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CHOLESTEROL LOWERING EFFICACY OF PLANT STEROLS: MECHANISMS OF ACTION

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Doctoral of Philosophy

ABSTRACT

Phytosterols occur naturally in the non-saponifiable material of plant oils. Sitostanol, the saturated derivative of ß-sitosterol, is found in negligible concentrations in plant sources and, hence, is almost absent from typical Western diets. Tall oil extracts, on the other hand, contain about 20% (w/w) sitostanol. Phytosterols have been shown to lower plasma total cholesterol levels in animals and humans while sitostanol exhibited stronger reducing effects. Several studies have suggested that phytosterols lower plasma total cholesterol levels by either inhibiting cholesterol absorption or altering the activities of enzymes critical in cholesterol metabolism and excretion. However, results obtained demonstrate inconsistency regarding the effects of phytosterols on cholesterol absorption rates and cholesterogenesis. In addition, few studies have determined quantitatively the changes in the rate of cholesterol absorption and biosynthesis. Hence, the objective of this thesis was to investigate further the impact of different sources of phytosterols on plasma lipid profiles and to develop a new methodology for simultaneous measurement of percent cholesterol absorption and cholesterol synthesis rates in animals and humans. The stable isotopes, ^{13}C-, ^{18}O-cholesterol, and deuterium oxide were utilized for the dual isotope plasma ratio and deuterium uptake methodologies. Results from the series of animal experiments conducted demonstrate (i) a gender effect of phytosterols in modulating plasma lipid profile in hamsters, (ii) that sitostanol was more potent in lowering plasma and hepatic lipid concentrations than ß-sitosterol in hamsters and rabbits and (iii) that this lowering effect was due to a reduction in fractional cholesterol absorption and an increase in cholesterol excretion rates. Concomitantly, an up-regulation in cholesterogenesis was observed in hamsters. Furthermore, in humans tall oil phytosterols lowered total cholesterol levels in hypercholesterolemic subjects fed low saturated fat/cholesterol diet, but neither endogenous cholesterol synthesis nor plasma campesterol and ß-sitosterol concentrations were altered. Finally, in another study, circulating lipid and campesterol concentrations decreased in hypercholesterolemic subjects given a HMG-CoA reductase inhibitor, which suggested a reduction in cholesterol absorption rate. In conclusion, phytosterols lower plasma lipid profile by inhibiting cholesterol absorption and increasing its excretion in feces.

RÉSUMÉ

Les phytostérols se trouvent naturellement dans la matière non-saponifiable des huiles végétales. Dans les plantes, on retrouve le sitostanol, un derivé saturé du ß-sitostérol, dans des concentrations négligeables, ce qui explique leur quasi-absence de la nourriture typique des Occidentaux. Par contre, les extraits d'huile de pin contiennent près de 20% (w/w) de sitostanol. Il a été démontré que les phytostérols ont pour effet de baisser les niveaux totaux de cholestérol sanguin chez les animaux et les humains, tandis que le sitostanol eut des effets réduisants encore plus forts. Plusieurs études ont suggéré que les phytostérols baissent les niveaux totaux du cholestérol plasmique en inhibant l'absorption du cholestérol ou en modifiant les activités des enzymes critiques dans l'excrétion et le métabolisme du cholestérol. Cependant, les résultats obtenus démontrent de l'inconsistance en ce qui concerne les effets des phytostérols sur l'absorption du cholestérol et la cholestérogénèse. En outre, quelques études ont déterminé quantitativement les changements dans la biosynthèse du cholestérol et les taux d'absorption. En conséquence, les objectifs de cette thèse furent d'étudier davantage l'impact de différents phytostérols sur les profils de lipides plasmatiques et de développer une nouvelle méthodologie où le pourcentage d'absorption du cholestérol et les taux de synthèse peuvent être mesurés simultanément chez les animaux et les humains. Les isotopes stables, ^{13}C -, ^{18}O - cholestérol et d'oxyde de deuterium, furent utilisés pour deux méthodologies: la double proportion plasmatique d'isotope et l'incorporation de deuterium. Les résultats de la série d'expériences faites sur des animaux démontrent (i) que l'effet du phytostérol sur le profil de lipides plasmatiques chez les hamsters dépend

du sexe, (ii) que le sitostanol était plus puissant que le ß-sitostérol dans l'abaissement des concentrations plasmatiques et hépatiques de lipides chez les hamsters et les lapins et (iii) que cet effet d'abaissement résultait d'une réduction dans l'absorption et d'un accroissement du taux d'excrétion du cholestérol. Simultanément, un accroissement de la cholestérogénèse a été observé chez les hamsters. De plus, chez les humains, les phytostérols derivés de l'huile de pin ont baissé les niveaux de cholestérol total chez les sujets hypercholestérolémiques qui furent alimentés d'un régime faible en cholestérol et gras saturés, toutefois ni la synthèse endogénique du cholestérol ni les taux plasmatiques du campésterol et ß-sitostérol n'ont été modifiés. Enfin, dans une autre étude, les taux de lipides et de campestérol circulants ont été diminués dans les sujets hypercholésterolémiques à qui l'on administra un inhibiteur de HMG-COA reductase. Cela suggère qu'il y eut une réduction dans le taux d'absorption du cholestérol. En conclusion, les phytostérols abaissent le profil lipidique sanguin en inhibant l'absorption du cholestérol et en accroissant son excrétion dans les selles.

PREFACE

In this thesis the effects of phytosterols and variable sitostanol concentrations derived from different sources of phytosterols on cholesterol metabolism, lecithin cholesterol acyl transferase (LCAT) activity, plaque development, cholesterol absorption, excretion and *de novo* cholesterol synthesis rates were examined in two animal models and in humans.

Results of topics are presented in manuscript format with pertinent literature reviews on each chapter of the thesis. Chapter 1 provides a broad view of the proposal including objectives and null hypotheses of the research. Chapter 2 deals with an extended literature review for all aspects of the thesis. Chapter 3 investigates the effect of gender on two sources of phytosterols, soybean and tall oil, cholesterol levels and fractional synthesis rates in male versus female hamsters for a 90-day trial. Chapter 4 further explores the impact of variable concentrations of sitostanol on plasma lipid and phytosterol profiles in male hamsters in a 45-day study. Chapter 5 discusses the effect of different sitostanol concentrations on rabbits' plasma lipid and phytosterol profiles. In addition, Chapter 5 examines the potential effects of sitostanol on plasma LCAT activity and cholesterol esterification rate as well as the development of plaque in coronary arteries. Chapter 6 combines the kinetic results of the 45-day hamster study and those of the rabbits and assesses the influence of phytosterols containing variable sitostanol levels on cholesterol absorption, excretion and synthesis rates using a new methodology that we developed. Stable isotopes are used to measure simultaneously cholesterol absorption and synthesis rates in hamsters and rabbits. Chapter 7 deals with a human study where 32

hypercholesterolemic subjects were fed low saturated fat/cholesterol North American versus similar but tall oil-supplemented diets. This chapter examines the impact of dietary changes and tall oil-phytosterol administration on plasma lipid and phytosterol profiles as well as cholesterogenesis in hypercholesterolemics. Finally, Chapter 8 addresses the question of cholesterol absorption in hypercholesterolemic patients using changes in plasma campesterol levels as an index of fractional cholesterol absorption. Those patients were given 40 or 80 mg/day simvastatin with no phytosterol supplementation.

The thesis ends with a summary and final conclusions to be drawn from the results that suggest the limitations of the whole thesis and the potential research to be conducted in the future. This thesis is based on three papers which are published or in press papers, and four submitted manuscripts to peer-reviewed research journals.

STATEMENT FROM THESIS OFFICE

According to the regulation of the Faculty of Graduate Studies and Research of McGill University, the following statement took out from the Guidelines for Thesis Preparation (McGill University October 10, 1997) is included:

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.

If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". The thesis must include: A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers.

ADVANCE OF SCHOLARLY KNOWLEDGE

1. **Original contributions to knowledge**

This thesis has added to our knowledge in the field of phytosterols and their impact on plasma lipid metabolism by:

a. Providing evidence that gender affects the mechanisms of action of phytosterols.

b. Confirming that sitostanol is more effective in lowering plasma lipid levels than ß-sitosterol or campesterol.

c. Demonstrating that sitostanol does not affect LCAT activity.

d. Showing that sitostanol delays plaque development in rabbits.

e. Proving that sitostanol lowers cholesterol absorption in the intestine, increases its excretion in the feces and consequently up-regulates cholesterogenesis in hamsters with similar potentials in rabbits by using the dual-isotope plasma ratio and deuterium uptake methodologies.

f. Showing that tall oil-phytosterols at 20% (w/w) sitostanol lowers plasma total cholesterol levels in humans but does not affect significantly cholesterogenesis or absorption when subjects were put on low saturated fat/cholesterol North American diets over 30 days.

g. Suggesting that a HMG-CoA reductase inhibitor (simvastatin) lowers cholesterol absorption in hypercholesterolemic patients.

2. Research publications in refereed scientific journals

1. Ntanios, F., Jones, P.J.H. Effects of graded sitostanol intake on plasma lipid profile and phytosterol metabolism in hamsters. *Biochimica Biophysica Acta,* 1390: 237-224, 1998.

2. Ntanios, F., Jones, P.J.H., Frohlich, J.J. Dietary sitostanol reduces plaque formation but not lecithin cholesterol acyl transferase activity in rabbits. *Atherosclerosis,* 1998 (in press).

3. Ntanios, F., MacDougall, D.E. Jones, P.J.H. Gender effects of tall oil versus soybean phytosterols as cholesterol lowering agents in hamsters. *Canadian Journal of Physiology and Pharmacology,* 1998 (in press).

4. Ntanios, F., Jones, P.J.H., Frohlich, J.J. The effect of simvastatin (HMG-CoA reductase inhibitor) on plasma sterol levels and cholesterol absorption in hypercholesterolemic subjects. *Metabolism,* 1998 (accepted pending revision).

5. Jones, P.J.H., MacDougall, D.E., Ntanios, F., Vanstone, C.A. Dietary phytosterols as cholesterol-lowering agents in humans. *Canadian Journal of Physiology and Pharmacology* 75(3): 217-227, 1997.

3. Research manuscripts submitted to refereed scientific journal

1. Ntanios, F., Jones, P.J.H. Dietary sitostanol reciprocally influences cholesterol absorption and biosynthesis in hamsters and rabbits. Paper submitted to *Atherosclerosis.*

2. Ntanios, F., Raeini, M., Jones, P.J.H. Tall oil-phytosterol feeding lowers plasma lipid levels but does not affect *de novo* cholesterol synthesis rate or plasma plant sterol concentrations in hypercholesterolemic subjects. Paper submitted to *Journal of Lipid Research.*

CONTRIBUTION OF CO-AUTHORS TO MANUSCRIPTS

The candidate was responsible for developing, execution and management of experiments in hamsters, rabbits and in part in humans. The candidate collected, analyzed the data, and wrote the research manuscripts. The candidate contributed in the review paper by making the tables, collecting articles, editing the manuscript, and by correcting the final revised version for publication. The candidate measured cholesterol synthesis rates in humans and supervised the analyses of their plasma phytosterols levels by gas liquid chromatography. The candidate analyzed phytosterol levels in blood samples from a subgroup of patients participating in a parallel multi-center study that examines the effect of simvastatin on circulating lipid levels in hypercholesterolemic subjects.

Dr. P.J.H. Jones, the candidate's supervisor, edited all the manuscripts and started the writing of the review article. Dr Jones developed with the candidate the study designs and conducted weekly meeting with him to monitor the progress of the work.

Dr. J.J. Frohlich provided the facilities to measure LCAT activity and cholesterol esterification rates, performed by the candidate, as well as the assistance in grading plaque development in the coronary arteries. Dr. Frohlich had valuable comments on the manuscripts where he is a co-author.

Dr. T. Johns and Dr. S. Kubow, the candidate's committee members, edited the final draft of the thesis.

Dr. M. Raeini-Sarjaz, (doctor fellow) measured phytosterol levels in human subjects fed tall oil-phytosterols.

Ms. D. MacDougall helped in collecting articles and writing the literature review manuscript and in the starting of the 90-day hamster study.

Ms. C. Vanstone contributed in editing the literature review manuscript.

ACKNOWLEDGMENTS

I would like to acknowledge my committee members; Dr. Tim Johns and Dr. Stan Kubow for their editorial input in this thesis. A sincere thank you to Dr. Frohlich for his support in conducting the LCAT assays and the histopathology work in his lab. He made my work in his laboratory fruitful and enjoyable. I am also grateful to Dr. Linda Wykes for her assistance in determining cholesterol enrichment in the standards. I would like to thank my friend Diane MacDougall for her help in the literature review paper and the 90-day hamster study. Thank you to Mahmoud for his technical help. I would like to thank my friends in the lab, school and Laird Hall for creating such a challenging and learning environment. Finally, a sincere thank you to my supervisor Peter Jones. His patience, trust and dedication helped me cross this road confidently and successfully.

Supervisors are like wine, they become better and better with time.

DEDICATIONS

I dedicate my thesis to my family. Their support was like the North star during my journey.

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CHAPTER 1

GENERAL INTRODUCTION AND STATEMENT OF PURPOSE

Phytosterols (plant sterols) have been shown to decrease plasma total and low density lipoprotein cholesterol levels in animals and humans since as early as 1953. From that time until late 1970, the potentials of phytosterols were investigated extensively. Early experiments demonstrated that dietary phytosterols, mostly ß-sitosterol (3 to 9 g/day), were effective in lowering plasma lipid levels at a range of 5 to 10% in humans (Beveridge et al., 1958; Grundy et al., 1969; Salen et al., 1970; Lees et al., 1977). At the same time, a group of powerful lipid lowering drugs such as statins and fibrates were under development. The advent of these drugs lowered the interest in phytosterols as cholesterol reducing agents until recently when Heinemann and his coworkers (1986) and Miettinen et al. (1995) investigated the cholesterol lowering potentials of hydrogenated ß-sitosterol in humans. They reported that dietary sitostanol (saturated ß-sitosterol) at low concentrations (1-3 g/day) is more effective than ß-sitosterol in reducing plasma cholesterol levels. Reportedly, pure sitostanol was utilized in their studies and was found to lower cholesterol levels by 10 to 14%. Unfortunately, the high cost of production of purified sitostanol adds significantly to the final cost of dietary products supplemented with sitostanol. However, other effective alternatives exist in nature. For instance, tall oil phytosterols contain about 20% (w/w) sitostanol. The abundance of tall oil-rich sitostanol versus pure sitostanol may promote, on a large scale, the use of naturally available tall oil.

To our knowledge, no published studies have compared the effects of pure sitostanol to that of tall oil-phytosterol extracts in animals or humans. Furthermore, the mechanisms by which phytosterol/phytostanol reduces plasma total cholesterol concentrations have not been fully characterized. This is in remarkable contrast to the numerous published data of the effects of phytosterol on plasma lipid profile. For example, it has been suggested that ß-sitosterol and sitostanol reduced plasma cholesterol levels by (i) competitively blocking cholesterol absorption from the intestinal lumen (Heinemann et al., 1986; 1988; 1993) (ii) displacing cholesterol from bile salt micelles (Child and Kuksis, 1986) (iii) increasing bile salt excretion (Salen et al., 1970) or (iv) by hindering cholesterol esterification rate in the intestinal mucosa (Ikeda and Sugano, 1983; Child and Kuksis, 1983a). The proposed changes in percent cholesterol absorption due to dietary phytosterols might alter cholesterol fractional synthesis rates. However, the effects of phytosterols on cholesterogenesis are not well determined. For example, dietary plant sterols either stimulated (Gerson et al., 1964; 1965; Grundy et al., 1969; Konlande and Fisher, 1969), inhibited (Kakis and Kuksis, 1984) or had no effect (Subbiah and Kuksis, 1973) on fractional cholesterol synthesis in humans or animals.

Intrinsic non-absorptive, hypocholesterolemic effects of plant sterols have also been reported. For instance, plant sterols lowered circulating cholesterol concentrations by modifying hepatic acetyl-CoA carboxylase (Laraki et al., 1993), 3-hydroxy-3-methylglutaryl-CoA reductase (Boberg et al., 1989) or cholesterol 7-α hydroxylase (Shefer et al., 1994) in animals. Moreover, ß-sitosterol primarily lowered cholesterol-rich lipoproteins via an accelerated esterification rate of the lecithin cholesterol acyl-transferase (LCAT) enzyme in humans (Weisweiler et al., 1984). Some studies have

postulated that gender may affect cholesterol and phytosterol levels in humans (Kempen et al., 1991). Phytosterol levels in low and very low density lipoproteins were shown to be higher in women than in men (Tilvis and Miettinen, 1986; Kempen et al., 1991). Conversely, other studies have suggested no influence of gender in the effect of phytosterol/phytostanol consumption on plasma cholesterol levels (Vanhanen et al., 1993; 1994; Miettinen and Vanhanen, 1994).

Changes in plasma campesterol levels in humans have been used as a marker for cholesterol absorption rates (Tilvis and Miettinen, 1986; Miettinen et al., 1990). Simvastatin, a HMG-CoA reductase inhibitor, lowers plasma lipid levels by inhibiting hepatic cholesterol synthesis at its rate limiting step (Plosker and McTavish, 1995). In addition, other mechanisms of simvastatin, such as inhibiting cholesterol absorption, have been suggested as contributing to lower circulating cholesterol levels (Miettinen, 1991). Consequently, variations in circulatory campesterol concentrations after administration of a HMG-CoA reductase inhibitor might indicate an inhibitory effect on cholesterol uptake in humans (Miettinen, 1991).

With this body of knowledge at hand, further investigations are warranted regarding the effect of variable sitostanol concentrations on plasma cholesterol metabolism and kinetics in animals and humans. Previous studies on the cholesterol lowering potency of phytosterol/phytostanol have addressed cholesterol absorption rates (Heinemann et al., 1986; 1988; 1991; 1993) and indirect measurement of cholesterol synthesis (Grundy et al., 1969; Miettinen et al., 1990; Miettinen and Vanhanen, 1994), but have not examined simultaneously the impact of different sitostanol concentrations on cholesterol metabolism, absorption and synthesis rates.

Accordingly, the objective of the current thesis was to develop a method to measure simultaneously cholesterol absorption and *de novo* cholesterogenesis using labeled ^{13}C- and ^{18}O-cholesterol, and cholesterol deuterium uptake. In addition, the aim of this study was to investigate the influence of gender on plasma lipid and phytosterol metabolism. Another objective of this thesis was to examine the effects of various sitostanol concentrations derived from soybean, tall oil and pure sitostanol on cholesterol absorption, excretion and synthesis in hamsters, rabbits and humans.

Project overall objectives

The overall objectives of the thesis were to investigate further, in animal and human experiments, the impact of various sitostanol concentrations derived from different phytosterol sources on cholesterol and phytosterol metabolism in blood and hepatic tissues. In addition, a further objective was to examine the impact of variable sitostanol concentrations on cholesterol absorption, excretion and synthesis rates measured using isotope ratio mass spectrometry methods.

1.1 Specific objectives

a. *In male and female hamsters for 90 days:*

Perform an efficacy study using tall oil- and soybean-phytosterols on plasma lipid and phytosterol levels and determine the *de novo* cholesterol synthesis rate using deuterium oxide.

b. *In male hamsters for 45 days:*

Investigate the action of variable sitostanol concentrations on the

circulating cholesterol and phytosterol profiles, and cholesterol kinetics in the intestine, liver and blood using ^{13}C- , ^{18}O-cholesterol and deuterium oxide.

c. *In male rabbits for 71 days:*

Measure the impact of variable sitostanol concentrations on circulating cholesterol and phytosterol levels, cholesterol absorption, cholesterogenesis, plaque development in coronary arteries, phytosterols effects on LCAT activity and fractional esterification rate of cholesterol.

d. *In hypercholesterolemic human subjects for 30 days:*

Examine the effect of dietary changes and tall oil-phytosterol administration on plasma lipid and phytosterol profiles and cholesterol absorption, using circulating campesterol levels as an index of fractional absorption rate, as well as cholesterogenesis in hypercholesterolemics using deuterium oxide.

e. *In hypercholesterolemic human patients given simvastatin for 168 days:*

Measure plasma phytosterol levels and monitor changes in circulating campesterol concentrations as an index of cholesterol absorption in hypercholesterolemic patients given 40 or 80 mg/day simvastatin.

1.2 Null hypotheses

Tissue and circulatory lipoprotein cholesterol and phytosterol levels, atherosclerosis development, cholesterol excretion, cholesterol absorption and

cholesterol fractional synthesis rates in hamsters and rabbits will not be affected by:

Gender and changes in phytosterol source

Changes in sitostanol concentrations

Plasma lipoprotein cholesterol and phytosterol levels and cholesterogenesis in hypercholesterolemic human subjects will not be affected by:

Diet with or without tall oil supplementation

Circulating lipoprotein cholesterol and phytosterol levels and cholesterol absorption rate in hypercholesterolemic human subjects will not be affected by:

Simvastatin and changes in its concentration

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Cardiovascular disease (CVD) accounts for about half of all mortaiity in the Western world, surpassing cancer, infectious and other diseases (Khachadurian, 1990). Epidemiological and experimental studies (Glueck and Connor, 1978; 1979; Anonymous, 1979; Lewis, 1980; Gartside and Glueck, 1995) have consolidated the positive association that exists between CVD and elevated plasma cholesterol levels. Lowering circulating cholesterol concentrations reduces significantly the incidence of CVD in hyperlipidemic patients (Anonymous, 1994b; 1996; Jukema et al., 1995; West et al., 1996). Although in 1979, data from the Framingham study have suggested that not all people who develop CVD have high cholesterol levels (Kannel et al., 1979; Kannel and Larson, 1993), recently several prospective clinical trials confirmed that lowering the lipid profile significantly reduces the death rate from CVD and related diseases (Anonymous, 1994b; 1996; Sacks et al., 1995).

Considering that low plasma cholesterol levels in humans were not significantly correlated with any major disease, primary preventions such as lowering lipid levels in a population are still necessary and beneficial (Sacks et al., 1995). Several approaches could also be adopted to lower plasma lipid levels. Circulating cholesterol concentrations may be decreased either through dietary and lifestyle changes or via the use of drugs (Glueck and Connor, 1978) including newer HMG-CoA reductase inhibitors such as

simvastatin. Cholesterol lowering drugs, however, are costly. Administration of the drug, Mevacor, for example costs approximately $1,000 per year (cost in 1997). The American Heart Association proposed through NCEP (Anonymous, 1993) an acceptable plasma cholesterol level of <200 or 160 mg/dl for the public. Consequently, the number of people whose plasma cholesterol fell into the high risk percentile increased drasticaily and the cost of treating these people with the appropriate drugs is considerable. Grundy (1990b), on the other hand, has suggested the development of a new type of medical economics that will weigh the cost of treating cholesterol medically against other CVD risk factors or early prevention. Evidently, the need for preventive approaches and effective alternatives to the expensive drugs for lowering plasma cholesterol levels in a population group is increasing.

From this body of knowledge, a question arises: How much lowering of plasma cholesterol concentrations would be beneficial in reducing the risk of CVD in hypercholesterolemics? "Do we need a sledgehammer to crack a nut?" The sledgehammer represents the drugs in the market. Cross comparison of pooled data from a large number of studies suggests that for every 1% decrease in plasma LDL-cholesterol level, there is a subsequent 2-3% decrease in CVD within a population (Muldoon et al., 1990). A difference of 0.6 mmol/l (about 10%) in total cholesterol concentration corresponds to a difference in mortality from ischemic heart disease of 17% (Grundy, 1990a; Law et al., 1994a; 1994b) and in middle aged men, the difference may reach 25-30% (Muldoon et al., 1990). Law et al. (1994a) reviewed 10 major cohort studies on the effect of plasma cholesterol on CVD. They presented strong evidence that lowering a person's plasma cholesterol concentration by diet or drugs results in substantial protection

from CVD. Accordingly, effective lipid lowering compounds with concurrent dietary modifications would lower significantly the risk of CVD in the population.

2.2 Cholesterol homeostasis in the body

Cholesterol homeostasis in a biological system is maintained through mechanisms that involve absorption, synthesis, storage and excretion. The need for cholesterol in the body is met either by the uptake of cholesterol from the extracellular environment or synthesis within a given tissue (Stange and Dietschy, 1984; Dietschy et al., 1993). In the non-growing adult animal, an equal amount of cholesterol absorbed and newly synthesized must be catabolized or excreted through the feces or other means to maintain a cholesterol homeostasis in the body (Dietschy et al., 1993).

2.2.1 Cholesterol absorption

The small intestine is the major site for cholesterol absorption. Total cholesterol entering the intestine originates from either bile or the diet. Normally about 50% is absorbed and the remainder ends up being excreted in the stools. Kaplan, Cox, and Tylor (1963) determined cholesterol absorption in 24 healthy volunteers to be as low as 150-300 mg/day. Wilson and Lindsey (1965) reported that cholesterol absorption was small (300 mg) even when the intake was very high (3,000 mg). In the metabolic "steady state", cholesterol absorption in the intestine is considered saturated because a large amount of cholesterol is present from endogenous sources such as in bile (Grundy et al., 1969). Nonetheless, the amount of dietary cholesterol absorbed is not insignificant. If other mechanisms fail to maintain homeostasis through decreasing either cholesterol

synthesis, increasing its catabolism or excretion through bile; cholesterol movement in the system and progressive accumulation in body pools might lead to plaque deposition and become a risk factor for atherosclerosis development (Grundy et al., 1969).

Different methodologies were used to determine cholesterol uptake in animals (Turley et al., 1994) and humans (Grundy et al., 1969; Zilversmit, 1972a; Bosner et al., 1993; Wong et al., 1995). Cholesterol absorption was measured using the sterol balance method (Grundy et al., 1969), an area ratio technique after pulse injection of dual radioactive isotopes (Zilversmit and Hughes, 1974) or an isotope ratio of oral to intravenously administered ^{3}H- and ^{14}C-labeled cholesterol at a single time point (Zilversmit, 1972a; Bosner et al., 1993; Wong et al., 1995). In this thesis, similar to the procedures of Zilversmit and Hughes (1974), percent cholesterol absorption was measured by an isotope ratio method at a single time point 72 hr after stable isotope administration. Injected cholesterol equilibrates in the rapidly miscible pool of body cholesterol at 72 hr (Turley et al., 1994; Wong et al., 1995). After that time, labeled cholesterol starts to decay slowly (Zilversmit and Hughes, 1974; Bosner et al., 1993). Nilsson and Zilversmit (1972b) have also shown that labeled cholesterol administered intravenously is quickly removed from the plasma and gradually released into the bloodstream. An important point to consider is that the dual isotope plasma ratio is based on the assumption that the portion of orally administered cholesterol equilibrates and is metabolized in the same manner as intravenously injected cholesterol (Zilversmit and Hughes, 1974). Also, the level of ^{18}O-cholesterol after 72 hr in the plasma is assumed to reflect the efficiency with which exogenous and endogenous cholesterol are absorbed (Bosner et al., 1993).

To our knowledge, no published work has previously measured simultaneously cholesterol absorption rate and cholesterogenesis using ^{13}C-, ^{18}O-cholesterol and deuterium uptake in hamsters and rabbits. This approach will provide a stronger understanding of the correlations that exist between absorption, excretion and synthesis in the regulation of the cholesterol pool in hamsters and rabbits fed phytosterols with different sitostanol concentrations.

2.2.2 Cholesterol synthesis

Mammalian cells, with rare exception, can synthesize adequate cholesterol for metabolic requirements. A series of more than 20 reactions is needed to convert cytosolic acetyl-CoA to cholesterol. Hydroxy methylglutaryl coenzyme A (HMG-CoA) reductase activity and several enzymes involved in cholesterol synthesis are feedback inhibited by cholesterol itself. The sensitivity of modulation of cholesterol synthesis and HMG-CoA reductase by exogenous cholesterol varies from one tissue to another for example between the liver and the intestine (Lutton, 1991). Various *in vivo* and *in vitro* methods have been utilized to determine the rate of cholesterol synthesis by the whole body (Dietschy and Gamel, 1971; Jones et al., 1992), by a particular tissue or by a tissue structure (Dietschy and Siperstein, 1967); few of them are truly quantitative. The techniques used include input-output analysis (Grundy et al., 1969; Samuel et al., 1978), labeled cholesterol precursors (Goodman et al., 1980; Ramakrishnan et al., 1981; Katz et al., 1982), measurement of hepatic enzymatic activity (Bjorkhem et al., 1987) and determination of circulating cholesterol precursors in humans (Popjak et al., 1979; Parker et al., 1984; Heinemann et al., 1991). The methods applied are either invasive, require

long periods of measurement, demand accurate measurement of intake and output or measure cholesterol synthesis indirectly. Conversely, the deuterium uptake technique utilizes deuterium oxide at concentrations that have been demonstrated to be safe with no side effects in humans (Jones et al., 1993), and measures cholesterol fractional synthesis rates directly (Dietschy and Spady, 1984).

a. Deuterium uptake methodology

Cholesterogenesis is determined *in vivo* after deuterium uptake into the circulatory-pool cholesterol. Labeled water equilibrates quickly between the intracellular site of cholesterogenesis and extracellular body fluid. Thus, newly synthesized cholesterol is derived from a pool of known enrichment that can be determined by measuring either urine, saliva or plasma water. Therefore, total body water becomes the precursor pool for whole-body studies of lipogenesis. The ratio of incorporation of deuterium versus protium into cholesterol during biosynthesis has been determined previously (Dietschy and Spady, 1984). Briefly, hydrogens contributing to the cholesterol molecule are derived from the three following sources: 7 hydrogens are obtained from body-pool water, 15 hydrogens are derived from nicotinamide adenine dinucleotide phosphate (NADPH) during cholesterol synthesis and the remaining 24 hydrogens are obtained from cytosolic acetyl coenzyme A. During the 24-hr period between tracer water administration and blood collection, hydrogen derived from NADPH and body-pool water equilibrates quickly while hydrogens from acetyl coenzyme A are unlabeled unless the period of measurement is prolonged (Jones et al., 1993). Thus, the ratio of 0.81 labeled protons per carbon is most representative of

cholesterol synthesis in humans that leads to 22 deuterium per 27 carbon atoms within a cholesterol molecule (Dietschy and Spady, 1984). As detailed previously by Jones et al. (1993), the correction factor of 0.478 calculated by Dietschy and Spady (1984) of deuterium over total hydrogen is used in this thesis for animal and human subjects who were administered deuterium over 1 to 24 hr.

Some limitations exist in the estimation of deuterium/carbon ratio when using deuterium oxide. For instance NADPH is derived from two sources. The first is the pentose phosphate pathway that does not exchange hydrogen with cellular water and thus does not contribute to enrichment of sterol during synthesis. The second is the malic enzyme system that may exchange protons with the body-water pool and may vary among tissues and species. A range of 21 to 25 ^{3}H per carbon atoms of a cholesterol molecule has been reported (Dietschy and Spady, 1984). This results in 10% error in apparent measures of cholesterol synthesis when using tracer water. Despite its limitations, the deuterium uptake method, validated versus cholesterol balance methodology (Jones et al, 1998), is a useful tool that measures directly cholesterogenesis and can be applied easily on free living subjects.

b. Cholesterol synthesis in tissues and its homeostasis

Nearly all cholesterol synthesized in the body is considered to come either from the liver or small intestine. The contribution of all other tissues in the synthesis of total sterol body pool was estimated to be negligible (Dietschy and Siperstein, 1967). On the other hand, Dietschy et al. (1993) have suggested that in humans less than 10% of cholesterol is synthesized in the liver and the remainder in other

tissues. In addition, Turley et al. (1981) reported that sterol synthesis has been disproportionately underestimated in non hepatic and non-intestinal tissues in rats. The quantitative contribution of extra-hepatic and intestinal tissues to the total body cholesterol synthesis appears to be more important than as previously reported (Turley et al., 1981).

Furthermore, a close interaction exists between cholesterol absorption and synthesis. It is believed that the concentration of cholesterol present in the extra-hepatic circulation could modify cholesterol synthesis rates in the liver (Dietschy and Gamel, 1971). However, circulating cholesterol levels did not affect intestinal absorption rates (Jones et al., 1994a). The homeostatic mechanisms by which cholesterol balance is maintained within the system are not fully characterized. Any disturbances in the system might lead to changes in cholesterol concentrations to abnormal levels that become an independent risk factor in the multifactorial CVD. In cases where abnormalities in circulating total cholesterol levels exist, dietary modifications, or in severe cases, drug interventions are recommended to reduce the risk of CVD.

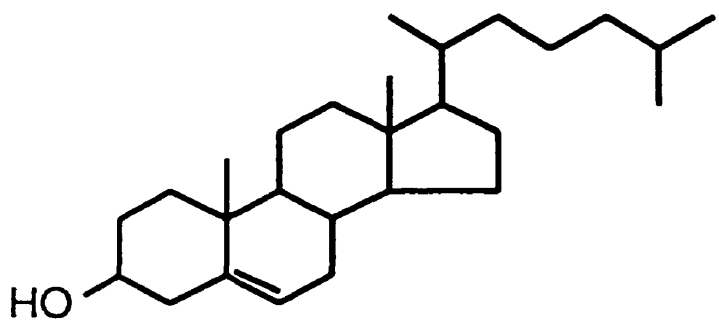
2.3 Phytosterols: A potential alternative to drugs

Some more natural alternatives to drugs for lowering plasma lipid levels have been investigated. Phytosterols are one of them. Phytosterols are naturally occurring compounds and are considered promising candidates for reducing plasma cholesterol concentrations.

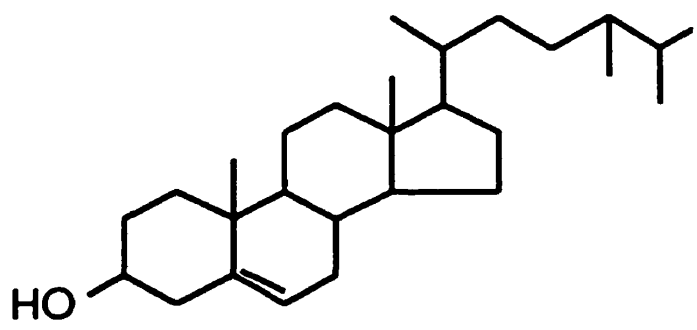
2.3.1 Structure and distribution of phytosterols

Phytosterols (plant sterols) are naturally occurring constituents of plants including ornamental and edible plants as well as herbs, shrubs and trees (Pollak and Kritchevsky, 1981). At least 44 sterols from seven different plant classes have been identified (Bean, 1973). The most abundant phytosterol is β-sitosterol, although phytosterol mixtures may include campesterol, stigmasterol and dihydrobrassicasterol. Most of plant sterols are similar to cholesterol in their basic skeleton structure except that they contain methyl, ethyl or other groups next to their C_{24} position on the aliphatic side chain of the compound (Figure 1) (Pollak and Kritchevsky, 1981). When phytosterols are saturated at the 5 α-position using commercial processes, compounds such as sitostanol are formed. Saturated phytosterol derivatives are not abundant in nature (Weihrauch and Gardner, 1978; Dutta and Appelqvist, 1996).

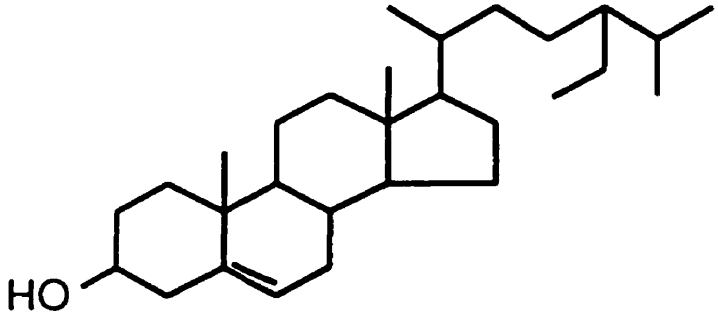
The presence and distribution of phytosterols across plant species have been extensively described by Pollak and Kritchevsky (1981). Briefly, phytosterols are naturally present in pure or esterified form, or conjugated as glycosides. They are detectable at various levels in the fat-soluble fraction of seeds,



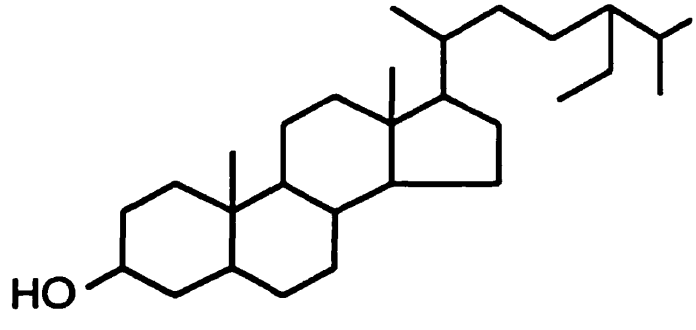
Cholesterol
(Δ 5-cholesten-3ß-ol)



Campesterol
(24α-methyl-Δ 5-cholesten-3ß-ol)



Sitosterol
(24α-ethyl-Δ 5-cholesten-3ß-ol)



Sitostanol
(24α-ethyl-5α-cholestan-3ß-ol)

Figure 1

roots, stems, branches, leaves and blossoms. Among other factors, the phytosterol content of any given plant depends on length of daylight, degree of soil alkalinity, and time of plant harvest.

2.3.2 Phytosterols/phytostanols as cholesterol depressants

a. Animal studies

Peterson et al. (1951), from his experiments in chickens, revealed the lowering potentials of plant sterols on cholesterol levels. Following his path, a number of investigators examined the effects of phytosterols on lipid levels in a range of animal models (Pollak and Kritchevsky, 1981). Peterson et al. (1951) fed chickens a chicken diet supplemented with either cholesterol, soy sterols or both sterols at a range of 0.5-1%. The addition of cholesterol to the diet increased the chicken plasma cholesterol levels from 196 to 942 mg/dl. However, on further addition of soy sterols, cholesterol levels did not increase. In another study, one-day-old White Leghorn cockerel chicks were fed either 1% cholesterol, 1% phytosterols or 1% of both sterols for a 4-week experiment (Chandler et al., 1979). The chicks fed the 1% cholesterol and phytosterols tolerated their diets and showed significant reduction in their serum and liver cholesterol values. Neither ß-sitosterol nor campesterol, both present in the phytosterol diet, could be detected in the serum. Chandler et al. (1979) have also found that stigmasterol, while apparently not exhibiting any deleterious effects, is ineffective as an antihypercholesterolemic agent in chicks. A study in rats by Alfin-Slater et al. (1954) examined the effects of soybean sterols on plasma, liver and carcass. No significant changes were seen in the plasma and carcass total cholesterol or total lipid values.

However, the hepatic total cholesterol and lipid levels in the group fed soybean phytosterols were both very much less than those found usually in the livers of rats fed cholesterol alone. Indeed, the values of hepatic cholesterol and lipids were very close to normal control levels despite the 1% cholesterol in the diet of these rats (Alfin-Slater et al. 1954). Ling and Jones (1995b) investigated the effects of tall oil-phytosterols that contain about 20% (w/w) sitostanol on circulating cholesterol and lipoprotein levels. They fed 5 groups of rats either the control diet alone, control diet with 1% cholesterol, control diet with 1% cholesterol and sitostanol mixtures or sitostanol-free mixtures at supplementation of 0.2, 0.5 and 1% (w/w), respectively. These investigators suggested that sitostanol-rich mixtures are more effective in lowering circulating lipoprotein cholesterol values than sitostanol-free mixtures. Similarly, Ikeda et al.(1981) in rabbits, and Sugano et al. (1977) in rats demonstrated that sitostanol was more potent in lowering lipid levels than ß-sitosterol alone. Ikeda et al. (1981) have reported that rabbits fed both 0.5% (w/w) cholesterol and sitostanol had markedly lower LDL-cholesterol levels than the group fed ß-sitosterol at the same concentrations as sitostanol. Moreover, Sugano et al. (1977) have shown that rats fed safflower or corn oil or lard with 0.5% (w/w) either unhydrogenated or hydrogenated ß-sitosterol (sitostanol), respectively, had lower lipid profiles than controls fed the same oils without the ß-sitosterol. Rats fed the sitostanol had the lowest lipid levels. However, both Ikeda et al. (1981) and Suagano and coworkers (1977) used either ß-sitosterol or sitostanol alone and did not investigate any additional synergistic effects of both sterols on lipid levels. These studies in different animal models indicate that phytosterols are effective in lowering cholesterol concentrations. The next task is to examine phytosterol/phytostanol effects in human trials.

b. Human studies

Dietary phytosterols as cholesterol-lowering agents in humans (Published in the *Canadian Journal of Physiology and Pharmacology, 1997, 75: 217-227*)

1. Abstract

Phytosterols, abundant in fat soluble fractions of plants, are consumed at levels of 200-400 mg/day in Western diets. Chemically resembling cholesterol, phytosterols inhibit the absorption of cholesterol. Phytosterol consumption in human subjects under a wide range of study conditions has been shown to reduce plasma total and low density lipoprotein (LDL) cholesterol levels, however, the response varies widely. Greater cholesterol lowering efficacy occurs with consumption of the saturated phytosterol, sitostanol, versus ß-sitosterol or campesterol. Most studies report no effect of phytosterol administration in high density lipoprotein (HDL) cholesterol or triglyceride levels, although evidence exists for an HDL cholesterol-raising effect of sitostanol. Phytosterol absorption is limited, although serum phytosterol levels have proven to be important indicators of both cholesterol absorption and synthesis. Serum phytosterols correlate with HDL cholesterol level. Also, higher phytosterol/cholesterol ratios appear in HDL versus LDL particles suggesting the existence of an intrinsic phytosterol action, besides the extrinsic effect on cholesterol absorption. In conclusion, addition to diet of the phytosterol sitostanol, represents an effective means of improving circulating lipid profiles to reduce risk of coronary heart disease.

2. Introduction

It has been identified for some time that ingestion of phytosterols results in favorable modification of circulating lipid profiles (Farquhar et al., 1956; Lees et al., 1977; Schlierf et al., 1978; Pollak and Kritchevsky, 1981; Pelletier et al., 1995), however, recently a new interest has focused on use of plant stanols in this manner, particularly sitostanol (Heinemann et al., 1986; Becker et al., 1992; Vanhanen and Miettinen, 1992; Becker et al., 1993; Gylling and Miettinen, 1994; Miettinen and Vanhanen, 1994; Ling and Jones, 1995b). The aim of the present review is to examine effects of plant sterol and stanol consumption on absorption, plasma concentration and excretion of cholesterol and phytosterols in humans. Potential mechanisms of action and toxicity will also be briefly described.

3. Typical phytosterol intakes

Dietary phytosterol levels among different populations vary greatly depending primarily on the type and amount of plant foods consumed. The Tarahumara Indians of Mexico who consume a diet containing unusually high amounts of beans and corn reportedly ingest over 400 mg of phytosterols per day (Cerqueira et al., 1979). In Japan, phytosterol intakes have remained at about 373 mg per day from 1957 to 1982, while cholesterol consumption simultaneously increased over twofold (Hirai et al., 1986). The most commonly ingested phytosterol is β-sitosterol (54%), while significant levels of campesterol (14%), brassicasterol (10%), and stigmasterol (7.5%) are also consumed (Hirai et al., 1986). Sitostanol is virtually absent from typical diets. Western diets typically contain lower levels of phytosterols than diets of many other parts of the world.

In 1991, the British consumed 104, 49, 10, and 4 mg per day of β-sitosterol, campesterol, stigmasterol and stigmastanol, respectively, representing a total phytosterol intake of 167 mg per day (Morton et al., 1995). The primary sources of phytosterols in the British diet are fats and oils, although breads and other cereals were also important sources (Morton et al., 1995). A trend was observed toward increased phytosterol intakes between 1987 and 1991 in Britain, possibly due to increased utilization of vegetable oils for cooking.

A comparison was made of phytosterol intakes of vegetarian and non-vegetarian Seventh Day Adventists (SDA) and nonvegetarians from the general population in the United States (Nair et al., 1984). Pure SDA vegetarians, lacto-ovo SDA vegetarians, and nonvegetarians who were SDA or from the general population ingested (ß-sitosterol + stigmasterol)/cholesterol in ratios of 16.0, 3.3, 1.0, and 0.5, respectively. In summary, although phytosterol intakes vary markedly depending on culture and food selection patterns, it is likely that phytosterol/cholesterol intake ratios have increased over the past several decades in Western countries.

4. Effect of phytosterol consumption on plasma total and low density lipoprotein cholesterol levels in humans

Phytosterol consumption in human subjects under a wide range of study conditions has been shown to reduce plasma total and low density lipoprotein (LDL) cholesterol levels within the range of 0.5-26% and 2-33%, respectively (Table 1). Notable is the wide variation in response. Clearly, certain study conditions promoted the cholesterol lowering efficacy of dietary phytosterols more than others. Several features of study design which may influence apparent phytosterol efficacy, including the serum

cholesterol modifying characteristics of the control diet, subject-specific type of lipid disorder or phytosterol dose and composition, are described in Table 1. Additional study considerations for which inconsistent or inconclusive responses to phytosterol administration were reported include effects of subject sex, age, health, form of administered phytosterol (suspension, crystal, capsule etc.) and duration of treatment.

4.1 Plasma cholesterol modification due to control diet alone

All phytosterol efficacy trials in humans except Schlierf et al. (1978) have involved a pretreatment and/or control diet. In some studies reduction in plasma cholesterol levels may have occurred due to consumption of the control and/or pretreatment diet alone, as many of these diets contained substantial amounts of unsaturated fat and/or phytosterols (Vanhanen and Miettinen, 1992; Vanhanen et al., 1993; 1994; Miettinen and Vanhanen, 1994; Miettinen et al., 1995). In fact, Miettinen and Vanhanen (1994) commented that the 8.6 mg/kg/d of phytosterols that subjects consumed with a rapeseed oil control diet compared with typical free-living intakes of phytosterols in this population (3.2 mg/kg/d) may have reduced the apparent cholesterol lowering efficacy of dietary phytosterols because the lower cholesterol absorption efficiency under basal conditions will result in a lesser reduction in cholesterol levels when additional phytosterols are consumed. Although many studies have failed to provide information on modification of plasma cholesterol levels by control diet alone, certain experiments involving substitution of 50 g of visible fat with an equivalent amount of fat from rapeseed oil reported plasma cholesterol reductions of 11% (Vanhanen et al., 1994) and 9.2%; 6 wk., n=24 (Vanhanen and Miettinen, 1992) due to

control diet alone. Other studies in hypercholesterolemic children receiving dietary intervention for 3 months before phytosterol treatment reported plasma cholesterol reductions of 3% (Becker et al., 1993) and 5% (Becker et al., 1992) due to diet treatment alone. These results underscore the importance of considering dietary pretreatment in evaluating data concerning effects of phytosterols on plasma lipid levels.

