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INFLUENCE OF PHYTOSTEROLS VERSUS PHYTOSTANOLS ON PLASMA LIPID LEVELS AND CHOLESTEROL METABOLISM IN HYPERCHOLESTEROLEMIC HUMANS

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For Mom and Dad

ABSTRACT

The objective of this research was to examine the effects of sitosterol and sitostanol supplementation on plasma cholesterol levels and cholesterol metabolism in hypercholesterolemic subjects consuming a fixed foods diet in a four-phase crossover design. It was hypothesized that addition of either phytosterols, phytostanols, or a 50:50 mixture of sterols and stanols to butter would reduce circulating cholesterol levels, despite butter's hypercholesterolemic effect, through actions involving cholesterol absorption, synthesis, and turnover rates. The data obtained indicate that in their free, unesterified form, plant sterols and stanols lower plasma LDL cholesterol equivalently in hypercholesterolemic subjects. Results of this study provide new data that phytosterols and stanols function by suppressing cholesterol absorption while increasing cholesterol synthesis, however, the de-suppression in synthesis cannot fully compensate for the decrease in absorption making the treatment effective, thus may assist in the development of a food which offers health-promoting advantages related to the prevention of cardiovascular disease.

RÉSUMÉ

L'objectif de cette recherche était d'examiner les effets de supplémentation de sitostérol et sitostanol sur les niveaux de cholestérol sanguin et métabolisme de cholestérol dans les sujets hypercholestérolemique consommants un régime de nourritures réparé dans une conception de crossover. Il a été posé une hypothêse qu'addition de ou phytostérols, phytostanols, ou une mélange de stérols et stanols à beurre réduirait les niveaux circulants de cholestérol, en dépit des effets hypercholestérolemic du beurre, par actions impliquant l'absorption, synthésis et roulement du cholestérol. Les données obtenu indique que dans leur libre, forme nonestérifient, plante stérols et stanols abaisse le plasma total et le cholesterol LDL équivalemment. Les résultats de cette étude fournit des nouveaux données en ce qui concerne la méchanisme d'action des stérols et stanols de plante en eliminant l'absorbation du cholestérol tandis que la synthèse croissante de cholestérol, alors peut aider dans le développement d'un nourriture qui offre des avantages promouvoir de santé relaté à l'empêchement de maladie cardiovasculaire.

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CHAPTER 1.

LITERATURE REVIEW

1.1 INTRODUCTION

Cardiovascular disease (CVD) is a broad term used to describe a group of chronic medical conditions, which ultimately affect the ability of the heart to function efficiently. These conditions include congestive heart failure, hypertension, angina pectoris, atherosclerosis, cerebral and peripheral vascular disease, venous insufficiency, and arrhythmia. Individually or together these conditions are considered extremely serious and have a relatively high degree of morbidity and mortality. High circulating total and LDL cholesterol concentrations have been identified as an important risk factor in the development of CVD (Willett, 1994; American Heart Association, 1990). Data from the Framingham Heart Study and the Multiple Risk Factor Intervention Trial confirm a continuous and graded relationship between serum cholesterol and CVD mortality (Wong et al., 1989; Multiple, 1982). From the NHANES III study, it has been estimated that 71% of the US adult population can be considered at risk for CVD by virtue of having total cholesterol levels higher than 181 mg/dl or 4.68mmol/l (Sempos et al., 1993). Despite the research derived knowledge that diet and lifestyle changes can modify risk as well as significant advances in technology and pharmaceutical preparations, CVD accounts for approximately 38% of deaths each year in the Canada (Laboratory Center for Disease Control, 1996).

It has been shown that lowering circulating total and LDL cholesterol concentrations significantly reduces death from CVD (Sacks et al., 1995). For every 1% decrease in the LDL serum cholesterol, there is a subsequent 2%-3% decrease in the risk

of CVD (Muldoon et al., 1990). Therefore many researchers have been searching for ways to decrease blood cholesterol levels, even modestly.

In the wake of the modern drug era, the use of herbal and nutraceutical remedies is increasing as people attempt to take their health into their own hands. Reasons for this trend, especially in the context of CVD, are the side effects and high cost associated with drug therapy. Plant sterols are not a new discovery but their use as a marketable functional food is state of the art. Since the 1950's much research has been done on the efficacy of phytosterols as cholesterol-lowering agents. Although results may vary between individuals, the bottom line is that when administered as part of a healthy diet, phytosterols have been shown to significantly reduce both total and LDL cholesterol levels. These products can be easily incorporated into the every day diet with little effort. Recently, phytosterols have been incorporated into margarine, cream cheese, and salad dressings as so-called functional foods in the prevention and treatment of hypercholesterolemia.

1.2 PHYTOSTEROLS

1.2.1 Structure of phytosterols

The term phytosterols or plant sterols, encompasses a small group of the numerous chemical components found in the non-saponifiable material of both edible and ornamental plant material such as wood, vegetable oils, corn, and rice. Phytosterols are not synthesized by animals or humans, and are only obtained from the diet. Daily dietary intake in North America varies from 160-360 mg/d (Miettinen et al., 1990). They are similar to cholesterol in their basic ring structure, while they differ in that they

contain either a methyl or ethyl side-chain substitution at the C-24 position. This feature apparently renders them unabsorbable or only minimally absorbable in animals and humans. Plant sterols naturally exist in both saturated and unsaturated forms, however the majority is unsaturated. The most abundant unsaturated phytosterol is sitosterol, although phytosterol mixtures may include campesterol, stigmasterol, and dihydrobrassicasterol (Pollack and Kritchevsky, 1981; Ling and Jones, 1995). Saturation of the sterol ring structure at the 5α-position results in the formation of saturated plant sterols or phytostanols such as sitostanol and campestanol, which are found in lesser amounts in foods (Dutta and Appelqvist, 1996).

1.2.2 Phytosterols as cholesterol lowering agents

It has been identified for some time that ingestion of phytosterols results in a favorable modification of circulating lipid profiles. Ingestion of sitosterol has been shown to moderately lower plasma total and LDL cholesterol concentrations in animals (Pollack, 1953; Sugano et al., 1976; Sugano et al., 1977; Pollack and Kritchevsky, 1981; Laraki et al., 1991; Ling and Jones, 1995; Moghadasian et al., 1997; Ntanios et al., 1998) and humans (Lees et al., 1977; Heinemann et al., 1986; Becker et al., 1992; 1993; Pelletier et al., 1995; Gylling et al., 1995; Jones et al., 1997; 1999; 2000; Sierksma et al., 1999; Hallikainen et al., 1999; 2000). Most studies report no effect of phytosterol consumption on HDL cholesterol or TAGs (Becker et al., 1992; 1993; Heinemann et al., 1986; Jones et al., 1999; 2000.

It has been suggested that phytosterols lower circulating cholesterol concentrations by (i) competitively blocking intestinal cholesterol absorption

(Heinemann et al., 1986; Miettinen and Vanhanen, 1994; Heinemann et al., 1988; Ikeda and Sugano, 1983, Heinemann et al., 1993) (ii) displacing cholesterol from bile salt micelles (Child and Kuksis, 1986) (iii) increasing bile salt excretion (Salen et al., 1970) or (iv) hindering cholesterol esterification rate in the intestinal mucosa (Ikeda and Sugano, 1983). The most widely accepted mechanism by which plant sterols and stanols inhibit cholesterol absorption, is by displacing cholesterol from micelles rendering it unable to cross the intestinal wall. Plant sterols are also esterified at a much slower rate in the intestinal mucosa compared to cholesterol, which may be an important limiting factor for its absorption (Salen et al., 1970). Sitosterol and sitostanol also appear to affect the absorption of other plant sterols. For instance, consumption of plant stanols significantly lowers cholesterol and circulating plant sterol concentrations indicating that both are competitively inhibited from being absorbed (Piironen et al., 2000).

Plasma cholesterol lowering efficacy of phytosterols among other factors varies according to the composition and dose of the phytosterol mix, the vehicle in which it is given and more importantly the degree of hydrogenation. At one time phytosterol doses of 6-18 g per day were thought necessary to significantly reduce plasma total and LDL cholesterol levels. Recently, studies have shown that considerably lower doses of phytosterols, specifically sitostanol, are as effective as larger phytosterol doses given in the past.

1.2.3 Free sitosterol and cholesterol reduction

The efficacy of sitosterol has been shown in numerous studies and under several study conditions. The rate at which phytosterols act and whether the action is maintained

over time was studied by Farquhar et al. (1956) who supplemented 15 post myocardial infarction men with 12-18 g/d sitosterol powder for 3-6 months. There was a maximal reduction of 17% total cholesterol, which was achieved after 3 weeks of treatment. Many other studies also report the greatest change in plasma cholesterol levels within the first three weeks of treatment. This reduction was maintained over the experimental period. They also showed that cholesterol levels rapidly return to near baseline levels upon cessation of treatment, which has been confirmed by other studies with the levels returning to baseline within one month.

Since there are several sources of plant sterols the question arose as to whether all sources are created equal in their ability to modify circulating cholesterol concentrations. Sierksma et al. (1999) studied the effects of feeding phytosterols from vegetable different sources blended into a spread to moderately hypercholesterolemic subjects for 9 weeks. Plasma total and LDL cholesterol concentrations were significantly reduced by 3.8% and 6%, respectively, at a dose of 0.8g per day, for the spread enriched with free soybean-oil sterols compared to control. The enriched spreads did not significantly affect HDL cholesterol concentrations.

One of the plant sources for phytosterols happens to be pine trees. During the processing of pine trees the pulp and paper industry creates a waste product called tall-oil soap, which contains large quantities of phytosterols. In 1999, our lab conducted a study to examine the impact of tall oil-phytosterol intake on circulating cholesterol and plant sterol metabolism in subjects fed precisely controlled diets, during which I served as the clinical study coordinator. The plant sterol composition of this tall oil derived phytosterol mixture included about 20% free sitostanol with the remainder largely as

sitosterol and campesterol. Thirty-two hypercholesterolemic men, divided into 2 groups, consumed a prepared foods diet alone or the diet plus 1.7 g/day sitostanol-containing plant sterols, blended in margarine, for 30 days in a parallel study design. Total cholesterol concentrations were not significantly different however LDL cholesterol decreased by 8.9% and 24.4% for diet and diet plus phytosterol treatments, respectively. Concentrations of HDL cholesterol and TAGs were unchanged. Also no changes were observed between control and phytosterol-fed subjects for fractional or absolute synthesis rates of cholesterol. Changes in circulating campesterol and sitosterol concentrations were also not significantly different between phytosterol-fed and control subjects (Jones et al., 1999).

