of significant changes in the measured serum enzymes and glucose indicate that the animals receiving the standard diet, and the diets with powder plant sterol and plant sterol containing MPS, maintained their hepatic cellular integrity. This finding is in agreement with earlier reports in which histopathological examination failed to uncover any untoward effects of long-term exposure to oral administration of β-sitosterol in experimental animals (Swell et al 1956; Sugano et al 1977; Malini & Vanithakumari 1990).

There are reports about inhibition of the mevalonate pathway by HMG-CoA enzyme inhibitors, i.e. statins, in humans leading to a decrease in the formation of cholesterol (Mortensen et al 1997; Miyake et al 1999). In humans coenzyme Q10 is a central component of the mitochondrial respiratory chain and an endogenous antioxidant, packaged into the LDL and VLDL fractions of cholesterol. The corresponding enzyme in rats is Q9. It has been suggested as an important protective factor against the development of atherosclerosis. Q9 levels have been shown to be lower in patients treated with statins than in untreated hypercholesterolaemic patients or healthy controls (De Pinieux et al 1996; Mortensen et al 1997; Miyake et al 1999). These findings support the fact that the main adverse effect of statins is a toxic myopathy, possibly related to mitochondrial

dysfunction (De Pinieux et al 1996). To our knowledge this is the first time that a lowering effect of dietary plant sterols on Q9 levels in rats has been reported. The lowering tendency of Q9 concentrations in groups receiving powdered plant sterol and MPS (Figure 2) thus suggests that the cholesterol-reducing effect of dietary phytosterols could be at least partly due to the inhibition of HMG-CoA (isoprene synthesis in the liver). Therefore, continued vigilance about possible adverse consequences from a reduction in ubiquinone seems to be important during long-term phytosterol therapy.

A significant decrease in serum BDC values was observed in the groups receiving plant sterol containing MPS and rapeseed oil. This lipid peroxidation diminishing effect is probably due to antioxidative components in the rapeseed oil in the MPS mixture. Diets rich in unsaturated fatty acids increase the need for antioxidants such as α-tocopherol and selenium (Horwitt 1974). Low-erucic acid rapeseed oil has been shown to decrease serum lipid levels in rats, especially with simultaneous supplementation with selenium (Watkins et al 1995). The fact that the mixture had little free radical scavenging activity might indicate that its action is due to factors other than free radical scavenging activity.

Conclusions

These findings indicate that unesterified plant sterols, such as a microcrystalline oil suspension, effectively reduce cholesterol absorption levels in obese Zucker rats.

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