4.2 Dietary cholesterol intake

It has been suggested that besides the indirect effect that a low cholesterol intake may have on apparent phytosterol efficacy via the serum cholesterol modifying effect of the control diet, a low level of dietary cholesterol may also directly influence the ability of phytosterols to reduce cholesterol absorption in the intestine (Denke, 1995). Oral administration of 3 g of sitostanol per day failed to reduce plasma cholesterol levels in 33 hypercholesterolemics consuming less than 200 mg of cholesterol per day, primarily from meat rather than eggs (Denke, 1995). Similarly, plasma cholesterol level was only slightly decreased by administration of 12 g of ß-sitosterol per day for 12 weeks in 16 hypercholesterolemic patients consuming less than 200 mg of cholesterol per day (Briones et al., 1984). Phytosterols are assumed to reduce plasma cholesterol levels by effectively precipitating dietary cholesterol from the intestinal oil phase during digestion. As it is believed that phytosterols may be less effective at precipitating choiesterol during the micellar phase, the absorption of dietary versus endogenous cholesterol would be more effectively inhibited by phytosterol consumption (Mattson et al., 1977). When humans were subjected to a dietary cholesterol overload (400-450 mg/d), dietary phytosterols significantly reduced plasma cholesterol levels, although the accompanying

phytosterol dose was low (0.74 g/d) and subjects were normocholesterolemic (Pelletier et al., 1995).

4.3 Phytosterol composition and dose

Plasma cholesterol lowering efficacy of phytosterols may also vary due to the composition and dose of the phytosterol mix. In the past, phytosterols were seen as unfavorable cholesterol lowering agents as doses of 6-18 g per day were thought necessary to reduce plasma cholesterol level significantly. Recently, studies have shown that lower doses of phytosterols, particularly sitostanol, are at least as effective as larger phytosterol doses given in the past. Three grams of tall oil or 6 g of soybean oil derived phytosterols per day reduced plasma cholesterol levels in hypercholesterolemic subjects as effectively as did 18 g of soybean oil derived phytosterols per day given in the past (Lees et al., 1977). Furthermore, ingestion of phytosterols composed mainly of sitostanol, ranging from 1.5 g (Heinemann et al., 1986; Becker et al., 1993) up to 3.4 g per day (Vanhanen et al., 1993; 1994; Gylling and Miettinen, 1994; Miettinen et al., 1995) reduced plasma total and LDL cholesterol levels. In fact, 1.5 g of dietary sitostanol was shown to reduce plasma total cholesterol level as effectively as β-sitosterol given in amounts four fold larger (Becker et al., 1993). Heinemann et al. (1986) have suggested that when phytosterol mixtures contain high concentrations of β-sitosterol, this phytosterol may displace sitostanol from its mode of action, and thus cholesterol absorption would be inhibited less than if β-sitosterol were present in a lower concentration. Miettinen et al. (1995) reported that, although consumption of 2.6 versus 1.8 g per day of sitostanol for 6 months reduced plasma cholesterol levels to a greater

extent, from a practical point of view, both doses were equally effective. At least 1 g of phytosterol per day should be consumed before adequate cholesterol lowering effects are observed (Vanhanen and Miettinen, 1992; Vanhanen et al., 1994; Miettinen et al., 1995). Below this level, efficacy of phytosterols high in either sitostanol or β-sitosterol does not differ appreciably (Vanhanen and Miettinen, 1992; Miettinen and Vanhanen, 1994; Vanhanen et al., 1994). Conversely, Denke (1995) saw no improvement in lipid profiles of hypercholesterolemic men given 3 g per day of sitostanol suspended in safflower oil.

4.4 Males versus females

Although it is well known that cholesterol metabolism differs in males and females, gender differences in plasma cholesterol lowering response to phytosterol feeding have not been observed in humans. Vanhanen et al. (1993; 1994) and Miettinen and Vanhanen (1994) commented on lack of gender specific response. On the other hand, gender was postulated to be a variable that responded differently to various phytosterol levels and sources (Kempen et al., 1991). Tow reports have shown that plasma plant sterol concentrations differ between males and females (Tilvis and Miettinen, 1986; Kempen et al., 1991).

4.5 Initiation and termination of phytosterol efficacy

Farquhar et al. (1956) reported that dietary phytosterols reduced plasma cholesterol levels within 2-3 weeks of initiation of treatment and maintained these reduced levels over 6 months of continued phytosterol ingestion. Heinemann et al. (1986) found that plasma total and LDL cholesterol levels fell from 10% and 11%,

respectively, after 3 weeks of phytosterol treatment to 15% and 15%, respectively, after 4 weeks. Beyond that, however, there was no indication that cholesterol levels were further reduced with extended phytosterol feeding. Miettinen et al. (1995) reported that although maximum cholesterol reduction was observed in the first 3 months of phytosterol treatment, a slight decrease in plasma total cholesterol level was observed with continued phytosterol administration. Lees et al. (1977) reported that treatment effects observed after 10 months were maintained for up to 3 years with continued feeding. Upon cessation of phytosterol consumption, cholesterol levels return to baseline within 2-3 weeks (Farquhar et al., 1956; Heinemann et al., 1986).

5. Effect of phytosterol feeding on plasma high density lipoprotein cholesterol and triglyceride levels in humans

Most studies examining plasma lipoprotein subfraction responses to plant sterol intakes have reported no changes in high density lipoprotein (HDL) cholesterol (Heinemann et al., 1986; Becker et al., 1992; Vanhanen et al., 1993; 1994; Denke, 1995; Miettinen et al., 1995; Pelletier et al., 1995) and triglyceride (TG) (Heinemann et al., 1986; Becker et al., 1992; Vanhanen et al., 1993; 1994; Gylling and Miettinen, 1994; 1994; Denke, 1995; Miettinen et al., 1995) levels with oral phytosterol administration. In some studies, HDL cholesterol (Gylling and Miettinen, 1994) and TG (Schlierf et al., 1978; Becker et al., 1993) levels increased moderately, while in others decreases in HDL cholesterol (Schlierf et al., 1978; Becker, 1993) or TG levels (Lees et al., 1977; study 2 and 4a) have been reported. However, a substantial increase in plasma HDL cholesterol level has been reported in rats (Ling and Jones, 1995b) and chicks (Newman et al., 1992) consuming phytosterols.

6. Mechanisms of action: phytosterol and cholesterol metabolism in healthy humans

6.1 Typical plasma phytosterol levels in health and disease

In healthy humans, plasma levels of campesterol and β-sitosterol are typically maintained within a range of 7-14 μmol/l and 4-8 μmol/l, respectively, (Table 2), while sitostanol is virtually nondetectable (Heinemann et al., 1986; Vanhanen et al., 1993). More phytosterol appears in HDL than LDL particles, measured per mol of cholesterol (Tilvis and Miettinen, 1986). Preferential accumulation of phytosterols in HDL may relate to reverse phytosterol transfer (Glueck et al., 1991). Plasma phytosterol levels vary four to tenfold between healthy humans, thus these levels show greater variability than that of cholesterol (Miettinen et al., 1990), and may relate to level of physical activity. Plasma campesterol levels in 14 male distance runners and 10 sedentary age/sex matched controls did not differ significantly, while the level of plasma β-sitosterol was 33% higher in the runners (Sutherland et al., 1991). β-sitosterol was no longer elevated when data were adjusted for body mass index (BMI).

Phytosterol levels have also been investigated in individuals with other health problems such as primary biliary cirrhosis (PBC), a chronic cholestatic liver disease that progresses from a nonsymptomatic clinical stage, through fibrosis, to end stage liver cirrhosis and finally to death within 10 to 15 years of diagnosis. The ratio of plasma campesterol to ß-sitosterol was found to decrease with progression of this disease (Nikkila et al., 1991), perhaps due to impaired biliary secretion rather than altered dietary absorption (Miettinen et al., 1995). Two year drug treatment with ursodeoxycholic acid or colchicine tended to retard the increase in plasma ß-sitosterol levels seen in these patients over time.

Individuals with non insulin dependent diabetes mellitus (NIDDM) had lower plasma campesterol (36% and 48% in men and women, respectively) and β-sitosterol (35% and 42% in men and women, respectively) levels than non-diabetic controls (Sutherland et al., 1992). In individuals with NIDDM, but not non-diabetic controls, fasting serum insulin level was inversely correlated with plasma campesterol and β-sitosterol levels suggesting that the absorption of dietary phytosterols and possibly cholesterol may be reduced in hyperinsulinemic diabetics (Sutherland et al., 1992). Plasma concentrations of campesterol and β-sitosterol were also reduced in individuals with coeliac disease, although the concentration of β-sitosterol was reduced to a greater extent than that of campesterol (Vuoristo et al., 1988). Plasma campesterol/ß-sitosterol ratio was positively correlated with percent absorption of cholesterol in individuals with coeliac disease indicating that the lower this ratio, the greater the impairment of cholesterol absorption. Clearly, differential metabolism of these two sterols exists in humans.

6.2 Correlations between dietary/metabolic factors and circulatory phytosterol levels

Many dietary factors, including dietary phytosterol intake, and metabolic factors, including cholesterol absorption, plasma cholesterol level and plasma cholesterol precursors have been investigated in relation to plasma phytosterol levels. To elucidate metabolic factors which regulate plasma phytosterol levels, Miettinen et al. (1990) analyzed diet, stool samples and blood samples in a randomly selected Finnish male population with normal dietary habits. Results indicated that serum phytosterol levels

were positively correlated with dietary components such as total fat, polyunsaturated fat, linoleic acid and phytosterol level as well as polyunsaturated to saturated fat ratio. Serum phytosterol levels were also positively associated with plasma linoleic acid level.

Serum phytosterol levels have also proven to be important indicators of cholesterol absorption and synthesis. When serum phytosterol levels are elevated, dietary cholesterol is efficiently absorbed and cholesterol synthesis is reduced (Miettinen et al., 1990; Vanhanen et al., 1993). Down-regulation of *de novo* synthesis may be a consequence of feedback inhibition in response to the increased absorption of cholesterol from the diet. Miettinen and Vanhanen (1994) found that circulating phytosterol levels in a randomly selected Finnish male population correlated with fractional and absolute absorption of dietary cholesterol. Interestingly, as serum cholesterol level rose, serum campesterol concentrations increased more than did those of ß-sitosterol. Thus, the serum campesterol/ß-sitosterol ratio was positively correlated with serum cholesterol concentration. Miettinen and Vanhanen (1994) also reported that serum levels of phytosterols were positively correlated with HDL cholesterol level and negatively correlated with cholesterol synthesis and very low density lipoprotein (VLDL) cholesterol level. Based on a stepwise multiple regression, serum campesterol level was dependent on cholesterol absorption efficiency, plant sterol intake, and biliary cholesterol secretion, in order of importance, but was not dependent on other dietary variables. In contrast, serum ß-sitosterol level was determined primarily by plant sterol intake, cholesterol synthesis, cholesterol absorption efficiency and biliary cholesterol secretion. The above mentioned variables explained approximately 60% of variance in serum phytosterol levels in this population.

Kempen et al. (1991) further elucidated factors which influence plasma phytosterol levels including age, sex, anthropometric parameters, plasma lipids and apo E phenotype in 160 healthy Dutch families. In 4 groups, consisting of either male or female parents or children, plasma cholesterol and lathosterol levels were significant independent predictors of plasma phytosterol levels in all groups; sex and apo E phenotype were significant predictors of phytosterol levels in only one or two of four groups, while age, weight, height and HDL cholesterol level were not significant predictors of phytosterol levels in any of the groups. Although a sex difference was not observed in the raw data, multivariate analysis indicated that sex did appear as a significant factor for both serum campesterol and β-sitosterol levels. The effect was opposite to that reported by Tilvis and Miettinen (1986) who found that women have higher phytosterol levels than men.

Plasma phytosterol levels have also been investigated in individuals with lipid disorders. Tilvis and Miettinen (1986) investigated plasma phytosterol levels in 17 individuals from 2 families with hypertriglyceridemia. As in healthy individuals, serum phytosterol levels were positively associated with fractional absorption of cholesterol and negatively associated with fecal neutral sterols of cholesterol origin and with cholesterol synthesis. Additionally, phytosterol levels in VLDL and LDL were higher in women than in men.

Neither age nor gender were significant predictive variables of plasma phytosterol levels in 595 hypercholesterolemic subjects (Glueck et al., 1991). A remarkable correlation was found in these hypercholesterolemics when serum phytosterol levels were compared with premature coronary heart disease (CHD). Individuals with elevated levels of either stigmasterol or campesterol were more likely to have had

premature CHD. Furthermore, in 34 first degree relatives of individuals identified as having elevated plasma phytosterol levels, history of premature CHD was twice as common as in the full cohort. Hyperphytosterolemia probands appeared to be a heritable marker of hypercholesterolemia (3.2 times more than full cohort in top decile), hyperapobetalipoproteinemia (7.9 times more than full cohort in top decile), and increased premature CHD. Furthermore, phytosterol levels in hyperphytosterolemic probands were correlated with total and LDL cholesterol and TG levels as well as apo B level in relatives. This is in contrast to obligate heterozygote relatives of individuals with sitosterolemia who tend to have normal cholesterol and phytosterol levels. Elevated absorption of phytosterol in hypercholesterolemics may identify those individuals, and their relatives, as being at increased risk for premature CHD (Glueck et al., 1991). Intervention for these individuals may also include reduced sources of phytosterol enriched foods.

6.3 Modification of plasma phytosterol level through variation in phytosterol intake

Reports in the literature regarding the effect of phytosterol intake on plasma phytosterol levels have been inconsistent. Tilvis and Miettinen (1986) reported that serum phytosterol levels appear to be independent of the amount of cholesterol and phytosterol in the diet. This report was based on a lack of correlation between serum phytosterols and fecal phytosterols in 17 subjects from 2 families with hypertriglyceridemia and on an assumption that on a normal diet, fecal phytosterols can be used to measure dietary phytosterol intake. Salen et al. (1970), showed that in order for healthy men to increase plasma β-sitosterol level twofold, more than eight fold

increase in dietary phytosterol intake was required. Many recent studies in humans, however, have shown that levels of phytosterols commonly ingested for the purpose of positively modifying plasma cholesterol levels do influence plasma phytosterol levels in a manner dependent on dietary phytosterol composition. Furthermore, individual plasma phytosterols are differentially influenced by dietary phytosterol intake (Table 3). Doses of 0.63 mg (Vanhanen and Miettinen, 1992), less than 1 g (Miettinen and Vanhanen, 1994), 6 g (Becker et al., 1992; Becker et al., 1993) and 12 g (Schlierf et al., 1978) of β-sitosterol increased plasma β-sitosterol level by approximately 40%, 40%, 22%, 13% and 68%, respectively, while consistently decreasing plasma campesterol level by approximately 17%, 23%, 23% and 27%, respectively (no value available for (Schlierf et al., 1978)). When sitostanol was orally administered, both plasma campesterol and ß-sitosterol levels were consistently and significantly decreased (Vanhanen and Miettinen, 1992; Becker et al., 1993; Vanhanen et al., 1993; 1994; Gylling and Miettinen, 1994; Miettinen and Vanhanen, 1994; Miettinen et al., 1995), while plasma sitostanol was virtually nondetectable (Heinemann et al., 1986; Vanhanen et al., 1993). Reduction in plasma campesterol and ß-sitosterol levels due to sitostanol ingestion are greater in apo E4 versus apo E3 alleles (Vanhanen et al., 1993). Vanhanen and Miettinen (1992) compared the ratio of campesterol to sitostanol in control and treatment diets and in pre-treatment and post-treatment plasma of hypercholesterolemic men and women. Individuals consuming a high rapeseed oil control diet (40% campesterol, 56% ß-sitosterol) increased plasma campesterol and ß-sitosterol by 65% and 17%, respectively, despite the fact that relative to normal intakes, ß-sitosterol intakes increased 30 times less than did campesterol intakes on this diet.

6.4 Modification of plasma phytosterol level and cholesterol absorption through variation in phytosterol intake

The intestinal absorbability of cholesterol and different phytosterols were compared in 10 healthy males using an intestinal perfusion technique (Heinemann et al., 1993). Cholesterol, campesterol, campestanol, stigmasterol, and ß-sitosterol were absorbed in percentages of 31, 10, 13, 5, and 4%, respectively (Heinemann et al., 1993). The effect of phytosterol administration on cholesterol absorption is described in Table 4. Also, using an intestinal perfusion technique, 9 healthy males received infusions of either ß-sitosterol or sitostanol (3.6 µmol/ml) into the proximal lumen. The ß-sitosterol infusion reduced cholesterol absorption from 32% to 16%, while the sitostanol infusion reduced cholesterol absorption from 29% to 5%. As sitostanol is barely absorbed, its inhibition of absorption of other plant sterols must occur at the level of micelle formation or incorporation into mucosal membrane rather than at the level of intestinal esterification (Vanhanen and Miettinen, 1992). Although phytosterol doses of at least 1 g per day have been recommended to decrease plasma cholesterol level, phytosterol doses smaller than 1 g have been shown to significantly reduce absorption of cholesterol and other sterols (Vanhanen and Miettinen, 1992). Greater inhibition of cholesterol absorption has been noted with apo E4 versus apo E3 alleles (Miettinen and Vanhanen, 1994).

6.5 Modification of apolipoprotein concentrations by phytosterol intake

Although no change in apolipoprotein A1 occurred (Becker et al., 1992; 1993), apolipoprotein B concentrations were found to respond slightly with dietary intervention (-6%) and significantly with ß-sitosterol administration after 3 months (-12%) and

sitostanol administration after 3 (-16%) and 7 (-23%) months (Becker et al., 1992). Similarly, Becker et al. (1993) found that apo B concentration changed with diet (-11%), and ß-sitosterol treatment (-12%).

6.6 Modification of fecal sterol excretion by dietary phytosterols

Becker et al. (1992) reported an increase in excretion of both neutral and acid sterols due to phytosterol feeding, suggesting that reduced absorption of both dietary and endogenous cholesterol must have occurred (Table 5). Furthermore, Becker et al. (1992) reported an increase in excretion of bile acids and speculated that this might be due to increased bile acid production resulting from either increased cholesterol synthesis or decreased plasma ß-sitosterol level. ß-sitosterol has been shown to inhibit 7 α-hydroxylase, the rate limiting enzyme in bile acid synthesis (Shefer et al., 1988).

7. Toxicity

In view of the potential therapeutic usage of phytosterols, it is imperative to determine any toxic side-effects that these compounds may exert. Oral administration of phytosterols in humans for the purpose of lowering plasma cholesterol levels has been associated with no obvious side-effect of any kind as indicated by subject perception (Farquhar et al., 1956; Heinemann et al., 1986; Miettinen et al., 1995) and physician examination (Becker et al., 1992; 1993). Furthermore, blood parameters remained within normal ranges (Becker et al., 1992; 1993). Lees et al. (1977) reported that subjects consuming up to 18 g per day of soy or tall oil-derived phytosterols for up to 3 years remained virtually free of side-effects, although a few individuals described constipation

upon questioning by a physician. Even chronic administration of very high doses of β-sitosterol subcutaneously in the rat were well tolerated with no indication of gross or microscopic lesions in the liver or kidney (Malini and Vanithakumari, 1990). However, a number of studies investigating the phytoestrogenic effect of β-sitosterol when given at very high doses (> 5 mg/kg body weight) subcutaneously or intravenously have shown an effect on testicular weight and sperm concentration in rats (Malini and Vanithakumari, 1991), irregularity in spermakinetics in immature rabbits (Ghannudi et al., 1978) and altered basal luteinizing hormone secretion in immature male and female rabbits (Register et al., 1995). Overall, oral administration of phytosterols in humans appears to be virtually free of negative side-effects. Only when phytosterols are administered at very high doses subcutaneously do negative side-effects become apparent.

8. Conclusion

Consumption of the phytosterol sitostanol reduces circulating cholesterol levels in humans when both phytosterol and cholesterol intakes are above a threshold concentration. Phytosterol absorption is limited, although serum phytosterol levels have been proven to be important indicators of both cholesterol absorption and synthesis. The absorption of sitostanol is essentially non-existent, and dietary sitostanol reduces the absorption of other phytosterols. Serum phytosterols correlate with HDL cholesterol level. Also higher phytosterol/cholesterol ratios appear in HDL versus LDL particles, suggesting the potential existence of an intrinsic phytosterol action, in addition to the extrinsic effect on cholesterol absorption. In conclusion, addition to diet of the phytosterol sitostanol, represents an effective means of improving circulating lipid profiles to reduce risk of coronary vascular disease.

Table 1. Percent change in plasma cholesterol and triglyceride levels due to phytosterol consumption.

Reference	Subjects	Pre-treatment diet (time)	Oral treatment dose	Treatment design/ (duration)	Total cholesterol (time)	LDL cholesterol (time)	HDL cholesterol TG (time)
Becker et al., 1993	9 children with heterozygous FH (10-14 years old)	intervention to ↓ plasma lipids (3 months)	6 g/day ß-sitosterol pastil	3 months	↓17.1%	↓19.5%	no change
			1.5 g/day sitostanol	7 months (post ß-sitosterol treatment)	↓25.7% (3 months) ↓23.5% (7 months)	↓33.2% (3 months) ↓29.2% (7 months)	HDL ↓12% TG ↑ (7 months)
Becker et al., 1992	7 children with heterozygous FH (5-10 years old)	intervention to ↓ plasma lipids (3 months)	6 g/day ß-sitosterol pastil	3 months	↓17%	↓17%	no change
Denke, 1995	33 polygenic hypercholesterolemic (M)	Step I Diet (< 30% fat, <0.20 g/day cholesterol) (3 months)	3 g/day sitostanol safflower oil dissolved - capsule	3 months	↓0.5%	↓2%	HDL ↑5% TG no change

Farquhar et al., 1956	15 men with myocardial infarction (some ↑ serum cholesterol)	some < 28% fat, 0.2-0.3 g/day cholesterol, some 40-48% fat, 0.9-1 g/day cholesterol (6-12 weeks)	12-18 g/day ß-sitosterol	non randomized, ß-sitostanol (12-24 weeks) placebo (10-16 weeks)	↓17%		
Gylling and Miettinen, 1994	11 hypercholesterolemic NIDDM (M)	none	3 g/d sitostanol *trans*-esterified with rapeseed oil and dissolved in margarine	randomized crossover, 30 g rapeseed oil margarine with or without sitostanol (6 weeks)	↓6%	↓9%	HDL ↓11% TG no change
Heinemann et al., 1986	6 hypercholesterolemic (M and F)	35% fat, < 0.30 g/day cholesterol (2 months)	1.5 g/day sitostanol sunflower oil dissolved - capsule	4 weeks	↓10% (3 weeks) ↓15% (4 weeks)	↓11% (3 wk) ↓15% (4 wk)	no change

Lees et al., 1977 Study 4b	18 type II hyper-cholesterolemic (M and F, adults and children)	↓ cholesterol ↓ SFA	6 g/day tall oil phytosterol suspension	average of 5 months	↓7% (↓5% in 11 adults and ↓ 10% in 5 children)		TG ↓9%
Lees et al., 1977 Study 1	12 type II hypercholesterolemic adults (M and F)	↓ cholesterol ↓ SFA	18 g/day soybean phytosterol (20% sterol suspension)	average of 10 months (1 for 3 years)	↓12%	↓17%	TG ↓6%
Lees et al., 1977 Study 3	9 type II hypercholesterolemic adults (M)	↓ cholesterol ↓ SFA	3 g/day tall oil phytosterol (5% campesterol powder)	average of 7 months	↓12%		TG ↓1%
Lees et al., 1977 Study 4a	31 type II hypercholesterolemic (M and F) (adult and child)	↓ cholesterol ↓ SFA	3 g/day tall oil phytosterol suspension	average of 6 months	↓7% (↓9% in 17 adults, ↓7% in 8 teens, ↓3% in 6 children)		TG ↓12%
Lees et al., 1977 Study 2	6 type II hyper-cholesterolemic adults (M)	↓ cholesterol ↓ SFA	18 g/day soybean phytosterol (powder form)	average of 10 months	↓12%		TG ↓14%

Miettinen et al., 1995	153 mildly hypercholesterolemic (M and F)	19-20 g/day rapeseed oil margarine fat substitution (6 weeks)	2.6 g sitostanol *trans*-esterified	randomized to diet or 2.6 g treatment for 1 year (n reduced after 6 months)	↓7.4% (6 months) ↓10.2% (1 year)	↓10.4% (6 month) ↓14.1% (1 yr)	no change
Miettinen and Vanhanen., 1994	31 hypercholesterolemic (M and F)	50 g rapeseed oil mayonnaise fat substitution - fat ~ 40%, cholesterol~ 0.30 g/day (6 weeks)	0.70-0.8 g/day dissolved in mayonnaise of either ß-sitosterol, sitostanol or sitostanol trans-esterified with rapeseed oil fatty acid	randomized diet or 1 of 3 treatments for 9 weeks	↓4.2 (phytosterol treatments pooled)	↓5.5 (phytosterol treatments pooled)	
Pelletier et al., 1995	12 healthy (M)	36% fat, 0.4-0.45 g/day cholesterol (1 week) (controlled feeding)	0.74 g/day soybean phytosterols mixed in butter	randomized crossover diet or treatment for 4 weeks	↓10%	↓15%	HDL ↓5%

Schlierf et al., 1978	12 familial type II hypercholesterolemic children (8-20 years old)	none	12 g/day ß-sitosterol granulate	randomized crossover, ß-sitosterol or placebo for 3 months	↓6%	↓7%	HDL ↓15% TG ↓23%
Vanhanen and Miettinen, 1992	24 hypercholesterolemic (M and F)	50 g rapeseed oil mayonnaise fat substitute (6 weeks)	0.63 g/day ß-sitosterol dissolved in rapeseed oil	randomized diet, ß-sitosterol or sitostanol for 9 weeks	↓5%		
			0.63 g/day sitostanol dissolved in rapeseed oil		↓1%		
Vanhanen et al., 1994	15 mildly hypercholesterolemic (M and F)	50 g rapeseed oil mayonnaise fat substitution (6 weeks)	0.8 g/day sitostanol *trans*-esterified	randomized diet (15 weeks) or 0.8 g sitostanol (9 weeks) followed by 2 g sitostanol (6 weeks)	slight ↓	slight ↓	no change
			2 g/day sitostanol *trans*-esterified		↓4.1% (from low dose), ↓9.3% (from diet)	↓10.3% (from low dose), ↓15.2% (from diet)	no change

Vanhanen et al., 1993	67 hypercholesterolemic (M and F)	50 g rapeseed oil mayonnaise fat substitute, 37% fat, 0.27 g/day cholesterol (4 weeks)	3.4 g/day sitostanol *trans*-esterified in rapeseed oil	randomized diet or treatment (6 weeks)	17.5% (from pre-treatment diet), 16.5% (from diet group)	110% (from pre-treatment diet), 19% (from diet group)	no change

* unless otherwise stated, pretreatment and/or control diets were also consumed during treatment phases.

M - male; F - female; NIDDM - non insulin dependent diabetes mellitus, FH - familial hypercholesterolemia, LDL - low density lipoprotein, HDL - high density lipoprotein, TG - triglycerides, SFA -saturated fatty acid

Table 2. Typical plasma plant sterol levels in humans.

Reference	Subject	Campesterol	ß-sitosterol
Glueck et al., 1991	595 hypercholesterolemics (some non-fasting)	2.10 µg/ml	2.98 µg/ml
Kempen et al., 1991	160 healthy Dutch families	Adults: E2/3, 11; E3/3, 12; E3/4, 14 µmol/l (according to Apo E phenotype) Children: E2/3, 10; E3/3, 12; E3/4, 14 (µmol/l (according to Apo E phenotype)	Adults: E2/3, 6; E3/3, 7; E3/4, 8 µmol/l (according to Apo E phenotype) Children: E2/3, 6; E3/3, 7; E3/4, 8 (µmol/l (according to Apo E phenotype)
Miettinen et al., 1995	69 patients with primary biliary cirrhosis	376 x 10^2 mmol/mol cholesterol	286 x 10^2 mmol/mol cholesterol
Miettinen et al., 1990	63 randomly selected healthy male Fins over 50 years old	9 µmol/l (range 3.5-21.4 µmol/L)	6 µmol/l (range 1.6-12.7 µmol/L)
Sutherland et al., 1991	male distance runners	1.82 mmol/mol cholesterol	2.21 mmol/mol cholesterol
	sedentary age matched controls	1.28 mmol/mol cholesterol	1.49 mmol/mol cholesterol
Tilvis and Miettinen, 1986	17 males and females from two families with type IV familial hyperlipidemia	(µmol/l) men women free 1.8 2.9 ester 5.3 7.6 total 7.1 10.5	(µmol/l) men women free 1.4 1.9 ester 2.9 4.1 total 4.3 6.0

Table 3. Percent change in plasma level of campesterol and ß-sitosterol due to phytosterol consumption.

Reference	Subject	Pretreatment diet (time)	Treatment dose	Treatment design/ duration	Plasma campesterol (% change)	Plasma ß-sitosterol (% change)
Becker et al., 1993	9 children with heterozygous FH (10-14 years)	intervention to ↓ plasma lipids (3 months)	6 g ß-sitosterol	3 months	↓ 27%	↓ 13%
			1.5 g sitostanol	7 months post ß-sitosterol treatment	↓ 49% (3 months) ↓ 51% (7 months)	↓ 50% (3 months) ↓ 53% (7 months)
Becker et al., 1992	7 children with heterozygous FH (5-10 years)	intervention to ↓ plasma lipids (3 months)	6 g ß-sitosterol	3 months	↓ 23%	↓ 22%
Gylling and Miettinen, 1994	11 hypercholesterolemic NIDDM (M)	none	3 g sitostanol ester	randomized crossover 30 g rapeseed oil margarine with or without sitostanol	↓ 44%	↓ 43%
Miettinen and Vanhanen, 1994	31 hypercholesterolemic (M and F)	50 g rapeseed oil mayonnaise fat substitute, fat ~ 40%, cholesterol ~ 0.3 g/d (6 weeks)	0.7 g/day ß-sitosterol 0.7 g/day sitostanol 0.8 g/day sitostanol-ester	diet or 1 of 3 treatments for 9 weeks	↓ 23% ↓ 23% ↓ 23%	↓ 40% ↓ 20% ↓ 30%

Miettinen et al., 1995	153 mildly hypercholesterolemic (M and F)	19-20 g rapeseed oil margarine fat substitute (6 weeks)	2.6 g sitostanol	randomized to diet or 2.6 g treatment for 6 months	↓ 36%	
Schlierf et al., 1978	12 familial type II hypercholesterolemic children (8-20 years)	none	12 g ß-sitosterol	randomized cross- over, ß-sitosterol or placebo for 3 months		↓ 68%
Vanhanen et al., 1994	15 mildly hypercholesterolemic (M and F)	50 g rapeseed oil mayonnaise fat substitute (6 weeks)	0.8 g sitostanol	randomized to diet (15 weeks) or 0.8 g sitostanol (9 weeks)	↓ 28.2%	↓ 23.4%
			2 g sitostanol	followed by 2 g (6 weeks)	↓ 30% (from low dose), ↓ 48% (from diet)	↓ 25.6% (from low dose), ↓ 41% (from diet)
Vanhanen et al., 1993	67 hypercholesterolemic (M and F)	50 g rapeseed oil mayonnaise fat substitute, 37% fat, 0.27 g/day cholesterol (4 weeks)	3.4 g sitostanol	randomized diet or treatment for 6 weeks	↓ 47.2%	↓ 31.4%
Vanhanen and Miettinen, 1992	24 hypercholesterolemic (M and F)	50 g rapeseed oil mayonnaise fat substitute (6 weeks)	0.625 g ß-sitosterol	randomized to diet, ß-sitosterol or sitostanol for 9 weeks	↓ 17%	↑ 41%
			0.630 g sitostanol		↓ 21	↓ 19%

NIDDM - non insulin dependent diabetes mellitus, FH - familial hypercholesterolemia, NEJM - New England Journal of Medicine.

Table 4. Change in percent absorption of cholesterol due to phytosterol consumption.

Reference	Subjects	Treatment	Study design	Cholesterol absorption (pre vs post treatment)	Measurement
Gylling and Miettinen, 1994	11 hypercholesterolemic NIDDM (M)	3 g sitostanol	randomized crossover, 30 g rapeseed oil margarine with or without sitostanol	25% vs 9%	labelled cholesterol and ß-sitosterol measured in 3 day stool collection
Heinemann et al., 1988	10 healthy males	90 mg/hr ß-sitosterol	continuous infusion of 36% fat diet (1 ml/kg/hr)	34% vs 17%	intestinal sample collection (intubated triple lumen tube)
		90 mg/hr sitostanol	20 mg/hr ß-sitosterol (stopped for 4 hr) randomized to either ß-sitosterol or sitostanol treatment for 5 hr	31% vs 6%	GC analysis

Mattson et al., 1977	9 adults no intestinal, hepatic or other clinical disorders that might alter cholesterol absorption	1 g ß-sitosterol (free sterol dispersed in food system) 2 g ß-sitosteryl oleate dissolved with diet fat (same amount of ß-sitosterol)	1 month apart feed one of three meals containing diet alone (492 mg cholesterol, 15 g fat) or treatments	46% vs 27% 46% vs 35%	labelled cholesterol and ß-sitosterol measured in 5 day stool collection
Miettinen and Vanhanen, 1994	31 hypercholesterolemic (M and F)	0.7 g/day ß-sitosterol 0.7 g/day sitostanol 0.8 g/day sitostanol ester	diet or 1 of 3 treatments for 9 weeks	25% vs 18% 25% vs 24% 29% vs 24%	labelled cholesterol and ß-sitosterol measured in 3 day stool collection
Vanhanen et al., 1994	15 mildly hypercholesterolemic (M and F)	0.8 g sitostanol	randomized diet for 15 weeks or 0.8 g sitostanol for 9 weeks	29% vs 23%	labelled cholesterol and ß-sitosterol measured in 3 day stool collection

NIDDM - non insulin dependent diabetes mellitus, M - male; F - female.

Table 5. Change in fecal excretion due to phytosterol consumption.

Reference	Subjects	Treatment	Neutral sterol excretion	Acidic sterol excretion
Becker et al., 1993	9 children with heterozygous FH (10-14 years)	6 g/day ß-sitostanol	↑ 88%	↑ 31%
		1.5 g/day ß-sitosterol	↑ 45%	↑ 16%
Gylling and Miettinen, 1994	11 hypercholesterolemic NIDDM (M)	3 g sitostanol ester	↑ 39%	↓ 2%
Miettinen and Vanhenen, 1994	31 hypercholesterolemic (M and F)	0.7g/day ß-sitosterol	↑ 16%	↑ 7%
		0.7 g/day sitostanol	↑ 23%	↑ 1%
		0.8 g/day ß-sitosterol ester	↑ 18%	↓ 12%
Vanhanen et al., 1994	15 mildly hypercholesterolemic (M and F)	0.8 g sitostanol	↑ 16.7%	↓ 9%

M - male; F - female; FH - familial hypercholesterolemia, NIDDM - non insulin dependent diabetes mellitus.

2.4 Phytosterol absorption

Despite the fact that great similarity exists between cholesterol and the majority of phytosterols, absorption of plant sterols by the mammalian intestine is much lower than cholesterol (Child and Kuksis, 1983b). Approximately 250 mg of phytosterols are ingested daily by an average human with 65% as ß-sitosterol, 30% as campesterol and 5% as stigmasterol (Weihrauch et al., 1978; Morton et al., 1995). Gould (1955) showed that ß-sitosterol is absorbed at 10% of the absorption rate of cholesterol in humans. Conversely, it has been suggested that ß-sitosterol absorption, measured using the balance method (absorption = difference between amount fed and amount recovered in feces), was about 22-32% (Ivy et al., 1955; Roth and Favarger, 1955). The later reported levels of ß-sitosterol absorption are questionable because of possible sterol breakdown in the intestine by the microflora. In another study in rats, phytosterol absorption rate was 5%; this value being independent of the amount of total sterols fed (Sylven and Borgstrom, 1969). Furthermore, Salen et al. (1970) have demonstrated that ß-sitosterol are absorbed at a rate of 5% in hypercholesterolemic human subjects. Data available in the literature consistently show that less than 5% of total phytosterols are absorbed in the human gut while 40 to 60% of dietary cholesterol is absorbed (Gould, 1955; Salen et al., 1970)

Several mechanisms were suggested to explain the dissimilarity observed between cholesterol and phytosterol absorption in animals and humans. For instance, Chijiiwa (1987) has shown that intermicellar concentration of ß-sitosterol is 1/100th of that of cholesterol in cholate micelles. He postulated that this significant difference in intermicellar concentrations of sterols might explain why cholesterol is more absorbed than phytosterols. On the other hand, Borgstrom (1967) has reported that the partition coefficient of cholesterol between

micellar and oil phases of intestinal content was similar to that of ß-sitosterol. Similarly, Tanaka et al. (1993) failed to demonstrate any difference between cholesterol and ß-sitosterol affinity to bile salt micelles. There was no differential influence of taurine or glycine conjugation on sterol absorption (Tanaka et al., 1993). These investigators suggested that the difference in absorption between cholesterol and ß-sitosterol in rats cannot be explained by their uptake rate into the micelle (Tanaka et al., 1993).

Moreover, the discrepancy in cholesterol and phytosterol absorption is believed to be due to the relative molecular weights of sterols. Kuksis and Huang (1962) examined the absorption rate of the various phytosterol subspecies in dogs. They showed that feeding ß-sitosterol and campesterol at a ratio of 60:40 increases the lymph content of campesterol over ß-sitosterol in dogs. Child and Kuksis (1983b) demonstrated that the uptake of 7-dehydrocholesterol by jejunal, brush border and red blood cells in rats decreases as more carbon atoms are added at C_{24} of the sterol skeleton. Similarly, Subbiah et al. (1970; 1971) observed higher campesterol absorption in pigeons as compared to ß-sitosterol. Conversely, sitostanol (24-ethyl-5α-cholestan-3ß-ol), a hydrogenated derivative of ß-sitosterol, is not absorbed by the gut in animals (Sugano et al., 1977) and humans (Heinemann et al., 1988; 1993).

In summary, despite the great similarity in the chemical structure between cholesterol and plant sterols, dietary phytosterols are poorly absorbed and they lower circulating cholesterol concentrations in animals and humans. Still, the mechanisms that mediate phytosterols' effects on cholesterol levels are not well understood.

2.5 Mechanisms of cholesterol reduction by phytosterols

Different hypotheses have emerged regarding the mechanisms involved in the hypocholesterolemic effect of phytosterols. Plant sterols are believed to form nonabsorbable micelles with cholesterol in the intestine, reduce micellar solubility of cholesterol, interfere with cholesterol uptake and esterification rate or alter the activities of some rate-limiting enzymes of cholesterol within the synthesis system. The proposed extrinsic effects of phytosterols are better documented than the intrinsic factors. Certainly, the low absorption of phytosterols and their virtual absence from tissues increases the likelihood that external mechanisms are involved in lowering circulating lipid levels.

Armstrong and Carey (1987) reported that phytosterols displace cholesterol and prevent it from binding to sodium taurocholate micelles with a favorable free energy difference of -0.6 Kcal/mol. Moreover, micellar solubilization of cholesterol was restricted by the addition of ß-sitosterol (Ikeda and Sugano, 1983) suggesting that the inhibitory effect of ß-sitosterol is on micellar cholesterol incorporation rather than on the brush border membranes. On the other hand, brush border membrane has been demonstrated to discriminate between cholesterol and ß-sitosterol absorption which results in lower cholesterol absorption (Ikeda et al., 1988b; Field and Mathur, 1983). The reasons proposed for this discrimination between cholesterol and ß-sitosterol absorption were that mucosal esterification enzymes (cholesterol esterase) (Ikeda et al., 1988b) and ACAT (Field and Mathur, 1983) worked less efficiently on ß-sitosterol as compared to cholesterol. Field and Mathur (1983) demonstrated that ß-sitosterol is a poor substrate for intestinal ACAT and that esterification rate for cholesterol was 60 fold greater than that of phytosterol. ß-sitosterol by competing with cholesterol to reach the brush border membrane inhibits cholesterol absorption (Field and Mathur, 1983). It has been also

shown that infusing ß-sitosterol in rats leads to a reduction in 7 α-hydroxylation of endogenous microsomal cholesterol (Boberg et al., 1989). In addition, phytosterols have been reported to alter plasma lecithin-cholesterol acyl transferase (LCAT) activity in humans (Weisweiler et al., 1984) or affect HMG-CoA activity in animals (Gerson et al., 1964). Kakis and Kuksis (1984), however, failed to demonstrate any inhibition of intravenously injected phytosterols on hepatic cholesterogenesis in male Wistar rats. The activation of hepatic cholesterogenesis was inhibited by the intravenous infusion of cholesterol in a 1:1 molar ratio with Intralipid rather than by phytosterols. This study implies that the feedback system of cholesterogenesis is sensitive to cholesterol molecules but not to cholesterol with methyl or ethyl groups at the C_{24} position such as campesterol, ß-sitosterol or sitostanol.

Another possible mechanism by which phytosterols may inhibit cholesterol absorption is by altering bile acid production in the liver. Bhattachryya and Eggen (1984) have shown in Rhesus monkeys that total fecal acidic steroid excretion was 113% higher with low versus high cholesterol diets during increased dietary intake of mixed plant sterols. They suggested that their observation might explain the action of intravenously injected phytosterols in animals. Finally, extrinsic and intrinsic effects of phytosterols have been reported. Despite this knowledge, the real mechanisms of action by which phytosterols lower plasma cholesterol levels are still not well characterized. Further investigations are warranted to determine these mechanisms and to test the effects of variable phytosterol concentrations on plasma lipid profile in animals and humans.

CHAPTER 3

GENDER EFFECTS OF TALL OIL VERSUS SOYBEAN PHYTOSTEROLS AS CHOLESTEROL LOWERING AGENTS IN HAMSTERS

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3.1 Abstract

To examine the effect of gender on the mechanisms of action of phytosterols extracted from tall oil (TO) and soybean (SB) on cholesterol and phytosterol metabolism, male and female hamsters were fed atherogenic diets containing 0.5 or 1% (w/w) TO- or SB-phytosterols for 90 days. Plasma lipoprotein cholesterol profile and tissue phytosterol and cholesterol biosynthesis levels were determined. Mean plasma total-cholesterol level in females fed 1% (w/w) SB was reduced ($p < 0.05$) by 44% while in males it was lowered ($p < 0.05$) by 25% compared to their respective controls. Conversely, while mean plasma total-cholesterol levels were reduced ($p < 0.05$) in male (-31%) and female hamsters (-32%) fed 1% (w/w) TO, no gender effect was observed. Cholesterol biosynthesis was higher ($p < 0.05$) by two fold in groups fed TO at 0.5 and 1% (w/w) concentrations, compared to SB. Hamsters fed TO at 0.5 and 1% (w/w) concentrations also had higher ($p < 0.05$) hepatic and enterocytic campesterol contents than SB-fed animals. These findings demonstrate gender differences in cholesterol metabolism in TO- and SB-fed hamsters. The results suggest that TO, conversely to SB-phytosterol, is an effective cholesterol lowering agent in male, but not in female hamsters, over a feeding period of 90 days.

3.2 Introduction

Phytosterols, found as mixtures of ß-sitosterol, campesterol, stigmasterol and other plant sterols, occur naturally as constituents of plant oils and differ structurally from cholesterol in ring or side chain configuration. The saturated derivatives of phytosterols such as sitostanol are found in negligible concentrations (Weihrauch and Gardner, 1978; Morton et al., 1995) and hence are almost absent from typical Western diets. Phytosterol mixtures extracted from tall oil (TO) contain about 20% (w/w) sitostanol while those extracted from soybean (SB) exhibit negligible sitostanol concentration (Ling and Jones, 1995b). Intestinal absorption of phytosterols is about 12 times less than that of cholesterol (Grundy et al., 1969) while sitostanol which is non-absorbable (Heinemann et al., 1993) is not found in tissues.

Dietary intake of phytosterols and phytostanols has been shown to reduce plasma total (Farquhar et al., 1956; Lees et al., 1977; Gylling et al., 1995) and low density lipoprotein cholesterol concentrations (Becker et al., 1993; Miettinen and Vanhanen, 1994; Pelletier et al., 1995) in humans and animals (Sugano et al., 1976; Ikeda et al., 1979). Several prospective clinical trials have confirmed that lowering plasma lipid levels significantly reduces the death rate from coronary vascular and related diseases (Anonymous, 1994; 1996; Sacks et al., 1995). A difference of 0.6 mmol/L (about 10%) in total-cholesterol concentrations corresponds to a difference in mortality from ischemic heart disease of 17% (Grundy, 1990; Law et al., 1994a; 1994b) and in middle aged men, the difference may reach 25-30% (Muldoon et al., 1990).

However, the mechanisms through wich phytosterols lower circulating cholesterol concentrations are not well-characterized. It has been suggested that phytosterols reduce plasma cholesterol levels by mainly inhibiting cholesterol absorption from the intestinal lumen (Shidoji et al., 1980; Ikeda and Sugano, 1983; Heinemann et al., 1988; Miettinen and

Vanhanen, 1994). Nonetheless, the effect of phytosterols on cholesterogenesis remains controversial, as studies have shown that ß-sitosterol lowers plasma cholesterol levels while simultaneously either stimulating (Gerson et al., 1964; Konlande and Fisher, 1969) or inhibiting (Kakis and Kuksis, 1984) cholesterol synthesis.

At present, whether the cholesterol-lowering ability and mechanisms of action of TO-phytosterols compared with phytosterol mixtures from SB are similar in both sexes has not been well-characterized. Although some studies suggest no influence of gender in the effect of phytosterol/stanol consumption on plasma cholesterol levels (Vanhanen et al., 1993; 1994; Miettinen and Vanhanen, 1994), other reports have shown that plasma phytosterol levels differ between males and females (Tilvis and Miettinen, 1986; Kempen et al., 1991).