The type and amount of fat into which plant sterols are mixed may also influence their efficacy. Pelletier et al. (1995) supplemented 12 healthy males with 0.74 g/day soybean phytosterols mixed in butter as part of a crossover designed controlled feeding trial for 4 weeks. This study was designed to examine the effects of a relatively small dose of phytosterols on plasma lipid levels in normal cholesterolemic subjects consuming a cholesterol overload, similar to the typical North American diet. Total and LDL cholesterol levels decreased by 10 and 15%, respectively, after four weeks compared to control despite the fact that phytosterol dose was low. They also reported a 5 % increase in HDL cholesterol, which had not been shown in other studies. The authors suggested that high levels of dietary cholesterol (400-450 mg/day) consumed from the butter might have attenuated the effectiveness of the phytosterols in the intestinal lumen.

To compare the cholesterol lowering effects of unsaturated and saturated phytosterols, Lees et al. (1977) conducted several studies in which hypercholesterolemics

were supplemented with both sitosterol and sitostanol in varying doses. Initially the authors reported that 18 g/day of soybean phytosterols in either a powdered or suspension form significantly reduced total cholesterol levels by 12%. Despite promising results, enthusiasm was tempered by the perception that large amounts of phytosterols were needed to obtain significant cholesterol lowering. Further studies showed that much smaller amounts, 3–6 g/day, of sitostanol containing tall oil derived phytosterols reduced plasma cholesterol levels in hypercholesterolemic subjects as effectively as did 18 g of soybean oil derived phytosterols per day given in the past. To try and elucidate the mechanism responsible for the cholesterol lowering, the authors also conducted a cholesterol balance study to measure changes in cholesterol absorption. It was demonstrated that 3 g/d of sitostanol containing tall oil sterol mixture decreased cholesterol absorption by 50%. This stemmed a new focus of interest on the use of plant stanols, particularly sitostanol as cholesterol lowering agents.

1.2.4 Free sitostanol and cholesterol reduction

Although sitosterol has been shown to lower plasma cholesterol levels, much research has been conducted with its saturated derivative sitostanol. Sitostanol has been shown to be virtually unabsorbed by the intestine and is believed to more effectively inhibit the absorption of cholesterol than sitosterol thus lowering lipid levels to a greater degree. Oral administration of sitostanol has been shown to be extremely efficacious in reducing circulating cholesterol concentrations in several human trials (Heinemann et al., 1986; Jones et al., 1997; Miettinen et al., 1995; Miettinen, and Vanhanen, 1994; Vanhanen et al., 1993; Heinemann et al., 1988; Heinemann et al., 1991; Ling and Jones,

1995b; Weststrate 1998; Gylling 1999; Jones et al., 1999).

To further compare the cholesterol-lowering efficacy of sitosterol and sitostanol, as well as attempt to understand the mechanism by which they work Becker et al. (1993) conducted a supplementation trial on 9 children with familial hypercholesterolemia. They compared sitosterol and sitostanol and demonstrated that sitostanol, even in a fourfold lower dose, reduces total and LDL cholesterol more effectively than does sitosterol. Following three months of treatment sitosterol (6 g/day) produced a decrease of 17% and 19.5% for total and LDL cholesterol, respectively. Moreover, the addition of a much smaller 1.5 g/day dose of sitostanol resulted in a 25.7% and 33.2% decrease in total and LDL cholesterol, respectively. The authors also looked at the effects of phytosterol feeding on hepatic cholesterol synthesis and intestinal cholesterol absorption. Neutral sterol excretion was increased 45% with sitosterol therapy and 88% with sitostanol therapy, which suggests that sitostanol is more effective in inhibiting cholesterol absorption. It was proposed that the addition of phytosterols could increase hepatic cholesterol synthesis, followed by an increase in bile acid formation. Another explanation was that the reduction of sitosterol in serum, found during sitostanol treatment, is related to an increase in bile acid synthesis and excretion of cholesterol and sitosterol into the bile acids.

To examine the effects of low dietary cholesterol intake on the cholesterol-lowering efficacy of a low sitostanol dose, Heinemann et al. supplemented hypercholesterolemic subjects with 1.5 g/d sitostanol. Subjects received a 500-mg supplement three times a day for 4 weeks. The sitostanol was partly dissolved and dispersed in sunflower oil and packaged in stomach-soluble capsules. Subjects were

instructed to maintain a low cholesterol diet (<300 mg/day) during the study period. Both total and LDL cholesterol concentrations decreased by 15% with sitostanol therapy while TAGs and HDL cholesterol concentrations were unaffected. There was no significant increase in plasma phytosterol levels and it was concluded that the reduction in total cholesterol levels was entirely due to the decline in LDL cholesterol levels. The authors also suggested that phytosterols competitively blocked intestinal cholesterol absorption, which lead to the decreased cholesterol levels.

In contrast to the numerous studies showing efficacy of sitostanol in the treatment of hypercholesterolemia a study by Denke in 1995 showed no significant effect of low-dose sitostanol therapy in 33 moderately hypercholesterolemic men supplemented with 3 g sitostanol per day. Lack of efficacy may be a result of many variables in the study design. Unesterified sitostanol was given as capsules emulsified in safflower oil. The free-living subjects were required to consume 12 capsules per day, a high number that may have lead to low patient compliance. Also, subjects were instructed to follow a low cholesterol diet (<200 mg/d) during the study, below that of normal individuals, which may explain the lower efficacy of sitostanol in decreasing plasma cholesterol levels in this population. In addition to the plasma cholesterol modifying effect that a low cholesterol intake may have, a low level of dietary cholesterol may also inhibit the ability of phytosterols to reduce cholesterol absorption in the intestine.

1.2.5 Sitostanol esters and cholesterol reduction

Over the past decade, the majority of research published in the area of phytosterols has focused on the efficacy of phytostanol esters, in the reduction of serum

total and LDL cholesterol levels. As sitostanol has been shown to be highly unabsorbable, it was considered that in order to obtain optimal effect, the insoluble sitostanol should be given in a fat-soluble form to inhibit the absorption of cholesterol during the oil phase of digestion. Esterification of the plant stanols to fatty acids was done in an attempt to increase plant sterol solubility and hopefully improve efficacy.

Vanhanen et al. (1993) supplemented hypercholesterolemic subjects with a sitostanol ester mayonnaise (3.4 g sitostanol esters/day) for 6 weeks. Total and LDL cholesterol decreased 7.5 and 10%, respectively while HDL and TAG concentrations remained unchanged. Reductions in plasma lipid levels during sitostanol feeding were related to increased serum cholesterol precursors, indicating increased synthesis and a decrease in plant sterols, indicating decreased absorption of cholesterol. Furthermore, a negative correlation between serum cholesterol precursors and serum phytosterols suggest that plant sterol and LDL cholesterol reductions were associated with the highest compensatory increase in cholesterol synthesis.

Miettinen and Vanhanen (1994) examined the cholesterol-lowering efficacy of much lower doses (<1 g/day) of sitostanol-esters, compared to free sitosterol and sitostanol, in hypercholesterolemic subjects. The authors found that small amounts of dietary phytosterols did interfere detectably with cholesterol absorption and cause a proportional fall in serum total and LDL cholesterol. Although, it is true that the decreases in serum total and LDL cholesterol levels were significant only in the sitostanol ester group, total phytosterol intakes were also higher in this group. It was concluded, however, that the reductions were too small for practical serum cholesterol lowering in hypercholesterolemics. They also proposed that phytosterols have less of an

ability to precipitate cholesterol during fat digestion when accompanied by a decrease in dietary cholesterol, as was the case in this study.

In an attempt to define the optimal dose of plant stanol ester Hallikainen et al. (2000) examined the effect of four doses of stanol ester margarine for 4 weeks. Doses ranged from 0.8 g - 3.2 g per day. Serum total and LDL cholesterol concentrations were significantly reduced with consumption of the 1.6 g, 2.4 g, and 3.2 g doses, compared to control. Similarly to Miettinen et al. (1995) the authors concluded that although the decreases were numerically greater with the 2.4 g and 3.2 g doses than with the 1.6 g dose, these differences were not statistically significant and that little benefit is gained at doses higher than 1.6 g per day.

In a landmark study, Miettinen et al. (1995) examined the long-term tolerability and cholesterol lowering effects of two doses of sitostanol ester margarine in mildly hypercholesterolemic humans. Margarine was enriched with sitostanol-ester, derived by hydrogenation of sitosterol from pine tree wood pulp followed by esterification of sitostanol to fatty acids. Sitostanol was supplemented at a level of 1.8 or 2.6 g/day for 6 months to one year resulting in significant reductions of 10 and 14% for total and LDL cholesterol concentrations, respectively. Although the 1.8 g dose produced a slightly less significant reduction, for practical purposes it was as effective as the 2.6 g dose.

Vanhanen et al. (1994) demonstrated that 2 g of sitostanol esters per day mixed in mayonnaise not only reduced total and LDL cholesterol levels by 9% and 15% but also lowered serum phytosterol levels and cholesterol absorption. They concluded that sitostanol mixed in dietary fat is not absorbed and reduces the cholesterol absorption efficiency so that serum lipid levels are reduced which subsequently stimulates a

compensatory increase in cholesterol synthesis.

Hallikainen and Uusitupa (1999) investigated 2 plant sterol ester-containing margarines enriched with wood or vegetable oil-based stanol esters for 8 weeks as part of a low-fat, low-cholesterol diet, following 4 weeks on a high-fat diet. Subjects were supplemented with approximately 2.2 g/day stanol esters. During the experimental period, the reduction in serum total cholesterol was 10.6% and 8.1% greater and in LDL cholesterol was 13.7% and 8.6% greater in the wood stanol ester group and vegetable oil stanol ester groups, respectively. It was suggested that phytosterols must inhibit not only the absorption of dietary cholesterol but also that of biliary cholesterol. Of particular interest in this study was the finding that the vegetable oil-based sitostanol ester mixture, in conjunction with a low fat diet, depressed LDL cholesterol concentrations by 24% over the 8 wk study period. This degree of decline was similar to that seen by Jones et al (1999) at a comparable dose, indicating similar efficacy of partially saturated and largely unsaturated phytosterol mixtures.

Similarly, Andersson et al. (1999) found that consumption of 2 g per day of stanol ester blended into margarine had additive effects on serum total and LDL cholesterol when given in conjunction with a lipid-lowering diet. Serum total and LDL cholesterol was reduced by 8% and 12%, respectively, compared to the control group. At this dose the authors concluded that stanol esters were an effective addition to a diet low in cholesterol aimed at lowering circulating lipid concentrations.