Thus, the objective of this study was to examine the influence of phytosterol/stanol composition, dose and gender on cholesterol metabolism in the hamster model. Specific aspects of cholesterol metabolism examined were circulating lipoprotein concentrations, cholesterol tissue levels and cholesterogenesis.

3.3 Materials and methods

3.3.1 Animals and diets

One hundred and twenty outbred Golden Syrian hamsters (60 males; 60 females; 80-100 g, Charles River Laboratories, Montréal, Canada) were individually housed in stainless steel mesh cages at 20-22°C with an alternating light-dark period of 12 hr (lights on 05:00 to 17:00 hr). Animals were given free access to plain rodent chow (Charles River Laboratories, Montréal, Canada) and water for 3 days. Following this acclimatization, hamsters were randomized into groups and fed a basal semi-purified diet (ICN Pharmaceuticals, Inc., Aurora,

Ohio) (Table 1) supplemented with cholesterol and phytosterols extracted from either TO (Forbes Medi-Tech, Vancouver, British Columbia) or SB (Sigma Chemical Company, St. Louis, MO) using cholesterol: phytosterol ratios of 1:0, 1:2 or 1:4. Diets were prepared from primary ingredients each week. Phytosterols, free from any fatty acids, were dissolved in a mixture of oil at 60°C containing equal amounts of saturated, monounsaturated and polyunsaturated fatty acids derived from coconut, olive and sunflower oil respectively. The control group received the same oil mix without phytosterols. Table 2 shows cholesterol and phytosterol additions to the basal diet, as well as group size. Phytosterol/stanol composition of the diet was determined by gas liquid chromatography (GLC). Tall oil and SB phytosterols contained 64% and 65% (w/w) ß-sitosterol, respectively. Sitostanol was found at a concentration of 21% (w/w) in the TO- and in a negligible amount in the SB-extracted phytosterols (Table 3). During the trial, animals had free access to water and food. Hamsters were weighed weekly and food intake was monitored daily by weighing food cups after each 24-hr-feeding period.

Animals were intraperitoneally injected with 0.4 ml deuterium (D) oxide (99.8% atom percent excess) and then deprived of food and water two hr before sacrificing. Thereafter, hamsters were anaesthetized with halothane and killed by cardio-puncture. Blood samples were withdrawn and centrifuged to obtain plasma. Liver and small intestine samples were quickly removed, weighed, frozen in liquid nitrogen and stored at -80°C for later analysis.

3.3.2 Lipid analyses

Plasma total-cholesterol, high density lipoprotein- (HDL) cholesterol and triglyceride levels were measured in duplicate using a VP Autoanalyzer in conjunction with commercial

enzymatic kits (Abbott Laboratories, Montréal, Québec). Heparin-manganese was used to precipitate the apo-B containing lipoproteins. Results are expressed as non-apolipoprotein-A (LDL + IDL + VLDL) levels instead of low density lipoproteins (LDL) because the Friedwald et al. (1972) equation may not be applicable in hamsters. The concentration of non-apo-A cholesterol was calculated by subtraction of HDL-cholesterol concentration from that of plasma total-cholesterol. Non-apo-A lipoprotein-cholesterol was taken as that associated with very low and low density lipoproteins. Plasma lipid levels were determined in duplicate against authenticated standards.

3.3.3 Hepatic cholesterol synthesis determination

Cholesterol biosynthesis was determined as the rate of incorporation of D from body water into sterol over 2 hr. Labeled water equilibrates quickly with intracellular and extracellular body-water pools and permits direct determination of cholesterol formation rates (Jeske and Dietschy, 1980). Deuterium enrichment was measured in hepatic free cholesterol and plasma water. Hepatic lipid extraction was performed in duplicate. Chloroform-methanol (2:1 v/v) was added to extract and homogenized for 2 min. The mixtures were centrifuged after shaking and supernatants were collected. The extraction procedures were then repeated and solvent layers combined. Water, at 20% of total volume, was combined with the supernatant, the sample was shaken again and the upper solvent layer was removed after centrifugation at 1500 rev/min. The lower phase was dried with nitrogen and the residue chromatographed on silica gel plates (20 x 20 cm, 250 mm, Whatman Inc., Clifton, NJ, USA) that were developed in petroleum-ether ethyl-acetic acid (70:30:1 v/v/v) and the free-cholesterol band identified. The free-cholesterol band was scraped from the plate and extracted

three times by shaking the silica in chloroform for 15 min followed by centrifugation. Dried cholesterol samples were transferred to 18-cm combustion tubes (Vycor, Corning Glass Works, Corning, NY). Cupric oxide (0.5 g) and a 2-cm length of silver wire were added and tubes were sealed under vacuum at less than 20 mtorr pressure.

The cholesterol sample was combusted for 4 hr at 520 °C and the water generated vacuum-distilled into Vycor tubes containing 60 mg zinc reagent (Biogeochemical Laboratories Indiana University, Bloomington, IN). To measure D enrichment of body water, additional plasma samples were diluted 25 fold with water to reduce D enrichment to within the normal analytical range while baseline samples were not diluted. Next, duplicate samples were vacuum-distilled into zinc containing Vycor tubes. Finally, both cholesterol and plasma water samples were reduced to hydrogen gas at 520 °C for 30 min. The D enrichments of cholesterol and plasma samples were measured by differential isotope ratio mass spectrometry using a triple inlet system with electrical H^{3+} compensation (VG Micromass, Cheshire, UK).

Hepatic fractional synthetic rate (FSR) values were calculated as cholesterol D enrichment in relation to that of the precursor body water pool adjusted for the fraction of hydrogens of cholesterol originating from labeled substrate. The hepatic FSR values were derived using the equation:

$$\text{FSR (per day)} = \frac{\delta \text{ cholesterol } (‰) \times 24\text{hr}/2 \text{ hr measurement}}{\delta \text{ plasma water } (‰) \times 0.478}$$

where δ cholesterol is D enrichment in parts per thousand (‰) over the 2 hr interval period between injection and sacrifice and δ plasma is the mean plasma D enrichment. FSR values were normalized to 24 hr. The multiplication factor of 0.478 accounts for the fraction of D

atoms obtained from body water during cholesterogenesis. The FSR data were expressed as a percentage and compared to the value obtained in of groups fed basal diet with added cholesterol.

3.3.4 Phytosterol analyses

Phytosterols, extracted from food, hepatic and intestinal samples, were quantitated by GLC (Hewlett Packard, 5890 Series II) as reported previously (Ling and Jones, 1995b). Briefly, samples were homogenized and the lipids extracted by the method of Folch et al. (1957). The internal standard, 5-α-cholestane, (Sigma Chemical Company, St. Louis, MO) was added to diet and tissue homogenates, and then saponified with 0.5 M methanolic KOH for 18 hr at 80°C. Sterols, from the non-saponifiable materials, were then injected into an RTx-1, 30 m capillary column, 0.25 mm ID, 0.25 μm thickness (Restek Corp. Bellefonte, PA) and detected as described earlier (Hamilton et al., 1992; Ling and Jones, 1995b).

3.3.5 Data analysis and statistics

The comparisons of cholesterol levels in both sexes are expressed as a percent response relative to the respective cholesterol control group, assuming the cholesterol group response to be 100%. Data are expressed as means ± SEM. The data across and between treatment groups in the experiment were analyzed using one- and two-way analyses of variance (ANOVA). Where a significance of p less than 0.05 was achieved, specific group differences were evaluated using Tukey's post-hoc comparison.

3.4 Results

3.4.1 Body weight gain and food intake:

Dietary intakes of hamsters did not differ among groups over the 90-day study duration (Table 4). Although body weight gains varied from 35 to 55 g in females and 32 to 45 g in males, there were no statistical differences between and within groups (Table 4).

3.4.2 Plasma lipid analysis: effect of phytosterol dose, phytosterol composition and gender

Plasma total, HDL and non-apo-A cholesterol as well as triglyceride levels are shown for male and female hamsters in Figure 1 and 2, respectively. Males fed 1% (w/w) TO-phytosterols had 32% lower ($p < 0.05$) levels of total cholesterol levels compared to control. A decrease ($p < 0.05$), but of lesser extent (≈25%), was observed when 0.5 and 1% (w/w) SB-phytosterols were provided to males. Plasma HDL-cholesterol concentrations were decreased ($p < 0.05$) in TO- (1% (w/w)) and SB- (0.5 and 1% (w/w)) phytosterol-fed male hamsters. Levels of non-apo-A lipoprotein cholesterol did not differ across diets in males. Plasma triglyceride levels in males were lower ($p < 0.05$) in the 1% (w/w) SB fed hamsters compared to those fed the control diet.

In females, circulating total cholesterol concentrations were lower ($p < 0.05$) in groups fed TO and SB at 0.5 and 1% (w/w) phytosterol compared to control. However, contrary to the effect observed in males, the SB mixture at 1% (w/w) resulted in a stronger lowering impact than the TO mixture at 1% (w/w) (Figure 2). Soybean-phytosterols at 1% (w/w) reduced ($p < 0.05$) non-apoA cholesterol compared to control. In addition, TO- and SB-phytosterols significantly lowered ($p < 0.05$) plasma HDL concentrations compared with the control diet supplemented with 0.25% (w/w) cholesterol without phytosterols. However, plasma

triglyceride levels did not differ across groups.

Figure 3 shows plasma levels of lipid, expressed as a percentage of total lipids, of animals fed TO- and SB- phytosterols. For total cholesterol, at 1% (w/w) phytosterol supplementation, the pattern of change over 90 days in males was similar for TO- and SB-phytosterol mixtures. However, females demonstrated improved total-cholesterol lowering effect of ≈17% with SB- over TO-phytosterols. The percent change in HDL-cholesterol to addition of phytosterols relative to control was similar in females regardless of the phytosterol sources. Conversely, males showed lower percent HDL-cholesterol changes in the group fed 0.5% (w/w) SB- than TO-phytosterols (Figure 3). It is not clear why such variation in percent HDL-cholesterol at 0.5% (w/w) SB was observed in males. Percent change in plasma non-apo A levels were lower (30-40%, $p < 0.05$) in males fed 1% (w/w) TO-phytosterols than other groups. On the other hand, females fed 1% (w/w) SB-phytosterols exhibited lower (20-32%, $p < 0.05$) percent changes in circulating non-apo A concentrations than the groups fed TO- or 0.5% (w/w) SB-phytosterols.

3.4.3 Cholesterol biosynthesis

Since no effect of gender on cholesterol synthesis rates across groups studied was observed, values from male and female hamsters consuming the same type of phytosterols were grouped together. Fractional synthesis rates of cholesterol in groups receiving TO- or SB-phytosterols are compared in Figure 4. Cholesterol biosynthesis was greater ($p < 0.05$) by two fold in both sexes consuming TO at 0.5% and 1% (w/w) levels versus 0.5% and 1% (w/w) SB.

3.4.4 Tissue phytosterol analysis.

Intestinal and hepatic phytosterol levels are presented in Table 5. Hepatic campesterol and sitosterol concentrations did not differ across groups in male and female hamsters. However, campesterol levels in enterocytes were higher ($p < 0.05$) in males and females fed 0.5% and 1% (w/w) TO compared to the other groups. Both females and males fed 1% (w/w) TO had elevated ($p < 0.05$) levels of sitosterol in their enterocytes compared to control animals.

3.5 Discussion

The current study evaluated the effects of TO- versus SB-phytosterol mixtures on plasma cholesterol levels and hepatic cholesterogenesis in male and female hamsters. Results demonstrated that dietary phytosterols given at 0.5 and 1% (w/w) exert an effect which differs between the genders in this species. Tall oil-phytosterols reduced plasma cholesterol levels more efficiently than those from SB- in males while SB-phytosterols were more effective in females. Although TO-phytosterols increased hepatic fractional synthetic rates of cholesterol in both sexes, SB-phytosterols did not alter their cholesterol synthesis.

Reports of gender effects on cholesterol and phytosterol metabolism in response to phytosterol administration have been inconsistent. For example, investigations in healthy Dutch families showed that although a gender effect was not observed in raw data, gender was a significant factor in the prediction of both plasma campesterol and ß-sitosterol levels in multivariate analysis (Kempen et al., 1991). In addition, Tilvis and Miettinen (1986) measured plasma phytosterol levels in 17 individuals from two families with hypertriglyceridemia and found that women have higher phytosterol levels than men. Conversely, Vanhanen et al. (1993) provided 67 hypercholesterolemic subjects with 3.4 g of sitostanol per day for 6 weeks

and observed no difference between plasma lipid response of males and females. Similar studies in 15 mildly- and 31 hyper-cholesterolemic subjects failed to identify differences due to gender (Vanhanen et al., 1994). However, those studies utilized one source of phytosterols, derived mostly from saturated TO and did not compare the effect of gender on the mechanisms of action of different sources of phytosterols on circulating lipid levels. In the present study, SB- showed stronger reducing effects on plasma total-cholesterol levels than TO-phytosterols in female but not in male hamsters. The probable reasons for not observing a similar reduction in plasma lipid levels in both groups are thus two fold. First, the reduction in plasma total-cholesterol concentrations in females may require a longer feeding period of SB- to exert similar effect to TO-phytosterols in males or vice versa in females. Second, a better internal compensation in cholesterol homeostasis may occur in male hamsters compared to females after a challenge with an atherogenic diets for 90 days.

Although not all mechanisms involved in the lowering effect of phytosterol on plasma cholesterol levels are well-understood, it has been suggested that phytosterols/stanols may reduce plasma cholesterol levels by intrinsic mechanisms (Malini and Vanithakumari, 1992; 1993) other than inhibiting cholesterol absorption. Since unsaturated phytosterols such as SB are more absorbable than saturated phytosterols (Gould et al., 1969; Vanhanen and Miettinen, 1992; Ling and Jones, 1995a) such as TO, a speculation of a hormonal-like action for ß-sitosterol might explain the gender discrepancy observed between TO- and SB-effects on plasma total-cholesterol levels in male and female hamsters. Sitostanol, found in TO-phytosterols, has been demonstrated to lower cholesterol and ß-sitosterol absorption simultaneously (Vanhanen et al., 1993). Although SB- and TO-phytosterol mixtures contain similar concentrations of ß-sitosterol, more ß-sitosterol from SB is most likely absorbed than

that from TO. Accordingly, Malini and Vanithakumari (1993) reported that ß-sitosterol acts as an estrogen-like agonist in rats. Both ß-sitosterol and estradiol increased the activities of carbohydrate metabolism *in uteri* of adult ovariectomised rats (Malini and Vanithakumari, 1992). In addition, ß-sitosterol increased uterine weight, RNA, DNA and protein concentrations in Wistar rats (Malini and Vanithakumari, 1993). However, further investigations addressing the metabolic and hormonal effects of atherogenic diets on females versus males and the action of phytosterols on sterol homeostasis in hamsters are needed.

The presently observed action of phytosterol mixtures on cholesterol biosynthesis is consistent with findings of work conducted in male rats where a compensatory increase in endogenous cholesterol synthesis was seen concurrent to a significant reduction in plasma total-cholesterol levels (Ling and Jones, 1995b). Although circulating lipoprotein patterns differ between rat and hamster models, cholesterol biosynthesis substantially increased with administration of TO-phytosterols in both species compared to control- and SB-fed groups. In most animals, a significant reduction in cholesterol levels in body compartments is accompanied by a raise in cholesterol synthesis, as seen in rats (Ling and Jones, 1995b) and now in hamsters. In this study, SB-phytosterols might act as hormonal agonists which de-suppress de novo hepatic cholesterol synthesis probably due to low availabiiity of free sterols or sterol-precursors. The increased hepatic FSR, measured using labeled water in TO-fed hamsters, can be explained by a possible reduction in cholesterol-feedback regulation by TO and hence a compensatory increase in cholesterol synthesis in the hepatocytes. The relatively lower plasma cholesterol levels in males fed TO- compared to SB-phytosterols might explain the latter suggestion. However, in females, circulating cholesterol concentrations were the lowest in the group fed SB-phytosterols. Thus, the discrepancy in cholesterol FSR between

TO- and SB-phytosterols is unclear. It might suggest that cholesterol FSR is independent of gender, but related to phytosterol sources and composition.

Another possible explanation for the difference in the influence of gender could be due to a potentially different intrinsic effect that might exist between TO- and SB-phytosterols. The detectable differences between TO and SB phytosterol compositions are in sitostanol and dihydrobrassicasterol (DHB) concentrations. Tall oil-phytosterols are rich in sitostanol which is not absorbed (Heinemann et al., 1988; 1991; Vanhanen and Miettinen, 1992) while SB-phytosterols are abundant in DHB. An intrinsic effect of absorbed phytosterols was suggested in several studies (Gerson et al., 1964; Konlande and Fisher, 1969; Malini and Vanithakumari, 1990). Intra-peritoneal and intravenous injections of high levels of ß-sitosterol decreased cholesterol-7-alpha-hydroxylase activity (Boberg et al., 1989; Shefer et al., 1994) which affects cholesterol homeostasis. We were unable to detect DHB in hepatic and enterocytic tissues. However, this does not exclude its potential existence in tissues at undetectable levels. To our knowledge, no data in the literature about DHB and its effects on cholesterol synthesis exist. Thus, further experimentation addressing the effect of DHB on cholesterol metabolism is needed.

Phytosterol levels in the enterocytes and hepatocytes exhibited large variability that resulted in no significance across groups. Several studies have reported similar variations in plasma phytosterol levels (Grundy et al., 1969; Tilvis and Miettinen, 1986; Miettinen et al., 1989) in human subjects but they were not well-explained. The possibility of procedural errors is excluded since the same methodology was applied to all samples analyzed. It is unclear if diurnal variation in cholesterol synthesis might affect phytosterol absorption or excretion rates in hamsters. Nevertheless, what was consistent across the groups is the campesterol level in

tissues. Interestingly, whereas ß-sitosterol was the major constituent in both TO- and SB-phytosterols, campesterol was present in greater concentration in liver and intestine. Such a discrepancy could be due to higher campesterol absorption compared with ß-sitosterol as has been shown by Kuksis and Huang (1962). The uptake of sterols by jejunal, brush border and red blood cells in animals decreases as more carbon atoms are added at C_{24} of sterol skeleton (Kuksis and Huang, 1962; Child and Kuksis, 1983). This discrepancy is likely due to the differential elimination rate of the two phytosterols from the liver. Since the hepatic esterification rate of campesterol is higher than that of ß-sitosterol, its elimination rate in the bile may have exceeded that of campesterol (Koivisto and Miettinen, 1988; Vanhanen and Miettinen, 1992).

In summary, the present results demonstrate enhanced efficacy of TO- versus SB-phytosterols as cholesterol-lowering agents in male hamsters while SB was more potent in female. Tall oil- increased FSR rates in both genders but SB-phytosterols, given at 0.5% and 1% (w/w), did not change hepatic cholesterol synthesis.

3.6 Figure legends

Figure 1.

Plasma total (TC), high density lipoprotein (HDL-C) and non-apolipoprotein-A cholesterol (N-apoA-C) and triglyceride (TG) levels in male hamsters fed semi-purified diets containing % (w/w) phytosterol mixtures from soybean and tall oil. Control group did not receive any phytosterol mixture. Values are means ± SEM. Values within each plasma lipid group carrying different letters are significantly different at p value < 0.05. TO: tall oil, SB: soybean, Chol: cholesterol.

Figure 2.

Plasma total (TC), high density lipoprotein (HDL-C) and non-apolipoprotein-A cholesterol (N-apoA-C) and triglyceride (TG) levels in female hamsters fed semi-purified diets containing % (w/w) phytosterol mixtures from soybean and tall oil. Control group did not receive any phytosterol mixture. Values are means ± SEM. Values within each plasma lipid group carrying different letters are significantly different at p value < 0.05. TO: tall oil, SB: soybean, Chol: cholesterol.

Figure 3

Percentage plasma total (TC), high density lipoprotein (HDL-C) and non-apoliporpotein-A cholesterol (N-apoA-C) levels in male and female hamsters receiving diet treatments for 90 days. % (w/w) phytosterol mixtures from soybean and tall oil. Data are expressed relative to the response of cholesterol group in the corresponding experiment. Values are means ± SEM.

Values within each gender group carrying different letters are significantly different at p value < 0.05. TO: tall oil, SB: soybean, Chol: cholesterol.

Figure 4

Effect of diet treatment on hepatic cholesterol fractional synthetic rate in both male and female hamsters. Control group did not receive any phytosterol mixture. Values are means ± SEM. Values carrying different letters are significantly different at p value < 0.05. % (w/w) phytosterol mixtures from soybean and tall oil. TO: tall oil, SB: soybean.

Table 1. Composition of experimental diets.

Ingredient	control %	soybean %	tall oil %
Casein	20.0	20.0	20.0
Corn starch	29.0	28.0	28.0
Sucrose	36.3	36.3	36.3
Oil mixture	5.0	5.0	5.0
DL-methionine	0.5	0.5	0.5
Mineral mixture	4.0	4.0	4.0
Vitamin mixture	1.0	1.0	1.0
Choline bitartrate	0.2	0.2	0.2
Cholesterol	0.25	0.25	0.25
Butyl hydroxy toluene	0.02% of oil	0.02% of oil	0.02% of oil
Cellulose	5.0	5.0	5.0
Phytosterols	0	0.5 or 1	0.5 or 1

The control diet contains <0.1% ß-sitosterol and campesterol.

The vitamins and salt mixture and vitamin-free casein were purchased from ICN Life Science Group, Nutritional Biochemicals Division, Cleveland, OH. Fat soluble vitamins were added to the oil mix which was a rich source of essential fatty acids.

Table 2. Dietary additions to basal diet.

	Groups	Sterol additions (% w/w) to basal diet		
		Cholesterol	**TO**	**SB**
60 females (n=12)	Cholesterol	0.25	0	0
60 males (n=12)	0.5% TO	0.25	0.5	0
	1.0% TO	0.25	1.0	0
	0.5% SB	0.25	0	0.5
	1.0% SB	0.25	0	1.0

TO: tall oil, SB: soybean, % w/w: percent of wet weight diet. The basal diet contained 0.025% w/w as cholesterol and <0.1% ß-sitosterol and campesterol.

Table 3. Percent composition of phytosterols in tall oil (TO) and soybean (SB).

Phytosterols	SB	TO
ß-sitosterol	65	64
Campesterol	20	16
Dihydrobrassicasterol	15	ND
Sitostanol	ND	21
Unknown phytoserols	ND	2.0

ND: non detectable

Table 4. Food intake and body weight gain at day 90, Mean ± SEM.

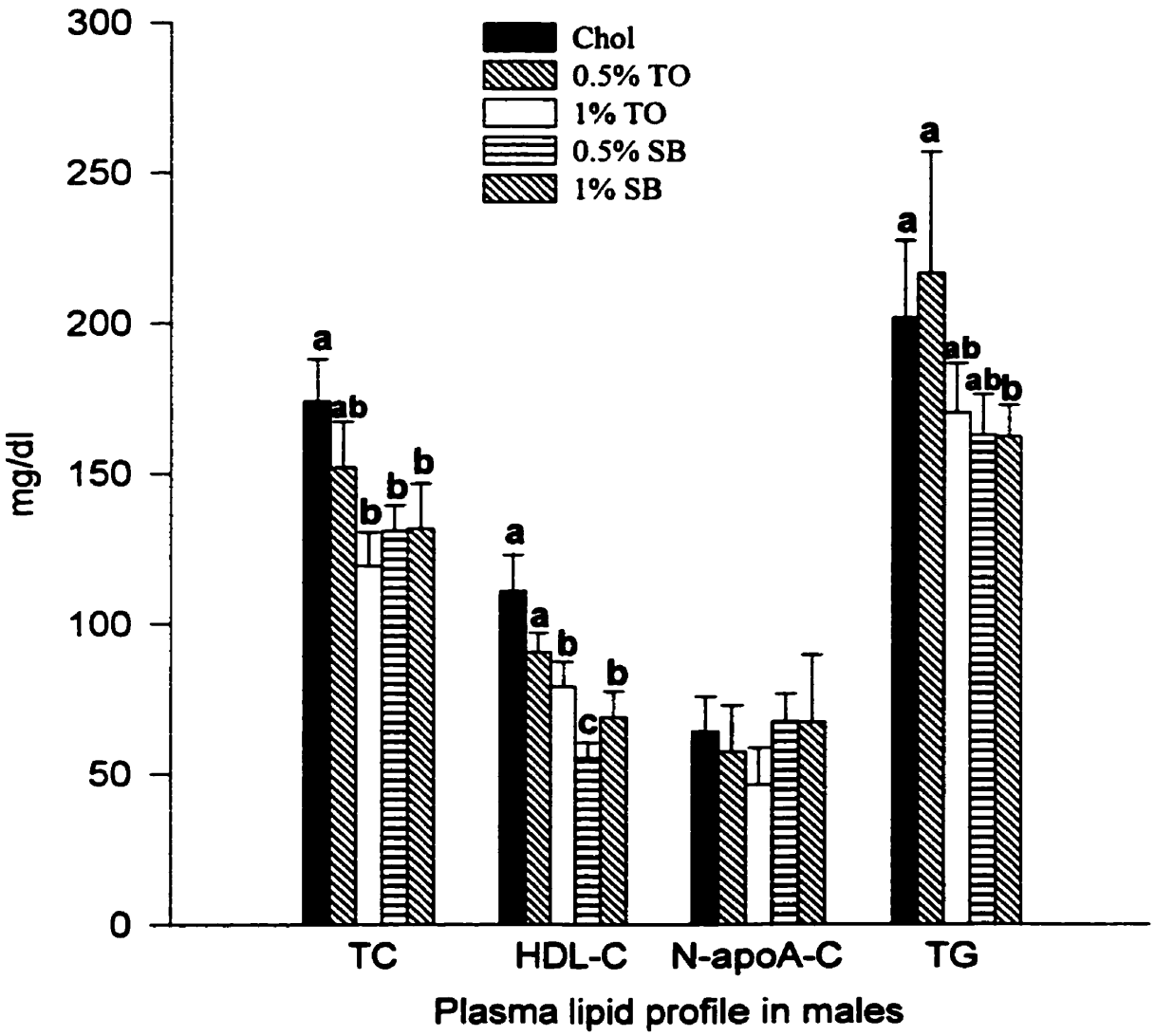
	Food intake (g)		Body weight gain (g)	
	females	males	females	males
Cholesterol	7.6±2.4	7.0±2.0	41±5	42±5
0.5% TO	8.0±1.3	8.0±2.0	37±6	32±5
1.0% TO	7.9±2.3	8.1±1.1	35±5	45±3
0.5% SB	7.0±2.6	7.3±1.2	43±6	37±6
1.0% SB	7.9±1.2	7.0±1.1	55±3	38±6

TO: tall oil, SB: soybean

Table 5. Phytosterols content in hepatocyte and enterocyte (μg/g ± SEM wet tissue).

Phytosterols	**Treatment groups**									
	Male hamsters					Female hamsters				
Hepatocytes	Chol	0.5% TO	1% TO	0.5% SB	1% SB	Chol	0.5% TO	1% TO	0.5% SB	1% SB
Campesterol	247±32	373±97	305±87	302±47	205±85	235±50	409±63	399±140	205±34	403±30
ß-sitosterol	137±19	241±92	356±88	144±39	296±162	160±97	256±40	268±95	127±53	65±17
Enterocytes										
Campesterol	8.1±4	114±58*	62±32*	2.6±1.9	1.3±0.8	1.3±1.2	25.3±22*	84.4±45*	5.3±2.7	9.3±3.4
ß-sitosterol	ND	5±4	33±12*	ND	ND	10±5	ND	21±17	12±2	9±6

Chol: cholesterol, TO: tall oil; SB: soybean, ND: not detectable, * $P<0.05$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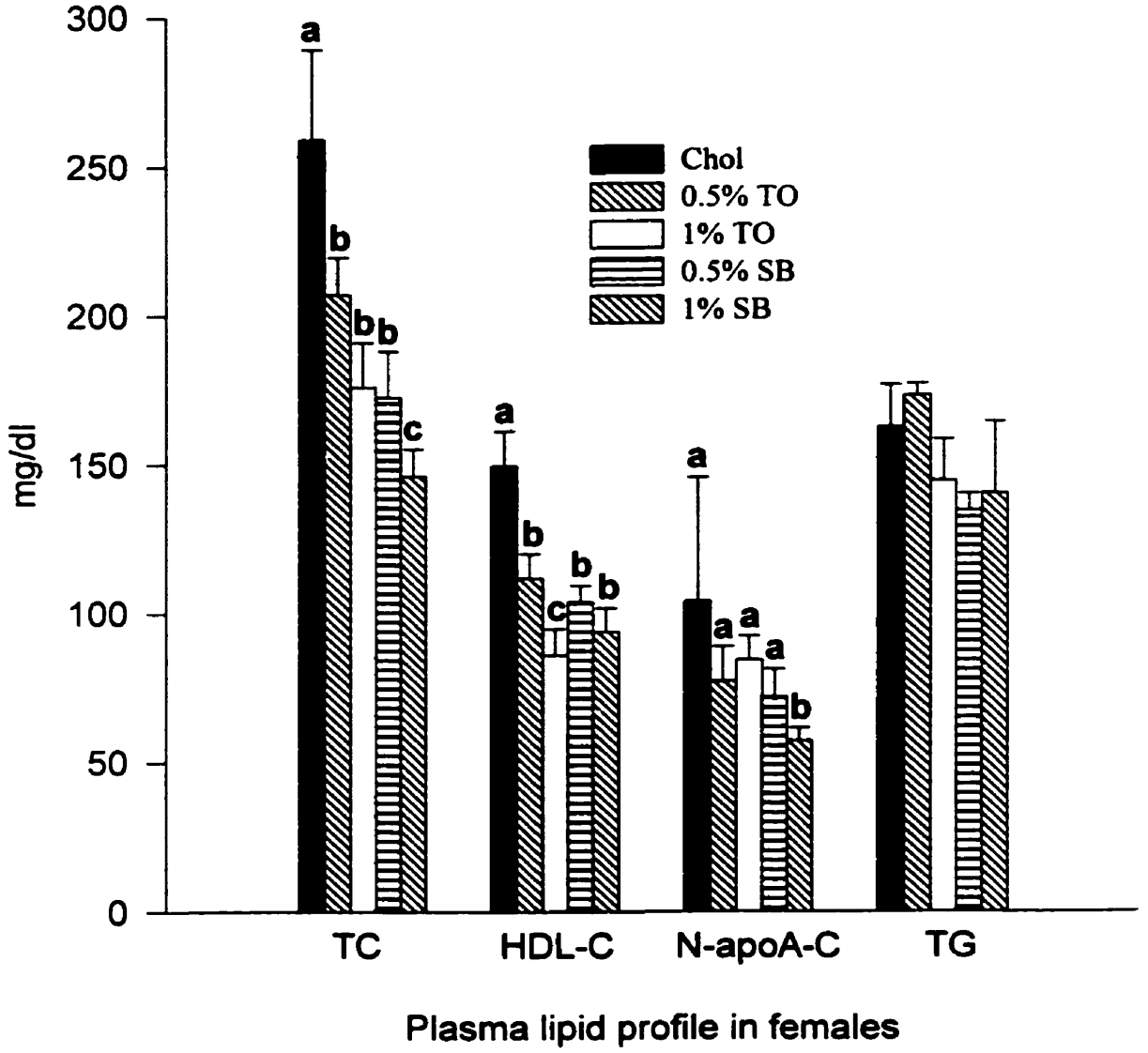
TC
HDL-C
N-apoA-C
TG
Plasma lipid profile in males

Figure 1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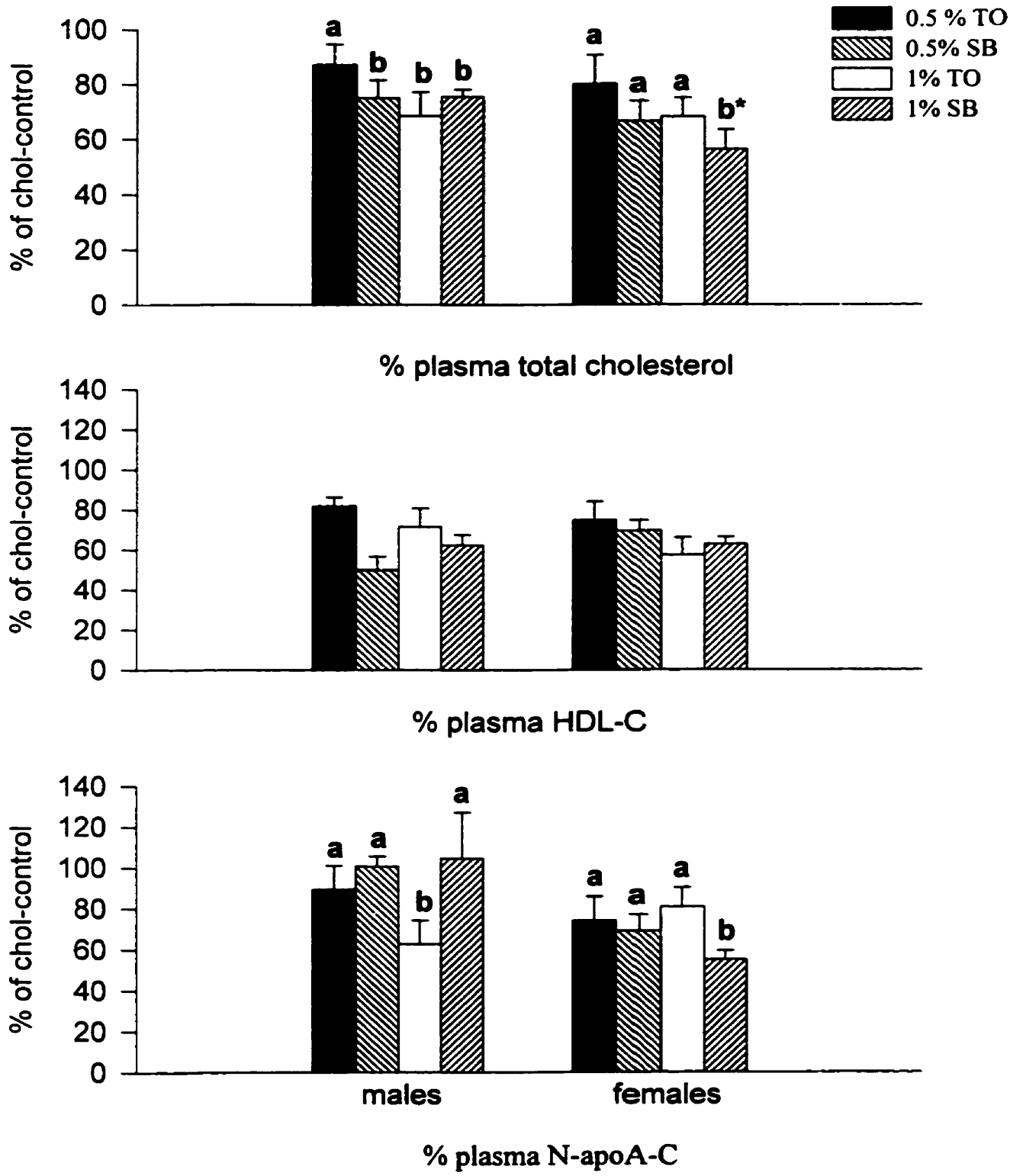
TC
HDL-C
N-apoA-C
TG
Plasma lipid profile in females

Figure 2


0.5 % TO
0.5% SB
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120
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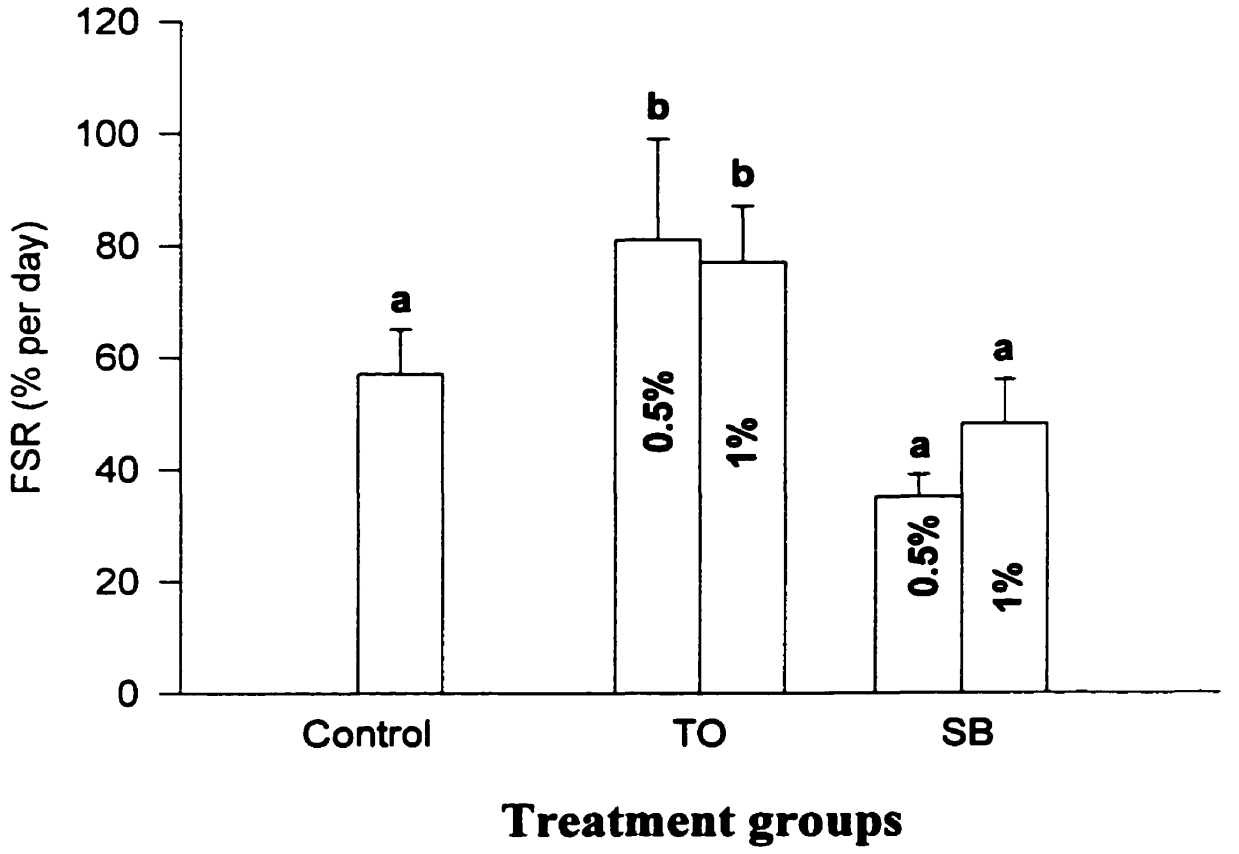
Figure 3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FSR (% per day)
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1%
Control
TO
SB
Treatment groups

Figure 4

TRANSITION

Results from the 90-day hamster study demonstrated that gender may affect phytosterol mechanisms of action. Both soybean- and tall oil-phytosterol extracts lowered plasma cholesterol levels in these animals. However, males were more responsive to tall oil while in females the soybean-phytosterols demonstrated a stronger cholesterol lowering efficacy than that of tall oil-phytosterols. The fractional synthesis rate of cholesterol did not change in the soybean-phytosterol fed hamsters. This was not the case with tall oil-phytosterols where a significant increase was noticed. The reasons for such a discriminatory impact of phytosterols on cholesterogenesis are not clear. We speculated an estrogen-like effect due to the absorbable ß-sitosterol found in the phytosterol mixtures. This study provided us with evidence that tall oil-phytosterols are effective in reducing total cholesterol levels in hamsters. The questions still to be answered are: which compound and what concentrations of sitostanol in the phytosterol mixtures are effective; and is there any synergistic effect between the different plant sterols in hamsters? To answer these questions, the following experiment covered the impact of variable sitostanol concentrations on tissue lipid and phytosterol concentrations for 45 days in hamsters.

CHAPTER 4

EFFECTS OF VARIABLE DIETARY SITOSTANOL CONCENTRATIONS ON PLASMA LIPID PROFILE AND PHYTOSTEROL METABOLISM IN HAMSTERS

Biochimica Biophysica Acta, 1390: 237-224, 1998.

4.1 Abstract

To examine how variable sitostanol (SI) levels in phytosterol-supplemented diets influence plasma and hepatic lipid concentrations, fifty hamsters were divided into five groups and fed semi-purified diets containing 0.25% (w/w) cholesterol for 45 days ad libitum. Four groups were fed this diet with 1% (w/w) phytosterol mixtures which contained 0.01% (w/w) SI derived from soybean, 0.2% (w/w) SI derived from tall oil, 0.2% (w/w) synthetic SI mixture (SIM) and 1% (w/w) pure SI, respectively. A control group did not receive phytosterols. Dietary SI supplementation at 1% (w/w) decreased total and non-apolipoprotein-A cholesterol levels in plasma by 34% ($p = 0.001$) and 55% ($p = 0.04$), respectively, whereas mean plasma total cholesterol level in the 0.2% (w/w) SI group was 23% ($p = 0.001$) lower than that of the control group. The effect of SI at 0.2% (w/w) on plasma lipid profile did not differ from 0.01% (w/w) SI derived from soybean. Liver weights were 15 and 20% ($p = 0.012$) higher in the control group compared with those fed 0.01% and 1% (w/w) SI, respectively, while the hepatic cholesterol content in the control group was greater ($p < 0.0001$) than that of all other groups. Plasma campesterol levels were higher ($p = 0.04$) in the 0.01% and 0.2% (w/w) SI fed groups than in the control, 0.2% (w/w) SIM and 1% (w/w) SI groups. Hepatic ß-sitosterol content was elevated ($p = 0.002$) in the SIM fed group compared to other groups. We conclude that dietary SI effect is proportional to its fractional concentration in phytosterol mixtures and in the diet. Dietary SI lowered plasma cholesterol levels at concentrations higher than 0.2% (w/w) in hamsters.

4.2 Introduction

Although recent studies (Shepherd et al., 1995; Sacks et al., 1996) have suggested longer life expectancy in hyper- and normal-lipidemic subjects treated with statins, dietary modifications are still the preferable and economical approach in reducing the risk of coronary vascular disease in population. For instance, phytosterols, abundant in the plant kingdom, have been considered promising candidates in the dietary treatment of mild hyperlipidemia (Pollak and Kritchevsky, 1981; Gylling et al., 1995; Miettinen et al., 1995; Ling and Jones, 1995b; Jones et al., 1997). No side effects have been reported with their usage (Pollak and Kritchevsky, 1981; Ling and Jones, 1995a).

The most abundant phytosterols in nature are ß-sitosterol, campesterol, stigmasterol, and dihydrobrassicasterol (Pollak and Kritchevsky, 1981; Ling and Jones, 1995a). Though, ß-sitosterol has been shown to lower plasma cholesterol levels (Alfin-Slater et al., 1954; Day, 1991; Newman et al., 1992) sitostanol (SI), the saturated ß-sitosterol, reduces circulating cholesterol levels more efficiently than ß-sitosterol in animals (Sugano et ai., 1977; Ikeda et al., 1979; 1989) and humans (Heinemann et al., 1986; 1991; Miettinen et al., 1995). Nevertheless, the cost of production of purified SI is high.

The effects of variable SI concentrations on plasma lipid profiles, hepatic cholesterol content, plasma and hepatic phytosterol levels have not been studied in hamsters. In addition, the possibility of using the more available and less expensive SI enriched phytosterol such as tall oil has not been systematically explored in view of the cost of pure SI production. Accordingly, the objective of the present study was to examine the effects of different dietary SI concentrations in phytosterol mixtures on plasma and hepatic cholesterol and phytosterol levels in Golden Syrian (GS) hamsters. The null hypothesis was that increasing the

concentration of SI in the phytosterol mixture will have no effect on cholesterol levels in plasma lipoproteins and liver tissue.

4.3 Materials and methods

4.3.1 Diet preparation and accommodation

Fifty GS hamsters weighing 80-100 g (Charles River Laboratories; Montréal Canada) and aged 48 days, were divided into five groups. Hamsters were accommodated for two weeks in stainless steel colony cages with wire mesh floor. The light period of 12 hr began at 11:00 a.m. Room temperature was set between 18 and 21 °C. During accommodation, hamsters had free access to water and were fed ad libitum a plain, pelleted chow diet (Charles River Laboratories, Montréal, Canada). Animals were then switched to semi-purified diets (ICN Pharmaceuticals, Inc., Aurora, Ohio) that were prepared every two weeks and stored at -80°C. Dietary composition is shown in Table 1. All diets contained 0.25% (w/w) cholesterol while non control diets also contained one of the following four mixtures of 1% (w/w) purified phytosterols: (i) soybean (65% ß-sitosterol, 20% campesterol and 15% dihydrobrassicasterol; Sigma Chemical Company, St. Louis, MO), (ii) tall oil (65% ß-sitosterol, 16% campesterol and 17% sitostanol; Forbes Medi-Tech. Inc., Vancouver, BC), (iii) a synthetic mixture prepared to resemble tall oil (54% ß-sitosterol, 17% campesterol, 12% dihydrobrassicasterol and 17% sitostanol; Sigma Chemical Company, St. Louis, MO), and (iv) pure SI (98% sitostanol; Sigma Chemical Company, St. Louis, MO). The synthetic mixture was similar to tall oil-derived phytosterols but contained dihydrobrassicasterol so that the potential effect of this phytosterol could be examined.

Phytosterol composition of each mixture was determined using gas liquid chromatography (GLC). No fatty acids were detected in the phytosterols. Phytosterols were

dissolved in a mixture of oil at 60°C containing equal amounts of saturated, monounsaturated and polyunsaturated fatty acids derived from coconut, olive and sunflower oil respectively. The control group received also the same oil mix without phytosterols. Food intake was measured every three days, and body weight once per week during the study period.

4.3.2 Sample collection and lipid measurement

After forty-five days on the semi-purified diets, hamsters were anaesthetized with halothane and blood samples collected by cardio-puncture. Liver, gall bladder, small intestine and large intestine were collected, immediately frozen in liquid nitrogen, and stored at -80°C. Plasma total, non-apolipoprotein-A and HDL-cholesterol and TG levels were measured in duplicate using a VG autoanalyzer with commercial enzymatic kits that precipitated the apo-B containing lipoproteins (Abbott, Montréal, PQ). Results are expressed as non-apolipoprotein-A levels instead of low density lipoproteins (LDL) because of the concern that Friedewald et al. (1972) equation may not be applicable in hamsters.

4.3.3 Phytosterol extraction and measurement

Plasma and hepatic phytosterol compositions were measured by GLC using a modification of the method of Hamilton et al. (1992). Briefly, an internal standard of 5α-cholestane (0.2 mg/ml plasma) was added to each sample (1 ml plasma and 1 g wet liver). Plasma and hepatic samples were saponified for 2 hr at 100°C with 0.5 M methanolic KOH in 15 ml Teflon capped glass tubes. After saponification, samples were left to cool at room temperature, then mixed with distilled water and petroleum-ether. Sample tubes were vortexed and the upper layers containing the non saponifiable materials transferred to clean

glass tubes. The extraction was repeated twice. Extracted samples were dried under nitrogen and resuspended in 300 µl of hexane. A sample volume of 2 µl was injected into the GLC (HP 5890 Series II, Hewlett Packard, CA). A 30 m Rtx-5 (Restek Corp. Bellefonte, PA) capillary column with an internal diameter of 0.25 mm and film thickness of 0.25 µm was used. Samples were injected at 80°C. The oven remained at this temperature for 1 min, then was increased to 285°C (20°C/min) where it remained for 20 min. Oven temperature was then increased to 320°C at a rate of 20°C/min and held at that level for 5 min before subsequent analyses. Injector and detector temperatures were set at 300°C and 320°C, respectively. The carrier gas (helium) flow rate was 1.2 ml/min with the inlet splitter set at 10:1. Phytosterol (campesterol, ß-sitosterol and sitostanol) peak identification was confirmed using authentic standards (Sigma Chemical Company, St. Louis, MO).

4.3.4 Analysis of data

All data were expressed as mean ± standard deviation (SD). Data was tested for normality by Kolmogorov-Smirnov test. One way analysis of variance (ANOVA) or Kruskal-Wallis ANOVA on ranks were used to determine whether SI altered plasma lipid and phytosterol levels and hepatic phytosterol concentrations in hamsters. The Newman-Keuls test was adopted where significance was observed for interaction measurement among treatment groups. For the correlation measurement between SI content in phytosterol and plasma cholesterol concentrations, the linear regression line of $y = ax + b$ was fitted by the method of least squares to the data from individual animals. The differences between the means were considered statistically significant where the P value was < 0.05.

4.4 Results

4.4.1 Food intake, body and liver weight measurement

No significant differences were observed in food consumption ($p = 0.130$) or body weight ($p = 0.43$) across treatment groups. Daily food intake across groups ranged from 8.84 to 9.34 g (Figure 1). During the 45-day trial, hamsters gained between 25 and 40 g in body weight. Liver weights (shown in Figure 1) varied significantly among the five groups. Mean liver weight of 0.01% (w/w) and 1% (w/w) SI fed hamsters was 15% and 20% lower ($p = 0.012$) than that of the control group, respectively. The ratio of liver weight over body weight in the control group (0.071 ± 0.009) was higher ($p = 0.0054$) than the 1% (w/w) SI (0.056 ± 0.0024) but did not differ from other groups. Cholesterol content in liver was significantly higher ($p < 0.0001$) in the control group compared with 0.2% SI, 0.2% SIM and 1% SI fed groups (Figure 2).

4.4.2 Plasma lipid profile

Compared with the control diet (226.9 ± 30.7 mg/dl), animals supplemented with 1% (w/w) SI (151.2 ± 25.8 mg/dl) had lower (37%) ($p = 0.001$) plasma total cholesterol levels (Figure 3). Mean plasma total cholesterol level in 0.2% (w/w) SI (175.2 ± 32.1 mg/dl) was also lower (23%) ($p = 0.001$) than that of the control group. However, the group consuming 0.2% (w/w) SIM (186.1 ± 30.6 mg/dl) was not statistically different from the control group. Similar to plasma total cholesterol response, non-apolipoprotein-A cholesterol level in the 1% (w/w) SI supplemented group was 55% lower ($p = 0.04$) than that of the control (Figure 4). However, no significant difference was observed between non-apolipoprotein-A cholesterol levels in 0.2% (w/w) SI versus control. Mean plasma HDL cholesterol ($p = 0.091$) and TG (p

= 0.824) levels did not differ across the five treated groups (Figure 3). The ratio of apolipoprotein-A (HDL) over non-apolipoprotein-A (Figure 4) did not vary (p = 0.315) across the different phytosterol treatments. A large variation was observed in this ratio which is most probably due to an additive compounding effect that results from two variables moving in opposite directions. A negative correlation of r=-3 (p = 0.046) was demonstrated between plasma total cholesterol levels and percent sitostanol concentrations in phytosterol mixtures.

4.4.3 Plasma and liver phytosterol levels

Figures 5 and 6 show plasma and liver phytosterol values, respectively, across the five treatments. Plasma campesterol was the highest in the 0.01% (w/w) SI fed group (p = 0.04) compared to the 0.2% (w/w) SIM and 1% (w/w) SI fed groups. Plasma ß-sitosterol, on the other hand, did not vary across groups with the exception of the 1% (w/w) SI treated group. Sitostanol was not detectable in plasma of hamsters fed control, 0.01% or 0.2% (w/w) SI diets. Negligible plasma concentrations of SI were seen in 1% (w/w) SI and 0.2% (w/w) SIM supplemented groups. Liver campesterol levels did not differ (p = 0.064) across groups. Conversely, ß-sitosterol content in 0.2% (w/w) SIM group was higher (p = 0.002) than the other groups. Negligible levels of SI were detected in liver tissue of the group fed 1% (w/w) SI.

4.5 Discussion

The current study evaluated the effectiveness of increased fractional sitostanol content in phytosterol mixtures on plasma lipid and phytosterol levels in hamsters. Dietary sitostanol at 1% (w/w) was more efficient in reducing plasma total cholesterol than phytosterol mixtures

containing lower SI concentrations (Figure 3). However, the production cost and difficulties of SI purification represent major obstacles in its usage as a wide scale food additive or supplement. Phytosterol mixtures with a SI concentration of 17 to 21% (w/w) showed a cholesterol-lowering action of about 67% that of pure SI when compared to control and thus may be a less expensive, yet effective agent to use as dietary supplement.

Although 0.2% (w/w) SI effect did not differ from that of soybean, results obtained from this study suggest that SI lowers plasma total and non-apolipoprotein-A cholesterol levels in hamsters in a dose-dependent manner. In agreement with our results, other investigators have shown that SI is more efficient in reducing plasma cholesterol than is ß-sitosterol in rats (Ikeda et al., 1979) and rabbits (Ikeda et al., 1981). Dietary SI, administered as esterified (Gylling et al., 1995; Miettinen et al., 1995) or non-esterified to margarine (Heinemann et al., 1986), has been demonstrated to lower plasma total cholesterol levels in mildly and hypercholesterolemic subjects. Conversely, Denke (1995) showed that SI in capsules administered orally did not affect plasma lipid profile in men with moderate hypercholesterolemia. The latter observation may be due to SI administration in capsules instead of mixing SI-fat with the diet, to the dose given and possibly to low subject compliance in taking the daily sitostanol capsule.

In contrast to plasma total-C and non-apolipoprotein-A response, phytosterols rich in SI did not exert any remarkable effect on plasma HDL-cholesterol or TG levels in hamsters. Several studies have shown that HDL-cholesterol and TG concentrations remained unchanged during phytosterol treatment (Day, 1991; Miettinen et al., 1995). However, Ling and Jones (1995b) and Newman et al. (1992) showed an increase in plasma HDL-cholesterol level with no effect on TG concentration in rats fed 1% (w/w) tall oil (21% (w/w) SI) and chickens fed

soybean phytosterols at 2 g/day, respectively. The present study, on the other hand, demonstrates that 1% (w/w) phytosterol supplementation from variable sources did not alter plasma HDL-C and TG levels in hamsters.

Sitostanol-free phytosterol did not affect plasma lipid profiles in this animal model, conversely to what has been reported by others in sea quail (Day, 1991), chickens (Newman et al., 1992) and rats (Laraki et al., 1991). Apparently, ß-sitosterol levels administered in the latter studies were higher than those (<0.6% (w/w) ≈ 0.05 g/day) given in our study. In this study, soybean (0.01% (w/w) SI) and the synthetically (0.2% (w/w) IS) prepared mixture (both containing dihydrobrassicasterol) did no affect plasma lipid profile. However, the 0.2% (w/w) SIM lowered plasma cholesterol concentration by 18%, although because of intra-individual variability, this effect was not statistically significant. When comparing the effect of 0.2% (w/w) SI to that of 0.01% (w/w) SI derived from soybean, no difference was observed between these two mixtures. Such results suggest that soybean-phytosterols at higher concentrations might be as effective as 0.02% (w/w) SI derived from tall oil. However, the data demonstrate the superiority of pure SI to both tall oil and soybean in lowering plasma total cholesterol. There is no strong evidence to suggest that other compounds than SI that might be found in tall oil but not in the synthetic mixture lowered plasma total cholesterol concentrations. The negative correlation between plasma total cholesterol levels and percent SI concentrations in the phytosterol mixtures suggest that SI is more potent than other phytosterol mixtures. It also suggest that SI is the active agent in lowering circulating cholesterol levels in hamsters.

Although not all mechanisms involved in lowering plasma cholesterol nor the dose dependent factors are well understood, SI was found safe to add to animal diets at the concentration of 1% (w/w). Histological examination of liver and duodenum tissue from

groups fed phytosterols showed no changes in tissue or cell structure (data not shown). SI has been shown to alter cholesterol uptake through the brush border membranes in animals (Ikeda et al., 1979; 1989) and humans (Heinemann et al., 1988; 1991). Heinemann et al. (1991) have suggested that SI, which is more hydrophobic than ß-sitosterol, potentially alters the micellar mixture more than unsaturated phytosterols and render it less miscible and absorbable. It has also been demonstrated that phytosterols alter micellar phospholipid (Child and Kuksis, 1986) and bile composition (Tanaka et al., 1993). Thus, SI apparently exerts its lowering effect on plasma cholesterol by inhibiting sterol absorption in the small intestine without having any intrinsic effect. Such suggestion is supported by the low to negligible plasma and tissue phytosterols concentrations.

Increased dietary phytosterol intake is usually associated with higher levels of plasma campesterol and ß-sitosterol (Gould et al., 1969; Salen et al., 1970; Lees and Lees, 1976; 1977). Kuksis and Huang (1962) demonstrated that dogs fed ß-sitosterol and campesterol (60:40 w/w) have increased lymph levels of campesterol compared with ß-sitosterol. It has been suggested that uptake of sterols by jejunal, brush border and red blood cells in animals decreases as more carbon atoms are added at C_{24} of the sterol skeleton (Kuksis and Huang, 1962; Child and Kuksis, 1983b). The higher plasma levels of campesterol versus ß-sitosterol could also be due to lower esterification rate of the latter in the enterocytes (Field and Mathur, 1983; Ikeda et al., 1988a) compared to campesterol. Moreover, plasma campesterol levels have been used as a marker of cholesterol absorption efficiency in humans (Tilvis and Miettinen, 1986; Miettinen et al., 1990; 1995; Vanhanen and Miettinen, 1992). It is uncertain if such relationship might also exist in hamsters. However, in this study, plasma campesterol level in the 1% (w/w) SI-fed group was the lowest suggesting that dietary SI at 1% (w/w)

decreased cholesterol absorption in hamsters. We are assuming that SI exerts its cholesterol lowering effect in the gut.

In the present study, plasma SI was undetectable except in the group fed pure SI where very low concentrations were observed. Furthermore, individual hepatic phytosterol contents were not significantly different across groups. Only in the group fed 0.2% (w/w) SIM did ß-sitosterol levels exceed those in other treated hamsters. However, such results do not confirm any preference for ß-sitosterol absorption over campesterol because the latter was not observed in the group fed 0.2% (w/w) SI or the group fed 0.01% (w/w) SI. Since liver concentrations of campesterol and ß-sitosterol were consistent across groups, it seems that 1% (w/w) dietary phytosterol mixtures from different sources do not considerably alter liver phytosterol concentrations. Such results suggest that phytosterols might have a high turnover rate in the liver and could be secreted into bile more readily than cholesterol. Moreover, higher liver weights and cholesterol content in the control group versus phytosterol treated groups imply that dietary supplementation with phytosterol decreases cholesterol deposition in the liver as compared to a phytosterol free diet.

In conclusion, independent of phytosterol concentration, pure dietary SI was superior to both tall oil and soybean-derived phytosterols and resulted in a more effective decrease in circulating total and non-apolipoprotein-A cholesterol levels in GS hamsters.

4.6 Figure legends

Figure 1.

Body weights, liver weights and food intake per day in hamsters fed semi-purified diet containing 1% (w/w) phytosterol mixtures with 0.01% (w/w) SI, 0.2% (w/w) SI, 0.2% (w/w) SIM and 1% (w/w) SI, respectively. Control group did not receive any phytosterol mixtures. Values within each group carrying different letters are significantly different at p value indicated. ANOVA and Kruskal-Wallis ANOVA. SI: sitostanol, SIM: sitostanol mixture. All groups have n=10.

Figure 2.

The correlation between plasma total cholesterol levels and % dietary sitostanol in hamsters fed semi-purified diet containing 1% (w/w) phytosterol mixtures with 0.01% (w/w) SI, 0.2% (w/w) SI, 0.2% (w/w) SIM and 1% (w/w) SI, respectively. Cholesterol content in wet liver tissue. Control group did not receive any phytosterol mixture. Values within each group carrying different letters are significantly different at p value indicated. SI: sitostanol, SIM: sitostanol mixture. All groups have n=10.

Figure 3.

Plasma total cholesterol, high density lipoprotein cholesterol and triglycerides levels in hamsters fed semi-purified diet containing 1% (w/w) phytosterol mixtures with 0.01% (w/w) SI, 0.2% (w/w) SI, 0.2% (w/w) SIM and 1% (w/w) SI, respectively. Control group did not

receive any phytosterol mixture. Values within each group carrying different letters are significantly different at p value indicated. ANOVA. SI: sitostanol, SIM: sitostanol mixture. To convert mg/dl cholesterol to mmol/L divide by 38.7. To convert mg/dl triglyceride to mmol/L divide by 88.2. All groups have n=10.

Figure 4.

Plasma non apo-A lipoproteins levels and the ratio of high density lipoprotein over non apo-A lipoproteins in hamsters fed semi-purified diet containing 1% (w/w) phytosterol mixtures with 0.01% (w/w) SI, 0.2% (w/w) SI, 0.2% (w/w) SIM and 1% (w/w) SI, respectively. Control group did not receive any phytosterol mixture. Values within each group carrying different letters are significantly different at p value indicated. ANOVA and Kruskal-Wallis ANOVA. SI: sitostanol, SIM: sitostanol mixture. To convert mg/dl cholesterol to mmol/L divide by 38.7. To convert mg/dl triglyceride to mmol/L divide by 88.2. All groups have n=10.

Figure 5.

Plasma campesterol, ß-sitosterol and sitostanol levels in hamsters fed semi-purified diet containing 1% (w/w) phytosterol mixtures with 0.01% (w/w) SI, 0.2% (w/w) SI, 0.2% (w/w) SIM and 1% (w/w) SI, respectively. Control group did not receive any phytosterol mixture. Values are means ± SD. Values within each group carrying different letters are significantly different at p value indicated. ANOVA. SI: sitostanol, SIM: sitostanol mixture, ND: non detectable. All groups have n=10.

Figure 6.

Liver campesterol, ß-sitosterol and sitostanol concentrations in hamsters fed semi-purified diet containing 1% (w/w) phytosterol mixtures with 0.01% (w/w) SI, 0.2% (w/w) SI, 0.2% (w/w) SIM and 1% (w/w) SI, respectively. Control group did not receive any phytosterol mixture. Values are means ± SD. Values within each group carrying different letters are significantly different at p value indicated. ANOVA. SI: sitostanol, SIM: sitostanol mixture, ND: non detectable. All groups have n=10.

Table 1. Composition of experimental diets.

Ingredient	control %	soybean %	tall oil %	synth. mix %	sitostanol %
Casein	20.0	20.0	20.0	20.0	20.0
Corn starch	29.0	28.0	28.0	28.0	28.0
Sucrose	36.3	36.3	36.3	36.3	36.3
Oil mixture	5.0	5.0	5.0	5.0	5.0
DI-methionine	0.5	0.5	0.5	0.5	0.5
Mineral mixture	4.0	4.0	4.0	4.0	4.0
Vitamin mixture	1.0	1.0	1.0	1.0	1.0
Choline bitartrate	0.2	0.2	0.2	0.2	0.2
Cholesterol	0.25	0.25	0.25	0.25	0.25
Butyl hydroxy toluene	0.02% of oil	0.02% of oil	0.02% of oil	0.02% of oil	0.02% of oil
Cellulose	5.0	5.0	5.0	5.0	5.0
ß-sitosterol	-	0.65	0.65	0.54	-
Campesterol	-	0.20	0.16	0.17	-
Dihydrobrassicasterol	-	0.15	-	0.12	-
Sitostanol	-	-	0.17	0.17	0.98
Unknown phytosterols	-	-	0.02	-	0.02

Synth. mix = Synthetic mixture.

The vitamins and salt mixture and vitamin-free casein were purchased from ICN Life Science Group, Nutritional Biochemicals Division, Cleveland, OH. Fat soluble vitamins were added to the oil mix which was a rich source of essential fatty acids. For detailed formulation of the vitamins and mineral mixes, please refer to the company's catalogue.

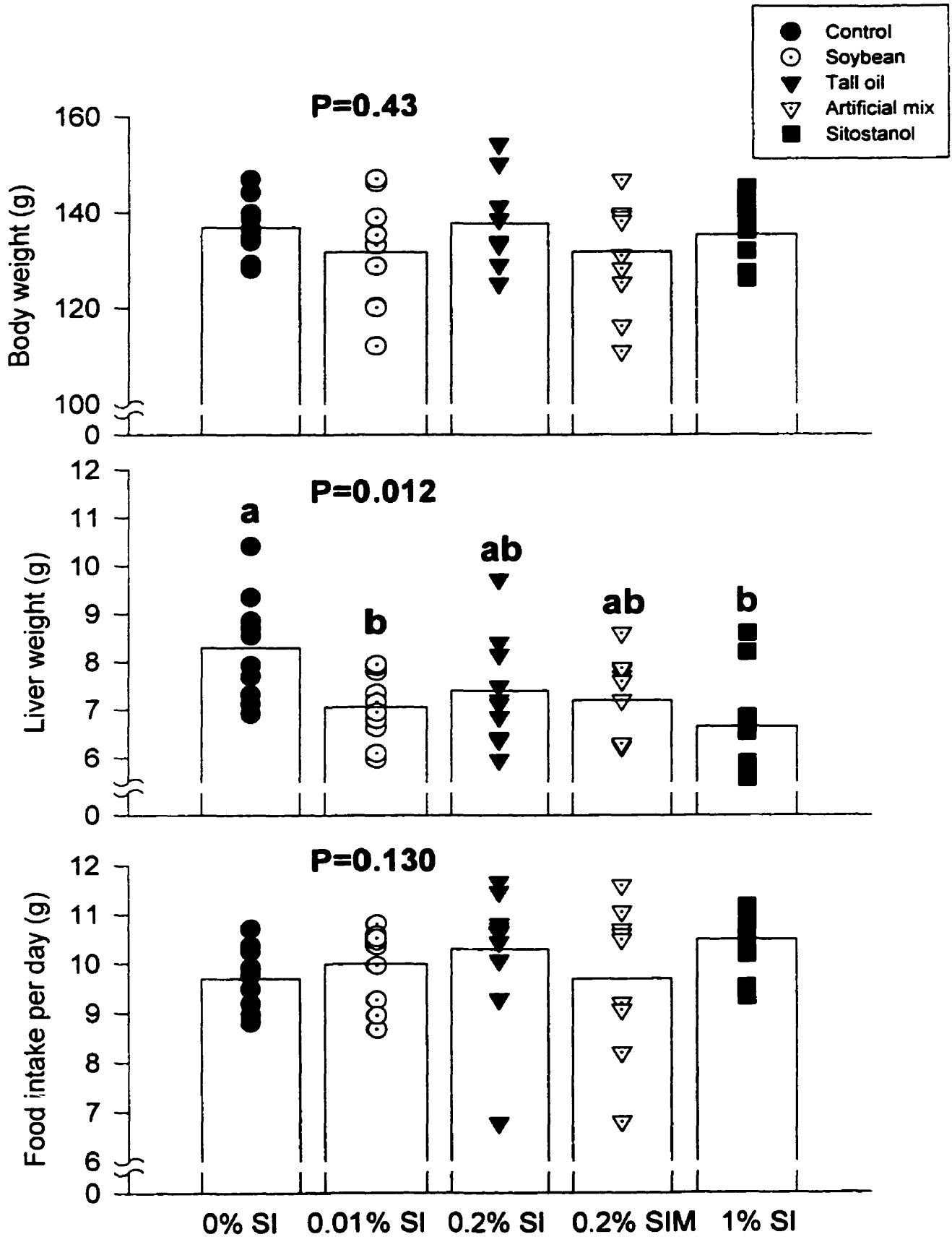
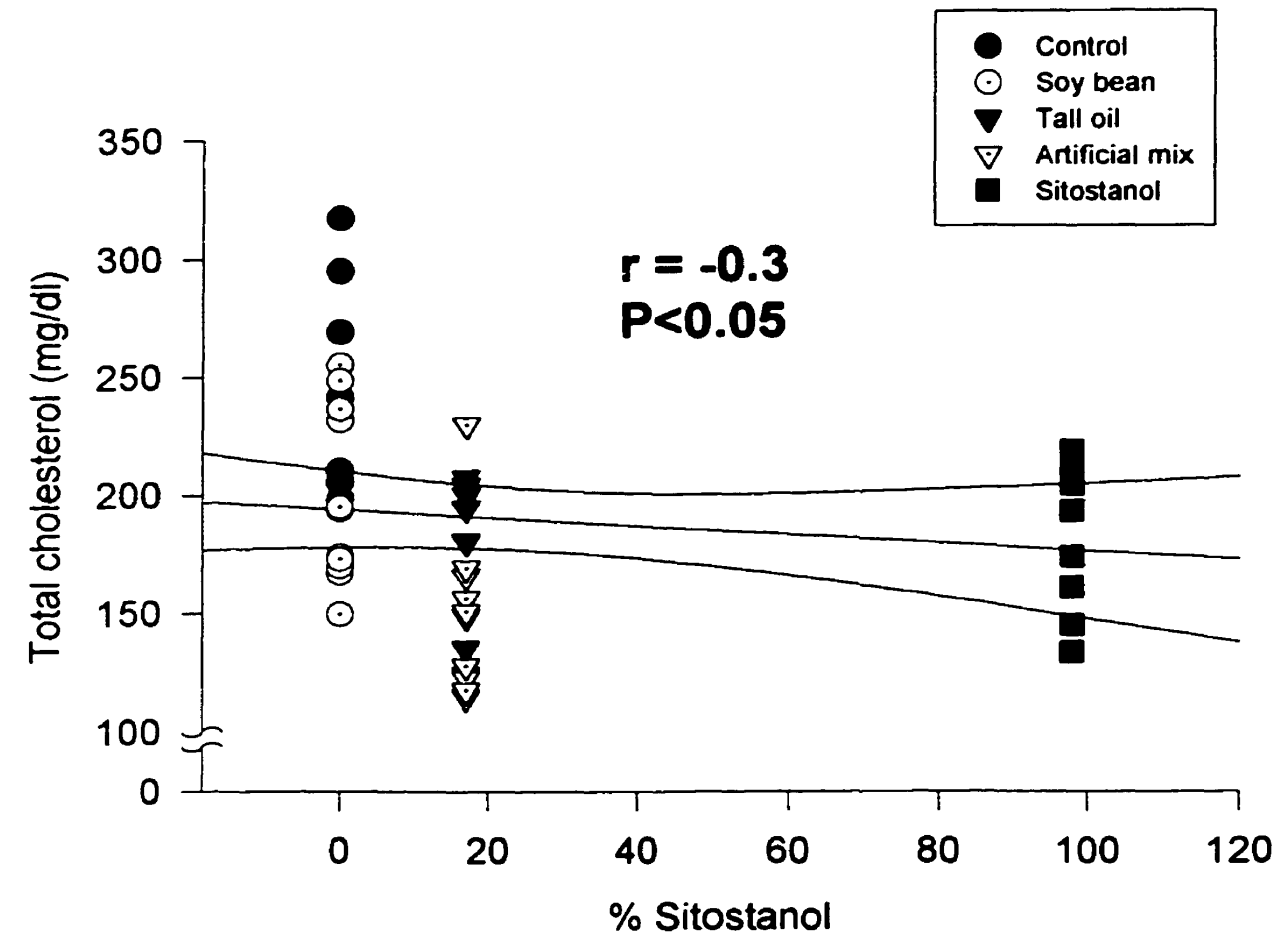

Control
Soybean
Tall oil
Artificial mix
Sitostanol
P=0.43
Body weight (g)
160
140
120
100
0
P=0.012
Liver weight (g)
a
b
ab
ab
b
12
11
10
9
8
7
6
0
P=0.130
Food intake per day (g)
12
11
10
9
8
7
6
0
0% SI
0.01% SI
0.2% SI
0.2% SIM
1% SI

Figure 1


Control
Soy bean
Tall oil
Artificial mix
Sitostanol
r = -0.3
P<0.05
Total cholesterol (mg/dl)
350
300
250
200
150
100
0
0
20
40
60
80
100
120
% Sitostanol

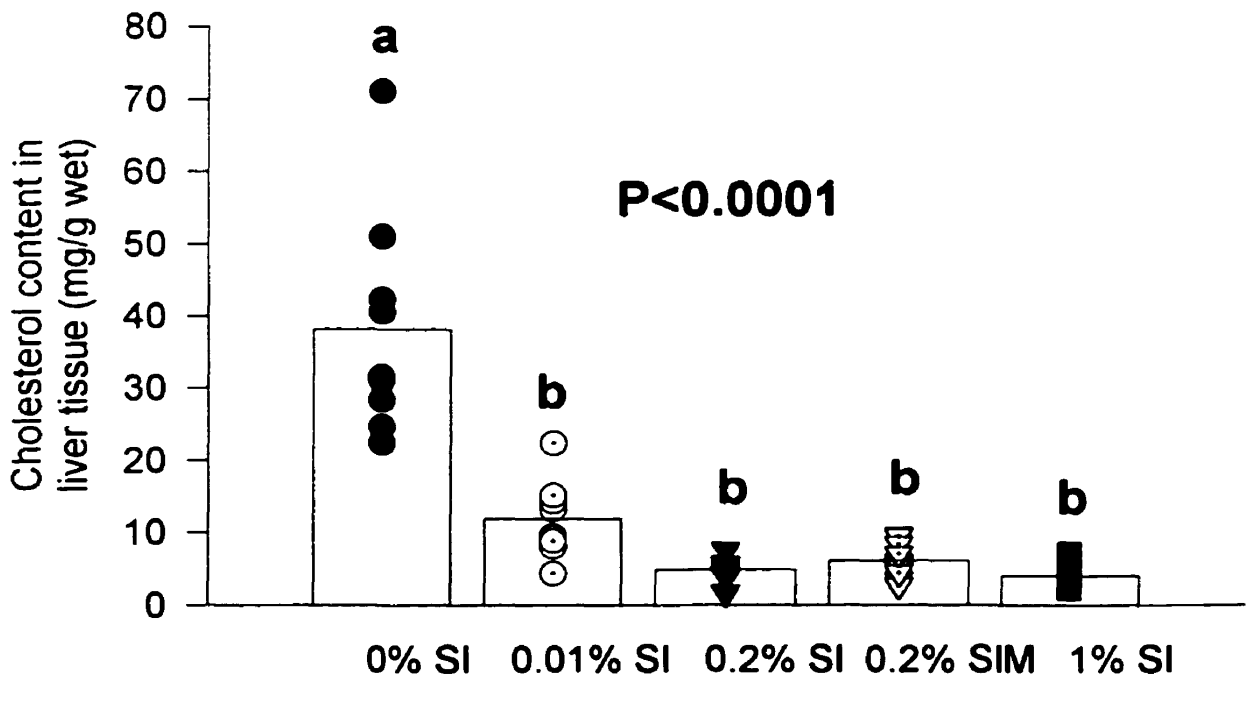

Cholesterol content in liver tissue (mg/g wet)
80
70
60
50
40
30
20
10
0
a
b
b
b
b
P<0.0001
0% SI
0.01% SI
0.2% SI
0.2% SIM
1% SI

Figure 2

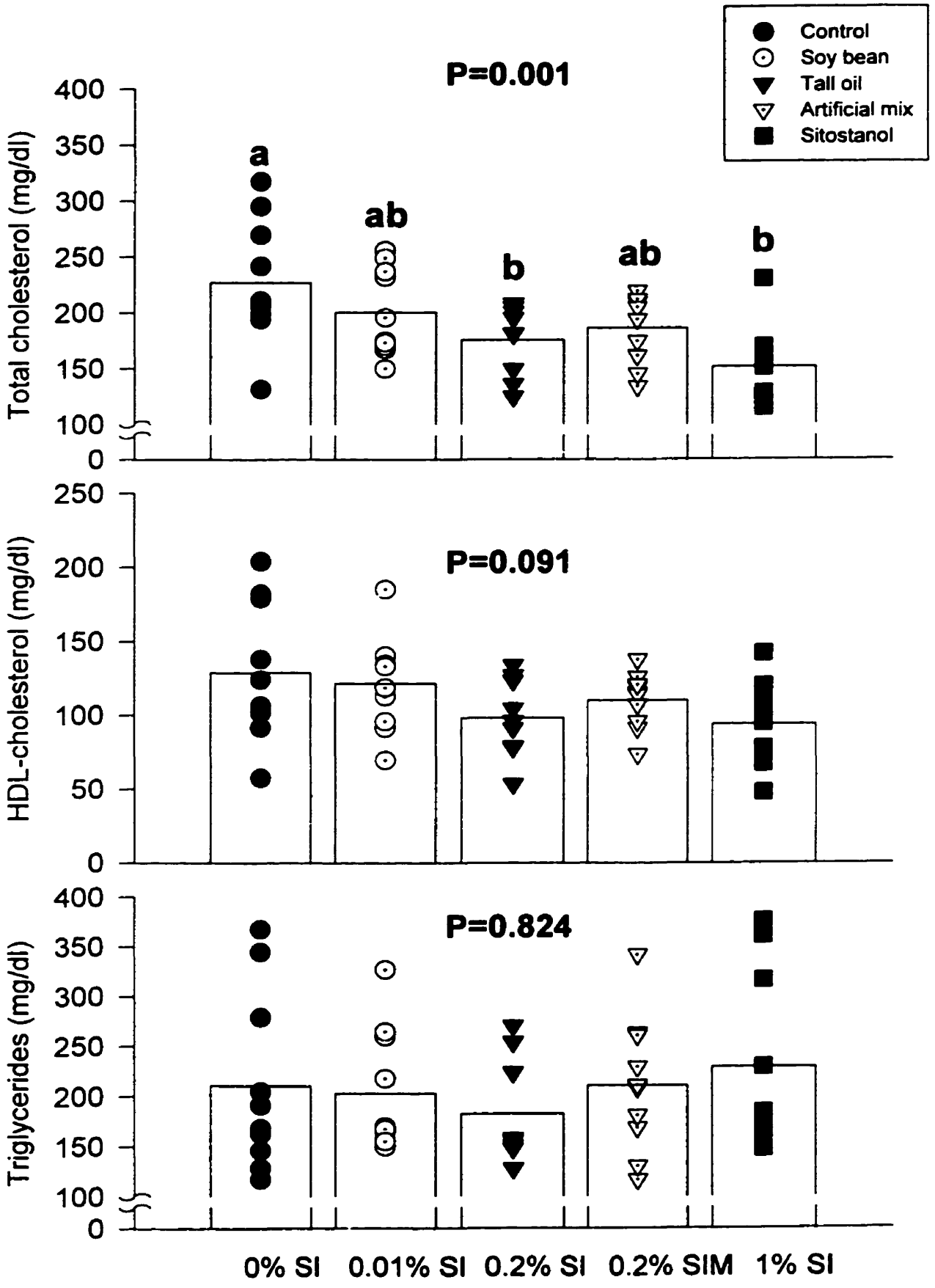

Control
Soy bean
Tall oil
Artificial mix
Sitostanol
P=0.001
a
ab
b
ab
b
Total cholesterol (mg/dl)
400
350
300
250
200
150
100
0
P=0.091
HDL-cholesterol (mg/dl)
250
200
150
100
50
0
P=0.824
Triglycerides (mg/dl)
400
350
300
250
200
150
100
0
0% SI
0.01% SI
0.2% SI
0.2% SIM
1% SI

Figure 3

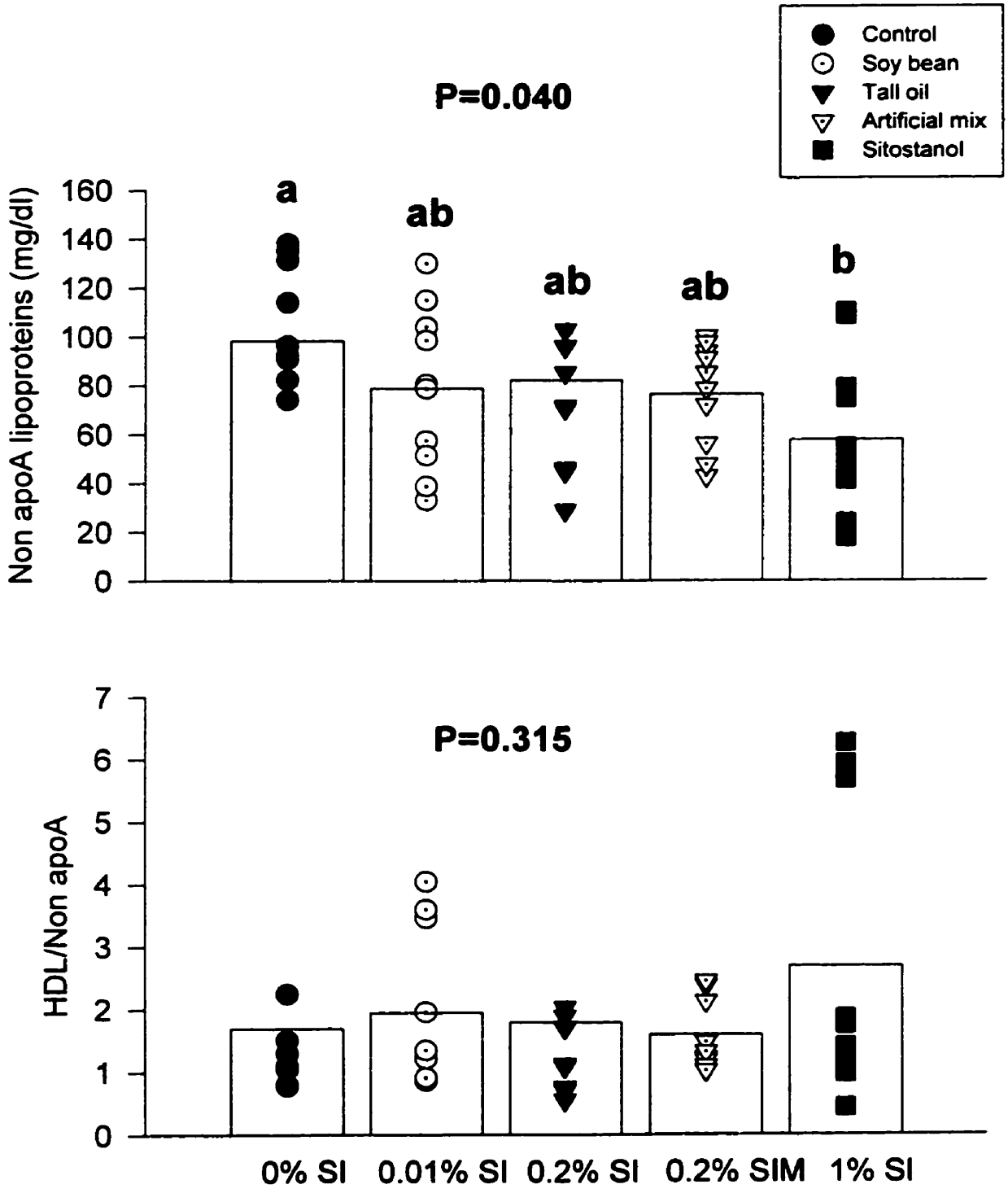

Control
Soy bean
Tall oil
Artificial mix
Sitostanol
P=0.040
Non apoA lipoproteins (mg/dl)
160
140
120
100
80
60
40
20
0
a
ab
ab
ab
b
P=0.315
HDL/Non apoA
7
6
5
4
3
2
1
0
0% SI
0.01% SI
0.2% SI
0.2% SIM
1% SI

Figure 4

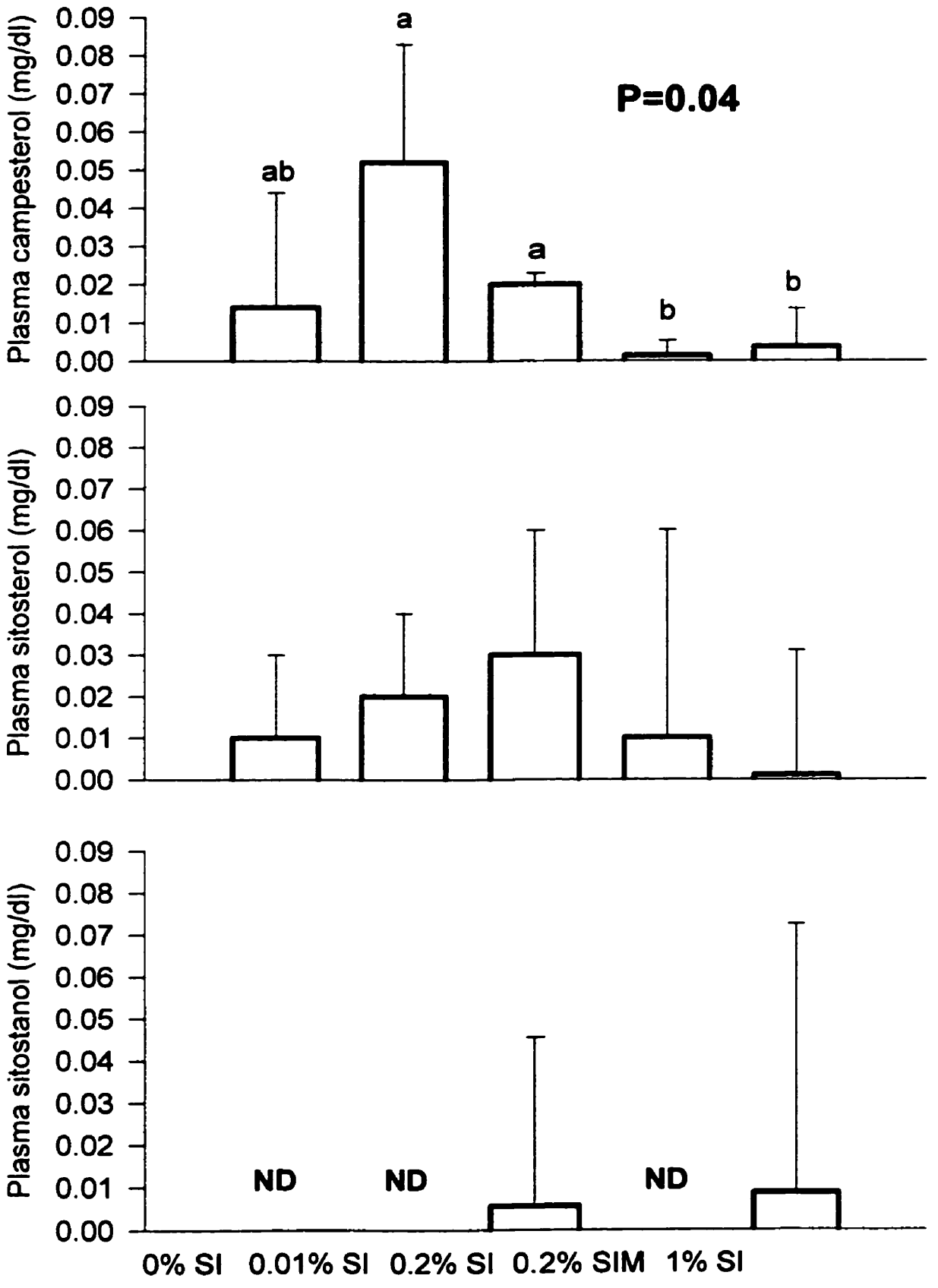

Plasma campesterol (mg/dl)
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
a
ab
a
b
b
P=0.04
Plasma sitosterol (mg/dl)
Plasma sitostanol (mg/dl)
ND
ND
ND
0% SI
0.01% SI
0.2% SI
0.2% SIM
1% SI

Figure 5

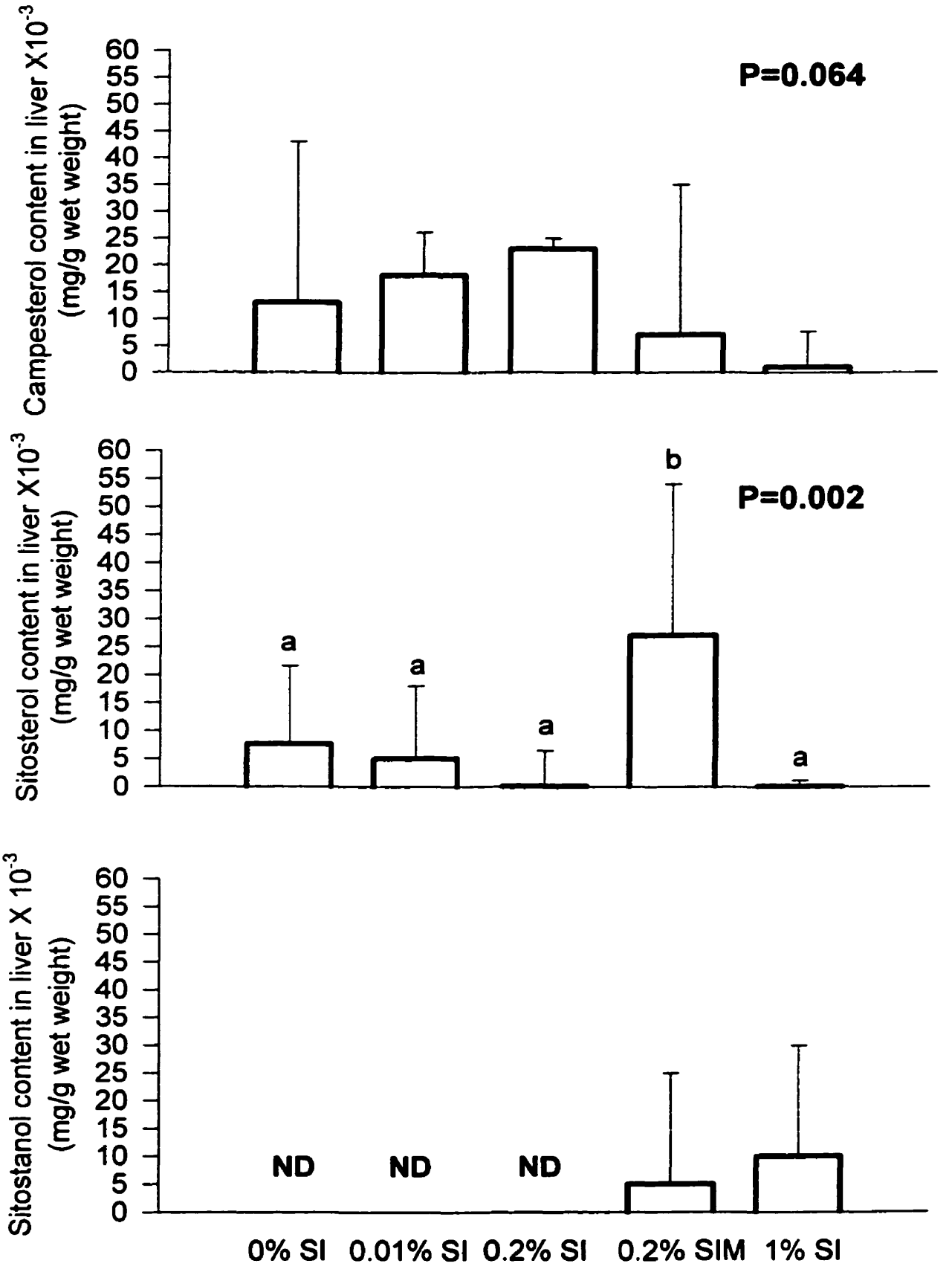

Campesterol content in liver X10-3 (mg/g wet weight)
P=0.064
Sitosterol content in liver X10-3 (mg/g wet weight)
P=0.002
a
a
a
b
a
Sitostanol content in liver X 10-3 (mg/g wet weight)
ND
ND
ND
0% SI
0.01% SI
0.2% SI
0.2% SIM
1% SI

Figure 6

TRANSITION

Data from the 45-day hamster manuscript showed that dietary sitostanol supplementation at 1% (w/w) decreased circulating total cholesterol levels compared to the control. In addition, mean plasma total cholesterol level in the 0.2% (w/w) sitostanol group was lower than that of the control group. Furthermore, liver weights and hepatic cholesterol contents were higher in the control group compared with those fed 0.01% and 1% (w/w) sitostanol. Thus, the results demonstrate that the effect of dietary sitostanol is proportional to its fractional concentration in phytosterol mixture and in diet. Sitostanol is more effective than other plant sterols in lowering circulating lipid levels in hamsters. However, the effects of sitostanol on plaque development and cholesterol esterification rate are not well characterized. In the following study, rabbits are used in order to examine the impact of phytosterols in another animal model as well as to measure the effects of phytosterols on plaque development and LCAT activity. The questions to answer are: How would sitostanol affect the end point of hyperlipidemia such as plaque development? And is there any intrinsic effect of plant sterols on LCAT activity? Rabbits were chosen because they are a good model to measure plaque development and LCAT activity.

CHAPTER 5

DIETARY SITOSTANOL REDUCES PLAQUE FORMATION BUT NOT LECITHIN CHOLESTEROL ACYL TRANSFERASE ACTIVITY IN RABBITS

Atherosclerosis, 1998 (in press).