Gylling and Miettinen (1999) supplemented twenty-three postmenopausal women to compare the cholesterol-lowering efficacy of a campestanol ester-rich margarine (3.18 g phytosterols/25 g margarine/day) and a sitostanol ester-rich margarine (3.16 g

phytosterols/25 g margarine/day) versus butter and a sitostanol rich butter (2.43 g phytosterols/25 g butter/day). Compared to baseline values, plasma total cholesterol concentration was reduced by 4% and 6% and LDL cholesterol concentration was reduced by 8% and 10% with the sitostanol and campestanol-ester rich margarines, respectively. Serum HDL levels were increased by approximately 5% with both stanol margarine mixtures. The butter phase alone increased both plasma total and LDL cholesterol levels by 4% and HDL cholesterol by 6%, however, the addition of sitostanol to butter decreased total and LDL cholesterol levels by 8% and 12% versus butter. This showed that sitostanol mixed with butter decreased circulating cholesterol as effectively as the respective stanol mixture in margarine, despite the smaller dose. This study revealed that sitostanol esters are able to decrease plasma total and LDL cholesterol concentrations even as part of a highly saturated fat diet.

In 1996 Gylling and Miettinen evaluated the effectiveness of a sitostanol ester margarine and pravastatin alone and in combination. They showed that the addition of 1 g/day sitostanol ester margarine significantly inhibited cholesterol absorption (-68%), and compensatorily increased hepatic cholesterol synthesis. However, despite increasing cholesterol synthesis, sitostanol lowered total and LDL cholesterol concentrations by 11 and 14%, respectively. Sitostanol ester feeding also significantly increased all cholesterol precursor sterol proportions in serum and fecal neutral sterol excretion.

Gylling et al. (1999) examined the effects of sitostanol-ester feeding on cholesterol and non-cholesterol sterol metabolism from a one-year study in mildly hypercholesterolemic subjects conducted previously (Miettinen et al., 1995). Stanol ester feeding significantly decreased serum cholesterol levels with a constant decrease in non-

cholesterol sterols, reflecting cholesterol absorption and with a constant increase in those reflecting cholesterol synthesis.

Miettinen et al. (2000) studied the effect of feeding margarine containing stanol ester on cholesterol and plant sterol metabolism. After only 7 days of treatment, serum cholesterol concentrations were reduced by 16% with consumption of 2 g per day stanol ester. Using the doubly labelled isotope fecal collection technique, they found that cholesterol absorption efficiency was also significantly decreased by approximately 40%.

1.2.6 Sitosterol esters and cholesterol reduction

Recently, the question has been raised as to whether saturation of plant sterols is really needed for optimal cholesterol-lowering efficacy; as studies have shown low dose sitosterol esters to be as effective as sitostanol esters in cholesterol reduction. To compare the efficacy of sitosterol-ester versus sitostanol-ester containing margarines, Weststrate and Meijer (1998) supplemented 100 healthy non-obese normocholesterolemic subjects. Subjects each completed four treatments of a five arm randomized placebo-controlled double-blind Latin square design matrix. During each period of 3.5 weeks, subjects consumed different margarines that replaced an equivalent amount of their normal intake. Margarines utilized in this study were either provided as a control, containing no plant sterols, or possessing one of 4 plant sterol mixtures including concentrates derived from (i) soybean oil, (ii) rice bran oil, (iii) sheanut oil or (iv) Benecol® margarine (Raisio Inc, Finland). Soybean-derived plant sterols consisted largely of sitosterol and campesterol esterified to fatty acids, largely from sunflower seed oil. Rice bran and sheanut oils contained 4,4'-dimethyl sterols esterified mostly to

ferulic, cinnamic and acetic acids, while Benecol® contained sitostanol and campestanol, esterified to rapeseed oil derived fatty acids. Total plant sterol intakes ranged from 1.7 to 3.2 g/day, due to variations in plant sterol levels across the supplemented spreads.

Plasma lipid data showed no difference in response due to baseline cholesterol level or gender. Consumption of both the Benecol® and soybean plant sterol containing spreads resulted in significantly lower total- and LDL-cholesterol concentrations. Weststrate and Meijer (1998) found that circulating total and LDL cholesterol concentrations were equally reduced by 8-13% with both the sitosterol-ester and sitostanol-ester margarines, compared to the control, which did not contain plant sterols. Margarines containing sterols derived from rice bran and sheanut oils showed no significant cholesterol lowering action compared with other margarines. No treatment effect on HDL-cholesterol was observed. The major finding of this study was that unsaturated plant sterols when esterified to fatty acids could be as effective as saturated plant sterols in lowering circulating cholesterol levels in humans.

Similarly, Jones et al. (2000) compared the efficacy of margarine enriched with phytosterol esters or phytostanol esters in fed to hypercholesterolemic subjects for 3 weeks. Plasma TC was significantly reduced by 9% following ingestion of 1.84 g per day sterol esters, compared to control. Stanol esters also reduced TC concentrations by 5.5%, however it was not significantly different from the control group. Both the sterol esters and stanol esters significantly reduced LDL cholesterol concentrations by 13% and 6%, respectively, compared to control. There were no significant changes in HDL cholesterol or TAG concentrations. Cholesterol absorption was significantly reduced by 36% and 26% with consumption of the sterol esters and stanol esters, compared to

control, while cholesterol synthesis was reciprocally increased 53% and 38% for sterol esters and stanol esters, respectively.

To further elucidate the mechanisms by which phytosterols and phytostanols lower circulating cholesterol concentrations, Normén et al. (2000) studied the effect of feeding soy derived sterol esters and stanol esters on small bowel cholesterol absorption. Since plant sterols and stanols are believed to lower LDL cholesterol by inhibiting cholesterol absorption in the small bowel, the aim of this study was to measure cholesterol absorption, sterol excretion, and hepatic cholesterol synthesis after intake of soy sterols esters and sitostanol esters.

They found that ingestion of 1.5 g per day for 3 days equally lowered the cholesterol absorption efficiency by 32% and 30% in the soy sterol ester period and stanol ester period, respectively. Normén et al. (2000) concluded that despite the different structures esterified soy sterols and sitostanol esters inhibited cholesterol absorption equally. This finding contradicts previous reports that saturated plant sterols are more potent inhibitors of cholesterol absorption than unsaturated plant sterols.

Dose response studies have also been conducted to assess the minimum required dose to achieve significant reductions in cholesterol. Hendriks et al. (1999) investigated the relationship between cholesterol lowering and three different relatively low intakes of soybean plant sterol esters (0.83, 1.61, and 3.24 g/day sitosterol esters) in a polyunsaturated fat margarine. Eighty hypercholesterolemic men and women consumed each of four margarine spreads and a butter control, each for a period of 3.5 weeks. Hendriks et al. (1999) showed that all three dosages of plant sterols significantly lowered plasma total and LDL cholesterol levels by 4.9-6.8% and 6.7-9.9%, respectively,

compared to the control margarine. The group consuming butter resulted in significantly higher lipid levels compared to all margarine groups. It was also noted that there were no significant differences between the groups however they concluded that 1.6 g/d of esterified plant sterols will beneficially lower plasma total and LDL cholesterol levels by 6 and 8.5%, respectively (Hendriks et al., 1999).

Reductions in circulating total and LDL cholesterol seen with the consumption of sterol esters appear to be comparable with that of stanol esters at the same dose. If there is no difference between sterol esters and stanol esters it may be worth revisiting the idea that there may be little difference in lipid lowering capacity between sterols and stanols in general. Plant sterols are also now being recommended as part of the new NCEP treatment guidelines, published in May 2001 (Expert panel, 2001). It states that consuming 2 g/day of plant sterols or stanols is recommended as one of the therapeutic lifestyle changes associated with LDL-lowering therapy, with no discrimination between sterols and stanols. On the basis of these new studies and the current recommendations, it appears that further investigation of the relative efficacy of saturated versus unsaturated plant sterols as cholesterol-lowering agents is required if these products are to be used most effectively.

1.3 DIETARY FAT AND MODULATION OF PLASMA CHOLESTEROL

Recent evidence suggests that fats of the type found in dairy products may have desirable effects in preventing obesity, a risk factor for hypercholesterolemia and CVD. However, contradictory evidence suggests that consumption of dairy fats raise circulating cholesterol levels. No other dietary ingredient exerts as great a modifying action on

circulating cholesterol levels than saturated fat (Mattson and Grundy 1985, McNamara et al. 1987). The cholesterol-raising action of saturated fatty acids exhibits chain-length dependency. Saturated fatty acids such as palmitic (C16:0) and myristic (C14:0) acid (Grundy 1991, Bonanome and Grundy 1988) are considered most hypercholesterolemic and are found abundantly in dairy fats like butter. Fatty acid chain length of may also be very important in regards to energy expenditure and obesity (White, 1999; Scalfi et al., 1991; Seaton et al., 1986). Short chain (2-4 carbons) and medium-chain fatty acids (6-12 carbons) can be directly absorbed into the portal circulation and carried to the liver for oxidation, and do not appear to be readily stored in adipose tissue. Long-chain fatty acids must travel through the peripheral circulation before reaching the liver and may end up being incorporated into adipose tissue. Recently, short and medium chain fatty acid feeding has been shown to increase energy metabolism in humans (White et al., 1999) and decrease fat deposition and weight gain in animals (Geliebter et al, 1983; Hill et al., 1993). Although, butter contains an appreciable amount of saturated fat and cholesterol, which contributes to its hypercholesterolemic effect, it is relatively rich in medium-chain fatty acids, and may be useful in the prevention of obesity. A higher saturated fat and cholesterol content of butterfat has been shown to improve the efficacy of phytosterols (Pelletier et al., 1995) and phytosterol esters (Miettinen and Vanhanen, 1994). At the present time, the impact of adding unesterified plant sterols versus stanols to diets containing higher levels of saturated fat has not been assessed.

1.4 DIETARY CHOLESTEROL AND MODULATION OF PLASMA CHOLESTEROL

Evidence also exists that dietary cholesterol intake moderately raises plasma lipid levels thus increasing the risk of CVD development, however, the relationship is less clear. High intakes of dietary cholesterol increase the number plasma LDL particles, enrich them in cholesteryl esters and suppress the activity of LDL receptors. Several controlled metabolic investigations have shown that the addition of cholesterol to the diet causes no change (Kestin et al. 1989, McNamara et al. 1987) or an increase (Schonfeld et al. 1982, Keys 1984, Gylling and Miettinen 1992, Hopkins 1992) in the concentration of serum total and LDL cholesterol levels. Furthermore, levels of HDL cholesterol also often increase (Nestel et al. 1992b) when cholesterol is added to the diet. Consensus opinion, however, is that modest increase in total and LDL cholesterol concentrations do occur with the addition of physiological quantities of dietary cholesterol. Kinetic experiments suggest that the percent of cholesterol absorbed and the quantity synthesized de novo appear to decrease in response to increasing levels of dietary cholesterol (McNamara et al. 1987, Quintao and Sperotto 1987). From this work, evidently there is some concern warranted in reducing dietary cholesterol to minimise CVD risk, however, cholesterol is no more important a dietary constituent compared with the nutrients with which it is mostly found such as dietary saturated fat. A higher cholesterol content of butterfat has been shown to improve the efficacy of phytosterols (Pelletier et al., 1995) and phytosterol esters (Miettinen and Vanhanen, 1994). At the present time, the impact of addition of plant sterols and stanols to diets containing higher levels of cholesterol on cholesterol metabolism has not been thoroughly assessed.

1.5 SUMMARY AND RATIONALE

Coronary heart disease remains the primary cause of morbidity and mortality in developed countries. Elevated levels of plasma total and LDL cholesterol concentrations have been firmly established as risk markers for the development of CVD (MRFITRG, 1982; Klag et al., 1993). Plant sterols, in various forms, have been shown to reduce total and LDL cholesterol concentrations and block intestinal cholesterol absorption efficacy in human feeding trials. Particularly controversial at present is the effect of degree of hydrogenation on cholesterol-lowering efficacy. Further experimentation is required to test whether unsaturated plant sterol mixtures possess the same cholesterol-lowering efficacy as saturated mixtures, specifically the degree of saturation of the mixture (i.e. the proportion of sterols to stanols). Moreover, to date the relative effectiveness of these materials in a dietary context where saturated fat and cholesterol intakes are at the higher end of the normal physiological range, under controlled dietary parameters, has not been assessed nor have mechanisms of action been fully explored.

Circulating lipid levels are modified by several factors including drugs, exercise, and dietary selection. Principal dietary factors associated with higher circulating lipid levels include saturated fat and cholesterol. Butter contains short and medium chain saturated fatty acids, which have been shown to be associated with more rapid energy expenditure thus potentially, lesser body adipose accretion in humans. In spite of this advantage, butter and its derivatives are sources of dietary saturated fat and cholesterol. Because of this unfavorable lipid-raising influence, butter is not considered to be a heart healthy food, despite its potentially positive attribute in promoting energy utilization. Thus, butter with added phytosterols and phytostanols should result in a marked

inhibition of cholesterol absorption and lowering of circulating cholesterol levels. However, both the action of plant sterols and the relative efficacy of phytosterols versus phytostanols in the context of saturated fat need to be assessed.

1.6 HYPOTHESES AND OBJECTIVES

1.6.1 Null Hypotheses

1. The addition of either phytosterols, phytostanols, or a 50:50 mixture of sterols and stanols to butterfat will not reduce its hypercholesterolemic effect through actions involving cholesterol absorption, synthesis, and turnover rates in mildly hypercholesterolemic human subjects.

2. The addition of either phytosterols, phytostanols, or a 50:50 mixture of sterols and stanols to butterfat will not influence circulating plant sterol concentrations in mildly hypercholesterolemic human subjects.

1.6.2 Objectives

I. To examine whether addition of either non-saturated plant sterols, partially-saturated plant sterols, or saturated plant sterols to butterfat results in a reduction in plasma TAG, and total, LDL, and HDL cholesterol levels versus butterfat alone in mildly hypercholesterolemic individuals.

II. To assess the influence of non-saturated plant sterols, partially saturated plant sterols, and saturated plant sterols added to butterfat on cholesterol synthesis, absorption, and turnover rates versus butterfat alone in mildly hypercholesterolemic individuals.

III. To assess the influence of non-saturated plant sterols, partially saturated

plant sterols, and saturated plant sterols added to butterfat on circulating plasma plant sterol concentrations versus butterfat alone in mildly hypercholesterolemic individuals.

CHAPTER 2.

MANUSCRIPT

Unesterified Plant Sterols and Stanols Lower Low-density Lipoprotein Cholesterol Levels Equivalently in Hypercholesterolemic Individuals

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2.1 ABSTRACT

Background: Plant sterols, in various forms, have been shown to reduce total and LDL cholesterol concentrations. Particularly controversial at present is the effect of degree of hydrogenation of plant sterols on cholesterol-lowering efficacy and the responsible mechanisms.

Objective: To examine the effect of unesterified plant sterol and stanol supplementation on plasma lipid and phytosterol concentrations, as well as cholesterol absorption, synthesis, and turnover.

Design: Fifteen otherwise healthy hypercholesterolemic subjects consumed each of four dietary treatments in a randomized crossover design. Unesterified sterols and stanols were blended into the butter component of the diet at a dose of 1.8 g/day. Diets contained (i) plant sterols (NS), (ii) plant stanols (SS), (iii) a 50:50 mixture sterols and stanols (NSS), or (iv) a control diet (control).

Results: Plasma total cholesterol concentration was decreased ($p<0.01$) by 7.8%, 11.9%, and 13.1% in NS, SS, and NSS groups, respectively, versus control. LDL cholesterol was decreased ($p<0.03$) by 11.3%, 13.4%, and 16.0% in NS, SS, and NSS groups, respectively, compared to control. Plasma TAGs and HDL cholesterol levels did not differ across diets. Cholesterol absorption efficiency was reduced ($p<0.001$) by 56.0, 34.4, and 48.9%, for NS, SS, and NSS, respectively, compared to control. Fractional synthesis rate was reciprocally increased ($p<0.003$) by 45.5% for NSS, relative to control. Plasma campesterol and sitosterol levels were increased ($p<0.01$) with NS and sitosterol was decreased ($p<0.01$) with SS.

Conclusions: These data indicate that in their free, unesterified form, sterols and stanols lower plasma LDL cholesterol equivalently in hypercholesterolemics by suppressing cholesterol absorption.

2.2 INTRODUCTION

Plant sterols and stanols, structural analogs of cholesterol, have been shown to substantially reduce total and low-density lipoprotein (LDL) cholesterol concentrations under a variety of study conditions. Several researchers have claimed that consumption of stanol- containing mixtures is more effective in reducing circulating cholesterol level, compared to sterols (Heinemann et al., 1986; 1988; 1991; Miettinen et al., 1995; Miettinen and Vanhanen, 1994; Vanhanen et al., 1993; Gylling and Miettinen, 1999; Jones et al., 1999). Recently, however, the paradigm has shifted to a position where sterol and stanol esters are viewed as comparable plasma cholesterol modulators. It was observed that circulating total and LDL cholesterol levels were equally reduced by 8-13% with both the sitosterol-ester and sitostanol-ester margarines at doses of 1.5 to 3.3 g/day (Weststrate and Meijer, 1998). Similar lowering of total and LDL cholesterol concentrations was reported with ingestion of sitosterol-esters compared to sitostanol-esters (Jones et al., 2000).

This controversy raises another important question as to whether unesterified (free) plant sterol and stanol mixtures, would possess the same cholesterol-lowering efficacy regardless of their degree of hydrogenation, or whether esterification and solubilization of plant sterol mixtures are responsible for their equal effectiveness. Comparison of free sitosterol and sitostanol in pastil form given to children with severe hypercholesterolemia showed that hydrogenation improved the LDL cholesterol lowering by increasing fecal neutral sterol output to a greater degree, compared to the sitosterol supplemented group (Becker et al., 1993).

Plasma cholesterol lowering efficacy of phytosterols varies according to the composition and dose of the phytosterol mix and the vehicle in which they are given. It

has been suggested that high intakes of saturated fat and cholesterol may improve the efficacy of phytosterols (Pelletier et al., 1995) and phytosterol esters (Miettinen and Vanhanen, 1994). To date the relative effectiveness of these materials in a dietary context where saturated fat and cholesterol intakes are at the higher end of the normal physiological range has not been assessed nor have mechanisms of action been fully explored. Therefore, the objective of this study was to examine the effect of unesterified sitosterol and sitostanol supplementation on plasma lipid and phytosterol concentrations as well as cholesterol absorption and synthesis in subjects consuming precisely defined diets.

2.3 SUBJECTS AND METHODS

Subjects

Ten male and six female otherwise healthy, free-living volunteers with primary familial hyperlipidemia between the ages of 35 and 58 years were recruited. Female subjects were either post-menopausal or had undergone a hysterectomy. Subjects were screened for total circulating cholesterol and triacylglycerol (TAG) concentrations. Inclusion criteria included a plasma total cholesterol concentration in the range of 5.2 to 9.0 mmol/L and TAG less than 3.5 mmol/L. Prior to acceptance, subjects were required to provide a medical history as well as undergo a complete physical examination. Fasting blood and urine samples were collected for serum biochemistry, hematology, and urine analyses. Subjects were screened for chronic illness, including hepatic, renal, thyroid, and cardiac dysfunction, prior to admission in the study. Subjects were required to refrain from using drug therapy for hypercholesterolemia during and for at least 8 weeks prior to the start of the study. Prior to study commencement, subjects received a thorough

explanation of the study protocol and were given the opportunity to discuss any queries with either the primary investigator, physician, or study coordinator before signing the consent form. Baseline characteristics of the study subjects are presented in **Table 2.1**. The experimental protocol was approved by the Human Ethical Review Committee of the Faculty of Agriculture and Environmental Sciences for the School of Dietetics and Human Nutrition at McGill University.

TABLE 2.1
Baseline characteristics of the subjects[1]

Variables	Men (n=9)	Women (n=6)	All (n=15)
Age (y)	46.4 ± 2.7	49.8 ± 2.6	47.8 ± 1.9
Weight (kg)	92.2 ± 3.5	82.4 ± 7.3	88.3 ± 3.6
Body mass index (kg/m^2)	30.5 ± 1.5	31.3 ± 2.5	30.8 ± 1.3
Lipids (mmol/L)			
Total cholesterol	6.48 ± 0.43	6.57 ± 0.28	6.52 ± 0.27
LDL cholesterol[2]	4.31 ± 0.44	4.29 ± 0.28	4.30 ± 0.26
HDL cholesterol[3]	0.93 ± 0.04	1.42 ± 0.08	1.16 ± 0.08
Triacylglycerols	2.53 ± 0.36	1.86 ± 0.26	2.26 ± 0.25

[1]Values are expressed as mean ± SEM.
[2]Low-density lipoprotein cholesterol
[3]High-density lipoprotein cholesterol

Experimental design and diets

The study was a randomized crossover double blind clinical trial. Subjects consumed each of four dietary treatments. Each dietary treatment phase consisted of 21 feeding days. Each feeding phase was separated by a four-week washout period during which time subjects returned to consuming their habitual diets. In order to reduce the error term associated with diet sequencing, subjects were randomly assigned to one of four

predetermined Latin squares, where each square possessed 4 sequenced phases and 4 subjects. In this manner, we ensured that the crossover design was balanced.