5.1 Abstract

The effects of graded amounts of dietary sitostanol (0.01%, 0.2% and 0.8% (w/w)) were examined on plasma lipid-profile, coronary artery plaque development and lecithin:cholesterol acyl transferase activity in male New Zealand White rabbits given semi-purified diets for 10 weeks. All diets provided <10% energy in the form of fat and contained 0.5% (w/w) cholesterol (C). Rabbits fed the semi-purified diet with 0.8% (w/w) (0.64 g/day) sitostanol had lower plasma total- (TC) ($p = 0.006$) (15.2 ± 4.80 mmol/L) and very low density lipoprotein-cholesterol (VLDL-C) ($p = 0.007$) (6.31 ± 3.11 mmol/L) levels compared to the atherogenic control group ($n = 6$) (29.6 ± 5.52 and 17.16 ± 7.43 mmol/L, respectively). Dietary sitostanol at 0.8% (w/w) depressed plaque accretion in coronary arteries ($p = 0.0014$) and ascending aorta ($p = 0.0004$) compared with the atherogenic control, 0.01% and 0.2% (w/w) sitostanol-fed groups. No differences ($p = 0.24$) in the activity of lecithin:cholesterol acyl transferase (LCAT) were observed across groups, although plasma cholesterol fractional esterification rate was higher ($p = 0.004$) in the 0.8% (w/w) sitostanol fed animals compared with the atherogenic control. Significant negative correlations were demonstrated between sitostanol intake and plasma TC, LDL-C and VLDL-C levels. Hepatic campesterol levels were correlated ($r = 0.3$, $p = 0.03$) with plasma but not hepatic TC concentrations. These results demonstrate that dietary sitostanol at a concentration of 0.8% (w/w) or 0.64 g/day lowered plasma cholesterol levels and depressed atherosclerosis development in rabbits but did not alter LCAT activity.

5.2 Introduction

Plant sterols have been shown to decrease plasma total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C) levels in animals (Sugano et al., 1976; 1977; Ikeda et al., 1981; Laraki et al., 1991; Ling and Jones, 1995b) and humans (Lees et al., 1977; Heinemann et al., 1986; Miettinen et al., 1995; Pelletier et al., 1995; Jones et al., 1997) with no known toxicity (Shipley et al., 1958). Previous studies examined the effect of high concentrations of plant sterols (>7 g/day) on plasma lipid levels and showed a reduction of <20% of cholesterol (C) in subjects consuming typical Western diets (Beveridge et al., 1958, Lees et al., 1976; 1977). More recent investigations (Heinemann et al., 1986; Vanhanen et al., 1993; Miettinen et al., 1995) have shown that low levels (1-3 g/day) of sitostanol (saturated β-sitosterol) are as effective as high concentrations of plant sterols in reducing plasma TC levels. Dietary sitostanol was virtually undetectable in the plasma of animals and humans (Sugano et al., 1977; Hassan and Rampone, 1979; Vanhanen and Miettinen, 1992). The mechanism of action by which sitostanol reduces plasma TC concentrations is not fully understood. It has been suggested that plant sterols reduce C absorption in the intestinal tract (Gerson et al., 1964; Ikeda et al., 1988a; Heinemann et al., 1991). However, intrinsic non-absorptive hypocholesterolemic effects of plant sterols have been reported (Konlande and Fisher, 1969; Boberg et al., 1989; Laraki et al., 1993; Shefer et al., 1994). For example, plant sterols altered C esterification rate in hypercholesterolemic subjects suggesting an effect on lecithin:cholesterol acyl transferase (LCAT, EC 2.3.1.43) activity (Weisweiler et al., 1984).

Lecithin:cholesterol acyl transferase enzyme is important in the process of reverse cholesterol transport which catalyzes the esterification of free cholesterol (FC) in plasma (Jonas, 1991). The circulating cholesterol ester (CE) content of lipoproteins relates best to their atherogenicity (Khachadurian, 1990; Frohlich et al., 1990). Moreover, Robins and Fasulo (1997) have recently shown that high density lipoproteins (HDL), but not other lipoproteins, provide the vehicle for sterol transport to bile. High density lipoproteins are a major component of the reverse cholesterol transport system. Thus, whether dietary sitostanol is effective in lowering LCAT activity and reducing atherogenic development needs to be investigated. Accordingly, the current study was conducted to further examine the effects of variable sitostanol concentrations from dietary plant sterol mixtures on plasma lipid levels and plaque development and to determine whether the mechanism of action of sitostanol is due to changes in plasma LCAT activity and C esterification rate (FER) in rabbits. The rabbit model is considered suitable for studying the effects of dietary supplementation on plasma C profile and plaque development in arteries over short periods of time (Constantinides et al., 1960; Caplice et al., 1995). In addition, rabbits fed semi-purified casein based diets or 0.125-0.5% C (w/w)-supplemented chow develop severe atherosclerotic lesions (Daley et al., 1994a; 1994b). We hypothesize that sitostanol, present in the plant sterol mixtures, will decrease C concentrations in plasma, retard plaque accretion and alter plasma LCAT activity in rabbits.

5.3 Materials and methods

5.3.1 Animals and diets

Twenty four male, pathogen-free NZW rabbits (1.6 ± 0.2 kg, 30 to 40 days old;

Charles River Laboratories; Montréal, Canada) were randomized into four groups (n=6). Rabbits were accommodated for two weeks in stainless steel cages with a wire mesh floor. Light was provided from 7:00 a.m. to 7:00 p.m.; the room temperature was maintained between 18 and 21 °C and the humidity was between 50 and 65%. During the first week, rabbits had free access to water and were fed ad libitum a pelleted chow diet (Charles River, Montréal, Canada). Subsequently, transition diet composed of a mixture of 50% (w/w) ground plain chow and 50% (w/w) purified dietary components, was mixed, pelleted and fed to the rabbits for 4 days. During the following two week weaning period, dietary chow content was decreased from 30 to 5% (w/w). Thereafter, rabbits received a 95% (w/w) semi-purified diet for 50 days. Adding the 5% chow to the diet was necessary to improve the diet palatability. Transition and semi-purified diets (ICN Pharmaceuticals, Inc., Aurora, Ohio) were prepared and pelleted in the animal facility (Table 1). The total feeding period was 10 weeks.

Rabbit chow plant sterol composition was measured using gas liquid chromatography (GLC) equipped with a flame ionization detector. Plant sterols in the rabbit chow were mostly constituted of ß-sitosterol and campesterol with traces of stigmasterol. No sitostanol was detected. All rabbits received 0.5% (w/w) C alone (atherogenic control) or this diet with one of three 1% (w/w) plant sterol mixtures derived from (i) soybean (Sigma Chemical Company, St. Louis, MO) containing 0.01% (w/w) sitostanol, (ii) tall oil (Forbes Medi-Tech. Inc., Vancouver, BC) containing 0.2% (w/w) sitostanol or (iii) tall oil (courtesy of Dr von Bergmann, Germany) containing 0.8% (w/w) sitostanol. Soybean-derived plant sterols are similar to tall oil but have negligible sitostanol concentrations. These were included in the study to provide a control for any

effects of the ß-sitosterol and campesterol that may have existed in tall oil-derived plant sterol mixtures. Sitostanol was not trans-esterified and no fatty acids were detected in any of the other plant sterol mixtures. Plant sterols were suspended in a mixture containing equal amounts of coconut, olive and sunflower oil at 60 °C. The atherogenic control group was given the same oil mix but without plant sterols. Food intakes and body weights were measured every three and seven days, respectively. After two months on the semi-purified diets, rabbits were anaesthetized with an intramuscular injection of Ketamine/Xylazine (1.5/0.4 ml/ kg body weight) 20 min before tissue collection. Blood was collected by cardiac puncture. Livers were washed with 0.9% saline and flash-frozen in liquid nitrogen. Collected hearts were perfused and stored in 4% formaldehyde at room temperature. The study was approved by the Animal Ethics Committee of McGill University.

5.3.2 Lipid analyses

Plasma TC, UC and TG were analyzed in duplicate using a VP autoanalyzer with commercial enzymatic kits (Abbott, Montréal, Canada). Plasma HDL-C concentrations were determined in plasma supernatant after precipitation of apo-B lipoproteins. Circulating LDL-C, VLDL-C and VLDL-TG levels were measured in duplicate after ultracentrifugation at 105,000 x g to separate VLDL at a density of <1.006 kg/L as previously described (Chung et al., 1986).

5.3.3 Assessment of atherosclerotic lesions

To measure the extent of plaque formation, hearts were perfused with 0.9% saline

and fixed with a buffered isotonic solution of 4% formaldehyde. The aortic arch of the heart was trimmed from surrounding fat tissues. Sections of 3 microns thickness, cut transversely from the ascending aorta and coronary arteries were embedded with paraffin after drying with water-ethanol (70% to 100% ethanol) and xylene. Sections were stained with haematoxylin and eosin then mounted on glass slides. Frozen sections were also prepared from additional heart sections from the same regions as described above. To determine the type of lesion and its relative fat enrichment, cryostat sections were stained with oil red O and the severity and type of lesions visually graded under a light microscope by blinded observers. The grading system was based on the area stained and coded as follows: grade 0; 0-5%, grade 1; 6-15%, grade 2; 16-33%, grade 3; 34-49% and grade 4; 50-100% (Gore and Tejada, 1957).

5.3.4 Lecithin:cholesterol acyl transferase assay

Lecithin:cholesterol acyl transferase activity and FER of C were measured in vitro using prepared ethanolosomes and plasma substrates labeled with [3H]-C, respectively as described by Dobiasova and Frohlich (1997). Two independent assay controls were tested simultaneously with the samples in order to evaluate the reproducibility of the LCAT assay. The control values obtained were 28.9 nmol/h/ml and 49.4 nmol/h/ml in human and rabbit (chow fed) plasma, respectively. They were similar to normal ranges 26.3 ± 5.9 nmol/h/ml in human (Dobiasova, 1983) and 45 ± 6 nmol/h/ml in animal plasma (Lacko et al., 1974; Dobiasova, 1983), as reported by others. Fractional esterification rate was measured as described previously (Albers et al., 1981; 1984). The radioassay assumes total equilibration of the label with the UC of the plasma lipoprotein

bilayers. Free to esterified C ratio was measured and calculated as reported by Dobiasova and Frohlich (1997).

5.3.5 Tissue plant sterol and cholesterol levels

Concentrations of plasma and hepatic plant sterols and liver C content were measured using GLC (HP 5890 Series II, Hewlett Packard, CA). An internal standard of 5α-cholestane (0.2 mg/ml plasma) was added to plasma, liver tissue and ascending aorta. Plasma and hepatic samples were saponified for 2 hr at 100°C and 16 hr at 80°C, respectively, with 0.5 M methanolic KOH in 15 ml Teflon capped glass tubes. After saponification, samples were left to cool at room temperature before 2.5 ml of distilled water plus 3 ml of petroleum-ether were added to the tubes. Tubes were then vortexed and the upper layer containing the non saponifiable materials transferred to a clean glass tube. This extraction was repeated twice. Combined extracts were dried under nitrogen and resuspended in 300 µl hexane. A sample volume of 2 µl was injected into the GLC. The carrier gas (helium) flow rate was 1.2 ml/min with the inlet splitter set at 10:1. A 30 m Rtx-5 (Restek Corp. Bellefonte, PA) capillary column with an internal diameter of 0.25 mm and film thickness of 0.25 µm was used. Samples were injected at 80°C. The oven temperature remained at 80°C for 1 min after injection, then increased to 285°C (20°C/min) for 20 min. Injector and detector temperatures were 300 and 320°C, respectively. Plant sterols including campesterol, ß-sitosterol and sitostanol were identified using authentic standards (Sigma Chemical Company, St. Louis, MO).

5.3.6 Analysis of data

Data are expressed as mean ± standard deviation (SD). One way analysis of variance (ANOVA) was used for the evaluation of changes in plasma C levels and LCAT activity and Kruskal-Wallis analysis was applied to assess severity of atherosclerosis as they related to the proportion of dietary sitostanol in the plant sterol mixture. Newman-Keuls test was used where significant interaction was observed among different groups. For the calculation of correlation between the content of sitostanol in dietary plant sterol and plasma C concentrations, Pearson correlation coefficient was calculated and the linear regression line of $y = ax + b$ fitted by the method of least squares to data from individual animals. Differences between means were considered statistically significant where $p < 0.05$.

5.4 Results

5.4.1 Food intake, body and liver weights

Food intake over the 10-week period did not differ among the four groups. Rabbits consumed the equivalent of 0, 0.008, 0.16 and 0.64 g/day of sitostanol in the atherogenic control, 0.01, 0.2 and 0.8% (w/w) sitostanol-fed groups , respectively. However, body weight was lower ($p = 0.04$) in 0% (atherogenic control; 2.5 ± 0.3 kg) compared with the 0.01% (2.93 ± 0.9 kg) or the 0.2% (w/w) sitostanol (2.74 ± 0.3 kg) fed rabbits at day 71. Liver weights were not different across the four groups.

5.4.2 Lipid profiles

Table 2 shows the lipid and lipoprotein concentrations obtained in the experiment.

Mean plasma TC, VLDL-C and UC levels were 49%, 63% and 57% lower, respectively, in rabbits supplemented with 0.8% (w/w) sitostanol compared with the atherogenic control group. Mean plasma TC level in the 0.8% (w/w) sitostanol-fed group was 15.2 ± 4.8 mmol/L ($p = 0.006$) versus 29.6 ± 5.52 mmol/L in the atherogenic control and 25.0 ± 5.52 mmol/L in the 0.01% (w/w) sitostanol-fed group. Mean VLDL-C level was lower ($p = 0.007$) in the 0.8% (6.31 ± 3.11 mmol/L) and 0.2% (9.23 ± 3.01 mmol/L) (w/w) sitostanol groups compared to the atherogenic control (17.16 ± 7.43 mmol/L). Mean plasma UC level was higher ($p = 0.04$) in the control (11.11 ± 3.25) than the 0.8% (4.75 ± 1.67 mmol/L) (w/w) sitostanol-treated groups. On the other hand, mean LDL-C levels showed a decrease of 37.3 and 23.6% in the 0.8% (8.06 ± 3.24 mmol/L) and 0.2% (9.81 ± 5.11 mmol/L) (w/w) sitostanol groups, respectively, compared to atherogenic control (12.96 ± 5.32 mmol/L), although these differences were not statistically significant.

Mean plasma HDL-C ($p = 0.37$), TG ($p = 0.51$) and VLDL-TG ($p = 0.33$) levels (Table 3) did not differ across the treatment groups. Dietary sitostanol intake was found to be correlated with plasma TC $r = -0.65$, $p = 0.0014$), LDL-C $r = -0.45$, $p = 0.041$) and VLDL-C $r = -0.60$, $p = 0.007$) concentrations. No such correlations were seen between sitostanol intake and plasma HDL-C, TG or VLDL-TG levels.

5.4.3 Atherosclerotic plaque formation

Average and median atheroma severity for each treatment group are presented in Table 4. The extent of atherosclerotic lesion formation differed across groups. Stained tissue showed less plaque development in the 0.8% (w/w) sitostanol-fed group (Panel a and c) compared to the control group (Panel b) in both the ascending aorta ($p = 0.0014$)

and coronary arteries (p = 0.0004), respectively, as shown in Figure 1. Animals in the 0.8% (w/w) sitostanol group had few or no atherosclerotic lesions. There were no significant differences among the 0.01%, 0.2% (w/w) sitostanol and control groups with regards to atherosclerosis in ascending aorta, coronary arteries or valvular region (Table 4). Foam cells were clearly visible in the intimal wall of both the ascending aorta and coronary arteries in these groups. Considerable intimal proliferation and abundant lipid deposition were also seen in intima of coronary arteries (Panel d).

5.4.4 Lecithin:cholesterol acyl transferase activity and cholesterol esterification rate

Plant sterol mixtures with increasing amounts of sitostanol had no effect (p = 0.24) on LCAT activity, nor did molar esterification rates (MER) of C differ (p = 0.14) across groups (Figure 2). However, the mean FER in plasma was 47% higher (p = 0.045) in the 0.8% (w/w) sitostanol group compared with the atherogenic control (Figure 2). The ratio of EC to UC was not different among groups and showed no correlation with LCAT activity. A correlation of r = -0.64 (p = 0.002) was observed between plasma UC levels and FER.

5.4.5 Tissue plant sterol and cholesterol levels

Plasma and hepatic plant sterol concentrations are shown in Table 5. Campesterol and ß-sitosterol were the only two plant sterols detectable in plasma. Sitostanol was either not observed or found only in trace amounts. Plasma and hepatic campesterol and ß-sitosterol concentrations, adjusted for C levels and presented as µmol/mol C, did not differ across groups. Campesterol levels in hepatic tissue were correlated r = 0.3, p =

0.03) with plasma but not hepatic TC concentrations. Conversely, ß-sitosterol concentrations were independent of both plasma and hepatic C levels. Virtually no plant sterols were detected in either arterial walls or plaque. Cholesterol contents of wet liver in rabbits were between 7 and 32 mg/g. The group fed 0.8% (w/w) sitostanol had the lowest median hepatic C level, although this was not significantly different from those of other groups (Figure 3).

5.5 Discussion

The current study evaluated the effectiveness of increased fractional sitostanol content on plasma lipid and plant sterols levels in rabbits. Dietary supplementation with 0.8% (w/w) sitostanol lowered plasma TC, VLDL-C and UC levels, increased FER and substantially reduced lesion developments in ascending aorta and coronary arteries more than diets consisting of plant sterol mixtures with lower or no sitostanol content. The effect of sitostanol on plasma lipid levels has been largely consistent across various animal (Sugano et al., 1976; 1977; Hassan and Rampone, 1979; Ikeda et al., 1981; Ling and Jones, 1995b; Moghadasian et al., 1997) and human (Heinemann et al., 1986; 1988; 1991; Vanhanen and Miettinen, 1992; Miettinen et al., 1995) studies. Sitostanol supplementation in humans (Jones et al., 1997) and animals (Sugano et al., 1976; Ikeda et al., 1981) over a wide variety of study designs has been shown to reduce plasma TC and LDL-C levels within the range of 5-26% and 10-33%, respectively with no remarkable lowering of plasma TG or raising of HDL-C levels. In keeping with other reports, the present study found that 0.8% (w/w) sitostanol did not alter plasma TG or HDL-C levels (Jones et al., 1997). Conversely, Denke (1995) showed that no changes in plasma lipid

profile occurred in men with moderate hypercholesterolemia who consumed 3 g/day sitostanol in capsules. These conflicting results are probably due to differences in study design. Subjects who participated in Denke's (1995) study were polygenic hypercholesterolemic (PH) and not familial hypercholesterolemic (FH). Patients with FH, conversely to PH, have lower bile acid synthesis rate (Simonen and Miettinen, 1987) which produces a favorable response to dietary plant sterols. Mattson et al. (1977) demonstrated that ß-sitosterol esters of acetate, decanoate, oleate and succinate and stigmasterol oleate have the same ability to lower cholesterol absorption in thoracic duct-cannulated rats. However, recent reports by Vanhanen et al. (1991; 1993; 1994) showed that sitostanol-ester is more efficient in lowering circulating cholesterol levels than unesterified sitostanol. The increased solubility of sitostanol ester in fat might decrease more readily the solubility of cholesterol in micelles compared with crystal or powder sitostanol. In Denke's (1995) investigation, unesterified sitostanol was given as capsules emulsified in safflower oil. In addition, the free living subjects were required to consume 12 capsules per day, a high number which may have led to low patient compliance. Finally, subjects in the latter study were put on a low cholesterol diet meaning that their dietary cholesterol baseline was below that of normal individuals. This might explain the lower efficacy of sitostanol in decreasing plasma C concentrations in these patients.

Although 0.2% (w/w) sitostanol lowered plasma VLDL-C levels, in this study, it did not apparently alter TC and LDL-C concentrations, an effect that has been observed in rats (Ling and Jones, 1995b) and apo-E deficient mice (TC only) (Moghadasian et al., 1997). In spite of the obvious species variation among rats and mice, these two species responded in a very similar manner to lower concentrations of dietary sitostanol (Ling and

Jones, 1995b; Moghadasian et al., 1997). This may not be true in rabbits. For example, insulin resistance in rats lowers their plasma lipid levels (Boivin and Deshaies, 1995; Hettiarachchi et al., 1996), but in Watanabe heritage hyperlipidemic rabbits, insulin resistance contributes to hyperlipoproteinemia and coronary atherosclerosis (Zhang et al., 1994).

Although plasma LDL-C levels were not apparently different between atherogenic control and 0.8% (w/w) sitostanol-fed groups, lesion developments in ascending aorta and coronary arteries were substantially lowered in the 0.8% (w/w) sitostanol-fed group compared with the atherogenic control animals. The descending aorta, including both the thoracic and abdominal aorta, were not examined in this study because the ascending aorta and coronary arteries presented convincing evidence of the atherogenic effect of dietary C as well as the potential benefit of sitostanol supplementation. It is likely that the lower concentration of C (15.2 mmol/L) in the 0.8% (w/w) sitostanol group, relative to other groups (>20 mmol/L), prevented atherosclerotic lesions from developing. Plaque development in rabbits is directly related to dietary C (Anitschkow and Chalatow, 1913; Constantinides et al., 1960). Chao et al. (1994) demonstrated that the amount of C in the aorta of a 3-year-old, chow-fed NZW rabbit was similar to that of the 3-month-old NZW rabbits prior to C feeding. No C accumulation in the aortae of rabbits occurred in the absence of dietary C (Chao et al., 1994). Moreover, in this study, plant sterols were undetectable in arterial walls and plaque deposits suggesting that they did not contribute to plaque regression or development. Thus, the strong association that exists between circulating C levels and plaque size in rabbits, noted by Daley et al. (1994b) may explain the results obtained in the present study.

Unlike the positive correlation between circulating C and plaque development, plasma C levels correlated negatively with dietary sitostanol concentrations. Data from this study suggest that C absorption was more efficiently inhibited in the 0.8% (w/w) sitostanol-fed group than in groups fed no or lower sitostanol concentrations. It has been suggested that plant sterols lower plasma lipid levels by inhibiting intestinal C absorption in animals (Gerson et al., 1964; 1965; Ikeda et al., 1988b) and humans (Heinemann et al., 1991; 1993) through alteration of C uptake (Gerson et al., 1965; Hassan and Rampone, 1979; Ikeda and Sugano, 1983) mainly by reducing micellar solubilization (Child and Kuksis, 1986; Armstrong and Carey, 1987; Tanaka et al., 1993) and thus enhancing C excretion in bile (Bhattacharyya and Eggen, 1984; Armstrong and Carey, 1987).

On the other hand, a number of studies have shown that plant sterols might lower circulating TC concentrations by modifying hepatic acetyl-CoA carboxylase (Laraki et al., 1993), 3-hydroxy-3-methylglutaryl-CoA reductase (Boberg et al., 1989) and/or cholesterol 7-α hydroxylase (Shefer et al., 1994) in animals. Weisweiler et al. (1984) showed an increase in mean FER (+105.7%) and MER (+60.5%) of C in hypercholesterolemic men fed ß-sitosterol. They concluded that ß-sitosterol primarily lowered cholesterol rich lipoproteins with a lower density range than LDL-C via an accelerated esterification rate of the LCAT enzyme. Conversely, the present study showed that the various dietary plant sterol mixtures had no effect on plasma LCAT activity measured in an artificial substrate even though plasma VLDL-C concentrations, which has a lower density range than LDL-C, were 65% less in the 0.8% (w/w) sitostanol-fed group than in the atherogenic control. Another possible explanation for the lack of difference in LCAT activity among groups could be due to the similar HDL levels in each

of the dietary treatments. High density lipoproteins, the main carriers of plant sterols to bile (Robins and Fasulo, 1997), are a key component of the reverse C transport system that also includes LCAT enzyme. Virtually the same plant sterol concentrations were present in circulating HDL suggesting that their potential effects on LCAT activity, if any, are similar in the different groups. The results suggest also that the LCAT protein mass was not altered by the dietary treatment. The LCAT enzyme is unlikely to have been a limiting factor in plasma C esterification rates in rabbits because no difference in LCAT activity among the groups and no correlation between plasma UC concentrations and LCAT activity were observed.

FER was highest in the 0.8% (w/w) sitostanol-fed animals, the group that showed the lowest UC concentration. A negative correlation was shown between FER and plasma UC levels suggesting that the absolute amount of C esterified (FER multiplied by plasma UC pool) was not affected by plasma C concentration. A complete equilibration of label with UC in plasma and an equal rate of exchange of UC among various lipoproteins is assumed. One possible interpretation of this finding is that the [3H]-C was more efficiently dispersed and had equilibrated with the endogenous UC in samples with lower VLDL-C and LDL-C concentrations compared with the hyperlipidemic groups. Consequently, the access of LCAT to the substrate may be hindered in the hyperlipidemic groups resulting in lower FER despite higher levels of UC. Free C transfer from the VLDL and LDL particles to the outer layer of HDL could be also altered by the hyperlipidemic milieu. The results obtained demonstrate that sitostanol lowers plasma TC levels but does not affect LCAT activity in rabbits.

Although increased dietary plant sterol intakes are usually associated with higher

levels of plasma campesterol and ß-sitosterol in rats (Gerson et al., 1964; Hassan and Rampone, 1979), neither circulating campesterol nor ß-sitosterol concentrations, presented as a ratio relative to C, differed across groups. Sitostanol has been demonstrated to decrease both plasma C and campesterol levels in humans (Vanhanen and Miettinen, 1992; Heinemann et al., 1993; Miettinen et al., 1995) without itself being detectable in plasma (Heinemann et al., 1988; 1993). Sitostanol's effect on inhibiting dietary campesterol and possibly ß-sitosterol absorption might explain why plasma and hepatic plant sterol concentrations did not vary in the 0.2 and 0.8% (w/w) sitostanol-fed groups compared with the atherogenic control. Moreover, Vanhanen and Miettinen (1992) suggested that changes in campesterol and ß-sitosterol proportions were positively correlated during sitostanol addition. Data showed a positive correlation between plasma campesterol and hepatic TC levels, but not with plasma TC or ß-sitosterol concentrations. It is unclear why a positive correlation between plasma campesterol and cholesterol levels was not seen in the present animal model. It is possibly a result of abnormal hyperlipidemia observed in these rabbits and the low absorption of campesterol due to the presence of sitostanol in the diet.

In summary, data obtained from our study demonstrated that dietary sitostanol at 0.8% (w/w) concentration or 0.64 g/day, lowered circulating C concentrations and significantly delayed plaque formation in the aorta of hyperlipidemic rabbits. High concentrations of sitostanol increased FER but did not affect LCAT activity in this species.

5.6 Figure legends

Figure 1.

Representative histological sections of aorta (a) from the 0.8% sitostanol fed group (x60). The arrow heads in panels a and c point to the intima of the aorta and coronary artery, respectively, where no fat depositions are visible. The arrow heads in panels b and d point to fatty deposition and foam cells shown in the intima of the aorta and coronary arteries of the control group (enlargements: x60, x120).

Figure 2.

Fractional esterification rate (FER) of free cholesterol and lecithin cholesterol acyl transferase (LCAT) activity in rabbits fed 0%, 0.01%, 0.2% and 0.8% sitostanol (SI). Dashed bars represent molar esterification rate (MER). Values are means ± SD. Columns labeled with different letters are significantly different at $p = 0.0045$, $n = 6$.

Figure 3.

Wet liver cholesterol content in rabbits fed 0%, 0.01%, 0.2% and 0.8% sitostanol (SI). Medians values are presented, $n = 6$.

Table 1. Composition of the semi-purified diet.

	Dietary treatment (% sitostanol w/w)			
Ingredients (% w/w)	control	0.01%	0.2%	0.8%
Casein	30.0	30.0	30.0	30.0
Dextrose	48.0	48.0	48.0	48.0
Cellulose	8.0	8.0	8.0	8.0
Oil mixture	1.0	1.0	1.0	1.0
Rabbit chow	5.0	5.0	5.0	5.0
DI-methionine	0.5	0.5	0.5	0.5
Mineral mixture	4.0	4.0	4.0	4.0
Vitamin mixture	2.0	2.0	2.0	2.0
Choline bitartrate	0.2	0.2	0.2	0.2
Cholesterol	0.5	0.5	0.5	0.5
Sitostanol	ND	0.01	0.2	0.8
ß-sitosterol	ND	0.65	0.63	0.09
Campesterol	ND	0.18	0.16	0.01
Dihydrobrassicasterol	ND	0.11	ND	ND
Unknown plant sterols	0.08	0.03	0.02	0.08
Total plant sterols	0.08	0.98	1.01	0.98
Total %	100%	100%	100%	100%

ND: Not detectable

The vitamins and salt mixture and vitamin-free casein were purchased from ICN Life Science Group, Nutritional Biochemicals Division, Cleveland, OH. Casein provided 30% protein in this low-fat diet. Fat soluble vitamins were added to the oil mix which was a rich source of essential fatty acids. For detailed formulation of the vitamins and mineral mixes, please refer to the company's catalogue.

Table 2. Plasma TC, LDL-C, VLDL-C and U-C levels in rabbits (mmol/L). Values are mean ± SD.

Group	TC	LDL-C	VLDL-C	UC
0% SI	29.6 ± 5.5 **a**	12.9 ± 5.3	17.2 ± 7.4 **a**	11.1 ± 3.3 **a**
0.01% SI	25.0 ± 5.5 **a**	13.5 ± 5.0	10.9 ± 2.4 **ab**	6.8 ± 1.4 **ab**
0.2% SI	19.7 ± 7.5 **ab**	9.8 ± 5.1	9.2 ± 3.0 **b**	7.24 ± 3.9 **ab**
0.8% SI	15.2 ± 4.8 **b**	8.1 ± 3.2	6.3 ± 3.1 **b**	4.75 ± 1.7 **b**
	p = 0.006	**p = 0.203**	**p = 0.007**	**p = 0.04**

Values within each group carrying different letters are significantly different at p given

SI: sitostanol, TC: total cholesterol, LDL-C: low density lipoprotein, VLDL-C: very low density lipoproteins, UC: unesterified cholesterol.

Table 3. Plasma HDL-C, TG and VLDL-TG levels in rabbits (mmol/L).
Values are mean ± SD.

Group	HDL-C	TG	VLDL-TG
0% SI	0.72 ± 0.44	1.18 ± 0.61	0.88 ± 0.57
0.01% SI	0.67 ± 0.25	1.10 ± 0.55	0.64 ± 0.35
0.2% SI	0.72 ± 0.21	0.96 ± 0.66	0.62 ± 0.35
0.8% SI	0.96 ± 0.31	0.68 ± 0.22	0.43 ± 0.10
	p = 0.37	**p = 0.51**	**p = 0.33**

SI: sitostanol, HDL-C: high density lipoproteins, TG: triglycerides, VLDL-TG: very low density lipoproteins-triglycerides.

Table 4. Grade of atheroma in ascending aorta and coronary arteries.

Group	No. Of rabbits	Grade of atheroma in aorta 0	1	2	3	4	Average atheroma		Median	25%	75%
0% SI	6	-	-	3	2	1	2.83	**a**	2.5	2	3
0.01% SI	6	-	1	5	-	-	1.83	**ab**	2.0	2	2
0.2% SI	6	-	1	3	2	-	2.17	**a**	2.0	2	3
0.8% SI	6	4	2	-	-	-	0.34	**b**	0.0	0	1
									p = 0.0014		
		Grade of atheroma in coronary arteries									
0% SI	6	-	-	1	3	2	3.00	**a**	3	3	4
0.01% SI	6	-	-	1	3	2	1.83	**ab**	3	3	4
0.2% SI	6	-	1	5	-	-	2.67	**a**	2	2	2
0.8% SI	6	3	3	-	-	-	0.50	**b**	0.5	0	1
									p = 0.0004		

Values within each group carrying different letters are significantly different at p given.

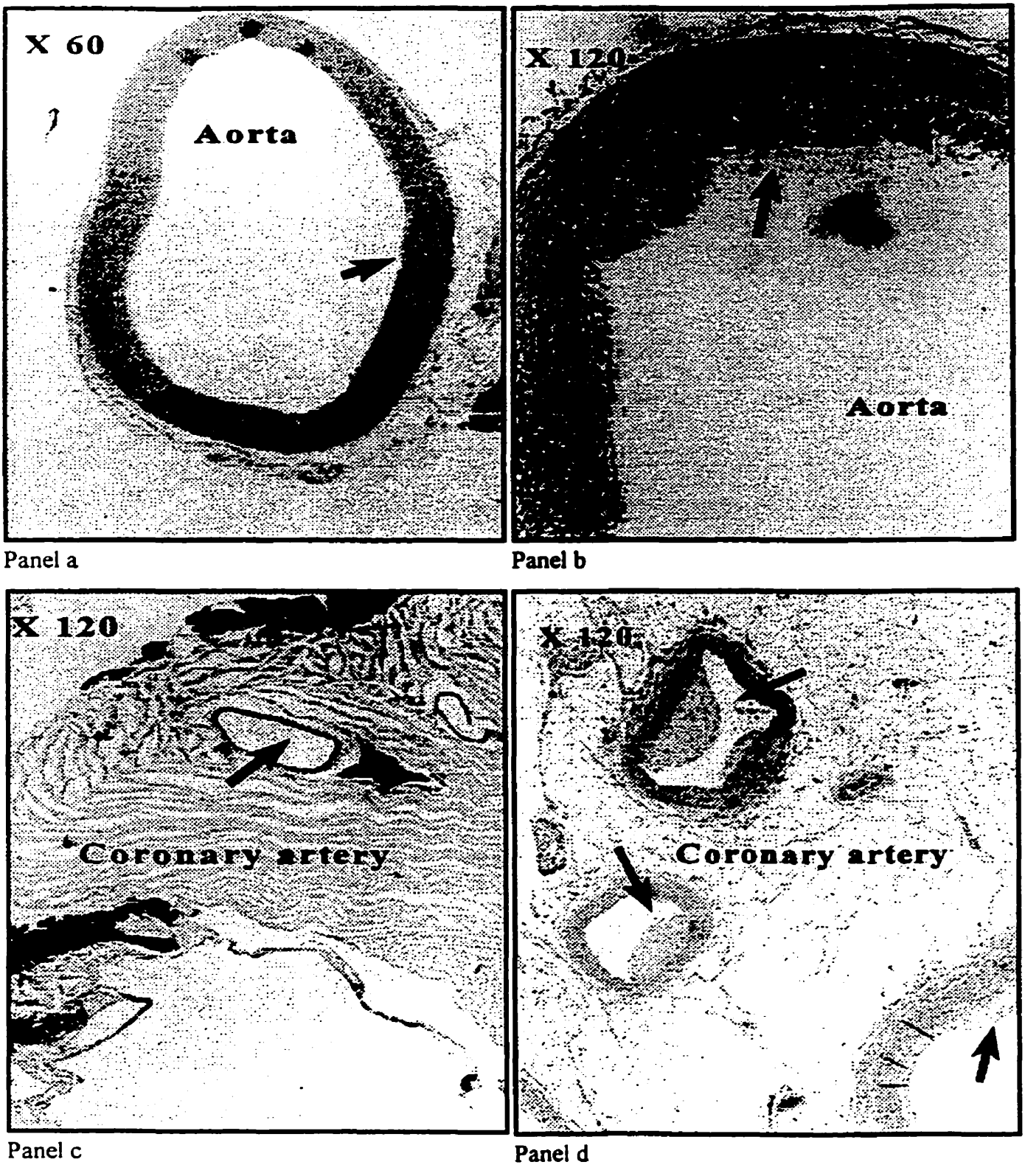
The grading system was based on the area stained and coded as follow: grade 0; 0-5%, grade 1; 6-15%, grade 2; 16-33%, grade 3; 34-49% and grade 4; 50-100%.

SI: sitostanol.

Table 5. Plasma and hepatic plant sterol levels in rabbits (μmol/mol cholesterol). Values are mean ± SD.

Group	Plasma campesterol	Hepatic campesterol ($x10^3$)	Plasma β-sitosterol	Hepatic ß-sitosterol ($x10^3$)
0% SI	142.0 ± 97.7	13.9 ± 8.3	196.0 ± 164.6	73.7 ± 47.8
0.01% SI	123.1 ± 53.6	27.5 ± 24.2	203.2 ± 90.4	77.1 ± 42.9
0.2% SI	171.0 ± 20.1	55.1 ± 48.9	107.2 ± 60.5	67.1 ± 45.6
0.8% SI	128.7 ± 43.8	16.3 ± 15.7	193.6 ± 97.7	64.1 ± 21.6
	p = 0.15	**p = 0.60**	**p = 0.41**	**p = 0.94**

Sitostanol was undetectable or found in trace amounts in only a few rabbits. SI: sitostanol.



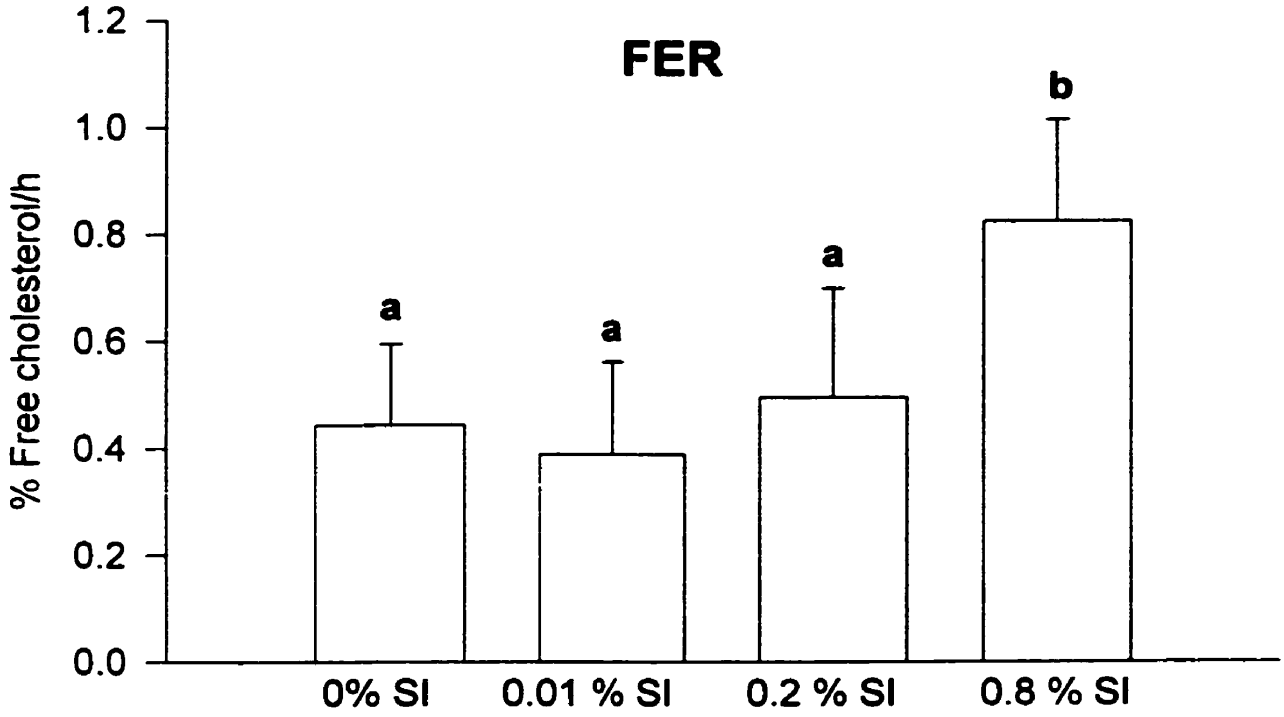
Panel a

Panel b

Panel c

Panel d

Figure 1


FER
% Free cholesterol/h
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
a
a
b
0% SI
0.01 % SI
0.2 % SI
0.8 % SI

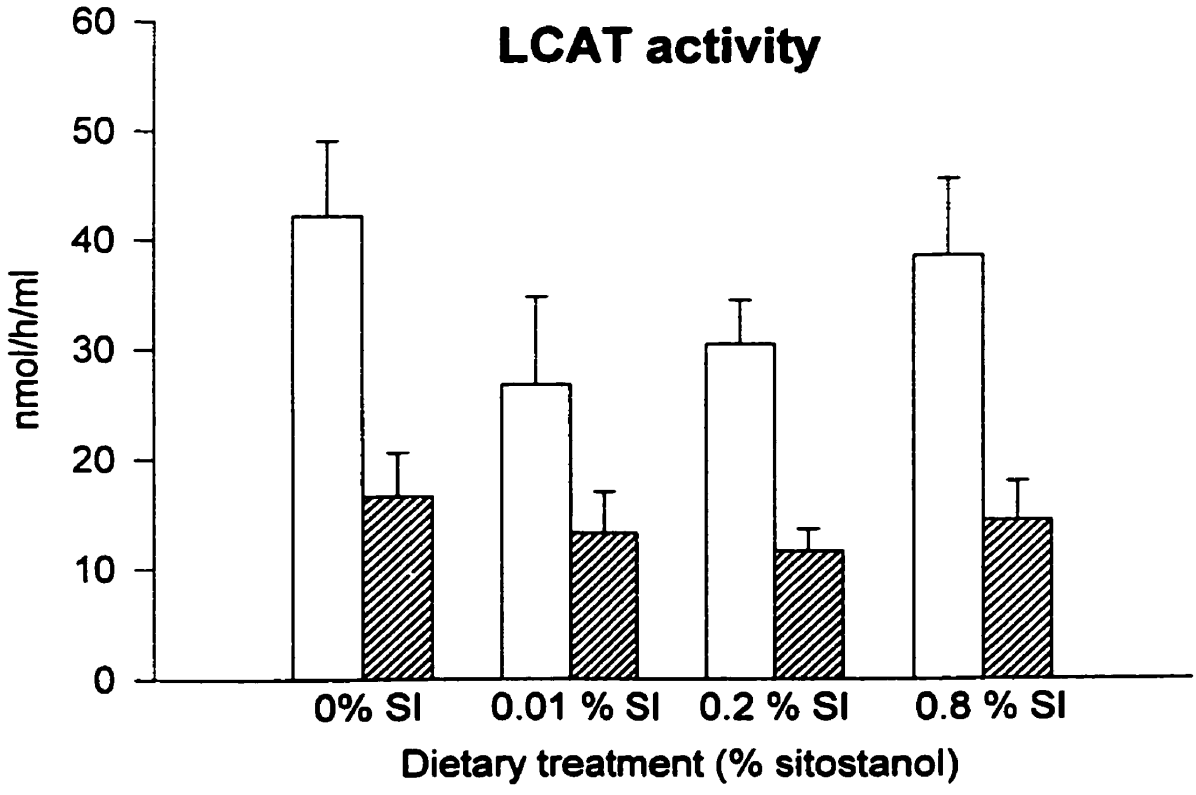

LCAT activity
nmol/h/ml
60
50
40
30
20
10
0
0% SI
0.01 % SI
0.2 % SI
0.8 % SI
Dietary treatment (% sitostanol)

Figure 2

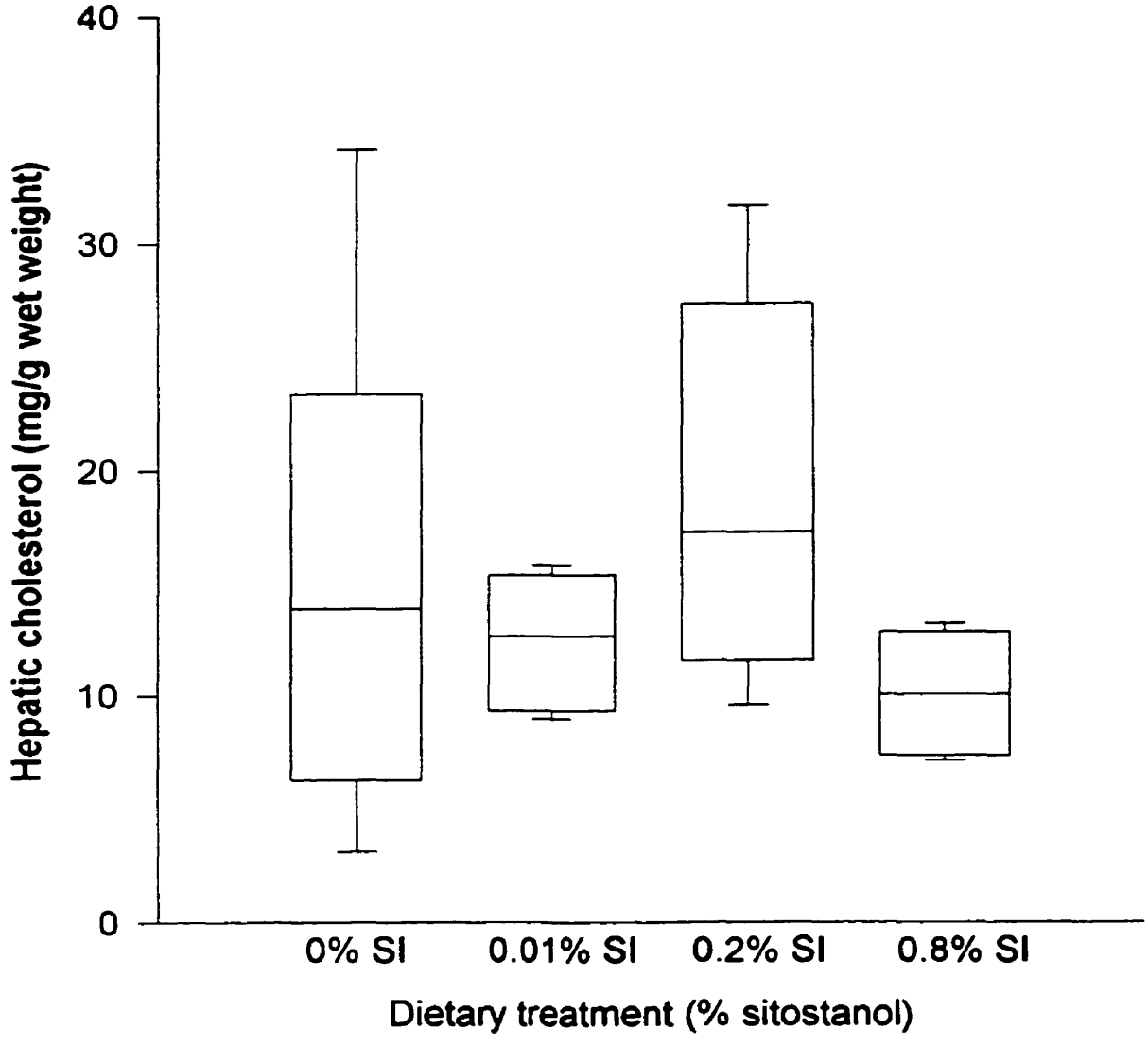

40
30
20
10
0
Hepatic cholesterol (mg/g wet weight)
0% SI
0.01% SI
0.2% SI
0.8% SI
Dietary treatment (% sitostanol)

Figure 3

TRANSITION

The previous manuscript demonstrated that sitostanol is effective in lowering plasma lipid levels and delaying plaque development but does not affect plasma LCAT activity in rabbits. The response of rabbits to phytosterol mixtures was similar to that of hamsters. This observation proves that the sitostanol effect is consistent across two animal models. The study involving the hamsters and rabbits consolidated the lipid lowering impact of phytosterol mixtures and, more precisely, that of sitostanol. However, the mechanisms of action were not examined and the phytosterol effects on cholesterol absorption, excretion and cholesterogenesis were not determined. Consequently, the second part of the study was to measure these variables. In the following manuscript, we included data from both hamster and rabbit studies in one chapter and discussed the results obtained.