The diets were comprised of solid foods, typical of those consumed in North America, and provided as three meals per day in a 3-day rotating menu. The nutrient content of the basal diet was calculated using Food Processor, a computerized dietary analysis system with a Canadian database. Diets were designed based on Recommended Nutrient Intakes for Canadians to provide 3000 kcal /70 kg individual /day. The Mifflin equation was used to estimate individual subject basal energy requirements (Mifflin et al., 1990), which was then multiplied by an activity factor of 1.7 to compensate for the additional energy needs of mildly to moderately active healthy adults. If subjects gained or lost weight during the first week of each treatment phase, energy adjustments were made to meet individual requirements and ensure that baseline body weights were maintained. Dietary carbohydrate, fat, and protein made up 50, 35, and 15% of ingested energy, respectively, with seventy percent of the fat provided as butter. Diets contained either plant sterols (NS), plant stanols (SS), 50:50 mixture plant sterols and stanols (NSS), or a control diet (control). The NS treatment consisted of purified phytosterols derived from soybeans and contained sitosterol (43%), campesterol (26%), stigmasterol (17%), and other identified phytosterols (14%). To achieve the SS treatment the same soybean phytosterols were hydrogenated to produce a composition of sitostanol (66%) and campestanol (33%). Equal parts of phytosterols and phytostanols were mixed together to create the NSS treatment. The control product was cornstarch, as it strongly resembled the white powdery phytosterol containing mixtures. The phytosterol and phytostanol mixtures, or cornstarch control, were blended into the butter component of the diet at a

dose of 1.8 g/day, warmed to 37°C, and administered equally across the three daily meals. To achieve double blinding, containers containing the plant sterols, stanols, and the cornstarch control were coded so that neither the researcher giving the test mixture, nor the subject receiving it, knew its true identity. Diets were prepared in the metabolic kitchen of the Mary Emily Clinical Nutrition Research Unit of McGill University. Subjects consumed a minimum of 2 of the three daily meals each day at the unit under supervision. It was required that all subjects consume breakfast at the unit and one of the other two meals was available for take out.

At the start (day 1) and end (day 22) of each dietary phase fasting blood samples were taken for determination of circulating lipid levels. Ninety-six hours prior to the end of each phase subjects provided a baseline blood sample prior to receiving an intravenous injection of 15 mg [25,26,26,26,27,27,27] D_7-cholesterol and a 75 mg oral dose of [3,4]^{13}C-cholesterol for cholesterol absorption determination. The ratio of ingested [3,4]^{13}C-cholesterol to injected [25,26,26,26,27,27,27] D_7-cholesterol enrichment in serum cholesterol after 24, 48, and 72 hrs was taken as an indicator of the fractional cholesterol absorption rate. The D_7-cholesterol isotope was prepared for injection by first, dissolving it in ethanol at a concentration of 5 mg/ml under sterile conditions at the Royal Victoria Hospital pharmacy. The isotope/ethanol mixture was then added drop-wise to an intravenous fat emulsion (Baxter Corp., Toronto, Ontario), for a total injectable volume of 9 ml. Cholesterol synthesis was also determined at the end of each diet period using the deuterium incorporation approach. Seventy-two hours following dosing with ^{13}C-cholesterol and D_7-cholesterol, subjects were dosed with 0.7 g/kg of estimated body water, deuterium oxide (D) (99.8% atom percent excess, CDN Isotopes, Montreal, Quebec).

Body water was estimated to be 60% for calculation of the dose. Deuterium oxide was given immediately following a fasting blood sample at approximately 08:00 hr on day 21 of each diet phase.

Analyses

Plasma lipid concentrations

Blood samples were centrifuged for 15 min at 1500 rpm to separate plasma from red blood cells (RBC) and stored at –80°C until analysis. Plasma total, HDL cholesterol, and TAG concentrations were analysed in quadruplicate with standardized reagents using a VP Autoanalyser (Abbott Laboratories, North Chicago, IL, USA). Calibration of the analyser prior to each run was performed as per the standardization protocol of the Canadian Reference Laboratory. The Friedewald equation was used to calculate LDL cholesterol levels (Friedewald et al., 1972).

Determination of cholesterol absorption

Free cholesterol extracted from RBCs was used to determine ^{13}C-cholesterol and D_7-cholesterol enrichments. Lipid was extracted from the RBCs in duplicate using a modified Folch extraction procedure (Folch et al., 1957). Thin layer chromatography (20x20 cm, 250 μ, Scientific Adsorbents Inc., Atlanta, GA, USA) was used to separate free cholesterol from cholesteryl ester. The free cholesterol band was then scraped from the silica gel plate and saponified with 0.5 M methanolic KOH to eliminate any fatty acid contaminants. Free cholesterol extracts were dried under nitrogen and transferred into 18 cm sealed combustion tubes (Vycor, Corning Glass Works, Corning, NY, USA). Cupric

oxide (0.6 g) and a 2 cm long piece of silver wire were added and tubes sealed under vacuum for at least 5 min at less than 20 mtorr pressure. Dual tracer labeled cholesterol samples were then combusted to D-enriched water and ^{13}C-enriched CO_2 over 4 hr at 520°C. The generated CO_2 was transferred under vacuum into Vycor tubes for measurement of ^{13}C enrichment and thereafter, water was vacuum-distilled into sealed tubes containing 0.06 g zinc (Biogeochemical Laboratories Indiana University, Bloomington, IN, USA) for D enrichment analysis. Tubes containing the water and zinc were then reduced to D-labeled hydrogen gas at 520°C for 30 min.

Nuclear magnetic resonance (NMR) was used to verify that the isotopic enrichments of the tracers [3,4]^{13}C-cholesterol and D_7-cholesterol (CDN Isotopes, Pointe Claire, Quebec) were greater than 99 atom percent excess. The ^{13}C enrichments of free cholesterol were measured by differential isotope ratio mass spectrometry (IRMS) using an automated dual inlet system (SIRA 12, Isomass, Cheshire, UK). Enrichments were then expressed relative to PeeDee Belemnite (PDB) limestone, which is used as the international reference standard for expressing carbon stable isotopic ratios, from the National Bureau of Standards (NBS). Linearity and gain of response of the SIRA IRMS instrument were assessed using a reference tank CO_2 and NBS standards of known isotopic enrichment. The D enrichments of free cholesterol were measured by differential IRMS using a manually operated dual inlet system with electrical H^{3+} compensation (VG Isomass 903D, Cheshire, UK). For D, enrichments were expressed relative to standard mean ocean water (SMOW) and a series of standards of known enrichment from the NBS, analysed concurrently on each day of measurement to correct for any variations in linearity of gain of response of the IRMS.

The average ^{13}C and D enrichments of 48 and 72 hr RBC free cholesterol relative to baseline (t=0) samples were used to calculate the cholesterol absorption coefficient (CAC) using the ratio of orally ingested ^{13}C-cholesterol to intravenously administered D_7-cholesterol as described by Bosner et al. (1993):

$$\text{CAC (pool/pool)} = \frac{del\ ^{13}C}{del\ D_7} \times \frac{\text{15 mg i.v. dose of } D_7\text{-cholesterol}}{\text{75 mg i.g. dose of } ^{13}C\text{-cholesterol}} \times \frac{7}{46} \times \frac{2}{27} \times \frac{0.0112}{0.000155} \quad \text{(Eqn 1)}$$

where *del* (‰) for ^{13}C and D_7 is the difference between the average of the enriched samples at 48 or 72 hr and the baseline abundance (at t =0) in parts per thousand relative to PDB and SMOW standards, respectively. The factors 7/46 and 2/27 reflect the ratios of labeled to unlabeled hydrogen and carbon atoms in the cholesterol tracers, respectively. The constants 0.0112 and 0.000155 represent factors converting the part per thousand units to equivalent atom percent excess for the PDB and SMOW scales, respectively.

Determination of cholesterol biosynthesis

Cholesterol biosynthesis was determined as the rate of incorporation of D from body water into RBC membrane free cholesterol over the period between 72 and 96 hr at the end of each feeding period. Deuterated water equilibrates quickly between intracellular and extracellular water pools and permits direct determination of cholesterol formation rates (Jeske and Dietschy, 1980). Deuterium enrichment was measured in both RBC free cholesterol and plasma water. To determine plasma cholesterol D enrichment, total RBC lipids were extracted and isolated using the same procedure described above.

To measure deuterium enrichment of plasma water, additional plasma samples were diluted 7 fold with water to reduce deuterium enrichment to within the normal analytical range. Baseline samples were not diluted. Triplicate samples were then vacuum-distilled into zinc-containing (0.06 g) Vycor tubes. Cholesterol and plasma water samples were then reduced to hydrogen gas at 520°C for 30 min and analysed by differential IRMS, as previously described above.

Cholesterol fractional synthesis rate (FSR) was taken to represent the RBC free cholesterol D enrichment values relative to the corresponding plasma water sample enrichment after correcting for the free cholesterol pool. The FSR represents that fraction of the cholesterol pool that is synthesized in 24 hours and was calculated as per the formula (Jones et al., 1993):

$$\text{FSR (pools/day)} = (del_{\text{cholesterol}} / del_{\text{plasma}}) \times 0.478 \qquad \text{Eqn (2)}$$

where *del* (‰) for D cholesterol is the difference between enriched free cholesterol and plasma water at 96 and 72 hr in parts per thousand relative to a SMOW standard. The factor 0.478 reflects the ratio of labeled H atoms replaced by D (22/46) during in vivo biosynthesis (Jones et al., 1993).

Cholesterol turnover measured by D_7-cholesterol decay

Plasma turnover of free unesterified cholesterol represents the rate of flux of incoming sterol from synthesis and diet relative to that being esterified, eliminated, or transferred into other pools. Turnover rates for RBC free cholesterol were determined

from the decay rate of D_7-cholesterol. Exponential curves were fitted to 24, 48, and 72 hr RBC D_7-cholesterol enrichments after subtraction of baseline D abundance at 0 hr.

Plasma phytosterol concentrations

Plasma phytosterol concentrations were determined duplicate by gas-liquid chromatography (GLC) from the nonsaponifiable material of plasma lipid as reported previously (Ntanios and Jones, 1998). Briefly, 1.0 ml plasma samples were saponified with 0.5 M methanolic KOH for 1 h at 100°C and the nonsaponifiable materials were extracted with petroleum ether. Samples with 250 μg of 5-α cholestane added as an internal standard were injected into a GLC equipped with a flame ionization detector (HP 5890 Series II; Hewlett Packard, Palo Alto, CA) and a 30-m capillary column (SAC-5; Supelco, Bellefonte, PA). Detector and injector temperatures were 310°C and 300°C, respectively. Duplicate samples were run isothermically at 285°C. Phytosterol peaks were identified by comparison with authenticated standards (Supelco, Bellefonte, PA).

Statistics

All data are expressed as the mean ± standard error mean (SEM). Lipoprotein cholesterol, TAG, and phytosterol concentration, absolute values at the beginning and end of each dietary period and % changes relative to baseline were compared using a crossover ANOVA design for determination of the diet effects. Although a four-week washout period separated each dietary period, a carryover term was employed in the model to reduce any error associated with dietary sequence (Cross-over experiments, 1993). When treatment effects were identified as significant, a Tukey test was used for identification of

significant effects between diets at particular time-points. Student's paired t-tests were used to compare baseline values with final time-points within each diet. Cholesterol absorption, synthesis, and turnover values were compared at the end of each treatment phase using a crossover ANOVA. When treatment effects were identified, a Tukey test was used for identification of significant effects of each diet treatment. Pearson's correlation coefficient analysis was used to test for relationships between variables. A level of statistical significance at $p<0.05$ was used in all analyses. The data were analysed using proc-General Linear Model SAS (version 6.12) software.

2.4 RESULTS

Sixteen subjects were enrolled into the study. One male subject dropped out at the end of the first feeding cycle due to difficulties with daily transportation to the unit each day. Therefore, complete data for nine men and six women were collected and analysed as per the study protocol. All individuals tolerated the diet without any reported adverse events. Subjects reported no abnormal or atypical smell, taste, color, or mouth-feel effects when consuming any of the four mixtures, thus were unable to distinguish between dietary treatments. There were no significant mean group weight changes across any of the three treatment phases. Blood and urine samples at the beginning and end of each phase for all 15 subjects were sent to LDS Diagnostic Laboratories (Pointe Claire, Quebec), where complete blood counts (CBC), biochemistry (sequential multiple analysis level C (SMAC)), and urinalyses were carried out. Results from all four phases of the feeding trial remained within normal ranges throughout the study period and regular physical exams revealed no suggestion of any clinical irregularities.

Circulating lipids in response to treatment

The concentrations of plasma lipids at the beginning and end of each treatment phase are shown in **Table 2.2**. Total cholesterol concentrations measured across all phases of the feeding trial showed significant variation between subjects. Plasma total cholesterol concentration was decreased ($p<0.01$) by 7.8%, 11.9%, and 13.1%, in the NS, SS, and NSS groups, respectively, versus control. LDL cholesterol levels were decreased ($p<0.03$) by 11.3%, 13.4%, and 16.0%, in the NS, SS, and NSS groups, respectively, compared to control. Plasma TAG and HDL cholesterol levels did not differ across diets. Over the study period, however, HDL cholesterol was lower ($p<0.05$) in the NSS group.

TABLE 2.2

Plasma lipid levels at day 0 and day 21 of each dietary period[1]

Lipid	NS	SS	NSS	C (control)
		mmol/L		
Total cholesterol				
Day 0	5.97 ± 0.26	6.23 ± 0.26	6.40 ± 0.31	6.14 ± 0.33
Day 21	5.54 ± 0.22 ^{b*}	5.57 ± 0.22 ^{b***}	5.59 ± 0.26 ^{b**}	6.15 ± 0.25
% Change	-6.3 ± 2.9 ^{b}	-10.4 ± 2.3 ^{b}	-11.6 ± 3.2 ^{b}	1.5 ± 3.2 ^{a}
% Relative to control	-7.8	-11.9	-13.1	
LDL-cholesterol[2]				
Day 0	4.0 ± 0.20	4.11 ± 0.18	4.18 ± 0.23	4.06 ± 0.26
Day 21	3.6 ± 0.17 ^{b*}	3.59 ± 0.18 ^{b*}	3.55 ± 0.17 ^{b**}	4.01 ± 0.20
% Change	-9.1 ± 2.9 ^{b}	-11.2 ± 3.0 ^{b}	-13.8 ± 3.2 ^{b}	2.2 ± 5.4 ^{a}
% Relative to control	-11.3	-13.4	-16.0	
Triacylglycerol				
Day 0	1.91 ± 0.22	2.10 ± 0.24	2.21 ± 0.35	1.94 ± 0.20
Day 21	1.85 ± 0.23	1.90 ± 0.20	1.99 ± 0.32	2.15 ± 0.33
% Change	2.2 ± 9.1	-5.4 ± 6.7	-3.1 ± 8.0	7.0 ± 6.3
% Relative to control	-4.8	-12.4	-10.1	
HDL-cholesterol[3]				
Day 0	1.14 ± 0.08	1.17 ± 0.07	1.20 ± 0.09	1.19 ± 0.07
Day 21	1.10 ± 0.07	1.14 ± 0.09	1.14 ± 0.09*	1.16 ± 0.08
% Change	-2.0 ± 2.9	-2.6 ± 3.7	-5.6 ± 2.5	-2.3 ± 2.1
% Relative to control	0.3	-0.3	-3.3	
LDL:HDL ratio[2,3]				
Day 0	3.71 ± 0.29	3.69 ± 0.26	3.81 ± 0.39	3.62 ± 0.34
Day 21	3.40 ± 0.25	3.48 ± 0.30	3.61 ± 0.42	3.73 ± 0.29
% Change	-7.9 ± 3.2	-6.4 ± 4.1	-6.7 ± 6.4	4.0 ± 6.0
% Relative to control	-11.9	-10.4	-10.7	

[1]Values are expressed as mmol/L ± SEM. Values carrying different superscript letters indicate a significant difference between diets ($p<0.05$). Percent change is based on individual data. Percent change relative to control is based on the mean of day 21. *P≤0.05; **P≤0.01; ***P≤0.001: significant differences within each diet(between day 0 and 21).

[2]Low-density lipoprotein cholesterol

[3]High-density lipoprotein cholesterol

Cholesterol absorption in response to treatment

Cholesterol absorption at the end of each feeding phase was taken as an average of the 48 hr and 72 hr measurements. Mean cholesterol absorption coefficient was decreased ($p<0.001$) following ingestion of the NS, SS, and NSS diets (0.200 ± 0.03, 0.298 ± 0.04, 0.232 ± 0.04 pool/pool, respectively), compared to the control diet (0.454 ± 0.04 pool/pool) (**Table 2.3**). Therefore, relative to control, absorption was reduced 56.0, 34.4, and 48.9 % for NS, SS, and NSS groups, respectively. Absorption values for NS and SS groups were significantly ($p<0.05$) different from each other, however, the NSS group was not significantly different from either the NS or SS group.

Cholesterol biosynthesis in response to treatment

Fractional synthesis rate in the control group was measured to be 0.040 ± 0.007 pool/d. Ingestion of NSS diet increased ($p<0.003$) fractional synthesis rate by 45.5% (0.064 ± 0.007 pool/day), compared to control. Consumption of both the NS and SS diets also increased fractional synthesis rates by 25.0% (0.055 ± 0.008 pool/day) and 34.1% (0.059 ± 0.01 pool/day), compared to control, however, this increase was not statistically significant from control (**Table 2.3**). There was no difference observed in synthesis between groups supplemented with phytosterols or phytostanols.

TABLE 2.3

Cholesterol absorption coefficients and fractional synthesis rates for each dietary period[1]

Phytosterol	NS	SS	NSS	C (control)
Absorption				
Efficiency[1]	0.200 ± 0.03^{c}	0.298 ± 0.04^{b}	0.232 ± 0.04^{bc}	0.454 ± 0.04^{a}
% Relative to control[¥]	-56.0	-34.4	48.9	
Synthesis				
Rate[2]	0.055 ± 0.008^{ab}	0.059 ± 0.01^{ab}	0.064 ± 0.007^{a}	0.040 ± 0.007^{b}
% Relative to control[¥]	37.5	47.5	60.0	

[1]Values are expressed as pool/pool ± SEM. [2] Values are expressed as pool/d ± SEM. Values carrying different superscript letters indicate significant differences between diets ($p<0.05$). [¥]Percent change relative to control diet for day 21.

Cholesterol turnover in response to treatment

Turnover rates of cholesterol were calculated from D_7-cholesterol enrichment values obtained during the 24 - 72 h period following injection of the isotope at the end of each treatment phase. Turnover rates of free cholesterol, extracted from RBCs, were 0.381 ± 0.05, 0.346 ± 0.06, 0.324 ± 0.04, and 0.364 ± 0.04 pools/day, for NS, SS, NSS, and control diets, respectively. There were no significant differences identified between any of the diets.

Plasma plant sterol levels in response to treatment

Plasma plant sterol concentrations and ratios relative to total cholesterol are presented in **Table 2.4**. Plasma campesterol and sitosterol concentrations were not different between groups at the beginning of each feeding phase. There were, however, changes between groups at the end of phytosterol supplementation. Plasma campesterol and sitosterol levels were higher ($p<0.01$) in the NS group compared to control, and SS

groups. Mean plasma campesterol and sitosterol concentrations were increased ($p<0.0001$) by 99.3% and 38.6%, respectively, compared to control with consumption of the NS diet. Sitosterol concentrations were reduced ($p<0.01$) by 23.6 % with SS, compared to control. Campesterol levels were also lower with SS feeding, however, this change did not reach significance. The NSS group produced little change in circulating plant sterol levels.

TABLE 2.4

Plasma plant sterol levels at day 0 and day 21 of each dietary period[1]

Phytosterol	NS	SS	NSS	C (control)
		μmol/L		
Campesterol				
Day 0	14.1 ± 1.2	17.3 ± 3.0	13.8 ± 1.5	15.6 ± 2.1
Day 21	24.3 ± 2.3 ^{a**}	8.5 ± 0.8 ^{c**}	17.7 ± 2.1 ^{b}	12.7 ± 2.4 ^{bc}
% Change	84.2 ^{a}	-43.1 ^{c}	39.2 ^{b}	-10.9 ^{bc}
% Relative to control	99.3	-27.9	54.6	
β-Sitosterol				
Day 0	8.6 ± 1.2	9.2 ± 1.0	7.2 ± 0.7	8.4 ± 1.1
Day 21	9.6 ± 1.0 ^{a}	4.8 ± 0.5 ^{c**}	7.4 ± 0.9 ^{b}	7.0 ± 0.9 ^{b}
% Change	26.3 ^{a}	-35.9 ^{c}	10.0 ^{ab}	-12.3 ^{b}
% Relative to control	38.6	-23.6	22.3	
β-Sitosterol:campesterol				
Day 0	0.12 ± 0.01	0.12 ± 0.02	0.09 ± 0.01	0.10 ± 0.01
Day 21	0.12 ± 0.01	0.06 ± 0.01	0.10 ± 0.01	0.11 ± 0.03
% Change	0.0	-50.0	11.1	10.0
% Relative to control	-10.0	-60.0	1.1	
Campesterol:total cholesterol				
Day 0	1.87 ± 0.18	2.25 ± 0.31	1.95 ± 0.12	1.93 ± 0.14
Day 21	2.65 ± 0.17	2.05 ± 0.32	2.53 ± 0.20	1.74 ± 0.18
% Change	41.7	-8.9	29.7	-9.8
% Relative to control	51.5	0.9	39.5	
β-Sitosterol:total cholesterol				
Day 0	0.20 ± 0.02	0.21 ± 0.03	0.17 ± 0.02	0.19 ± 0.02
Day 21	0.30 ± 0.03	0.10 ± 0.01	0.25 ± 0.03	0.21 ± 0.06
% Change	50.0	-52.4	47.1	10.5
% Relative to control	39.5	-62.9	36.6	

[1]Values are expressed as mmol/L ± SEM. Values carrying different superscript letters indicate significant differences between diets ($p<0.05$). Percent change is based on individual data; percent change relative to control diet for day 21.