CHAPTER 6

DIETARY SITOSTANOL DECREASES CHOLESTEROL ABSORPTION AND INCREASES EXCRETION AND SYNTHESIS RATES IN HAMSTERS AND RABBITS USING DUAL ISOTOPE RATIO AND DEUTERIUM INCORPORATION METHODS

Paper submitted to *Atherosclerosis (1998).*

6.1 Abstract

The aim of this study was to examine the efficacy of variable dietary sitostanol (SI) concentrations on cholesterol absorption, synthesis and excretion rates in two animal models. Hamsters and rabbits were fed semi-purified diets supplemented with cholesterol and 1% (w/w) phytosterols containing either 0.01, 0.2 or 1% (w/w) SI. The control (0% (w/w) SI) groups consumed the same diets but no phytosterols were added. The dual-isotope plasma ratio of ^{13}C- and ^{18}O-cholesterol and deuterium incorporation methods were applied to measure simultaneously cholesterol absorption and fractional synthesis, respectively. Plasma cholesterol levels were lower in hamsters and rabbits fed 1% (w/w) SI as compared to their controls. Percent cholesterol absorption was lower ($p = 0.03$) in hamsters fed 1% (w/w) SI (42.5% ± 13.3) than control (65.1% ± 13.4). Moreover, cholesterol excretion in the feces was 77 and 57% higher ($p = 0.017$) in the 1% (w/w) SI- relative to control- and 0.2% (w/w) SI-fed groups, respectively. In rabbits, cholesterol excretion was 64% higher ($p = 0.018$) in 1% (w/w) SI- compared with control-fed groups. Fractional synthesis rate was higher ($p = 0.033$) in hamsters fed 1% (w/w) SI (0.116 ± 0.054) as compared to control (0.053 ± 0.034), however, cholesterol synthesis rates did not vary among groups fed variable concentrations of SI. In rabbits, percent cholesterol absorption and its fractional synthesis rate varied but did not reach significance. Plasma total-cholesterol levels in hamsters ($r = 0.33$, $p = 0.02$) and rabbits ($r = 0.4$, $p = 0.059$) correlated with percent cholesterol absorption. Similarly, fractional synthesis rate in hamsters was correlated ($r = -0.32$, $p = 0.03$) with percent cholesterol absorption. In conclusion, dietary SI exhibited a dose-dependent action in inhibiting cholesterol absorption while increasing cholesterol excretion and biosynthesis in hamsters resulting in lower circulating lipid levels.

6.2 Introduction

Phytosterols, derived from plant sources (Weihrauch and Gardner, 1978) and not synthesized in humans (Salen et al., 1970) have been suggested as promising compounds in the dietary treatment of mild hyperlipidemia (Heinemann et al., 1988; Miettinen et al., 1995; Jones et al., 1997). No side effects have been reported with their usage (Pollak and Kritchevsky, 1981). Sitostanol (SI), the saturated derivative of ß-sitosterol, is not present in significant amounts in plants (Weihrauch and Gardner, 1978; Dutta and Appelqvist, 1996) and thus is virtually absent from typical North American diets. Phytosterol mixtures extracted from tall oil (TO), however, contain about 20% (w/w) SI (Ling and Jones, 1995). Although ß-sitosterol lowers plasma cholesterol levels significantly (Lees et al., 1977), SI is more efficient in reducing circulating cholesterol concentrations than is ß-sitosterol in animals (Sugano et al., 1977; Ikeda et al., 1979; 1989) and humans (Heinemann et al., 1986; 1988).

The mechanisms of action of phytosterol mixtures on plasma total-cholesterol (TC) concentrations are still not well-characterized, in remarkable contrast to the numerous published data (Sugano et al., 1977; Heinemann et al., 1986; 1988; Miettinen et al., 1995) of the effects of phytosterols on plasma lipid profile. Several mechanisms of action of phytosterols on lipid metabolism have been suggested. For instance, ß-sitosterol and SI reduced plasma TC levels by competitively blocking cholesterol absorption from the intestinal lumen (Heinemann et al., 1986; 1988; 1993); displacing cholesterol from bile salt micelles (Child and Kuksis, 1986); increasing bile salt excretion (Salen et al., 1970) or by hindering cholesterol esterification rate in the intestinal mucosa (Ikeda and Sugano, 1983; Child and Kuksis, 1983). Other uncertainties regarding the mechanisms

by which phytosterols lower plasma TC are shown through reports that suggest that ß-sitosterol lowers plasma cholesterol levels while simultaneously either stimulating (Gerson et al., 1964; 1965; Grundy et al., 1969; Konlande and Fisher, 1969); inhibiting (Kakis and Kuksis, 1984) or exhibiting no effect (Subbiah and Kuksis, 1973) on cholesterol synthesis. The lack of agreement in the results of the different experiments warrants further investigation of the action of phytosterols on cholesterol absorption, excretion and synthesis rates measured simultaneously.

Accordingly, the aim of this study was to examine concurrently the changes in cholesterol absorption, excretion and synthesis in hamsters and rabbits fed variable SI concentrations using ^{13}C- and ^{18}O-cholesterol isotope-plasma ratio, fecal elimination and deuterium uptake methods, respectively.

6.3 Materials and methods

6.3.1 Diet preparation and accommodation

a. Hamsters

Fifty GS hamsters weighing 80-100 g (Charles River Laboratories; Montréal, QC, Canada) and aged 48 days, were randomly divided into five groups. Hamsters were acclimatized for two weeks in stainless steel colony cages with wire mesh floors. The light period of 12 hr began at 11:00 a.m. Room temperature was set between 18 and 21 °C. During accommodation, hamsters had free access to water and were fed *ad libitum* a plain, pelleted chow diet (Charles River Laboratories, Montréal, Canada). Animals were then switched to semi-purified diets (ICN Pharmaceuticals, Inc., Aurora, Ohio, USA) prepared every two weeks and stored at -80°C. Dietary composition is shown in

Table 1. All diets contained 0.25% (w/w) cholesterol while non-control diets also contained one of the following four mixtures of 1% (w/w) purified phytosterols: (i) soybean (0.01% (w/w) SI; Sigma Chemical Company, St. Louis, MO, USA), (ii) tall oil (0.2% (w/w) SI; Forbes Medi-Tech. Inc., Vancouver, BC, Canada), (iii) sitostanol (1% SI; Sigma Chemical Company, St. Louis, MO, USA) or (iv) a synthetic mixture prepared to resemble tall oil (0.2% (w/w) SI; Sigma Chemical Company, St. Louis, MO, USA). The synthetic mixture was similar to tall oil-derived phytosterols but contained dihydrobrassicasterol so that the potential effect of this phytosterol could be examined.

b. Rabbits

Twenty-four male, pathogen-free NZW rabbits (1.6 ± 0.2 kg, 30 to 40 days old; Charles River Laboratories; Montréal, QC, Canada) were randomized into four groups based on weight. Rabbits were accommodated for two weeks in stainless steel cages. Light period was from 7:00 a.m. to 7:00 p.m.; room temperature was between 18 and 21 °C and humidity was between 50 and 65%. During the first week, rabbits had free access to water and were fed ad libitum a pelleted chow diet (Charles River, Montréal, QC, Canada). Transition and semi-purified diets (ICN Pharmaceuticals, Inc., Aurora, Ohio, USA) were prepared and pelleted in the animal facility (Table 2). The transition diet, a mixture of 50% (w/w) ground plain chow and 50% (w/w) purified dietary components, was mixed, pelleted and fed to the rabbits for 4 days. Subsequently, during the weaning period of two weeks, dietary chow content was decreased from 30 to 5% (w/w). Afterwards, rabbits received a 95% (w/w) semi-purified diet for 50 days. Adding the 5% chow to the diet was necessary to improve the diet palatability. Phytosterols in

rabbit chow consisted mostly of ß-sitosterol and campesterol with traces of stigmasterol. No SI was detected in the chow

All rabbits received 0.5% (w/w) cholesterol alone (control) or this diet with one of three 1% (w/w) plant sterol mixtures derived from: (i) soybean (0.01% (w/w) SI; Sigma Chemical Company, St. Louis, MO, USA), (ii) tall oil (0.2% (w/w) SI; Forbes Medi-Tech. Inc., Vancouver, BC, Canada) or (iii) sitostanol-mix (0.8% (w/w)) SI; courtesy of Pr. von Bergmann, Germany). Soybean-derived phytosterol mixtures have the same composition as tall oil but contain a negligible SI concentration and significant amount of dihydrobrassicasterol. The rabbit study lasted for 71 days.

For both experiments, phytosterol compositions were measured using gas liquid chromatography (GLC) (HP 5890 Series II, Hewlett Packard, CA, USA) equipped with a flame ionization detector. Phytosterols were not trans-esterified and no fatty acids were detected in any of the mixtures. Phytosterols were suspended in a mixture of equal amounts of coconut, olive and sunflower oil at 60°C. The control group was given the same oil-mix but without phytosterols. Food intakes and body weights were measured every 3 and 7 days, respectively. The study was approved by the Animal Ethics Committee of McGill University.

6.3.2 Intravenous and intra-gastric administration of labeled cholesterol

a. Hamsters

At day 42 and 72 hr before the end of the trial, hamsters were intravenously (i.v.) injected with 0.18 mg ^{13}C-cholesterol contained in 0.5 ml Intralipid (20%, Kabi Pharmacia Inc., Clayton, NC, USA) and followed directly by intra-gastric (i.g.) injection

of 0.44 mg ^{18}O-cholesterol suspended in 0.6 ml of equal amounts of coconut, olive and sunflower oil-mix as reported previously (Turley et al., 1994). Preparations of labeled cholesterol mixtures were described elsewhere (Turley et al., 1994). Hamsters were anaesthetized with halothane and a small incision was made next to the jugular vein where the Intralipid was slowly injected using a calibrated plastic tuberculin syringe fitted with a 30G needle. The incision was then closed with sterilized surgery staples. After that, each hamster was immediately administered i.g. the ^{18}O-cholesterol in the oil-mix. The handling time between i.v. and i.g. injection was about 10 min. Hamsters were then returned to their cages and provided their diets and water as usual. At day-45 of the trial, animals were intraperitoneally injected with 0.4 ml deuterium oxide (99.8% atom percent excess) 1 hr before anaesthetization with halothane and sacrificed by cardio-puncture. Hamsters were deprived of food and water for 1 hr time before sacrificing.

b. Rabbits

Rabbits were handled similarly as reported above for hamsters. Rabbits were injected i.v. 3.4 mg ^{13}C-cholesterol in 1 ml Intralipid in their ear-vein after warming and dilating the vein. Then, 8.6 mg ^{18}O-cholesterol in 2 ml oil mix were administered i.g. Three hr before sacrificing, rabbits were intraperitoneally injected with 2.5 ml deuterium oxide (99.8% atom percent excess). They were deprived of food and water during the 3-hr period. Rabbits were anaesthetized with an intramuscular injection of Ketamine/Xylazine (1.5/0.4 ml/ kg body weight) 20 min before killing by cardio-puncture.

The effects of handling-time and the vehicle used to administer labeled cholesterol on cholesterol absorption rates were considered either negligible or at least similar across the different treatments (Turley et al., 1994). The interval between i.v. injection and i.g. administration was about 7-10 min. This time difference between the two isotopes injections is minimal when compared with the 72-hr equilibration period.

The purity of labeled cholesterol and its respective tracer enrichment with ^{13}C or ^{18}O were determined by gas chromatography-negative ion chemical ionization mass spectrometry (GC-MS) (HP 5988, Hewlett Packard, CA, USA). Pentafluorobenzoyl chloride, pyridine and toluene (5:20:200; v:v:v) were added to pure cholesterol. Later, derivatized cholesterol was extracted with petroleum ether, dried under nitrogen and resuspended in toluene (Ostlund et al., 1996). Samples were injected into the GC-MS equipped with a SAC-5 capillary column (Supelco, Bellefonte, PA, USA).

6.3.3 Determination of plasma lipid profile and percent cholesterol absorption

In both animals, blood samples were collected by cardio-puncture and RBC were separated from plasma and stored at -80°C. Plasma total-, LDL-, non-apolipoprotein-A and HDL-cholesterol and TG levels were measured in duplicate using a VP autoanalyzer with commercial enzymatic kits that precipitated the apo-B containing lipoproteins (Abbott, Montréal, QC, Canada). Measurements of phytosterol levels in hamsters (Ntanios and Jones, 1998a) and rabbits (Ntanios et al., 1998b) have been reported previously. Briefly, plasma and liver tissues were saponified with 0.5M methanolic KOH and the unsaponifiable materials were extracted with petroleum ether. Samples were dried under nitrogen and afterwards resuspended in hexane and injected into GLC.

Free cholesterol extracted from RBC was used to determine ^{13}C- and ^{18}O-cholesterol enrichment. Briefly, lipids were extracted from RBC in duplicate and thin layer chromatography (20 x 20 cm, 250 mm, Whatman Inc, Clifton, NJ, USA) was used to separate free cholesterol from cholesteryl esters. The free cholesterol band was scraped from the silica gel plate and saponified with 0.5 M methanolic KOH to eliminate any TG 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 (0.5 g) and a 2-cm length of silver wire were added and tubes were sealed under vacuum at less than 20 mtorr pressure. Cholesterol samples were combusted for 4 hr at 520°C. The generated CO_2 was transferred under vacuum into Vycor tubes and thereafter, water was vacuum-distilled into sealed tubes containing 60 mg zinc reagent (Biogeochemical Laboratories Indiana University, Bloomington, IN, USA). The water was used for measurement of deuterium incorporation as described below.

The ^{13}C and ^{18}O enrichments of free cholesterol were measured by differential isotope ratio mass spectrometry (IRMS) using a triple inlet system with electrical H^{3+} compensation (SIRA 12, Isomass, Cheshire, UK). Mean internal and external precision (SD) levels of the IRMS were 0.18 and 2.0 per mil ($^{o}/_{oo}$), respectively. The instrument was calibrated using CO_2 and standards of known isotopic composition. Samples for each animal were analyzed concurrently using a single set of standards. Baseline enrichments of ^{13}C- and ^{18}O-cholesterol in RBC were measured in non labeled animals. The amount of the combusted free cholesterol (4 - 8 mg/sample) provided sufficient gas to be measured by the IRMS. Standards with different enrichment levels at 0, 1, 2, 8 and 20 mmol:mol ^{18}O:^{16}O-cholesterol were measured, and a regression line of $r = 0.9989$ ($p < 0.0001$) and a

slope a = 2.33 were obtained (Figure 1). Figure 1 suggests a constant rate of oxygen incorporation into CO_2 during combustion, and shows that at the current concentrations at room temperature, exchange of oxygen between CO_2 and H_2O was nonexistent or at least nondetectable.

The ^{13}C and ^{18}O enrichment levels in baseline control samples were utilized to calculate percent cholesterol absorption using the ratio of orally ingested ^{18}O- to intravenously administered ^{13}C-cholesterol at a single time point as described by Bosner et al. (1993) (Eq. 1):

$$\text{Cholesterol absorption (\%)} = \left[\frac{\delta\ ^{18}O}{\delta\ ^{13}C}\right] X \left[\frac{\text{i.g. dose of } ^{18}\text{O-cholesterol}}{\text{i.v. dose of } ^{13}\text{C-cholesterol}}\right] X\ 100$$

where $\delta(^{o}/_{oo})$ of either ^{18}O or ^{13}C is the difference between the enriched sample and that of natural abundance. The dilution factors of ^{18}O and ^{13}C during cholesterol combustion and their natural abundance in the organic compounds were corrected for as follows:

The combustion reaction of cholesterol follows an oxidation-reduction pathway where alkenes and alkanes are oxidized by oxygen derived from the CuO. The first step leads to the breakdown of all double bonds in the molecule and further to the development of free radical compounds. Usually, free radicals are unstable and they disintegrate quickly in an unknown mechanism to produce CO_2 and H_2O. The presence of silver is important as a catalyst in the reaction.

$$76\ CuO + C_{27}H_{46}O \xrightarrow{Ag} 23\ H_2O + 27\ CO_2 \qquad \text{(Eq. 2).}$$

The linear curve (Figure 1) of known enrichments (r = 0.9989, slope a = 2.33) suggests that at the current concentrations, the labeled oxygen on the cholesterol molecule is

exclusively incorporated into CO_2. Hence, for 27 CO_2 molecules 53 oxygens are derived from CuO and one oxygen from cholesterol. The combustion of ^{13}C-cholesterol will produce 26 unlabeled CO_2 and one $^{13}CO_2$. The natural abundance of ^{13}C and ^{18}O were corrected for automatically by the IRMS computer when measuring their consecutive enrichments in the samples.

6.3.4 Determination of cholesterol synthesis

Cholesterol biosynthesis was determined as the rate of incorporation of deuterium from body water into sterol. Labeled water equilibrates quickly with intracellular and extracellular water body pools and permits direct determination of cholesterol formation rates (Jeske and Dietschy, 1980). Deuterium enrichment was measured in liver and RBC free cholesterol, and plasma water. Lipid extraction was performed in duplicate as described elsewhere (Jones et al., 1988; 1992).

To measure deuterium enrichment of plasma water, additional samples were diluted 40 fold with water to reduce deuterium enrichment to within the normal analytical range. Baseline samples were not diluted. Duplicate samples were vacuum-distilled into zinc-containing Vycor tubes and afterwards, cholesterol and plasma water samples were reduced to hydrogen gas at 520 °C for 30 min. The deuterium enrichments of cholesterol and plasma samples were measured by differential IRMS using a triple inlet system with electrical H^{3+} compensation (VG Isomass 903D, Cheshire, UK). Fractional synthetic rate (FSR) values were calculated as previously described (Jones et al., 1988; 1992). FSR values were normalized to a rate reflecting a 24-hr period.

6.3.5 Determination of cholesterol excreted in feces

Cholesterol concentrations in feces were measured by GLC. Briefly, an internal standard of 5α-cholestane (0.2 mg/g feces) was added to each sample. Samples were saponified for 1 hr at 100°C with 0.5 M methanolic KOH in 15 ml Teflon capped glass tubes. After saponification, samples were left to cool at room temperature, then mixed with distilled water and petroleum-ether. The upper layer containing the non-saponifiable materials was transferred to a clean glass tube. The extraction was repeated twice. Extracted samples were dried under nitrogen and resuspended in 300 µl of hexane. A sample volume of 2 µl was injected into the GLC. A 30 m SAC-5 capillary column with an internal diameter of 0.25 mm and film thickness of 0.25 µm was used (Supelco, Bellefonte, PA, USA). The oven temperature was 285°C. Injector and detector temperatures were set at 300°C and 320°C, respectively. The carrier gas (helium) flow rate was 1.2 ml/min with the inlet splitter set at 10:1. Phytosterol (campesterol, ß-sitosterol and SI) peak identification was confirmed using authentic standards (Sigma Chemical Company, St. Louis, MO, USA).

6.3.6 Analysis of data

All data were expressed as mean ± standard deviation (SD). Data were tested for normality by Kolmogorov-Smirnov test. One way analysis of variance (ANOVA) was used to determine whether phytosterols altered cholesterol absorption, excretion and synthesis in hamsters and rabbits. The Newman-Keuls test was adopted where significance was observed for interaction measurement among treatment groups. For the correlation measurement between cholesterol absorption, excretion and synthesis, the

linear regression line of $y = ax + b$ was fitted by the method of least squares to the data from individual animals. The differences between the means were considered statistically significant if p value was < 0.05.

6.4 Results

6.4.1 Hamsters

a. Lipid and phytosterol profiles

Plasma lipid and phytosterol profiles, as well as hepatic cholesterol content, food consumption and body weights have been reported previously elsewhere (Ntanios and Jones, 1998a). Briefly, no significant differences were observed in food consumption ($p = 0.130$) or body weight ($p = 0.43$) across treatment groups. Daily food intakes across groups ranged from 8.8 to 9.3 g. During the 45-day trial, hamsters gained between 25 and 40 g in body weight. Mean liver weights of 0.01 and 1% (w/w) SI-fed hamsters were 15% and 20% lower ($p = 0.012$) than that of the control group, respectively. Cholesterol content in liver was significantly higher ($p < 0.0001$) in the control group compared to all other groups. Dietary SI at 1% (w/w) supplementation decreased total and non-apolipoprotein-A-cholesterol levels in plasma by 34% ($p = 0.001$) and 55% ($p = 0.04$), respectively, whereas mean plasma TC level in the tall oil (0.2% (w/w) SI group was 23% ($p = 0.001$) lower than that of the control (0% (w/w) SI). However, the effect of 0.2% (w/w) SI on plasma lipid profile did not differ from 0.01% (w/w) SI.

Plasma campesterol levels were higher ($p = 0.04$) in the 0.01 and 0.2% (w/w) SI (tall oil) fed-hamsters than in the control, 0.2% (w/w) (artificial mix) and 1% (w/w) SI groups. Hepatic sitosterol content was elevated ($p= 0.002$) in the artificial-mix-fed

animals (0.2% (w/w) SI) compared to other groups. A negative correlation ($r = -3.0$, $p = 0.046$) was demonstrated between plasma TC levels and percent SI concentrations in phytosterol mixtures.

b. Cholesterol absorption and synthesis

Percent cholesterol absorption was significantly lower ($p = 0.03$) in hamsters fed 1% (w/w) SI (42.5.% ± 13.3) as compared to control (65.1% ± 13.4) (Figure 2). No difference was, however, observed between 1% (w/w) SI-fed animals and all phytosterol supplemented groups. Similarly, cholesterol excretion in the 1% (w/w) SI-fed animals was 77 and 57% higher ($p = 0.017$) than control and artificial-mix (0.2% (w/w) SI) groups, respectively (Figure 3). No differences in cholesterol excretion were noticed between 0.2, 0.01 and 1% (w/w) SI-fed hamsters. Fractional synthesis rate of hepatic free cholesterol was higher ($p = 0.033$) in the 1% (w/w) SI (0.12 ± 0.05 day^{-1}) compared to control (0.06 ± 0.02 day^{-1}) group (Figure 4). Furthermore, FSR determined from RBC-free cholesterol exhibited similar profile to that of hepatic FSR but was border line significant ($p = 0.052$). No significant changes in hepatic or RBC FSRs were identified between the phytosterol fed-groups. The correlation ($r = 0.033$, $p = 0.02$) between plasma TC levels and percent cholesterol absorption was significant (Figure 5). However, the correlations between TC and percent FSR (Figure 6), between percent FSR and percent absorption (Figure 7), between percent cholesterol excretion and percent absorption, and percent FSR and percent excretion of cholesterol did not reach significance.

6.4.2 Rabbits

a. Lipid and phytosterol profile

Data regarding plasma lipid levels, phytosterol concentrations, hepatic cholesterol content, lecithin cholesterol acyl transferase activity (LCAT), plaque development in the coronary arteries, food intake and body weights have been presented previously (Ntanios et al., 1998b). Briefly, rabbits fed the semi-purified diet supplemented with 0.8% (w/w) SI had lower TC ($p = 0.006$) (15.2 ± 4.80 mmol/L) and very low density lipoprotein-cholesterol ($p = 0.007$) (6.31 ± 3.11 mmol/L) levels compared to the control group (29.6 ± 5.52 and 17.16 ± 7.43 mmol/L, respectively). Dietary SI at 0.8% (w/w) depressed plaque accretion in coronary arteries ($p = 0.0014$) and ascending aorta ($p = 0.0004$) compared to control, 0.01 and 0.2% (w/w) SI-fed groups. No differences ($p = 0.24$) in the activity of LCAT were observed across groups, although plasma cholesterol fractional esterification rate was higher ($p = 0.004$) in the 0.8% (w/w) SI fed animals relative to control. Significant negative correlations were demonstrated between SI intake and plasma TC, LDL-C and VLDL-C levels. Hepatic campesterol levels were positively correlated with plasma but not hepatic TC concentrations.

b. Cholesterol absorption and synthesis

Results observed in rabbits exhibited a similar trend of effects to those observed presently in hamsters. Rabbits, however, showed larger variability in values within each group. Rabbits supplemented with 0.8% (w/w) SI had lower mean percent cholesterol absorption (49.9% ± 26.8) versus control (78.1% ± 14.1) and more than double the mean FSR (0.044 ± 0.021 day^{-1}) than control (0.019 ± 0.024 day^{-1}), but values did not reach

significance (Figure 2 and 4). The 0.8% (w/w) SI-fed rabbits had higher ($p = 0.018$) cholesterol excretion compared to all other groups (Figure 3). A positive correlation ($r = 0.4$, $p = 0.059$), however, was observed between plasma TC and percent cholesterol absorption (Figure 5). Conversely, the correlation ($r = -0.35$, $p = 0.09$) between percent FSR and percent cholesterol absorption did not reach significance (Figure 7).

6.5 Discussion

The current study is the first report applying the dual isotope-plasma-ratio and deuterium uptake methods to measure simultaneously cholesterol absorption and *de novo* cholesterogenesis in two species. Labeled ^{13}C- and ^{18}O-cholesterol and deuterium oxide were chosen because of their safety and potential use in humans (Wong et al., 1995; Jones et al., 1998). Hamsters fed 1% (w/w) SI had lower cholesterol absorption rates, higher cholesterol excretion and FSR compared to control. No differences in plasma lipid profile and cholesterol metabolism were observed, however, between 1% (w/w) SI-fed hamsters and those provided with 0.01 and 0.2% (w/w) SI-phytosterol. Similar trends seen in hamsters were observed in rabbits. However, due to inter-animal large variability statistically significant differences were not attained. Nonetheless, it was of interest, in our opinion, to observe a similar pattern of response in rabbits as seen in hamsters.

As it has been reported previously from these two experiments (Ntanios and Jones, 1998a; Ntanios et al., 1998b), SI supplementation at 0.8-1% (w/w) was more effective than 0.01 and 0.2% (w/w) SI in lowering plasma lipid levels in hamsters and rabbits. The lipid lowering effects of 0.8-1% (w/w) SI is in accord with works reported elsewhere in animals (Sugano et al., 1976; Ikeda et al., 1979; 1981) and humans (Heinemann et al.,

1986; Becker et al., 1992; Gylling et al., 1995; Miettinen et al., 1995). We can conclude based on the present work that the reduction in circulating cholesterol levels in hamsters fed 1% (w/w) SI was due to an inhibition in cholesterol absorption and an increase in fecal excretion rate.

Percent cholesterol absorption was determined at a single 72 hr-time point using stable isotope administration similar to that of the procedures of Zilversmit and Hughes (1974). The 72-hr-interval was chosen because injected ^{13}C-cholesterol is quickly removed from the plasma and gradually released into the bloodstream (Zilversmit, 1972) where it starts to turnover slowly (Zilversmit and Hughes, 1974; Bosner et al., 1993) after 72 hr (Turley et al., 1994; Wong et al., 1995). The isotope-plasma-ratio methodology (Zilversmit, 1972; Bosner et al., 1993; Wong et al., 1995) adopted in this study was modified to allow measurement of percent cholesterol absorption and cholesterogenesis simultaneously. An important assumption of the dual isotope plasma ratio method is that the portion of orally administered cholesterol equilibrates, and is metabolized, in the same manner as is the intravenously injected cholesterol (Zilversmit and Hughes, 1974). Also, the level of ^{18}O-cholesterol after 72 hr in the plasma is assumed to reflect the efficiency with which exogenous and endogenous cholesterol are absorbed (Bosner et al., 1993). The absorption efficiencies observed, which resemble those obtained in other studies (Bosner et al., 1993; Turley et al., 1994), suggest these assumptions are reasonable.

Concurrent to the lower cholesterol absorption in the 1% (w/w) SI-fed hamsters, percent cholesterol excretion was the highest in this group as compared to control. A significant negative correlation was observed between cholesterol absorption rate and percent cholesterol excretion in hamsters fed cholesterol and SI. Still the precise

mechanisms of action through which SI lowers cholesterol absorption and increases its excretion are not well-defined. Heinemann et al. (1988; 1991; 1993) have suggested that SI, which is more hydrophobic than cholesterol, alters the composition of the micellar mixture by replacing cholesterol and rendering it less miscible and absorbable. An alternative mechanism was also proposed by Mattson et al. (1977) who suggested that phytosterol-cholesterol absorption depends on total sterol concentrations in the oil and the micellar phases as well as in the solid state as sterol monohydrate. The accumulation of free-sterols in the oil phase in the lumen of the intestine because of lipolysis and the incorporation of added SI-cholesterol further augments the precipitation rate of the sterols. Thus, regardless of any other factor that may influence cholesterol uptake, it is unlikely that a sterol in the solid state can undergo absorption (Mattson et al., 1977). Regarding this point, it is interesting to know that SI differs from cholesterol by having an ethyl group at position 24, and the ß ring saturated. Nonetheless, SI, unlike cholesterol, is almost completely unbsorbable as we reported previously (Ntanios and Jones, 1998a) and inhibits cholesterol absorption in hamsters. With this body of knowledge, still more studies are required at the molecular level to determine why SI, having almost similar structure to cholesterol, is not absorbed and simultaneously inhibits cholesterol uptake in the lumen.

Subsequently to lowering cholesterol absorption and increasing its excretion in the feces, an up-regulation in cholesterol FSR in hamsters fed 1% (w/w) SI was observed as compared to control. The deuterium uptake method has been validated against the sterol balance technique and shown to be sensitive to diet induced changes in cholesterol synthesis rates (Jones et al., 1998). Ling and Jones (1995) reported a similar increase in

cholesterol FSR in male Wistar rats fed tall oil-phytosterol extracts with a SI concentration of 0.2% (w/w) SI as compared to control. Rats, however, exhibit a different lipid profile than hamsters. The ratio of HDL to LDL in rats is higher than hamsters and their plasma cholesterol is mostly transported in HDL (Mills and Taylaur, 1971). The difference in lipid profiles between these two animal models could explain why cholesterol FSR in hamsters fed 0.2% (w/w) SI was not significantly increased as in rats supplemented with the same phytosterol-mix. A trend of elevated FSR, however, exists in hamsters fed 0.2% (w/w) SI, but it is not significant. In addition, the fact that there was no significant difference in FSR between 0.2 and 1% (w/w) SI- fed hamsters support the later argument. The upregulation in *de novo* cholesterogenesis is likely due to lower circulating levels and higher excretion rates of cholesterol in the hamsters fed 1% (w/w) SI. The significant correlation observed between plasma TC levels and percent cholesterol absorption suggests that plasma cholesterol concentrations are directly affected by the rate of cholesterol absorption which influences cholesterol fractional synthesis rates. Cholesterol synthesis is feedback inhibited and hence lower circulating cholesterol levels upregulates cholesterogenesis (Grundy et al., 1969). Nonetheless, the increase in cholesterol biosynthesis was not large enough to compensate to lower circulating cholesterol levels in hamsters. Our data support the notion that plasma cholesterol concentration is a complex balance between absorption and endogenous synthesis on one hand and excretion and catabolism on the other hand.

In summary, dietary SI exhibited a dose-dependent effect in lowering plasma lipid levels and cholesterol absorption as well as increasing fecal cholesterol excretion and upregulating cholesterogenesis in hamsters. Sitostanol at 1% (w/w) can be considered as an effective dietary adjunct that lowers circulating cholesterol levels which are a risk factor in coronary vascular disease.

6.6 Figure legends

Figure 1.

Correlation between cholesterol enrichment and δ/‰ reading. δ: ((Sample enrichment/reference enrichment) - 1) x 1000.

Figure 2.

Percent cholesterol absorption in hamsters and rabbits fed different phytosterols. Groups carrying different letters are significantly different at p value given (ANOVA).

Figure 3.

Percent cholesterol excretion in feces in hamsters and rabbits fed different phytosterols. Groups carrying different letters are significantly different at p values given (ANOVA).

Figure 4.

Cholesterol fractional synthesis rate (FSR per day) in hamsters and rabbits fed different phytosterols. Groups carrying different letters are significantly different at p value given (ANOVA).

Figure 5.

Correlation between plasma total cholesterol and percent cholesterol absorption in hamsters and rabbits fed different phytosterols.

Figure 6.

Correlation between cholesterol fractional synthesis rate (FSR per day) and percent cholesterol absorption in hamsters and rabbits fed different phytosterols.

Figure 7.

Correlation between plasma total cholesterol and fractional synthesis rate (FSR per day) in hamsters and rabbits fed different phytosterols.

Table 1. Composition of the semi-purified diets fed to hamsters.

Ingredients (% w/w)	control %	soybean %	tall oil %	synth. mix %	sitostanol %
Casein	20.0	20.0	20.0	20.0	20.0
Corn starch	29.0	28.0	28.0	28.0	28.0
Sucrose	36.3	36.3	36.3	36.3	36.3
Oil mixture	5.0	5.0	5.0	5.0	5.0
DI-methionine	0.5	0.5	0.5	0.5	0.5
Mineral mixture	4.0	4.0	4.0	4.0	4.0
Vitamin mixture	1.0	1.0	1.0	1.0	1.0
Choline bitartrate	0.2	0.2	0.2	0.2	0.2
Cholesterol	0.25	0.25	0.25	0.25	0.25
Butyl hydroxy toluene	0.02% of oil	0.02% of oil	0.02% of oil	0.02% of oil	0.02% of oil
Cellulose	5.0	5.0	5.0	5.0	5.0
ß-sitosterol	ND	0.65	0.65	0.54	ND
Campesterol	ND	0.20	0.16	0.17	ND
Dihydrobrassicasterol	ND	0.15	ND	0.12	ND
Sitostanol	ND	ND	0.17	0.17	0.98
Unknown phytoserols	ND	ND	0.02	ND	0.02

ND: Not detectable

Synth. mix = Synthetic mixture

Table 2. Composition of the semi-purified diet fed to rabbits.

Ingredients (% w/w)	control %	soybean %	tall oil %	sitostanol %
Casein	30.0	30.0	30.0	30.0
Dextrose	48.0	48.0	48.0	48.0
Cellulose	8.0	8.0	8.0	8.0
Oil mixture	1.0	1.0	1.0	1.0
Rabbit chow	5.0	5.0	5.0	5.0
DI-methionine	0.5	0.5	0.5	0.5
Mineral mixture	4.0	4.0	4.0	4.0
Vitamin mixture	2.0	2.0	2.0	2.0
Choline bitartrate	0.2	0.2	0.2	0.2
Cholesterol	0.5	0.5	0.5	0.5
Sitostanol	ND	0.01	0.2	0.8
ß-sitosterol	ND	0.65	0.63	0.09
Campesterol	ND	0.18	0.16	0.01
Dihydrobrassicasterol	ND	0.11	ND	ND
Unknown plant sterols	0.08	0.03	0.02	0.08
Total plant sterols	0.08	0.98	1.01	0.98
Total %	100%	100%	100%	100%

ND: Not detectable

The vitamins and salt mixture and vitamin-free casein were purchased from ICN Life Science Group, Nutritional Biochemicals Division, Cleveland, OH. Casein provided 30% protein in this low-fat diet. Fat soluble vitamins were added to the oil mix which was a rich source of essential fatty acids.

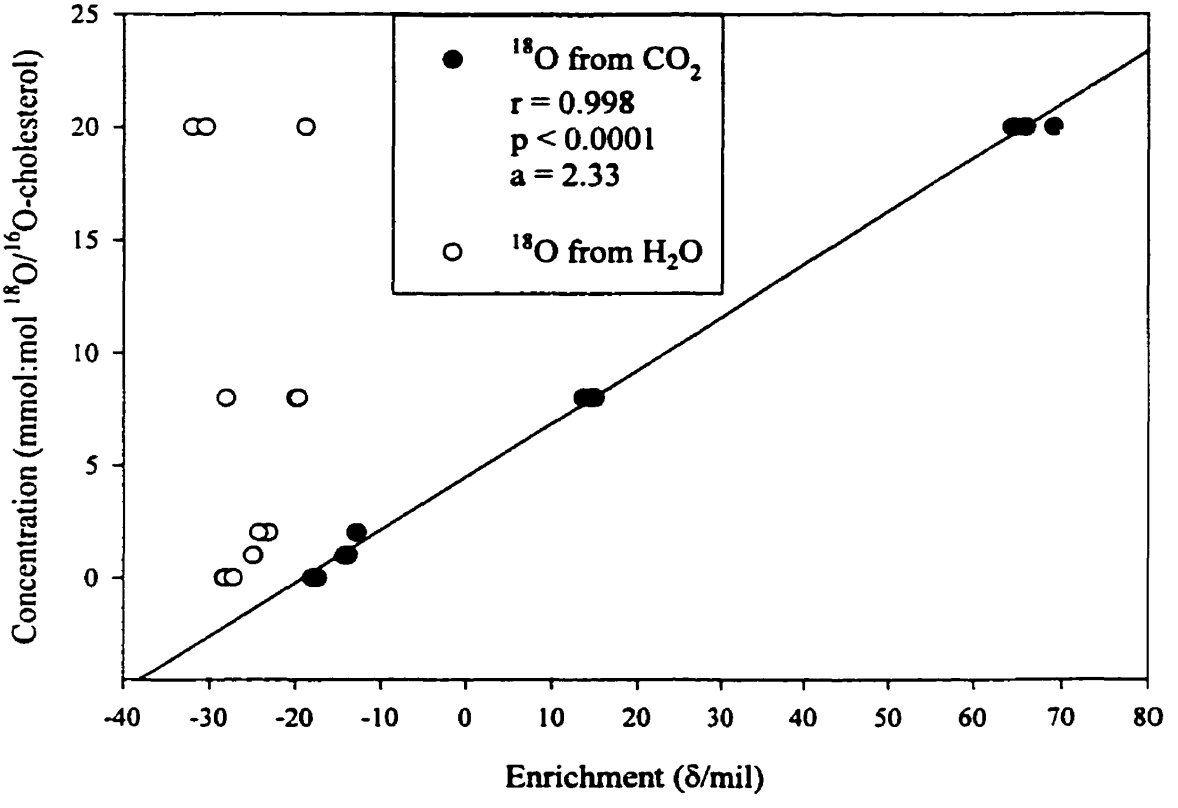

25
20
15
10
5
0
-40
-30
-20
-10
0
10
20
30
40
50
60
70
80
Concentration (mmol:mol $^{18}O/^{16}O$-cholesterol)
Enrichment (δ/mil)
^{18}O from CO_2
r = 0.998
p < 0.0001
a = 2.33
^{18}O from H_2O

Figure 1

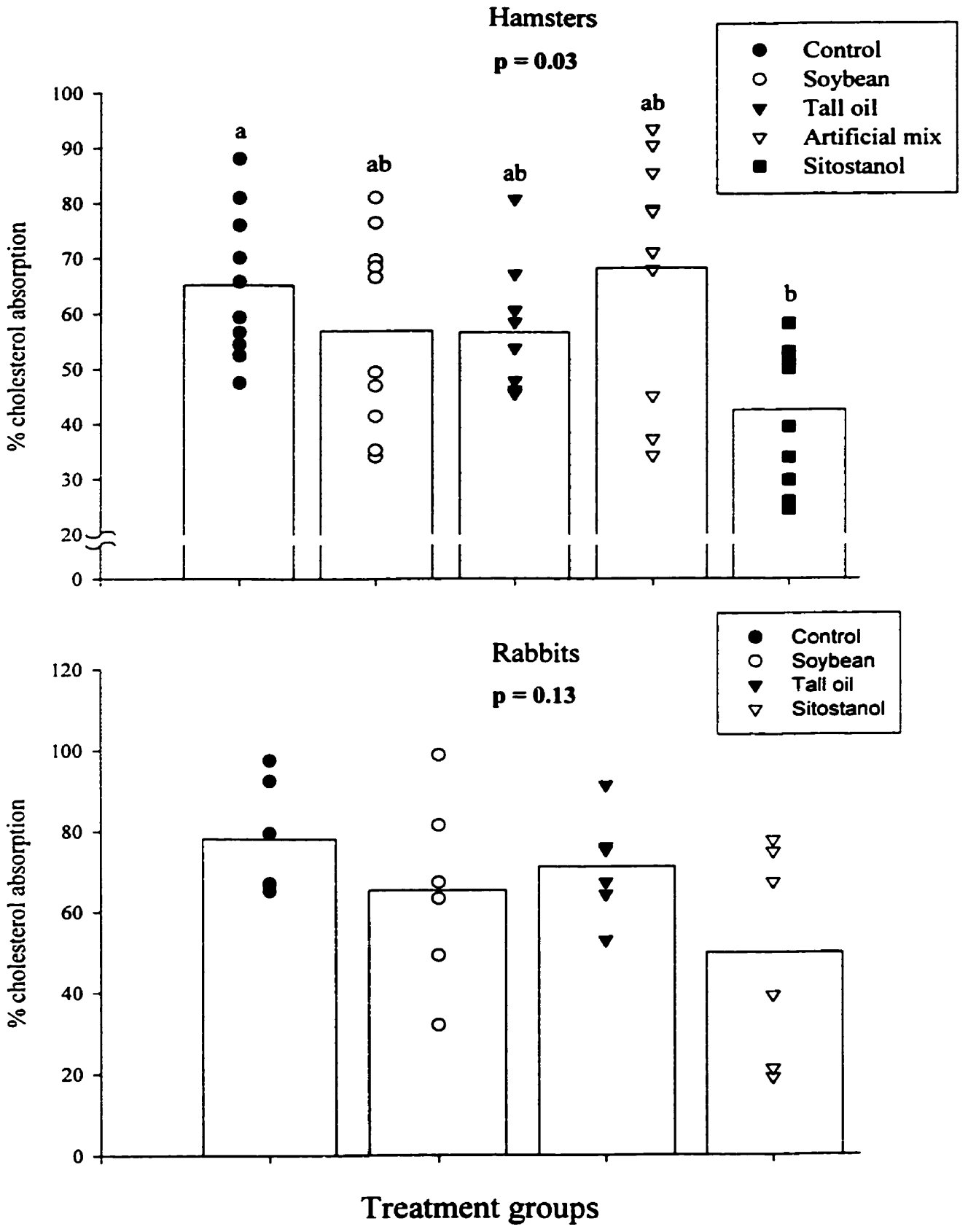
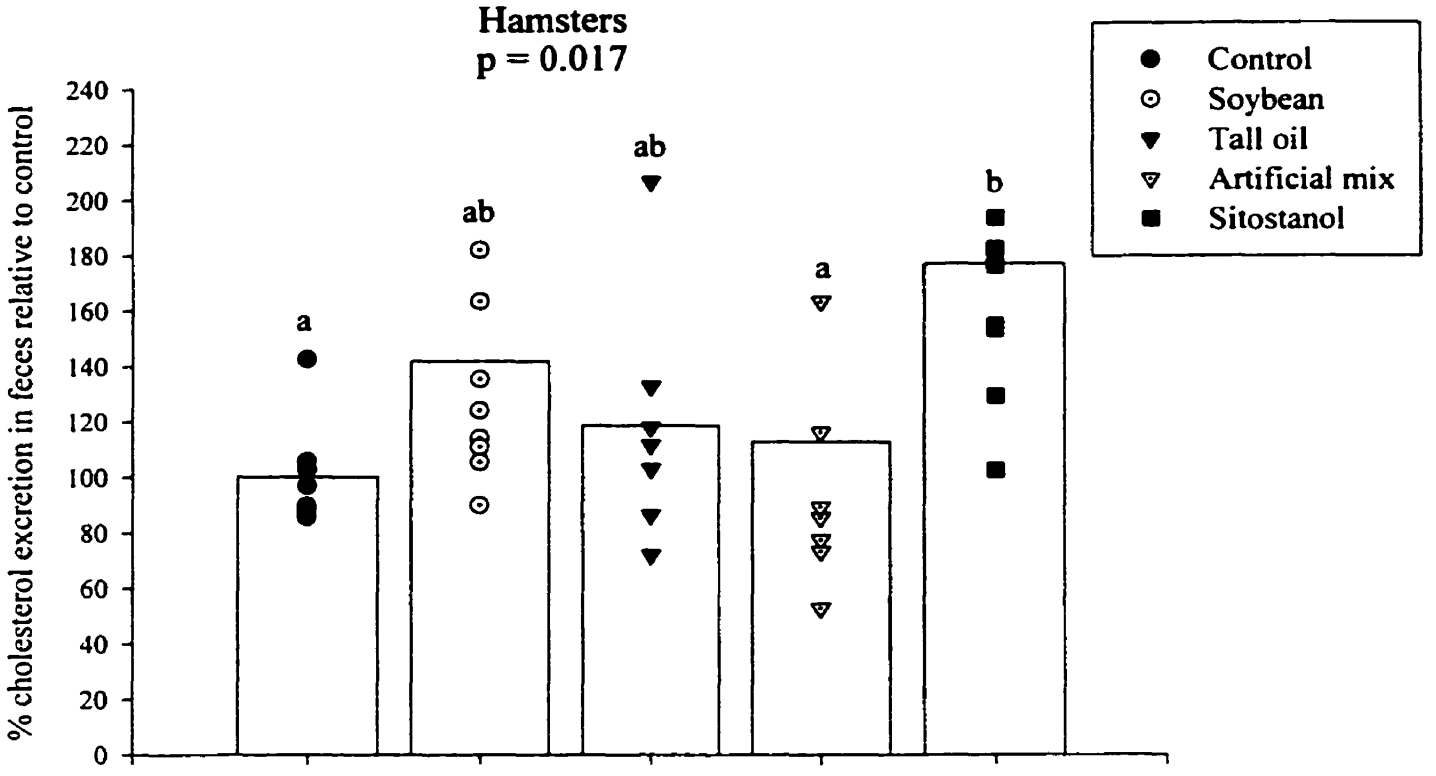