*P<0.05; **P<0.01; ***P<0.001: significant differences within each diet (between day 0 and day 21).

Associations between plasma lipid levels and kinetic measurements

Across all subjects, both plasma total ($r = 0.42$, $P < 0.001$) and LDL cholesterol ($r = 0.35$, $p<0.006$) levels varied directly with cholesterol absorption coefficient. Similarly, β-sitosterol ($r = 0.40$, $p<0.002$) levels were found to vary directly with circulating LDL cholesterol concentrations. Fractional synthesis rate varied inversely with LDL cholesterol ($r = -0.29$, $p<0.03$) levels, further supporting the compensatory relationship between cholesterol lowering and increased synthesis rates. The sitosterol:campesterol ratio correlated directly with plasma LDL cholesterol ($r = 0.49$, $p<0.0001$) and inversely with FSR ($r = -0.42$, $p<0.0008$). Notably, neither campesterol nor the campesterol:cholesterol ratio correlated with cholesterol absorption coefficient.

2.5 DISCUSSION

The major novel finding of the study is the demonstration that non-saturated, saturated, and an equal part mixture of non-saturated/saturated phytosterols, in their unesterified form, significantly and equally reduce both plasma total and LDL cholesterol concentrations. The degree of cholesterol lowering observed is entirely due to the action of the plant sterols and stanols, not the basal diet, since plasma total and LDL cholesterol concentrations marginally increased on the control diet. This reduction in circulating cholesterol concentrations was achieved through inhibition of intestinal cholesterol absorption as evidenced by lower absorption coefficients, however, these reductions were accompanied by a partial compensatory de-suppression of cholesterol synthesis, which may be an indication that other mechanisms are also at work.

Despite the relatively high content of saturated fat and cholesterol in the basal diet, it was shown that sterols and stanols are efficacious in lowering circulating total and LDL cholesterol concentrations. It has been postulated that elevated intakes of dietary fat and cholesterol (400-450 mg/day) may increase the effectiveness of the phytosterols in the intestinal lumen. Unesterified plant sterols blended in butter and supplemented at a level of 0.74 g for 4 were shown to decrease total and LDL cholesterol levels by 10 and 15 %, respectively, despite a phytosterol dose of less than 1g/day (Pelletier et al., 1995). These authors attributed their results to the high cholesterol intake obtained from butter. Several researchers, however, have achieved similar degrees of cholesterol suppression when the total fat and cholesterol contents of the diet were much lower (Andersson et al., 1999; Hallikainen and Uusitupa, 1999; Gylling and Miettinen, 1999). These and other studies have shown efficacy of plant sterols and stanols when blended into a fat source such as margarine, butter, mayonnaise, or vegetable oils prior to supplementation (Jones et al., 1997). Conversely, when provided as a powder-filled capsule as part of a low fat diet, plant stanols failed to exert any lipid modulating effect (Denke, 1995), suggesting that the amount of fat and cholesterol in the diet are not strong modulators in the effectiveness of plant sterols. It is more likely that the effectiveness of plant sterols and stanols is more dependent on the vehicle in which they are matrixed and added to the diet rather than the composition of the diet.

Although structurally very similar to cholesterol, plant stanols are believed to be negligibly absorbed by the intestine (Heinemann et al., 1986), therefore, do not enter the cell and displace cholesterol at the level of the micelle, interrupting absorption (Piironen et al., 2000). Several methods exist to directly measure cholesterol absorption, however,

many require fecal collections and/or radio-labeled cholesterol administration (Grundy et al., 1969; Turley et al., 1994). The current study is one of few to employ the dual-stable isotope methodology. A coefficient of absorption for cholesterol is derived through a time-step comparison of the proportion of an oral labeled bolus of tracer cholesterol appearing in blood, relative to the appearance of a bolus labeled with a second tracer administered intravenously (Zilversmit and Hughes, 1974; Bosner et al., 1993). The pattern of decay of the intravenous tracer permits correction of the oral tracer response in plasma for loss into routes of excretion or deeper metabolic pools. Previously, selected ion monitoring mass spectrometry has been used to measure isotope enrichments (Bosner et al., 1993), whereas this study used more sensitive isotope ratio mass spectrometry to improve precision with lower isotopic dosages. The cholesterol absorption coefficient was determined by calculating the average of the 48 and 72 hr time points using the approach described by Bosner et al. (1993), who showed that the plasma ratio of oral and intravenous tracers becomes constant between 48 and 72 hrs after dosing which therefore allows for accurate assessment of intestinal cholesterol absorption.

The relative effect of unesterified sterols and stanols versus a control group on cholesterol absorption has not been previously studied particularly in the context of a rigidly controlled dietary paradigm. A cholesterol absorption efficiency of 45.4 % in the control group is comparable to values reported elsewhere (Mattson et al., 1982; Bosner et al., 1993; Normén et al., 2000). Similarly, a decrease in cholesterol absorption rates of 34-56 %, following sterol and stanol supplementation, is in accordance with data using sterol and stanol esters reported elsewhere in humans (Normén et al., 2000; Jones et al., 2000; Heinemann et al., 1991; Mattson et al., 1982). Present data indicate that the decrease in

circulating cholesterol concentrations in subjects supplemented with unesterified phytosterols or phytostanols was due to this inhibition in the cholesterol absorption efficiency.

Cholesterol absorption varied directly with both total and LDL cholesterol concentrations, suggesting that circulating cholesterol levels are dependent on the uptake of cholesterol in the intestine and that plant sterols and stanols effectively inhibited cholesterol absorption. The group with the lowest absorption coefficient was not, however, the group with the greatest degree of cholesterol lowering. The sterol group lowered cholesterol absorption 56 %, and raised synthesis by 25 %, however, a smaller impact on cholesterol lowering was seen, compared to the other groups. Although, the stanol group decreased the absorption coefficient by 34.4 % and raised synthesis by the same amount (34.1 %), cholesterol levels fell more dramatically. Interestingly, the 50:50 mix of sterols and stanols, decreased cholesterol absorption decreased 48.9 % and synthesis increased almost 50 %, yet lowered cholesterol levels to the greatest degree. Free sitosterol has been shown to more effectively lower cholesterol absorption compared to sitosterol ester (Mattson et al., 1982). However, most previous reports conclude that sitostanol more effectively inhibits cholesterol absorption compared to sitosterol (Heinemann et al., 1986; 1991; Gylling and Miettinen, 1999) or results in equal reductions in cholesterol absorption efficiency (Normén et al., 2000; Jones et al., 2000). Although results are in contrast to those previously reported, the present protocol study enforced a strict dietary regimen ensuring that all subjects consumed identical foods, in equal proportions, while maintaining a steady weight. This regimen minimized several dietary confounders making the comparisons between groups more accurate.

Consumption of plant sterols and stanols significantly induced changes in circulating plant sterols as well cholesterol concentrations, indicating mutually competitive inhibition between all sterol forms (Piironen et al., 2000). On this basis, plasma plant sterol concentrations have been used as indicators of compliance. Absolute values and percent changes in campesterol and sitosterol levels were similar to those previously reported following phytosterol feeding (Jones et al., 2000; 1999; 1997), signaling that the subjects were in fact consuming the treatment. Plasma plant sterol concentrations have also been used as an indirect measure of cholesterol absorption. Specifically, serum campesterol concentration and the campesterol:cholesterol ratio have been shown to correlate positively with intestinal cholesterol absorption. This association would be expected to reflect cholesterol absorption under static dietary conditions. However, different plant sterols are variably absorbed and metabolized, therefore, it is unclear whether the use of campesterol is appropriate for measuring cholesterol absorption under conditions where plant sterol/stanol intakes are changing. Supplementation with stanols inhibits cholesterol absorption and has consistently produced decreases in sterol concentrations (Jones et al., 2000; 1997; Gylling and Miettinen, 1999). During sitosterol feeding, however, sitosterol and campesterol levels have been shown to increase (Jones et al., 2000) or remain unchanged (Becker et al., 1993) despite a clear inhibition in the cholesterol absorption efficiency making this correlation inapplicable as a method of estimating cholesterol absorption in any situation where phytosterol intakes would be expected to change. In the present study plasma sterol levels decreased with stanols and increased with sterols while levels remained similar to control with a 50:50 mix of sterols and stanols. Furthermore cholesterol absorption coefficient was not associated with either

campesterol concentration or the campesterol:cholesterol ratio, as would be predicted from investigating stanols alone.

In summary, the present study demonstrates that in free form, sterol and stanol feeding results in equal reductions in total and LDL cholesterol concentrations. Cholesterol absorption was reduced in response to sterol and stanol feeding, and found to vary directly with reductions in LDL cholesterol concentration. Cholesterol synthesis was increased, however, not to an extent which prevented cholesterol lowering. In conclusion, both unesterified plant sterols and stanols favorably lower LDL cholesterol, independent of the degree of hydrogenation in hypercholesterolemic individuals.

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GENERAL CONCLUSION

This study examined the cholesterol-lowering efficacy and responsible mechanisms of action in response to feeding unesterified plant sterols and stanols, matrixed into margarine, to hypercholesterolemic males consuming precisely controlled diets. The novelty of approach of the present study was the combination of use of dual stable-isotope-plasma-ratio and deuterium uptake methods to simultaneously obtain indices of cholesterol absorption and *de novo* cholesterogenesis, respectively. The major finding of the present study is the demonstration that free sterols and stanols possess comparable efficacy in lowering total cholesterol concentrations, likely largely as a result of their ability to reduce cholesterol absorption, despite the compensatory de-suppression in cholesterogenesis. The fact that the final circulatory total cholesterol level was positively associated with the cholesterol absorption coefficient, particularly in the group showing the most pronounced cholesterol lowering, underscores the importance of inhibition of absorption as a chief mechanism in the cholesterol-modulating effect of plant sterols.

In conclusion, the present study confirms previous demonstration that in unesterified forms, phytosterol efficacy in cholesterol lowering is not influenced by the saturation state of the plant sterol constituents. Moreover, reduction of absorption of dietary cholesterol appears to exist as a mechanism contributing to the action of plant sterols in lowering total and LDL cholesterol concentrations. Cholesterol biosynthesis is de-suppressed, but not to an extent that plasma cholesterol levels are normalized. In summary, both unesterified sitosterol and sitostanol are efficacious in favourably reducing circulating cholesterol concentrations in hyperlipidemic males.

Future research should include examination of the exact pathway by which plant

sterols and stanols inhibit absorption. From this research it is clear that sterols and stanols are not metabolised in the same way, as evidenced by the differences in circulating sterol levels following their consumption as well as the differences in absorption coefficients and synthesis rates. We need to know which changes evoked by the consumption of sterols or stanols are the most beneficial if we are to make the best cholesterol lowering therapy recommendations to the public.

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APPENDIX 1.

September 1998

Ethics Review Form

Plant Sterols as Cholesterol-lowering Agents in Butter

CONSENT BY SUBJECT OF RESEARCH PROTOCOL

Protocol #: _____ Patient Name: _____

Influence of Phytosterols versus Phytostanols in Butterfat on Plasma Cholesterol Levels, Absorption and Synthesis Rate in Hypercholesterolemic Humans
School of Dietetics and Human Nutrition, Macdonald Campus of McGill University
Clinical Research Coordinator: Catherine Vanstone, Phone: 398-7527 day.
Researcher: Dr. Peter Jones, Phone: 398-7547 day / 457-5122 eve.
Physician: Dr. William Parsons, Pager: 897-5213, Home: 694-4869.

I, _____, the undersigned, hereby consent to participate as a subject in the above-named research project conducted by McGill University. The nature of the procedure or treatment, its risks and/or benefits, and possible alternatives, follow:

I. NATURE AND DURATION OF PROCEDURE:

The aim of this study is to examine how different types of plant sterols added to the diet influence the body's levels, rate of production and absorption of circulating cholesterol. Before the study, you will be examined by a physician to ensure that you are in good health. A blood sample (5 ml or _ tablespoon) will be taken for the laboratory to confirm the absence of health abnormalities and to measure your blood lipid levels. You will also need to ensure that you refrain from consuming any lipid lowering drugs for at least 8 weeks prior to initiation or during the study. A dietary assessment questionnaire will be provided to you before study commencement also.

When you start the trial, you will consume test diets provided by the Metabolic Kitchen within the Clinical Research Laboratory for 4 periods each of 21 days. A one month washout period will separate each diet, where you will resume consumption of your typical intake. Each test diet will contain normal foods and be fed to you as three meals per day. All diets will contain butter as the main fat source. To three of the diets will be added at a level of approximately 1.6 g/day phytosterols, a material resembling cholesterol obtained from plant sources. These plant sterols are tasteless and odourless. At the beginning (day 1) and end (day 21) of each of the four 21 day periods you will be asked to provide 34 ml (2.5 tablespoons) of blood for cholesterol level and metabolite analysis. On day 18 of each period, you will be given both by mouth and by arm vein, a small amount of cholesterol labelled with a "stable isotope" tag for assessment of cholesterol absorption. In addition, on day 18 you will be required to provide 20 ml (1.5 tablespoons) of blood at breakfast and 10 ml (1 tablespoon) at both lunch and dinner. Also you will be required to provide 10 ml (1 tablespoon) of blood on the mornings of day 19 and 20.

Plant Sterols as Cholesterol-lowering Agents in Butterfat

CONSENT BY SUBJECT OF RESEARCH PROTOCOL

On day 21 of each period, you will also be requested to drink approximately 25 ml water labelled with a "stable isotope" tag called deuterium for assessment of cholesterol synthesis. Finally on day 22 of each period you will be requested to provide 20 ml (1.5 tablespoons) of blood for similar analyses.

At the beginning and end of each trial you will be examined by a physician to ensure that you are in good health. A portion of the blood sample taken across the study will be used to again confirm the absence of health abnormalities. The total blood volume required for each phase of this trial will be 148 ml. Prior to each blood draw your blood pressure and pulse will be taken. Also, at the midpoint and end of each of the feeding phases you will be asked to complete a questionnaire concerning perception of taste, texture, smell and mouth-feel of the diet, particularly the diet fat.

II. <u>POTENTIAL RISKS AND/OR BENEFITS</u>:

There are no known hazards associated with the use of the stable labelled tags in the present procedures. A slight chance exists that you will experience transitory dizziness after drinking the labelled water. There are no risks of the procedure other than that normally associated with blood-taking. The plant sterol mixtures added to the diet at the proposed level has been shown to have no negative effects on health in previous animal and human experiments. In case you feel any discomfort during the experimental trial, a physician Dr. Parsons will be available to contact at any time. Dr. Parsons can be reached at Pager: 897-5213, Home: 694-4869.

The substance of the project and procedures associated with it have been fully explained to me, and all experimental procedures have been identified. I have had the opportunity to ask questions concerning any and all aspects of the project and any procedures involved. I am aware that I may refuse to participate as well as withdraw my consent at any time. I acknowledge that no guarantee or assurance has been given by anyone as to results to be obtained. Confidentially of records concerning my involvement in this project will be maintained in an appropriate manner.

I understand that, in compensation for the inconvenience of the study schedule, I will receive $1,200 at completion of the trial and subsequently be provided access to my results concerning the lipoprotein and cholesterol synthesis assessment, when they become available. If I decide to withdraw before completion, I will receive an appropriate pro-rated fraction of this amount.

I acknowledge receiving a copy of this consent form and all appropriate attachments.

Signature of Subject: _____

Doctor: _____ Witness: _____

Date: _____ Time: _____ a.m./p.m.

APPENDIX 2.

Consentement pour Sujet de Protocole de Recherche

Protocole # _____ Nom du Patient _____

Régulation de l'absorption et du métabolisme des lipides par les stérols végéteaux dans la diète, chez les sujets humaines

École de Diététique et de Nutrition Humaine Campus Macdonald, Université McGill

Chercheurs: Dr. Peter Jones, Tél: 398-7841 jour / 457-5122 soir.
Catherine Vanstone, Tél: 398-7527jour / 626-0058 soir.
Médecin: Dr. William Parsons, Tél: 897-5213 pagette / 696-4869 domicile.

Je, _____ sous-signé, consent à participer en tant que sujet dans le projet de recherche ci-haut mentionné, conduit par l'Université McGill. Je comprends la nature des procédures, les risques et/ou bénéfices et alternatives possible.

I. NATURE ET DURÉE DES PROCÉDURES:

Le but de cette étude est d'examiner comment les différents stérols végétaux, ajoutés à la diète, peuvent influencer le niveau sanguin et la vitesse de l'absorption et de production du cholestérol plasmique. Avant l'étude le sujet doit subir un examen physique afin d'établir un bilan de santé. Un échantillon sanguin (5 ml) sera prélevé afin de confirmer l'absence d'abnormalitées et pour mesurer les niveaux de lipides sanguins. Une questionnaire alimentaire sera rempli avant le commencement de l'étude.

Par la suite, le sujet devra consommer une diète fourne par la cuisine du laboratoire clinique pour 4 étapes de 21 jours chaques. Chaque étape sera separée par une période de un mois pendant laquelle vous retournez à votre diète habituelle. La diète étudié consiste d'aliments 'normaux'. Le beurre est la source primaire de gras dans les diètes. À trois des diètes sera ajoutée une substance 'stérol végétale' obtenue des huiles végétaux a un niveau de 1.6 g par jour. Cette substance est inodore et sans saveur et sera incorporé dans le beurre. Au commencement (jour 1) et la fin (jour 21) de chacune des quatres étapes, un échantillon sanguin sera prélevé (34 ml) pour déterminer le niveau sanguin de lipides et assurer un bilan de santé. Au jour 18 de chaque étape on vous demandera de manger et d'avoir une injection de cholestérol pour déterminer l'absorption du cholestérol. En plus, au jour 18 un échantillon sanguin (20 ml) sera prélevé avant le déjeuner, et 10 ml sera prélevé avant le dîner et le souper. Aux jours 19 et 20 de chaque étape, un échantillon sanguin (10 ml) sera prélevé pour déterminer le niveau sanguin de lipides. Au jour 21 de chaque étape, on vous demandera de boire (25 ml) d'eau deutérisée (eau lourde) pour pour déterminer la vitesse de production du cholestérol. Au jour 22 de chaque étape un échantillon sanguin (35 ml) sera prélevé pour les analyses.

À la commencement et à la fin de chaque étape vous serez à nouveau examiné par

le médecin afin d'assurer un bilan de santé. Le volume total de prélèvement sanguin s'élève à 148 ml au cours de chaque étape. Avant chaque prélèvement votre tension arteriele et poux sera mesuré. Aussi au milieu et fin de chaque étape on vous demendera de completé une questionnaire de votre perception de la diète.

2. Risques / Bénéfices Potentiels

Il n'y a aucun risque de santé associé à la prise de l'eau lourde dans la procédure de l'étude. Cependant, vous pouvez ressentir un léger étourdissement après son injestion. Il n'y a aucun risque, autre que ceux normalement associés à la prise de sang. Le mélange de 'stérols végétaux' aditionné à votre nourriture n'a démontrée aucun effets négatifs pour la santé lors des nombreuse études précédentes chez les animaux et les humaines. En tout temps, si vous ressentez un malaise durant la période d'étude vous pouvez rejoindre Dr. Parsons à son téléchasseur 897-5213 ou à la maison (514) 694-4869.

Les détails et procédures de l'étude me fûrent bien expliqués et toutes les procédures expérimentales ont été identifiées.

J'ai eu l'occasion de poser mes questions concernant fait aspect et procédure de l'étude. Je suis conscient que je peux refuser de participer et/ou retirer ma candidature en tout temps. Je comprends qu'il n'y a aucune garantie quand aux résultats obtenus. La confidentialité des résultats et de ma participation sera maintenue. Je comprends qu'en guise de compensation pour les inconvénients de l'étude, je vais recevoir $1,200 à la fin de l'étude et que j'aurai accès à mes résultats concernant la synthèse des lipoprotéines et du cholestérol. Si je décide de retirer ma candidature avant la fin de l'étude, je recevrai un pro-rata, au montant approximatif du montant total.

Je reconnais avoir lu et reçu une copie du consentement.

Signature du sujet:_____

Témoin:_____

Date:_____ Heure:_____ am / pm

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APPENDIX 4.

Visit Number	Study Number DAIRY	Subject Code
Date of Visit _____/_____/_____ MM DD YR	**Investigator** Dr. Peter Jones	**Subject Number** **Subject Initials**

BLOOD DRAW FORM

A. Vital Signs

Blood Pressure (seated): _____/_____ mm Hg
syst diast

Heart Rate: _____ bpm **Body Weight**: _____ lbs

B. Lab Work

Date samples were collected: _____/_____/_____
mm dd yr

Time samples were collected: _____:_____
(24 hr clock)

Time of last meal: _____:_____ (if less than 12 hrs, reschedule patient)

Were lab samples sent to LDS Laboratories? Yes No (circle one)

Notes:_____

_____