Hamsters
p = 0.03
Control
Soybean
Tall oil
Artificial mix
Sitostanol
% cholesterol absorption
100
90
80
70
60
50
40
30
20
0
a
ab
ab
ab
b
Rabbits
p = 0.13
Control
Soybean
Tall oil
Sitostanol
% cholesterol absorption
120
100
80
60
40
20
0
Treatment groups

Figure 2


Hamsters
p = 0.017
% cholesterol excretion in feces relative to control
240
220
200
180
160
140
120
100
80
60
40
20
0
a
ab
ab
a
b
Control
Soybean
Tall oil
Artificial mix
Sitostanol

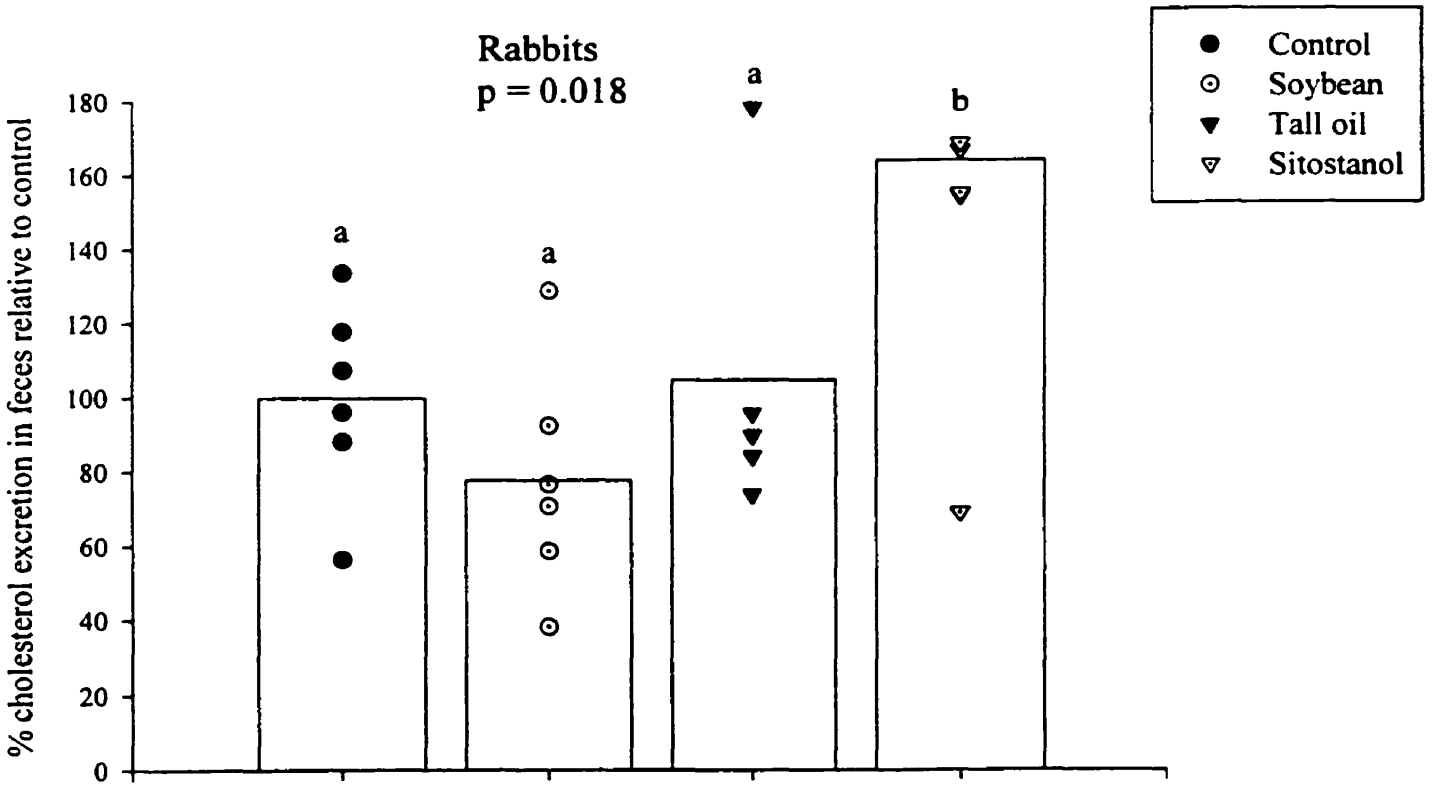

Rabbits
p = 0.018
% cholesterol excretion in feces relative to control
180
160
140
120
100
80
60
40
20
0
a
a
a
b
Control
Soybean
Tall oil
Sitostanol

Figure 3

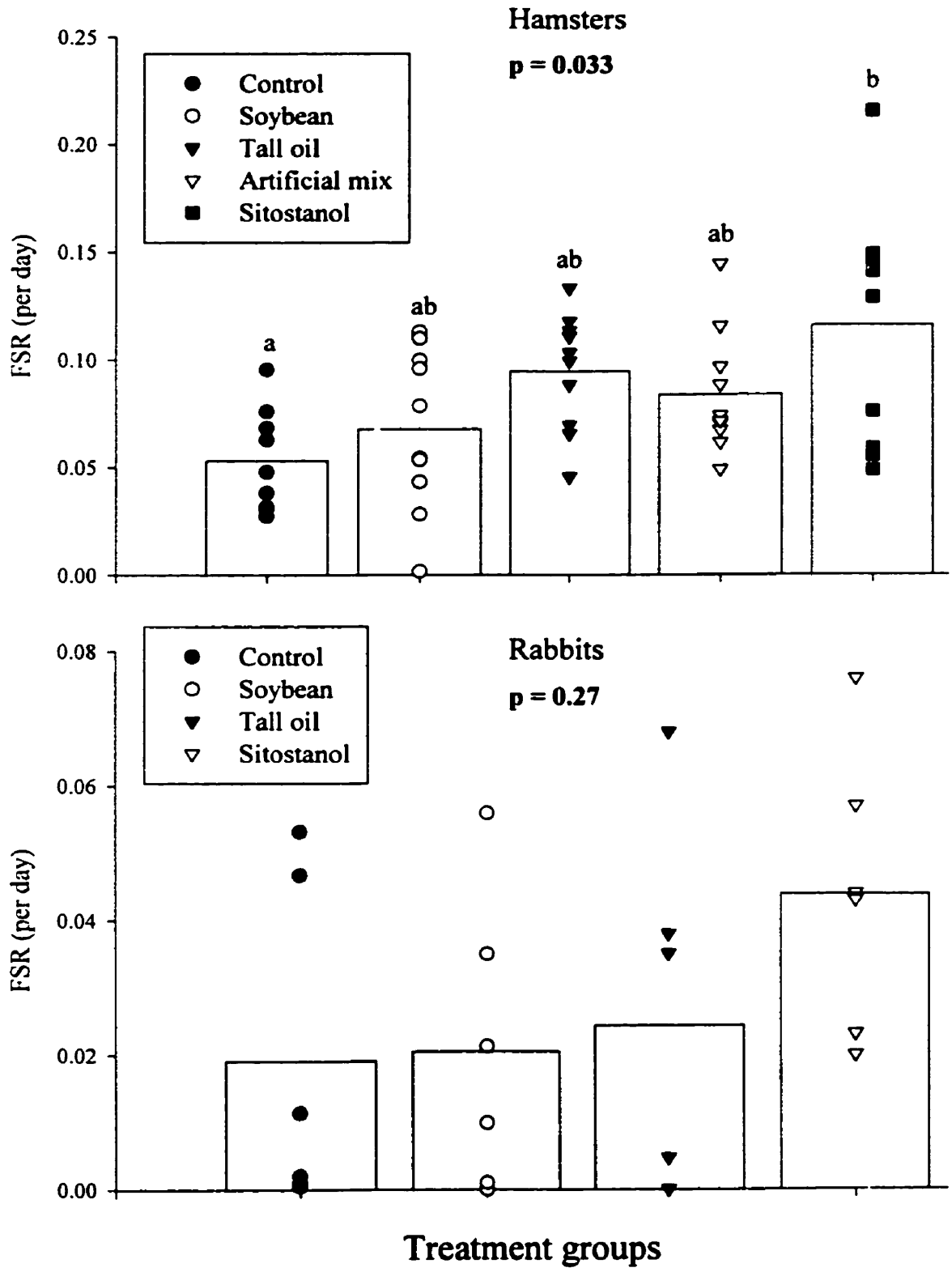
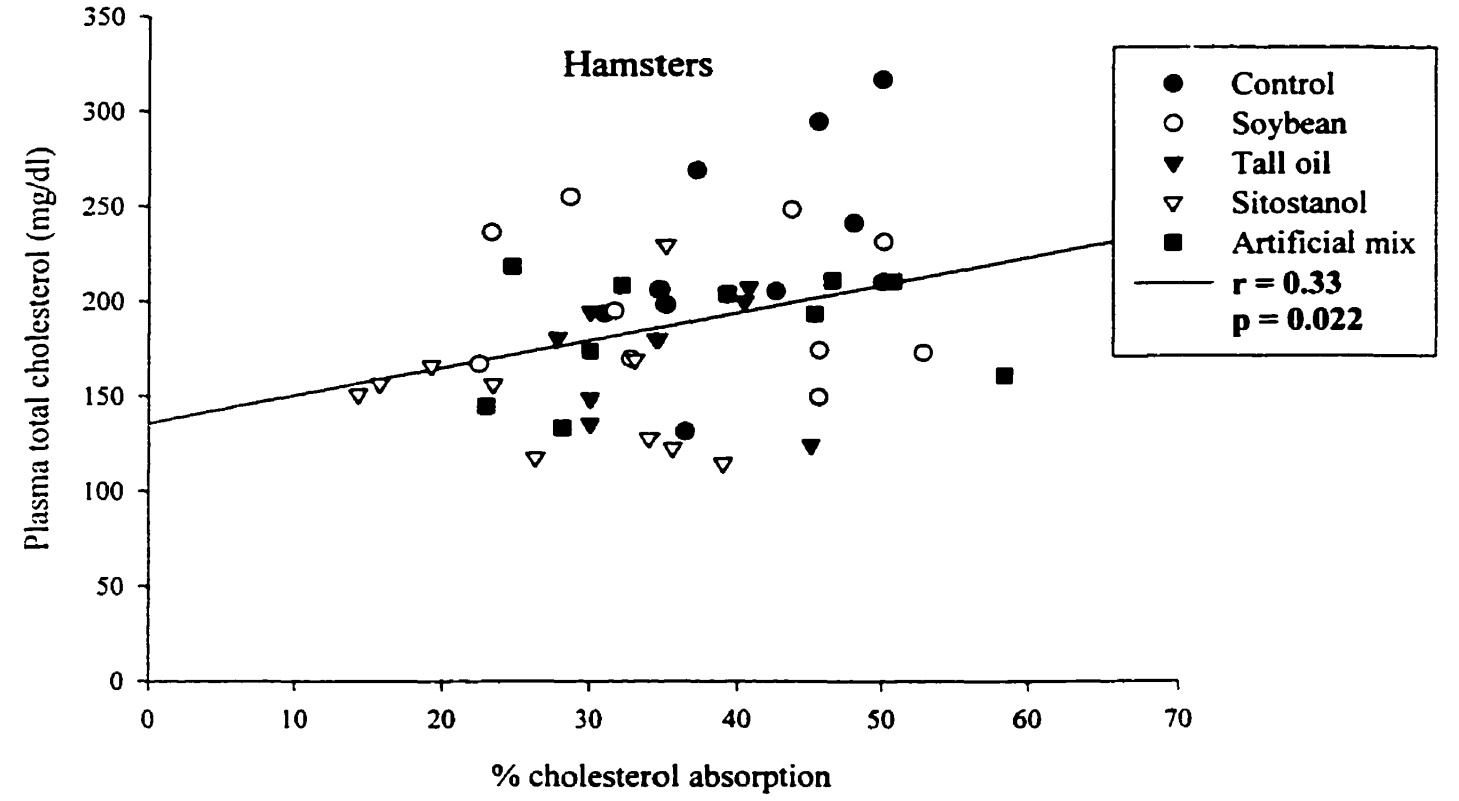

Hamsters
p = 0.033
Control
Soybean
Tall oil
Artificial mix
Sitostanol
FSR (per day)
0.25
0.20
0.15
0.10
0.05
0.00
a
ab
ab
ab
b
Rabbits
p = 0.27
Control
Soybean
Tall oil
Sitostanol
FSR (per day)
0.08
0.06
0.04
0.02
0.00
Treatment groups

Figure 4


Hamsters
Plasma total cholesterol (mg/dl)
% cholesterol absorption
Control
Soybean
Tall oil
Sitostanol
Artificial mix
r = 0.33
p = 0.022
0
50
100
150
200
250
300
350
0
10
20
30
40
50
60
70

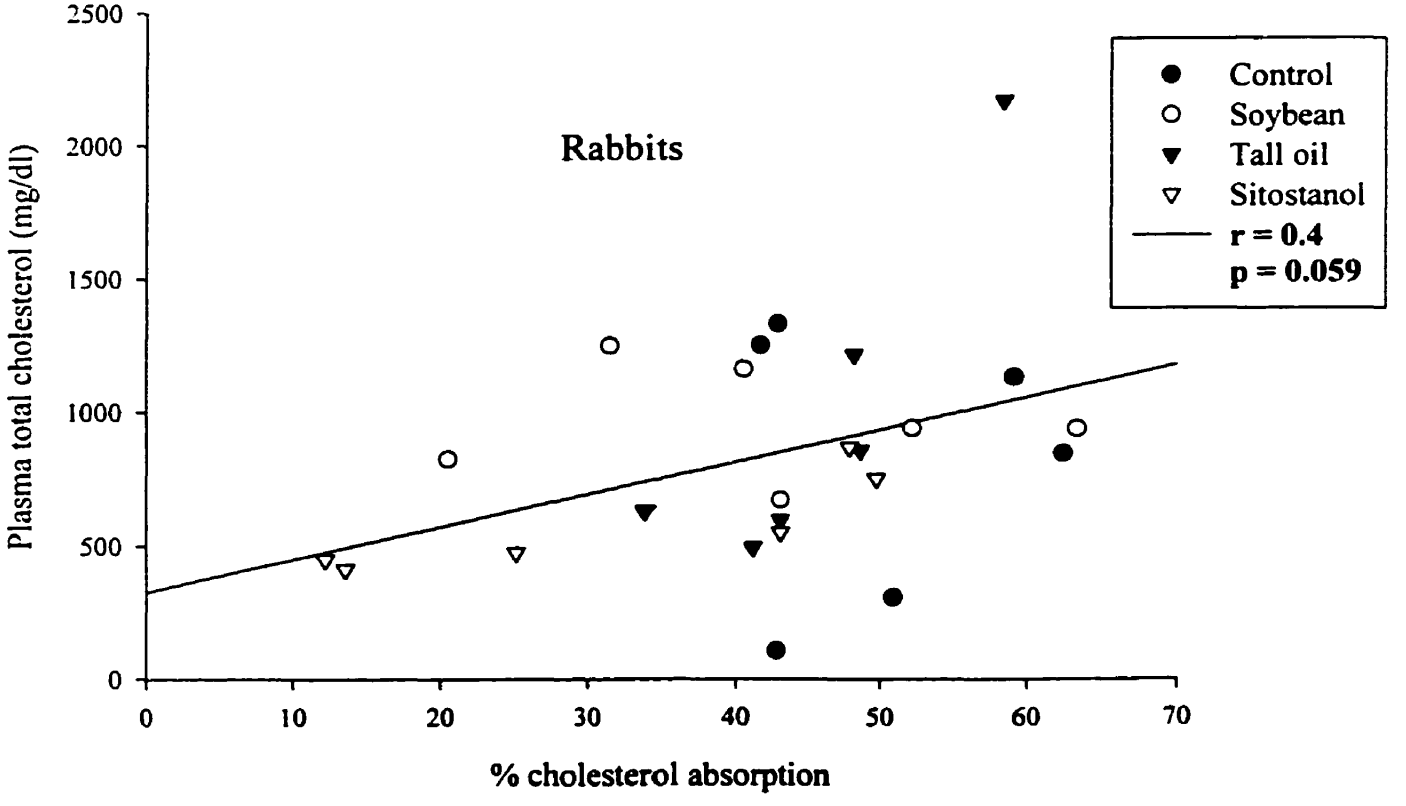
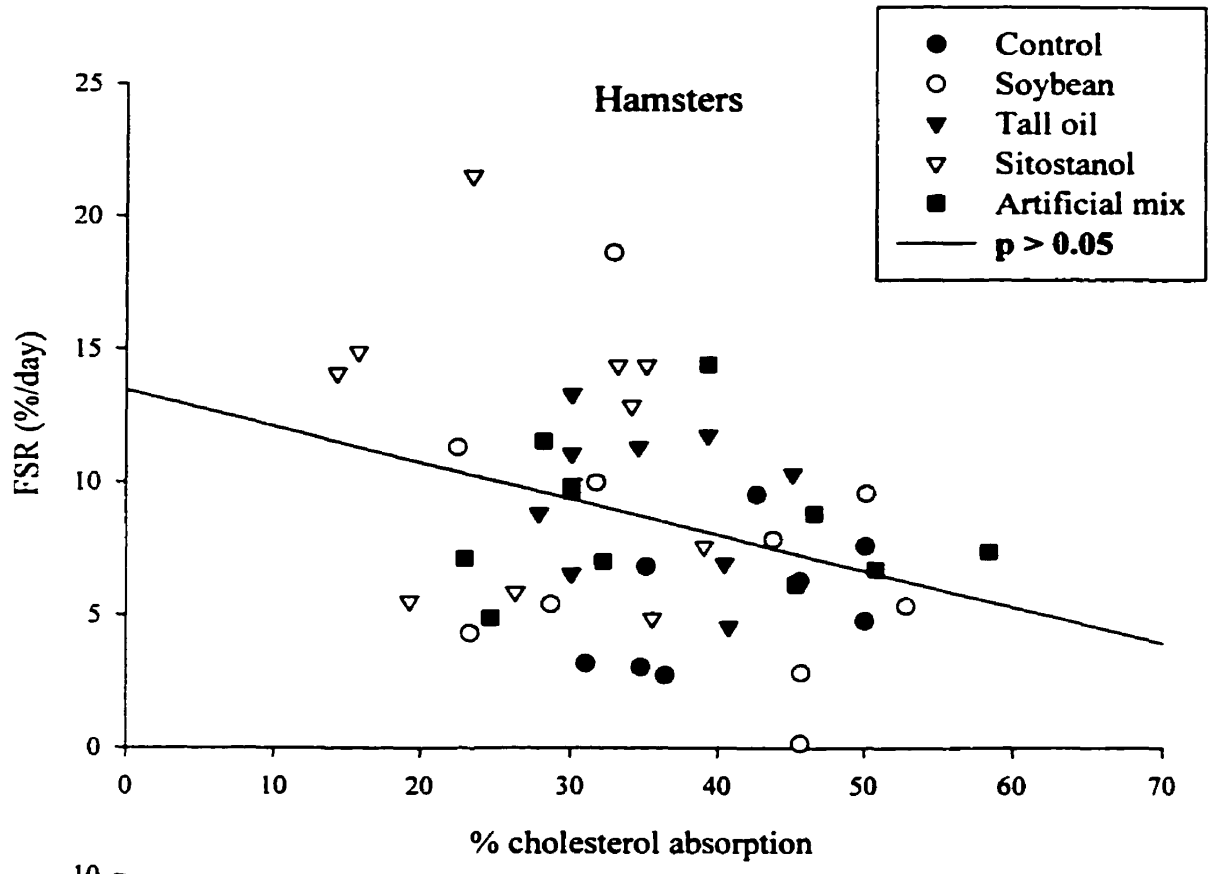

Rabbits
Plasma total cholesterol (mg/dl)
% cholesterol absorption
Control
Soybean
Tall oil
Sitostanol
r = 0.4
p = 0.059
0
500
1000
1500
2000
2500
0
10
20
30
40
50
60
70

Figure 5


Hamsters
Control
Soybean
Tall oil
Sitostanol
Artificial mix
p > 0.05
FSR (%/day)
% cholesterol absorption
0
5
10
15
20
25
0
10
20
30
40
50
60
70

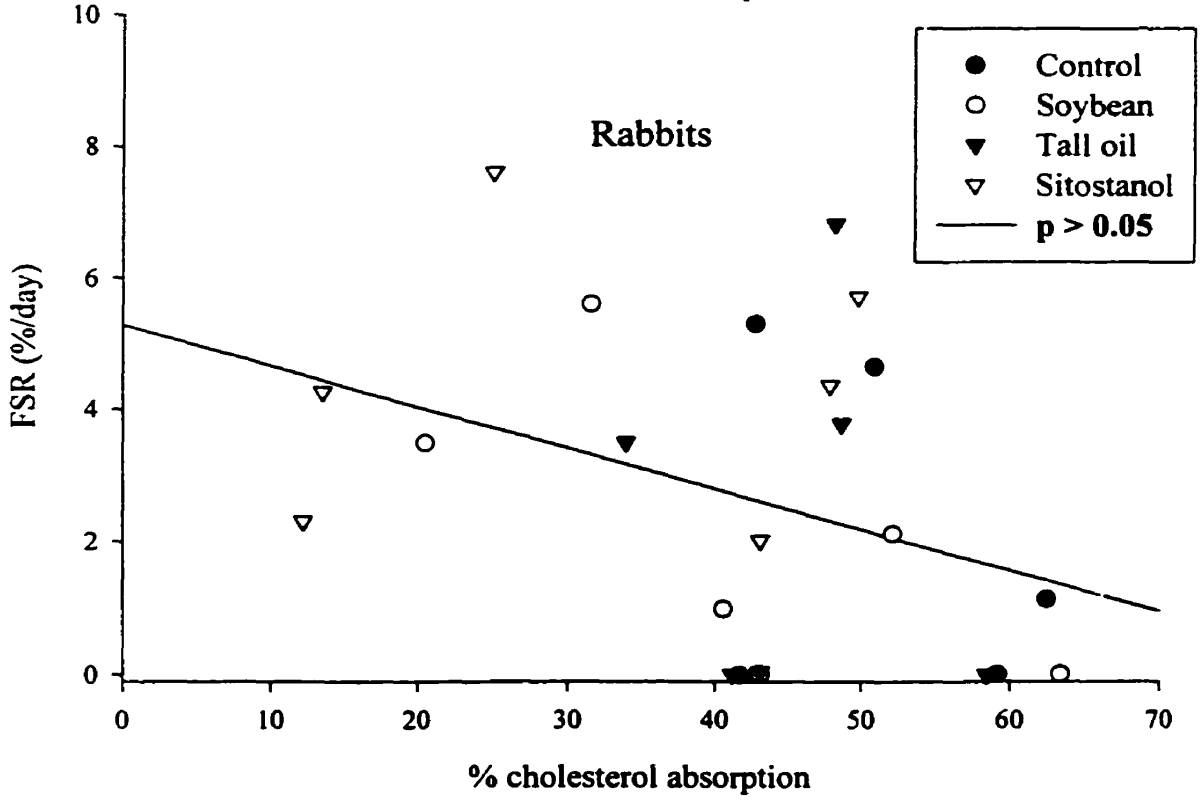
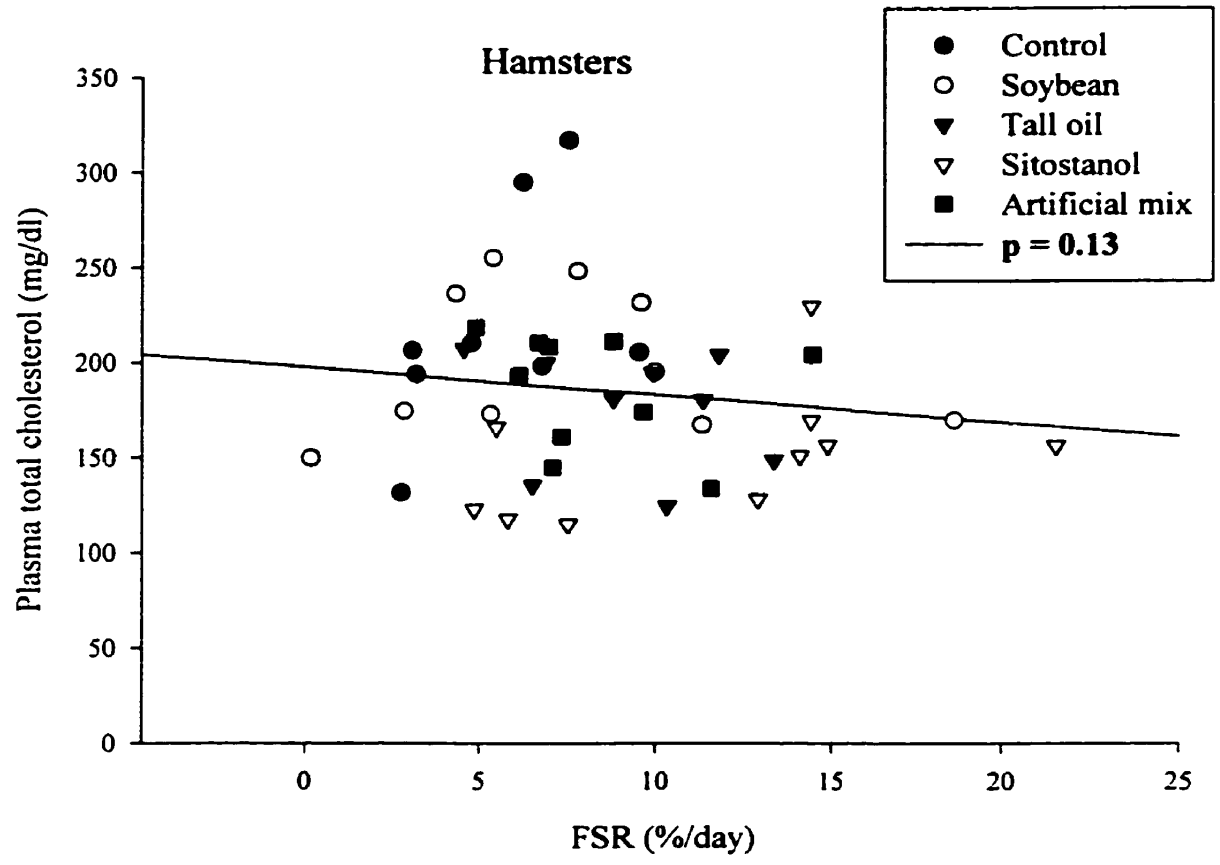

Rabbits
Control
Soybean
Tall oil
Sitostanol
p > 0.05
FSR (%/day)
% cholesterol absorption
0
2
4
6
8
10
0
10
20
30
40
50
60
70

Figure 6


Hamsters
Control
Soybean
Tall oil
Sitostanol
Artificial mix
p = 0.13
Plasma total cholesterol (mg/dl)
350
300
250
200
150
100
50
0
0
5
10
15
20
25
FSR (%/day)

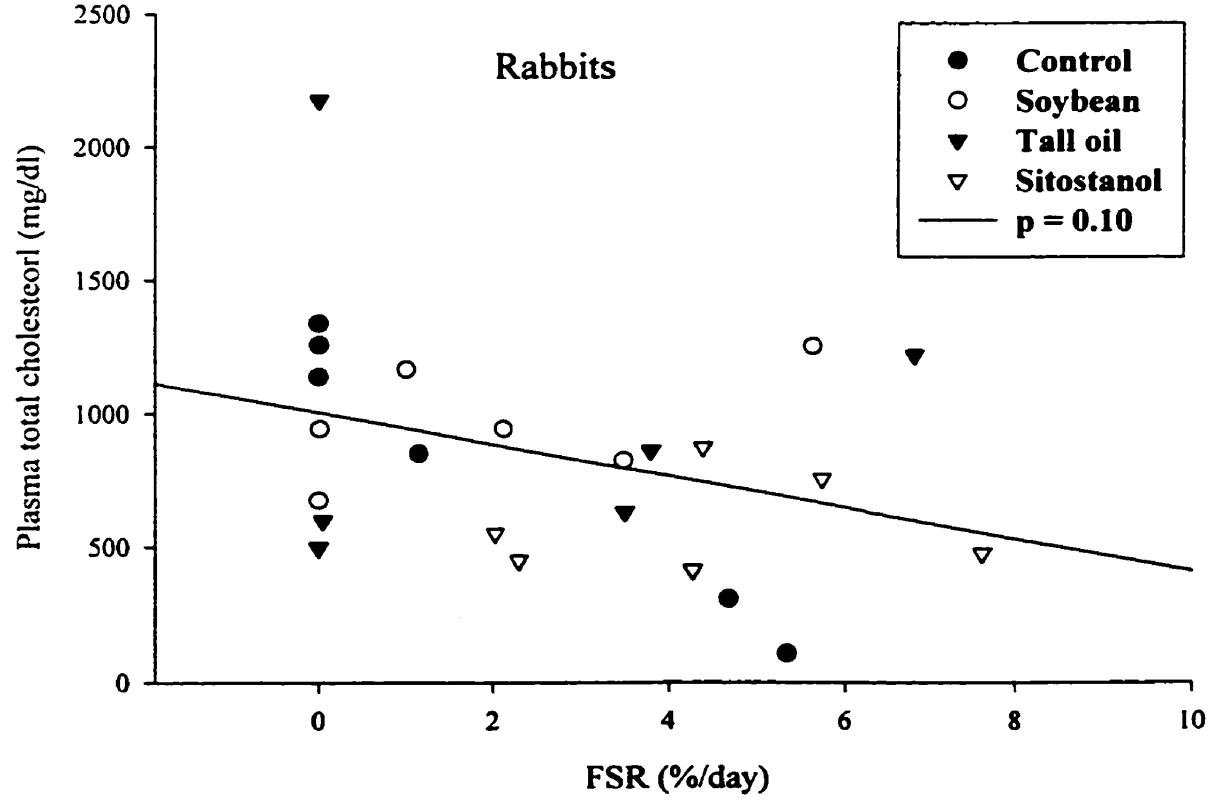

Rabbits
Control
Soybean
Tall oil
Sitostanol
p = 0.10
Plasma total cholesteorl (mg/dl)
2500
2000
1500
1000
500
0
0
2
4
6
8
10
FSR (%/day)

Figure 7

TRANSITION

Results from the previous manuscript demonstrated that sitostanol inhibited cholesterol absorption rate more than other phytosterol mixtures in hamsters. In addition, cholesterol excretion was higher in both hamsters and rabbits fed sitostanol. Moreover, cholesterogenesis was up-regulated in the hamster group fed sitostanol. The newly developed methodology allowed for simultaneous measurement of cholesterol absorption and synthesis from the same tissue. After this series of animal experiments, the next step was to assess the efficacy of plant sterols in hypercholesterolemic humans and their mechanisms of actions on cholesterol metabolism. However, not all the measurements and analyses done in hamsters and rabbits could be performed in humans. Nonetheless, cholesterogenesis and measurement of plasma plant sterol concentrations would be conducted in humans. In the following study, the effects of dietary modification and concurrent addition of tall oil-phytosterols on plasma lipid and phytosterol profile as well as on cholesterogeneis were examined in hypercholesterolemic subjects over 30 days.

CHAPTER 7

THE EFFECTS OF TALL OIL PHYTOSTEROLS ON *DE NOVO* CHOLESTEROL SYNTHESIS AND PLASMA PLANT STEROL LEVELS IN HYPERCHOLESTEROLEMIC SUBJECTS FED A PRUDENT NORTH AMERICAN DIET OVER 30 DAYS

Paper to be submitted to *Journal of Lipid Research (1998)*.

7.1 Abstract

Dietary phytosterols have been shown to lower plasma lipid levels, however, the impact of tall oil-phytosterol intake, however, on endogenous cholesterol synthesis rate and plant sterol metabolism has not been well-characterized in hypercholesterolemic subjects. The aim of this study was to investigate effects of phytosterols extracted from tall oil on plasma lipid concentrations, *de novo* cholesterol synthesis and phytosterol levels in hyperlipidemic patients consuming precisely controlled diets. In a double-blind, case-control design, subjects were given either 22 mg/kg body weight phytosterols (n =16) or placebo (n =16) suspended in margarine for 30 days, while consuming a 35% fat prepared-food diet as 3-meals/day. After subtraction of the effect of the diet alone, dietary phytosterols lowered plasma total- (-8.4%, $p = 0.046$) and low density lipoprotein- (-15.8%, $p < 0.03$), but did not change high density lipoprotein-cholesterol levels. Combined diet and phytosterol treatment reduced low density lipoprotein-cholesterol concentrations by -25.4%, $p < 0.01$. No differences were observed between control and phytosterol-fed subjects for fractional (0.091 ± 0.028 and 0.091 ± 0.026 day^{-1}, respectively) and absolute synthesis (0.61 ± 0.24 and 0.65 ± 0.23 day^{-1}, respectively) rates of cholesterol measured by deuterium uptake using isotope ratio mass spectrometry. Cholesterol absolute synthesis rate was correlated ($r = -0.36$, $p = 0.045$) with plasma total-cholesterol levels of all subjects. In addition, fractional synthesis rate was correlated with plasma campesterol ($r = -0.39$, $p = 0.027$) and ß-sitosterol ($r = -0.39$, $p = 0.03$) levels as well as with campesterol:cholesterol ($r = -0.37$, $p = 0.038$) and ß-sitosterol:cholesterol ratios ($r = -0.34$, $p = 0.05$). Circulating campesterol and ß-sitosterol concentrations did not change in phytosterol-fed compared to control subjects. In conclusion, tall oil-

phytosterols reduced plasma total- and low density lipoprotein-cholesterol concentrations by mechanisms that did not result in changes in endogenous cholesterol synthesis in hypercholesterolemic subjects fed a prudent Western diet.

7.2 Introduction

Phytosterols occur naturally as non-saponifiable components of plant oils. While ß-sitosterol, campesterol, stigmasterol and dihydrobrassicasterol are the most abundant (Pollak and Kritchevsky, 1981), sitostanol, the saturated derivative of ß-sitosterol, is found in negligible concentrations and hence is almost absent from typical Western diets (Morton et al., 1995). Phytosterols extracted from tall oil (TO), however, contain about 20% (w/w) sitostanol (Ling and Jones, 1995).

Oral administration of phytosterols has been shown to reduce plasma total- (Lees et al., 1977; Gylling et al., 1995) and low density lipoprotein- (LDL) cholesterol levels in humans (Becker et al., 1993; Miettinen and Vanhanen, 1994; Jones et al., 1997) and animals (Sugano et al., 1976; Ikeda et al., 1979), with sitostanol being more effective than non-saturated plant sterols (Heinemann et al., 1991; Becker et al., 1993). ß-sitosterol and sitostanol are believed to reduce plasma cholesterol levels extrinsically by competitively blocking cholesterol absorption from the intestinal lumen (Heinemann et al., 1986; 1993), displacing cholesterol from bile salt micelles (Child and Kuksis, 1986), increasing bile salt excretion (Salen et al., 1970) or hindering cholesterol esterification rate in the intestinal mucosa (Ikeda and Sugano, 1983; Child and Kuksis, 1983). Besides these extrinsic effects, several investigators have also suggested that ß-sitosterol may lower plasma cholesterol levels through intrinsic actions including modification of hepatic acetyl-CoA carboxylase (Laraki et al., 1993) and/or cholesterol 7-α hydroxylase (Shefer et al., 1994) in animals and humans.

At present, whether the cholesterol-lowering ability of sitostanol-rich phytosterol mixtures would affect cholesterogenesis has not been well-addressed. Several studies

have shown that ß-sitosterol lowers plasma cholesterol levels while simultaneously stimulating (Grundy et al., 1969; Konlande and Fisher, 1969), inhibiting (Kakis and Kuksis, 1984) or exerting no effect (Subbiah and Kuksis, 1973) on cholesterol synthesis. Previous reports that examined the effect of phytosterols on cholesterogenesis in humans have determined synthesis rates indirectly (Grundy et al., 1969; Miettinen et al., 1990; Miettinen and Vanhanen, 1994). However, none of those studies examined the impact of a precisely controlled prudent Western diet with simultaneous supplementation of TO-phytosterols on cholesterogenesis measured directly by the deuterium uptake method in hypercholesterolemic subjects.

Therefore, the objective of this trial was to examine the effect of TO-phytosterol extracts on cholesterol metabolism in hypercholesterolemic males. The null hypotheses tested were that circulating lipoprotein concentrations, *de novo* cholesterol synthesis rate and plasma phytosterol levels as an index of cholesterol absorption would not be influenced by TO-phytosterol supplementation.

7.3 Subjects and methods

7.3.1 Subjects

Thirty two hypercholesterolemic males were recruited, stratified and randomly divided into control (n=16) and treatment (n=16) groups using a double-blind, case-control design. Subjects (29-60 years) were admitted into the study based on plasma total cholesterol (TC) > 5.5 mmol/L, LDL-cholesterol > 3.5 mmol/L, triglyceride (TG) ≤ 5.2 mmol/L and 18 < body mass index (BMI) < 37. Subjects reported no hypertension, diabetes and coronary heart problems. In addition, plasma chemical profile, hematology

and urinalysis were performed. Subjects were not suffering from renal failure and not taking lipid lowering medications. The experimental protocol was approved by the Ethics Committee of the Faculty of Agriculture and Environmental Sciences and School of Dietetics and Human Nutrition at McGill University. The study was conducted in accordance with the principles of the Helsinki Declaration and all subjects completed and signed relevant consent forms.

7.3.2 Study design

The study was conducted for a 30-day period during which hypercholesterolemic males received either TO-extract phytosterols (Forbes Medi-Tech, Vancouver, British Columbia) or starch as placebo. Plasma TC, LDL-, high-density lipoprotein- (HDL) cholesterol and TG were measured at days 0, 10, 20 and 30 of the trial. Blood samples were taken after subjects had fasted for 12 hours. During the 30-day trial, subjects consumed a prudent Western diet (15-16% energy as protein, 50% as carbohydrate and 35% as fat: saturated- 11%, polyunsaturated- 10% and monounsaturated- 14% fatty acids, 140 mg cholesterol/1000 Kcal). Diets were prepared every day at the Metabolic Unit in a three-day cycle. Subjects consumed 3 meals/day in the Metabolic Unit except for a few occasions where meals were prepared for take-out. Subjects were instructed to consume only the food and beverages provided to them by the Unit.

Phytosterols and the starch placebo were both suspended in margarine at 1:20 (w/w). Phytosterol composition of the diet was determined by gas liquid chromatography (GLC). The tall oil-extract phytosterol mixture used contained approximately 20% (w/w) sitostanol. Caloric intakes of subjects were calculated according to individual

requirements determined using the predictive equation of Mifflin et al. (1990). When fluctuations in body weight were seen, adjustments to caloric intakes were made during the initial 10-day period of the trial. On day 29 of the trial, subjects were given orally 0.7 g deuterium oxide (D) (99.8% atom percent excess) per kg body water at 8 a.m. Deuterium uptake into cholesterol was measured over the following 24 hr. Blood samples were collected just prior to, and 24 hr after D dosing for red blood cells (RBC) free cholesterol and water D enrichment measurement.

7.3.3 Lipid and phytosterol analyses

Plasma TC, HDL-cholesterol and TG concentrations were measured in duplicate using a VP Autoanalyzer and commercial enzymatic kits (Abbott Laboratories, North Chicago, IL, USA). High-density lipoprotein-cholesterol concentrations were measured in plasma after precipitation of apoB-lipoproteins with dextran sulfate/magnesium chloride (Warnick et al., 1982). The concentration of LDL-cholesterol was calculated according to the methods of Friedewald et al. (1972). Plasma phytosterol concentrations were determined in duplicate by GLC from the non-saponifiable material of plasma lipid. Briefly, 0.5 ml plasma samples were saponified with 0.5 M methanolic KOH for one hour at 100°C and the non-saponifiable materials were extracted with petroleum ether. Five alpha cholestane was used as an internal standard. Samples were injected into a GLC equipped with a flame ionization detector (HP 5890 Series II, Hewlett Packard, CA, USA) using a 30-m capillary column, SAC-5 (Supelco, Bellefonte, PA, USA). Detector and injector temperatures were 310 and 300°C, respectively. Duplicate samples were run isothermically at 285°C. Phytosterol peaks were identified by comparison with authenticated standards (Supelco, Bellefonte, PA, USA).

7.3.4 De novo cholesterol synthesis determination

Cholesterol biosynthesis was determined as the rate of incorporation of D from body water into free sterol over 24 hr. Labeled water equilibrates quickly with intra- and extracellular water body pools and permits direct determination of cholesterol synthesis rates (Jeske and Dietschy, 1980). Deuterium enrichment was measured in RBC free cholesterol and plasma water as reported previously (Jones et al., 1988; 1990; 1993). A free exchange of sterols exist between plasma and RBC (Salen et al., 1970). Briefly, lipids were extracted from 3 g RBC in duplicate and thin layer chromatography (20 x 20 cm, 250 mm, Whatman Inc, Clifton, NJ, USA) was used to separate free cholesterol from cholesteryl ester. The free cholesterol band was scraped from the silica gel plate and saponified with 0.5 M methanolic KOH to eliminate any TG contaminants. Free cholesterol extracts were dried under nitrogen and thereafter samples were transferred to 18-cm combustion tubes (Vycor, Corning Glass Works, Corning, NY, USA). Cupric oxide (0.5 g) and a 2-cm length of silver wire were added and tubes sealed under vacuum at less than 20 mtorr pressure. Cholesterol samples were combusted for 4 hr at 520°C and the generated water was vacuum-distilled into Vycor tubes containing 60 mg zinc reagent (Biogeochemical Laboratories Indiana University, Bloomington, IN, USA).

To measure D enrichment of body water, plasma samples were diluted 10-fold with distilled water to reduce D enrichment to within the normal analytical range. Baseline samples were not diluted. Duplicate samples were vacuum-distilled into zinc containing Vycor tubes. Plasma water samples and water derived from cholesterol were reduced to hydrogen gas at 520 °C for 30 min. The D enrichments of cholesterol and plasma samples were measured by differential isotope ratio mass spectrometry (IRMS)

using a triple inlet system with electrical H^{3+} compensation (VG Isomass 903D, Cheshire, UK). Mean internal and external precision levels of the IRMS were 0.18 and 2.0 per mil ($^{o}/_{oo}$), respectively. The instrument was calibrated using water standards of known isotopic composition. Samples for each subject were analyzed concurrently using a single set of standards

Cholesterol fractional synthesis rate (FSR) was determined as incorporation of precursor D into plasma TC relative to the maximum theoretical enrichment using the linear regression model as described previously (Jones et al., 1988; 1990; 1993). Absolute synthesis rate (ASR = FSR multiplied by M_1 pool) was calculated according to the model of Goodman et al. (1980). M_1 pool is equal to 0.287 weight + 0.0358 cholesterol - 2.40 TGGP (TGGP is a variable which is equal to 1, 2 or 3 depending on serum TG concentration: <2.267, 2.267-3.401 or > 3.401 mmol/L, respectively).

7.3.5 Statistical analysis

Values are presented as mean ± standard deviation (SD) or as percent change. The effects of TO-derived phytosterols on the measured variables, plasma lipid profile, phytosterol levels, FSR and ASR values were tested using repeated measures analysis of variance and unpaired Student's t-test (SAS software, USA). The relationship between the variables, plasma TC levels, phytosterols, ASR and FSR were determined using Pearson Product-Moment Correlation Coefficients.

7.4 Results

No adverse clinical side effects from dietary phytosterols were reported by subjects to physicians conducting regular monitoring examinations. Mean body weight, age and BMI values did not differ between control and treatment. In addition, mean body weight and BMI did not change significantly over the 30-day trial within and between groups. Individual values of body weight, age and BMI are presented in Table 1.

Mean plasma lipid profiles and their percent changes during the 30-day study are shown in Table 2 and Figure 1 and 2, respectively. At days 10, 20 and 30, mean circulating TC levels were significantly lower ($p < 0.05$) from day 0 in both control and treatment groups (Table 2). The respective percent changes in mean circulating TC concentrations in phytosterols-fed subjects were 4.3 and 5.0% lower ($p \geq 0.13$) than control at days 10 and 20 but did not reach significance (Figure 1). However, at day 30, the treatment group had 8.4 % lower ($p = 0.046$) TC levels as compared to control (Figure 1). At day 10, 20 and 30, mean plasma LDL-cholesterol levels were lower ($p < 0.032$) by 8.7, 9.0 and 15.8%, respectively, in the phytosterol-supplemented group as compared to control (Table 2). The absolute values of percent changes in LDL-cholesterol concentrations were significantly higher ($p = 0.004$) at day 30 in the treatment group as compared to control (Figure 1). Absolute values of percent changes in LDL-cholesterol levels within the treatment group were greater ($p = 0.018$) from days 10-30 (Figure 1). Mean plasma TG levels were higher ($p < 0.034$) in the phytosterol-fed group compared to control at days 10, 20 and 30 (Table 2). On the other hand, percent change in TG levels as shown in Figure 2 did not differ within and between the groups during the trial period. Similarly, mean plasma HDL levels did not vary between treatment and control, and within each group (Figure 2 and Table 2).

Individual values for cholesterol FSR and ASR are shown in Table 3. Fractional synthesis rates did not differ between control (mean ± SD: 0.0911 ± 0.0280 per day, range: 0.0408 - 0.1420) and treatment (mean ± SD: 0.0914 ± 0.0250 per day, range: 0.0487 - 0.1320). In addition, no difference was noted in ASR between control (mean ± SD: 0.613 ± 0.243 per day, range: 0.200 - 1.10) and phytosterol-fed subjects (mean ± SD: 0.647 ± 0.234 per day, range: 0.274 - 1.060).

Percent changes in campesterol/ß-sitosterol ratio were higher ($p = 0.003$) while percent change in ß-sitosterol was lower ($p = 0.016$) at day 30 within the treatment group (Figure 3). Mean plasma campesterol and ß-sitosterol levels in the control did not vary from those in the treatment except for ß-sitosterol at day 10 which was higher ($p < 0.05$) than day 0 in the treatment group (Figure 4). However, when correcting for variations in TC levels and expressing the values of campesterol as mmol:mol cholesterol, both control and treatment groups had higher ($p < 0.022$) levels at day 30 as compared to day 0 (Figure 5). Moreover, significant correlations between circulating plant sterols and various parameters such as TC, FSR, ASR, body weight and BMI have been observed in this study. For instance, plasma campesterol levels (day 30) were correlated with TC concentrations in the control ($r = 0.62$, $p = 0.0107$) but not in the treatment group. Conversely, the correlation of percent changes in plasma campesterol levels with percent changes in TC concentrations was not significant during the treatment period. Circulating campesterol levels were negatively correlated with M_1-pool size at day 30 in control ($r = -0.68$, $p = 0.004$) and treatment ($r = -0.48$, $p = 0.05$). Fractional synthesis rate was negatively correlated with campesterol ($r = -0.4$, $p = 0.027$), ß-sitosterol ($r = -0.39$, $p = 0.03$), campesterol:cholesterol ($r = -0.37$, $p = 0.038$) and ß-sitosterol:cholesterol (r = -

0.34, p = 0.05) concentrations. A negative correlation (r = -0.36, p = 0.045) was also found between ASR and TC levels for pooled data of the two groups (Figure 6). Finally, body mass index correlated with plasma TC (r = 0.34, p = 0.04), campesterol (r = -0.41, p = 0.02), ß-sitosterol (r = -0.452, p = 0.009) and percent change in campesterol at day 30 (r = 0.425, p = 0.015) in all subjects.

7.5 Discussion

To our knowledge, the current trial represents the first study where hypercholesterolemic subjects consumed phytosterol enriched precisely controlled diets, in contrast to previous studies that have employed self-selection dietary approaches (Gylling et al., 1995; Miettinen et al., 1995). Supplementation of 22 mg/kg body weight TO-phytosterols per day was effective in lowering circulating TC and LDL-cholesterol concentrations in these subjects without changing either endogenous cholesterol synthesis or phytosterol levels. Although the reduction in TC and LDL-cholesterol levels observed in this study is in accord with that demonstrated by other investigators (Becker et al., 1993; Miettinen and Vanhanen, 1994), the observation of no change in cholesterogenesis does not agree with previous reports (Grundy et al., 1969; Vanhanen et al., 1993). No compensatory increase in endogenous cholesterol synthesis was detected between control and treatment.

For instance, Vanhanen et al. (1993) examining effects of dietary sitostanol ester on cholesterol synthesis in hypercholesterolemic subjects with different apo-E phenotypes, found that sitostanol intake increased cholesterogenesis by 10% (Vanhanen et al., 1993). Here, changes in plasma cholesterol precursor of squalene, Δ^5-cholestenol,

desmosterol and lathosterol levels were taken as indirect indices of cholesterol synthesis rate (Vanhanen et al., 1993). On the other hand, Grundy et al. (1969) used cholesterol balance studies in males fed different type of oils as well as different concentrations of cholesterol and phytosterols to determine the relationship that exists between cholesterol absorption and synthesis. A feedback control of cholesterol synthesis was observed when the diets contained large quantities of phytosterols. In addition, absorption of both endogenous and exogenous cholesterol was greatly reduced and a compensatory increase in cholesterol synthesis occurred in the hypercholesterolemic patients (Grundy et al., 1969). In this study, no changes in cholesterogenesis were detected due to TO-phytosterol supplementation.

A possible reason for our failure to detect differences in cholesterogenesis across groups was that cholesterol synthesis was compared between placebo- and phytosterol-fed subjects at day 30 of the trial and not between days 0 and 30. Our data have shown that dietary modifications exert a significant impact on plasma cholesterol levels. In the present instance, changing the diets of these subjects into a prudent North American one rich in polyunsaturated fatty acids and low in cholesterol significantly improves their lipid profile. Caloric intake was calculated to maintain body weight throughout the trial. Moreover, dietary supplementation with 22 mg/kg body weight of TO-phytosterols per day resulted in additional decreases of 8.4 and 15.8% in plasma TC and LDL-cholesterol levels, respectively. This added reduction in circulating TC concentrations, however, did not apparently reach a threshold that desuppressed cholesterogenesis in these subjects. Although cholesterol homeostasis regulation is substantially different between animals and humans, rats fed similar TO-phytosterols showed an increase in hepatic FSR

compared to control (Ling and Jones, 1995). The upregulation in hepatic FSR in rats was likely due to a notably lower mean plasma TC level in the treatment compared to the control group (Ling and Jones, 1995).

In another clinical trial by Jones et al. (1994), using the same technique as presently employed, subjects fed typical Western diets exhibited cholesterol FSR values about 40% lower than those observed at the end of this study. Although FSR values from this study cannot be directly compared to those previously reported (Jones et al., 1994), the values obtained (Jones et al., 1994) suggest that if cholesterol FSR in this study was measured at day 0 and compared to day 30 in both groups, a potential difference in cholesterogenesis could have been found. Therefore, the lack of response of cholesterol FSR and ASR between groups presently could be mostly attributed to the impact of the diet itself.

The deuterium incorporation methodology has been validated and proven to be sensitive to small changes in endogenous cholesterol synthesis (Jones et al., 1998). It is assumed that total body cholesterol is considered as one pool because of rapid equilibration of deuterium in body water pool as suggested by Jeske and Dietschy (1980). The techniques applied by Vanhaenen et al. (1993) and Grundy et al. (1969) are considered non-invasive and relatively easy to conduct, however, neither measures cholesterol synthesis directly. The cholesterol precursor measurement does not provide quantitative data and may be affected by the inherent variability in cholesterol synthesis among subjects (Vanhanen et al., 1993). By the same token, the sterol balance method requires that internal sterol pools reach equilibrium which takes longer time than deuterium uptake in order to ensure accuracy in using this technique (Grundy et al.,

1969). Since study designs and methodologies have varied among trials, it is difficult to make a meaningful comparison of cholesterol synthesis measurements between our work and that of others (Grundy et al., 1969; Vanhanen et al., 1993; Ling and Jones, 1995).

Plasma phytosterol levels, notably those of campesterol, have been used as an index of fractional cholesterol absorption rate in humans (Miettinen et al., 1990; Heinemann et al., 1991). In this study, significant negative correlations were demonstrated between FSR and both campesterol and ß-sitosterol levels. Indeed, the negative correlation between FSR and campesterol levels might suggest that lower cholesterol absorption could lead to an increase in *de novo* cholesterol synthesis rate (Grundy et al., 1969). This was not, however, the case in this investigation. A negative correlation was found between ASR and plasma TC in all subjects. This association suggests that endogenous cholesterol synthesis varies with plasma TC levels which might support the concept of cholesterol feed-back mechanism (Grundy et al., 1969). However, the later correlations between FSR and campesterol levels as well as ASR and TC concentrations do not provide sufficient evidence that endogenous cholesterol synthesis was significantly different between control- and treatment-subjects.

Plasma campesterol levels did not change over study duration in either control or treatment groups. Phytosterol concentrations were comparable with those previously reported in hypercholesterolemics (Miettinen et al., 1992; 1995; Vanhanen, 1994). These data are also consistent with results of Salen et al (Salen et al., 1970) who reported that more than eight-fold increase of normal intake (250-500 mg/day) in dietary ß-sitosterol is needed to less than double plasma levels in healthy subjects. Similarly, Lees et al (Lees et al., 1977) demonstrated that administration of 3 g/day dietary TO (93% ß-sitosterol) to

25 hypercholesterolemic patients over one month period did not increase their plasma phytosterol levels. On the other hand, Miettinen et al. (1994; 1995) have reported that sitostanol simultaneously lowers circulating lipid and phytosterol levels in hypercholesterolemic subjects. Miettinen and coworkers (1994; 1995) have used the variations in plasma campesterol levels as an index of cholesterol absorption rate. A reduction in circulating campesterol levels indicates lesser cholesterol absorption in humans (Tilvis and Miettinen, 1986). The possible reasons for not observing significant reductions in campesterol levels in the present study groups are thus two-fold. First, the reduction in plasma TC levels were not large enough to be reflected in significant changes in campesterol concentrations. Second, it has been shown that subjects fed phytosterols might absorb about 5% of dietary campesterol and ß-sitosterol found in the phytosterol mixtures (Salen et al., 1970; Heinemann et al., 1993). Consequently, subjects fed TO-phytosterols would exhibit plasma campesterol levels that include also the campesterol absorbed from the diet. Hence, it becomes difficult to assume that circulating campesterol concentrations in these subjects accurately reflect their cholesterol absorption rate. Nonetheless, a tendency toward lower plasma campesterol levels is shown in this study. This is likely due to the effect of 20% (w/w) sitostanol found in TO-phytosterols. Uncertainties exist regarding the use of changes in plasma ß-sitosterol levels as an index of cholesterol absorption rates. The correlation between cholesterol absorption rates and plasma ß-sitosterol levels was not as strong as the one with campesterol (Tilvis and Miettinen, 1986; Miettinen et al., 1995).

Finally, triglyceride concentration was higher in the TO-phytosterol-fed than in the control group. Five subjects in the treatment group were by chance

hypertriglyceridemic (4-7 mmol/L); their values increased the mean of their group significantly (Table 2). The difference in TG levels between control and treatment was most likely due to the starting baseline concentrations. Indeed, data presented as percent change from day 0 in plasma TG levels, which corrects for the difference in the starting baseline values, showed no changes within and between groups in TG concentrations. Furthermore, none of the subjects with starting normal TG level became abnormal after 30 days in the trial. Another, but perhaps less likely, explanation for the observed changes in plasma TG levels could be due to the modification in the subjects' diets. The prudent North American diet given to the subjects had a higher proportion of carbohydrates and lower saturated fatty acids than the typical Western diet which may have influenced their plasma TG levels.

In conclusion, precisely controlled dietary modification with concurrent TO-phytosterol supplementation lowered plasma TC and LDL-cholesterol concentrations in hypercholesterolemic subjects. However, the decrease in TC and LDL-cholesterol concentrations in the TO-phytosterol fed group did not result in changes in *de novo* cholesterogenesis in the hypercholesterolemic subjects.

7.6 Acknowledgments

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7.7 Figure legends

Figure 1.

Percent changes in plasma lipid profile in control and treatment groups. TC: total-cholesterol and LDL: low density lipoprotein-cholesterol. Values carrying the letters ***a*** and ***b*** are significantly different within the same group at $p < 0.05$. Values carrying the letters ***x*** and ***y*** are significantly different between control and treatment groups at $p < 0.05$.

Figure 2.

Percent changes in plasma lipid profile in control and treatment groups. HDL: high density lipoprotein-cholesterol and TG: triglyceride.

Figure 3.

Percent changes in plasma phytosterol levels and ratio in control and treatment groups. Camp: campesterol, sitos: ß-sitosterol. Values carrying the letters ***a*** and ***b*** are significantly different within the same group at $p < 0.05$. Values carrying the letters ***x*** and ***y*** are significantly different between control and treatment groups at $p < 0.05$.

Figure 4.

Plasma campesterol and ß-sitosterol levels in the control and treatment groups. Values carrying the letters ***a*** and ***b*** are significantly different within the same group at $p < 0.05$. Values carrying the letters ***x*** and ***y*** are significantly different between control and treatment at $p < 0.05$.

Figure 5.

Plasma campesterol and ß-sitosterol levels presented as mmol:mol cholesterol in the control and treatment groups. Values carrying the letters ***a*** and ***b*** are significantly different within the same group at $p < 0.05$. Values carrying the letters ***x*** and ***y*** are significantly different between control and treatment at $p < 0.05$.

Figure 6.

The correlation between plasma total cholesterol levels and absolute synthesis rate (ASR) in hypercholesterolemic subjects. Filled circles represent subjects in the control group. Open circles represent subjects in the treatment group.

Table 1. Intake of placebo, age, body weight, body mass index and percent change in body weight between day 0 and day 30 in hypercholesterolemic subjects in the control group (n=16).

Subject No	Age (year)	Body weight (kg)	Body mass index (kg/m^2) at day 0	% change in body weight (kg)
1	54	74	22.9	-2
2	55	83.2	25.8	-1.3
3	51	91.6	30.7	-2.9
4	35	101.8	26.3	-0.5
5	35	73.6	26.4	-0.5
6	47	58.4	18	-0.2
7	46	75.9	26.2	-0.9
8	60	68.6	28.7	-1.4
9	59	83.6	29	-1.9
10	55	77.7	27	-2.3
11	57	81.8	28.6	-0.9
12	47	79.1	25.8	0
13	60	82.3	27.2	-0.4
14	43	82.3	24.2	0.5
15	48	80	27	0.7
16	44	69.1	29.5	-0.9
Mean	49	78.9	26.5	-0.9
SD	8	9.5	2.9	0.9

Table 1 (continued). Intake of phytosterols, age, body weight, body mass index and percent change in body weight between day 0 and day 30 in hypercholesterolemic subjects in the treatment group (n=16).

Subject No	Age (year)	Body weight (kg)	Body mass index (kg/m^2) at day 0	% change in body weight (kg)
17	54	83.2	28.3	-3.2
18	53	93.2	30.6	-3.6
19	51	81.8	29.4	-0.7
20	33	105.9	37.4	0
21	43	84.5	26.3	-0.5
22	56	87.5	25.8	-3
23	48	66.4	22.6	-0.6
24	60	79.5	26.9	0.4
25	41	90.9	28	-1.4
26	46	75.5	24.6	-1.8
27	40	77.3	23.6	0.9
28	53	95.9	29.9	-0.5
29	29	55	24	-0.7
30	40	84.5	28.9	-1.4
31	46	111.8	33.4	-0.9
32	55	114.5	33.8	-1.1
Mean	47	86.7	28.3	-1.1
SD	8	15.1	3.9	1.2

Table 2. Plasma lipid concentrations in the two study groups (unpaired Student's t-test).

Plasma lipid and study days	Study groups	
	Control	Treatment
	mmol/L (mean ± SD)	
Total cholesterol		
0	6.81 ± 1.30	6.73 ± 1.15
10	6.43 ± 1.39*	6.06 ± 1.05**
20	6.22 ± 1.60**	5.78 ± 1.19*
30	6.10 ± 1.45***	5.42 ± 0.92***
LDL-cholesterol		
0	5.00 ± 1.27	4.45 ± 1.37
10	4.89 ± 1.30[a]	3.94 ± 0.95**[b]
20	4.59 ± 1.42*[a]	3.66 ± 0.84**[b]
30	4.56 ± 1.35*[a]	3.40 ± 0.94***[b]
HDL-cholesterol		
0	0.64 ± 0.18	0.75 ± 0.24
10	0.64 ± 0.19	0.71 ± 0.26
20	0.63 ± 0.21	0.71 ± 0.25
30	0.62 ± 0.19	0.67 ± 1.80
Total triglycerides		
0	2.55 ± 1.20	3.33 ± 1.23
10	2.00 ± 0.60[a]	2.97 ± 1.09[b]
20	2.18 ± 0.90[a]	3.08 ± 1.14[b]
30	2.06 ± 0.66[a]	3.00 ± 1.60[b]

Values carrying * as superscript are significantly different at $p < 0.05$ from day 0 within the group.
Values carrying [a-b] as superscripts are significantly different at $p < 0.05$ between groups.
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 3. Individual plasma total cholesterol levels at day 0 and 30, M_1-pool, fractional and absolute synthesis rates of free cholesterol from red blood cells in hypercholesterolemic subjects in the control group (n=16).

Subject No	TC (mmol/L) at day 30	TG (mmol/L) at day 30	TG factor	M_1-pool	FSR (per day)	% FSR (per day)	ASR (per day)
1	5.24	1.1	1	6.34	0.06	6.27	0.4
2	6.11	1.78	1	7.23	0.07	7.17	0.52
3	6.49	3.29	2	7.24	0.14	13.89	1.01
4	4.77	1.97	1	9.00	0.12	12.24	1.1
5	5.24	2.1	1	6.30	0.11	10.99	0.69
6	11.03	2.97	2	4.12	0.05	4.85	0.2
7	5.85	2.23	1	6.53	0.14	14.23	0.93
8	5.78	1.28	1	5.83	0.11	11.12	0.65
9	6.71	2.97	2	6.48	0.07	6.80	0.44
10	6.31	1.29	1	6.71	0.04	4.08	0.27
11	6.24	2.13	1	7.10	0.09	9.21	0.65
12	7.13	2.71	2	6.05	0.11	10.97	0.66
13	4.31	1.12	1	7.12	0.08	8.32	0.59
14	5.74	1.72	1	7.14	0.08	7.78	0.56
15	5.66	2.06	1	6.92	0.09	8.74	0.60
16	5.04	1.55	1	5.87	0.09	9.02	0.53
Mean	6.10[a]	2.02[a]		6.62	0.09	9.11	0.61
SD	1.46	0.66		0.98	0.03	2.88	0.24

Table 3 (continued). Individual plasma total cholesterol levels at day 0 and 30, M_1-pool, fractional and absolute synthesis rates of free cholesterol from red blood cells in hypercholesterolemic subjects in the treatment group (n=16).

Subject No	TC (mmol/L) at day 30	TG (mmol/L) at day 30	TG factor	M_1-pool	FSR (per day)	% FSR (per day)	ASR (per day)
17	2.92	1.12	1	7.19	0.11	11.02	0.79
18	5.86	1.6	1	8.19	0.13	13.01	1.06
19	4.89	2.14	1	7.08	0.08	7.80	0.55
20	6.59	4.13	3	8.61	0.07	6.75	0.58
21	5.52	2.41	2	6.55	0.13	12.85	0.84
22	4.95	4.32	3	6.83	0.09	8.57	0.59
23	6.63	1.58	1	5.63	0.07	7.02	0.40
24	6.08	4.84	3	6.08	0.13	13.21	0.80
25	5.58	7.08	3	6.36	0.07	7.15	0.45
26	5.10	2.54	2	5.68	0.05	4.87	0.28
27	6.16	2.95	2	5.87	0.09	9.22	0.54
28	6.52	5.02	3	6.85	0.10	9.96	0.68
29	5.56	1.65	1	4.53	0.06	6.05	0.27
30	4.51	1.69	1	7.34	0.11	10.95	0.80
31	5.03	2.76	2	9.16	0.11	10.96	1.00
32	4.82	2.25	1	10.21	0.07	6.87	0.70
Mean	5.42[a]	3.00[a]		7.01	0.09	9.14	0.65
SD	0.92	1.58		1.41	0.03	2.57	0.23

TC: total cholesterol, TG: triglyceride, FSR: fractional synthesis rate, ASR: absolute synthesis rate, M_1-pool: 0.287 weight + 0.0358 total cholesterol - 2.40 TGGP (TGGP is a variable which is equal to 1, 2 or 3 depending on serum TG concentration: <2.267, 2.267-3.401 or > 3.401 mmol/L).
To convert mmol/L TG to mg/dl multiply by 88.2.
Values carrying [a] as superscript are significantly different at $p < 0.05$ from day 0.

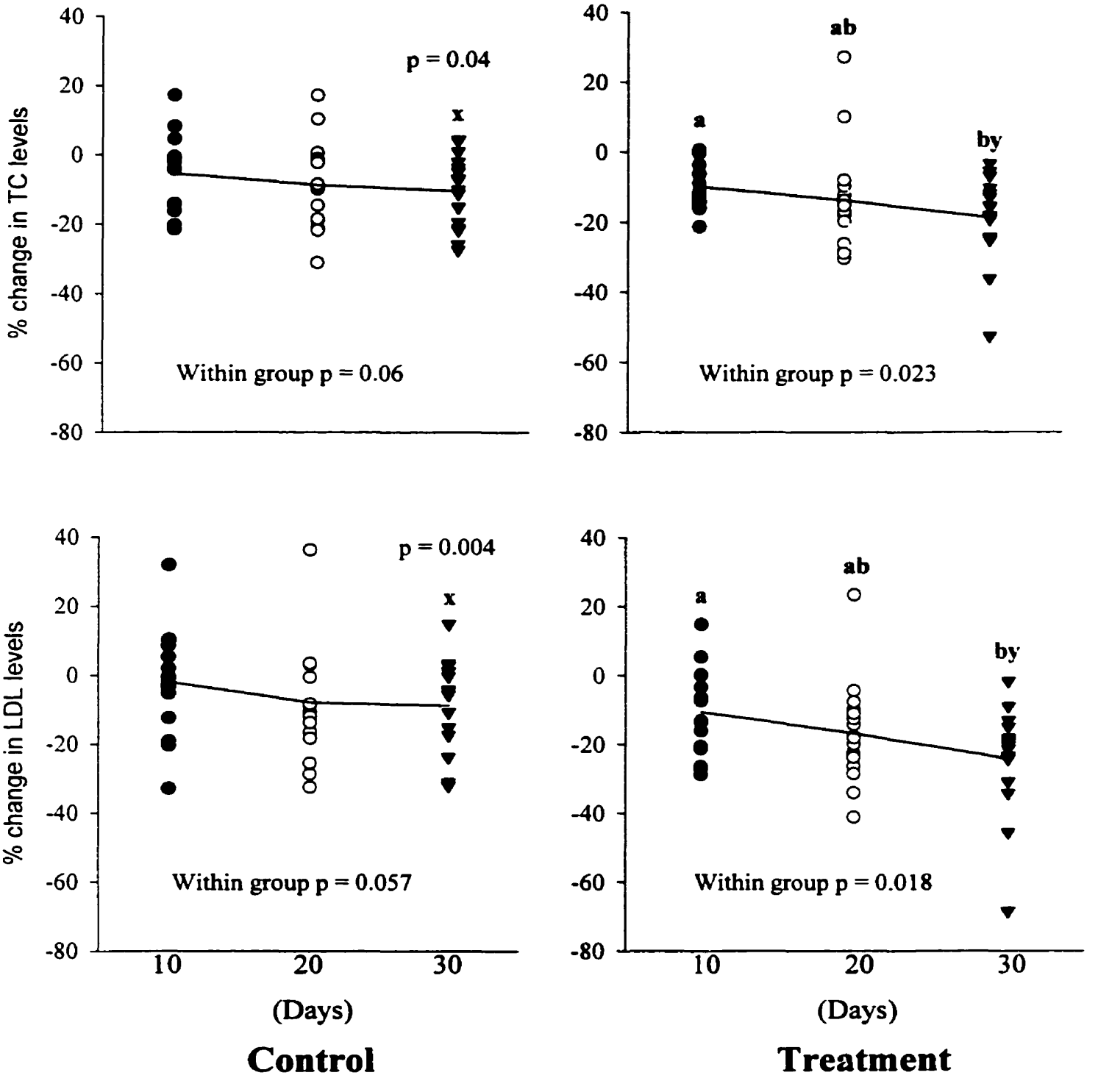
40
20
0
-20
-40
-60
-80
% change in TC levels
p = 0.04
x
Within group p = 0.06
ab
a
by
Within group p = 0.023
% change in LDL levels
p = 0.004
x
Within group p = 0.057
a
ab
by
Within group p = 0.018
10
20
30
(Days)
Control
Treatment

Figure 1

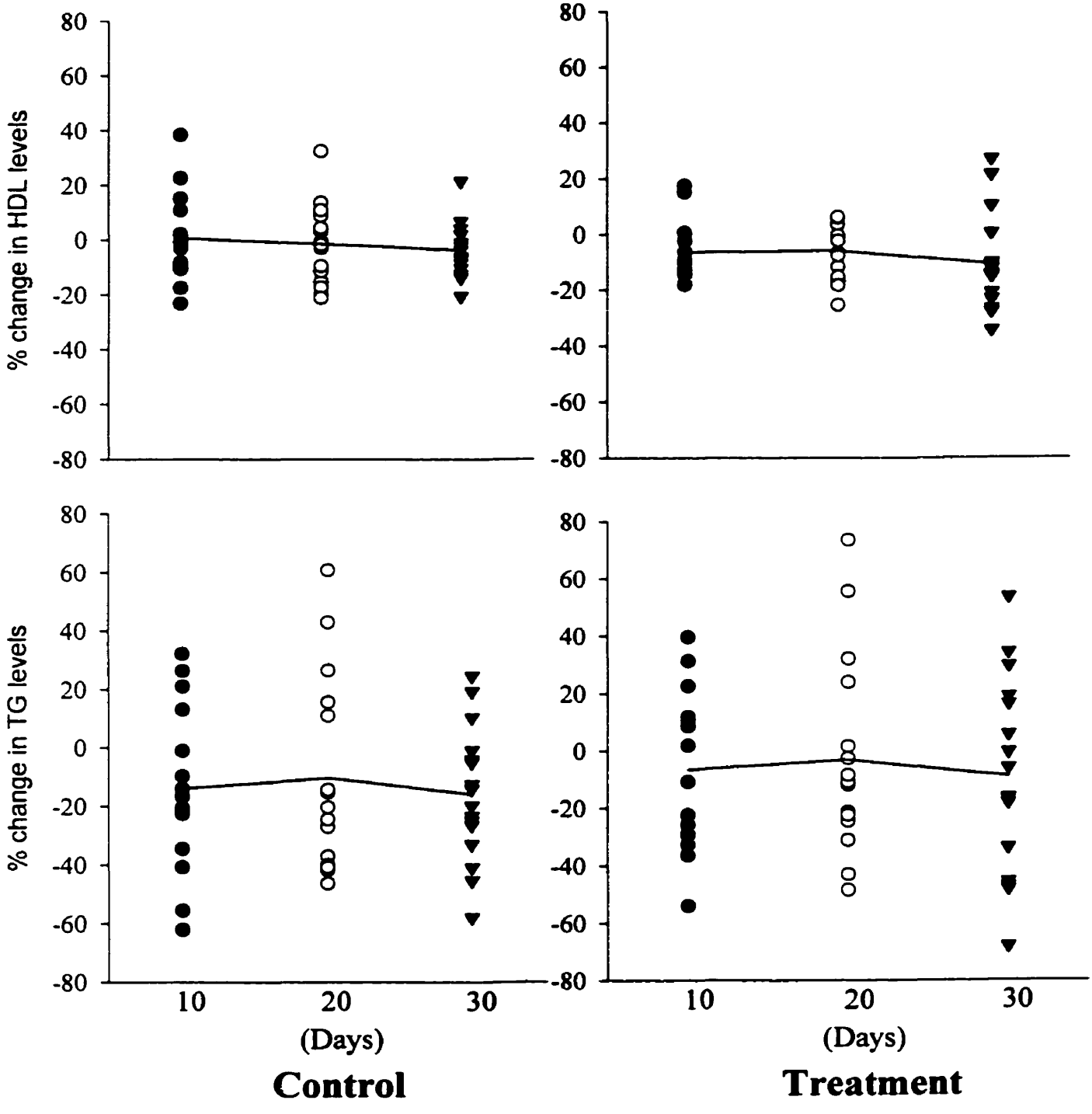
% change in HDL levels
% change in TG levels
80
60
40
20
0
-20
-40
-60
-80
10
20
30
(Days)
Control
Treatment

Figure 2

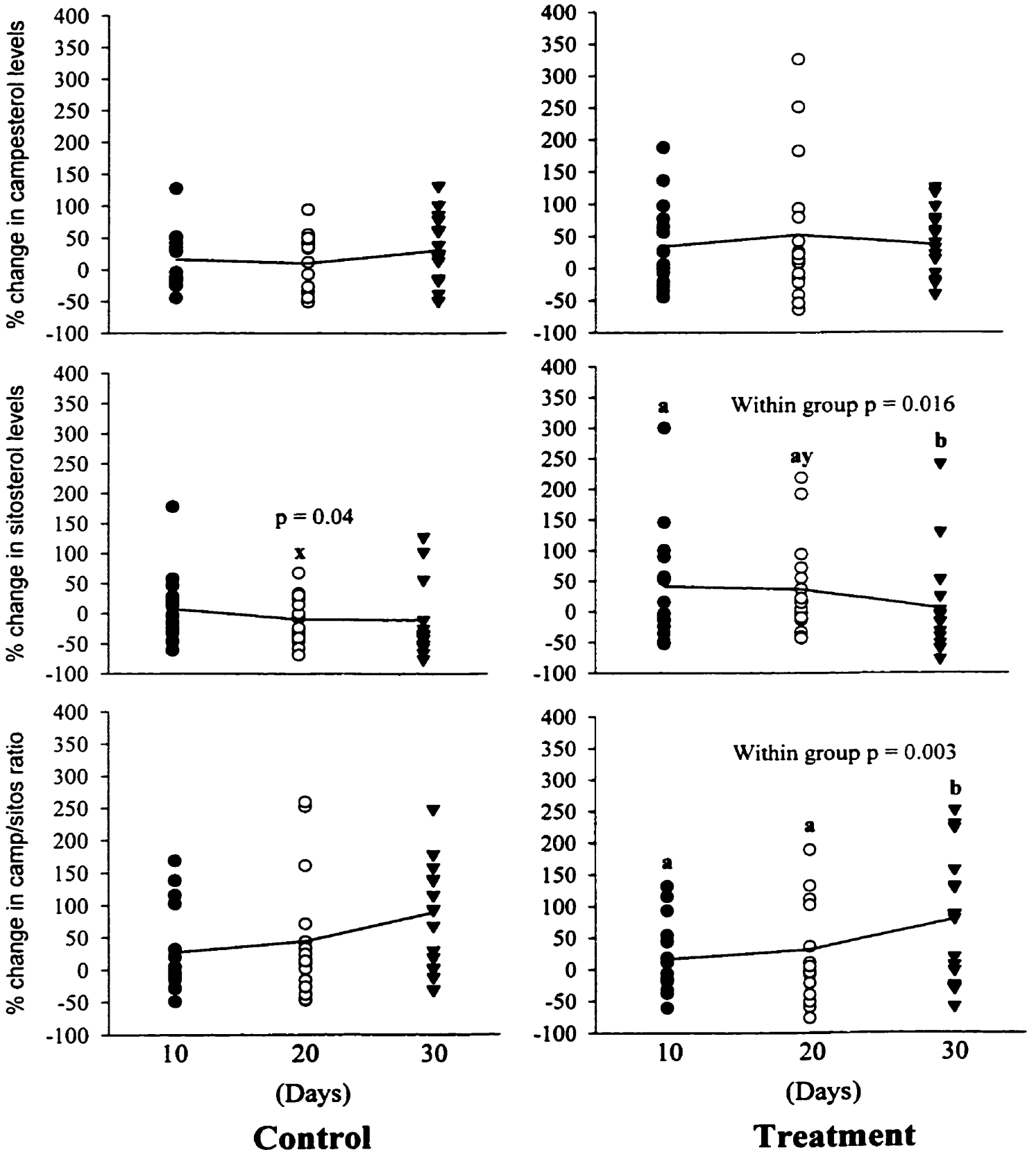
% change in campesterol levels
% change in sitosterol levels
% change in camp/sitos ratio
400
350
300
250
200
150
100
50
0
-50
-100
p = 0.04
x
Within group p = 0.016
a
ay
b
Within group p = 0.003
a
a
b
10
20
30
(Days)
Control
Treatment

Figure 3

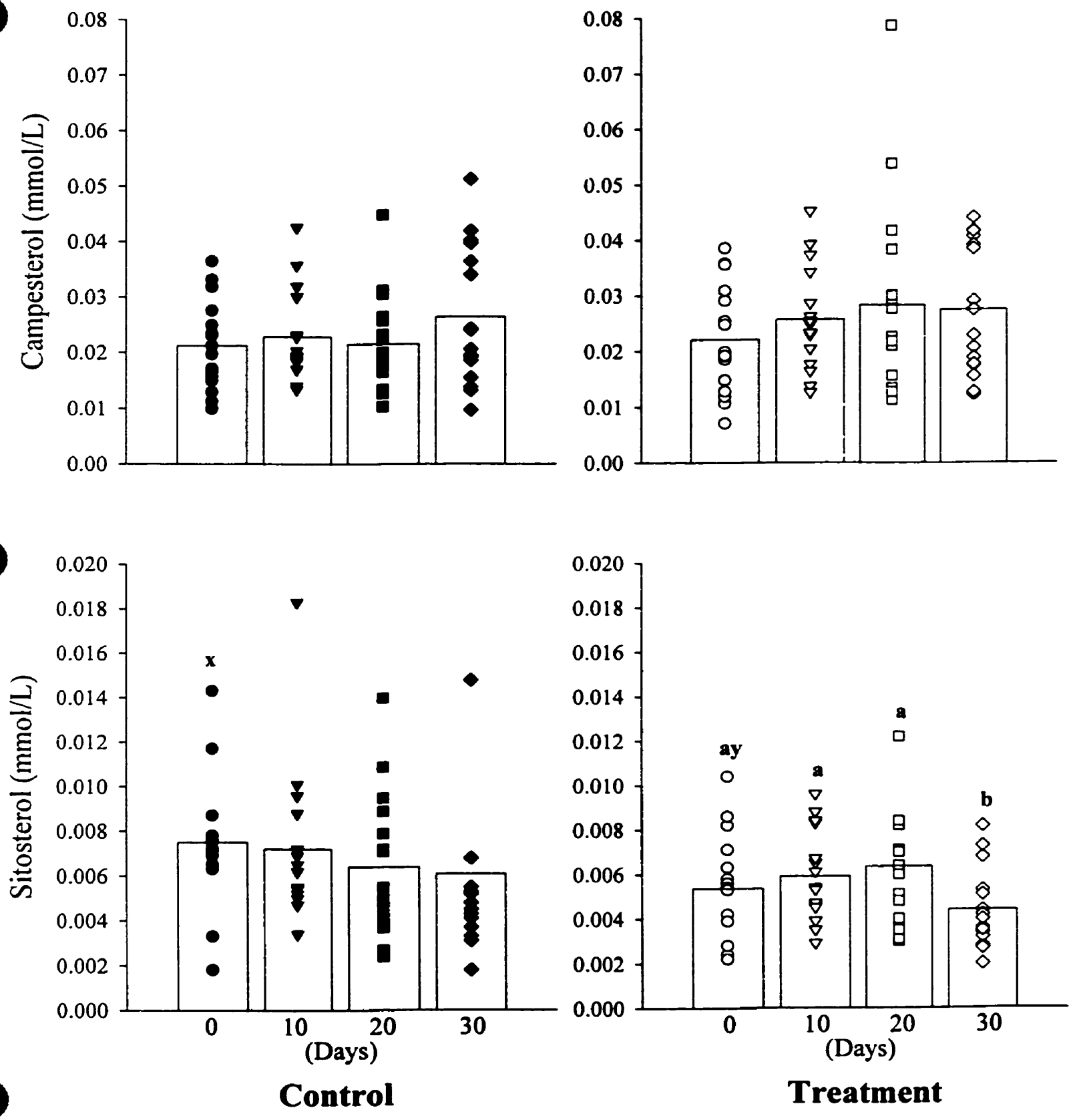
Campesterol (mmol/L)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Sitosterol (mmol/L)
0.020
0.018
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
x
ay
a
a
b
0
10
20
30
(Days)
Control
Treatment

Figure 4

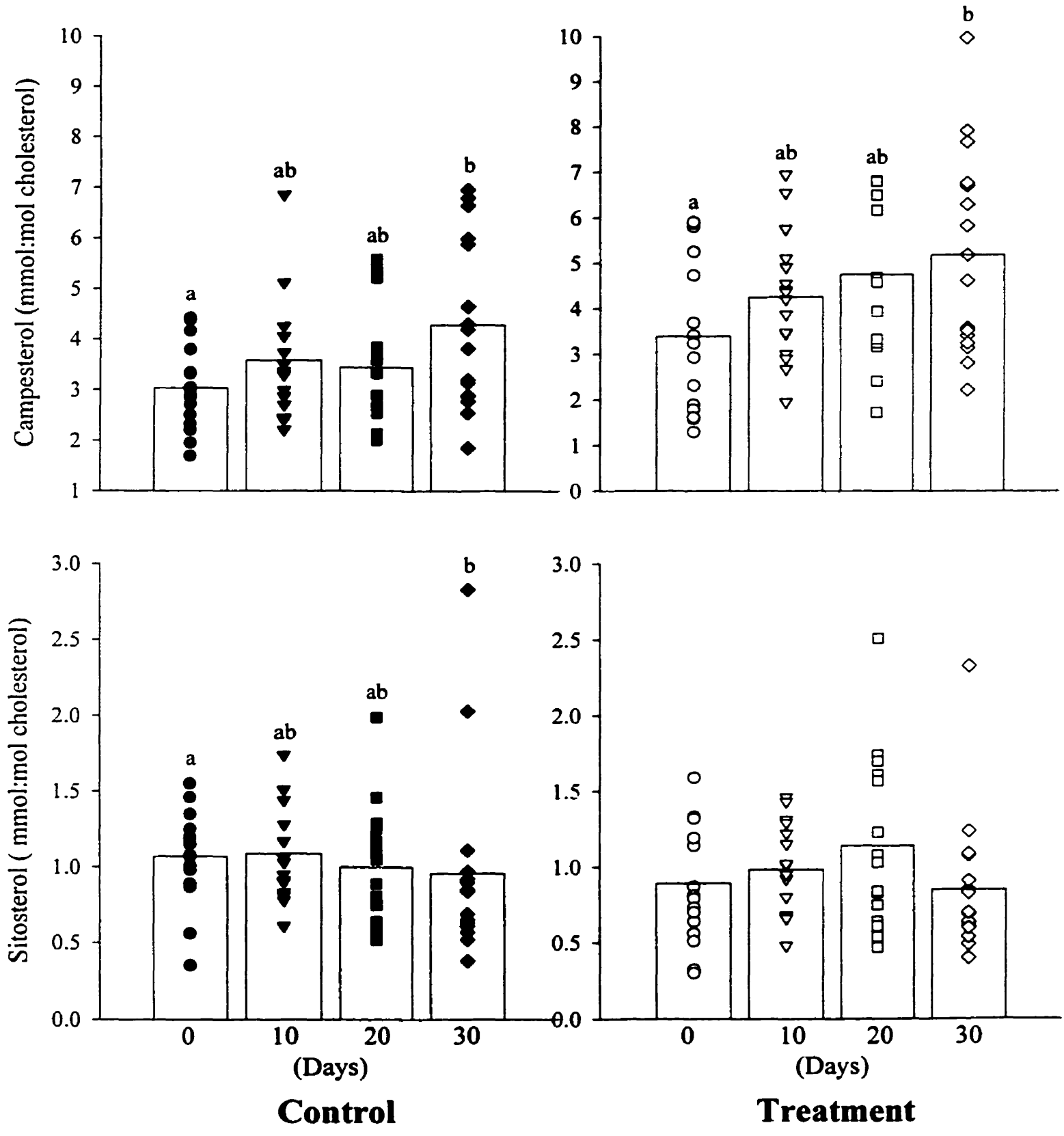
Campesterol (mmol:mol cholesterol)
Sitosterol (mmol:mol cholesterol)
a
ab
ab
b
(Days)
Control
Treatment

Figure 5

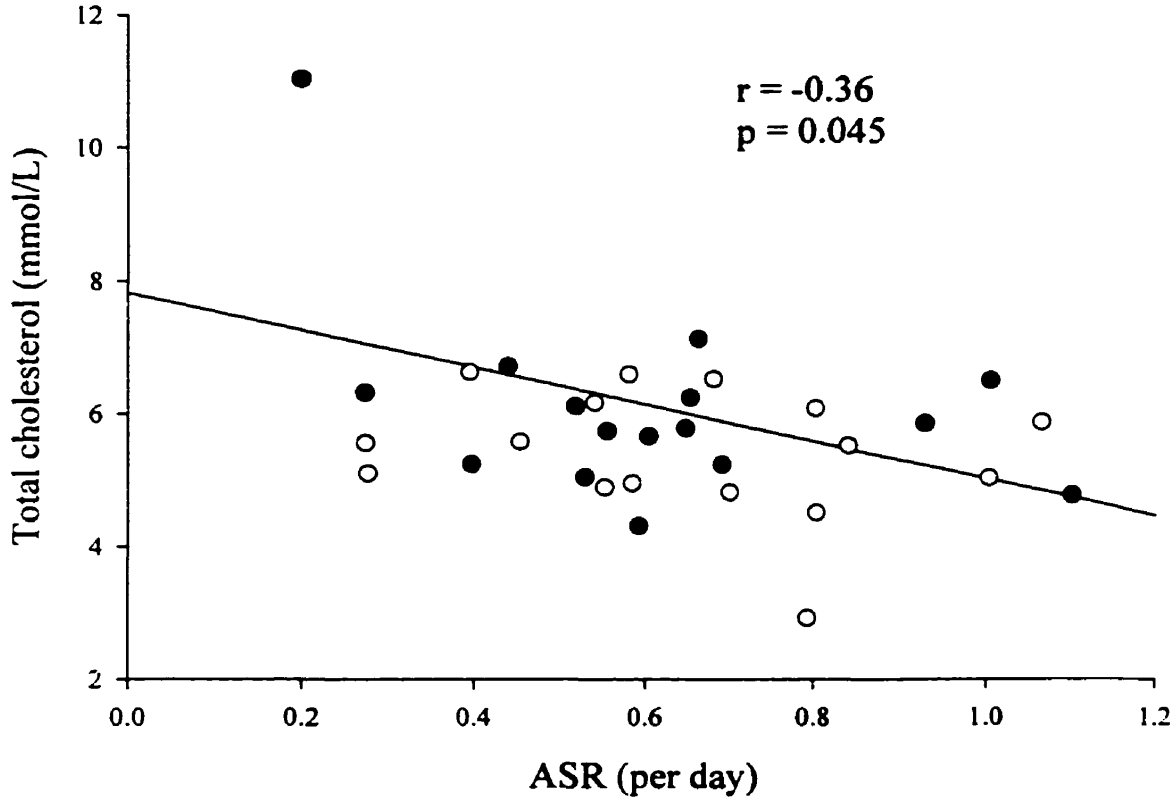
r = -0.36
p = 0.045
Total cholesterol (mmol/L)
ASR (per day)
12
10
8
6
4
2
0.0
0.2
0.4
0.6
0.8
1.0
1.2

Figure 6

TRANSITION

Data from the 30-day human study consolidated what we demonstrated previously in the animal trials. Indeed, tall oil-phytosterol mixtures lowered plasma cholesterol levels in hypercholesterolemic subjects. Moreover, dietary modification of the enrolled subjects resulted in a significant lowering in circulating lipid levels. Although subjects fed tall oil-phytosterols demonstrated lower total cholesterol levels than the control, the decrease in plasma total cholesterol levels did not reach a threshold that might allow for detection of changes in cholesterogenesis. Plasma campesterol levels could not be used as an index of cholesterol absorption in these subjects since they were fed tall oil-phytosterols which made the usage of variations in campesterol concentrations unreliable in this study. For this reason, in the following study, human subjects were not fed phytosterols. Instead, they were given a HMG-CoA reductase inhibitor (simvastatin) while they maintained their Step One diet. It has been suggested that simvastatin lowers plasma total cholesterol levels by suppressing cholesterol absorption in animals, in addition to inhibiting the activity of HMG-CoA reductase. The changes in circulating campesterol levels would thus indicate any modification in fractional cholesterol absorption in the hypercholesterolemic patients.

CHAPTER 8

THE EFFECT OF HMG-CoA REDUCTASE INHIBITOR ON STEROL ABSORPTION IN HYPERCHOLESTEROLEMIC SUBJECTS

Paper submitted to the journal *Metabolism (1998)*.

8.1 Abstract

To investigate potential effects of high doses of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA) inhibitor on plasma campesterol, total cholesterol (TC), low density lipoproteins (LDL)-, high density lipoproteins (HDL)-cholesterol and triglyceride levels, hypercholesterolemic subjects were given 40 or 80 mg/day simvastatin in a 24-week prospective clinical trial. Plasma lipid levels were analyzed enzymatically and plasma phytosterol concentrations determined using gas liquid chromatography (GLC). Changes in plasma campesterol levels are used as an indicator of cholesterol absorption. Simvastatin treatment reduced plasma campesterol concentrations 24% ($p = 0.017$) but did not affect circulating stigmasterol and ß-sitosterol levels. A dose of 80 mg/day simvastatin lowered ($p = 0.032$) plasma campesterol level more than did 40 mg/day. There was a positive correlation between plasma campesterol and TC concentrations both before ($r = 0.54$, $p = 0.027$) and after ($r = 0.63$, $p = 0.009$) treatment. Simvastatin treatment reduced circulating TC and LDL-cholesterol, and triglyceride levels by 40%, 50% and 33% ($p < 0.007$), respectively. Plasma lipid levels did not differ between groups given 40 or 80 mg/day simvastatin. No significant effect of simvastatin was seen on plasma HDL-cholesterol concentrations, but the ratio of HDL/LDL increased 1.3 fold ($p < 0.0001$). In conclusion, this HMG-CoA reductase inhibitor reduces plasma campesterol level, a marker of cholesterol absorption, which might contribute to the mechanism by which simvastatin may lower circulating cholesterol levels.

8.2 Introduction

Simvastatin (MK-733) is among the most effective agents for the treatment of hypercholesterolemia in humans. The Scandinavian Simvastatin Survival Study (Scandinavian Simvastatin Survival Study Group, 1994) demonstrated a significant reduction in overall morbidity and mortality with this lipid-lowering agent. Simvastatin is a potent inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA). It decreases hepatic cholesterol biosynthesis by a competitive and reversible inhibition of HMG-CoA reductase activity which leads to an increased expression of low density lipoproteins (LDL) receptors in the liver. These receptors bind LDL particles and remove them from the circulation and thus lower plasma total cholesterol (TC) levels (Plosker and McTavish, 1995).

Other mechanisms by which HMG-CoA inhibitors may lower plasma TC have been proposed. As the benefits seen were manifest before plaque stabilization or regression may have occurred, it has been suggested that statins in general (Vaughan et al., 1996; Martinez et al., 1996; Gaw, 1996a), and simvastatin in particular (Negreaminou et al., 1997; Odriscoll et al., 1997; Human et al., 1997; Morita et al., 1997; Abbott et al., 1997), exert effects other than inhibiting HMG-CoA reductase activity. Simvastatin has been shown to decrease synthesis of apo-B lipoproteins (Gaw et al., 1993; 1996b; Ryomoto et al., 1996), reverse endothelial dysfunction seen in hypercholesterolemia (Kobayashi et al., 1989), improve the arterial topographical morphology after four years in the Multicentre Anti-Atheroma Study (MAAS investigators, 1994) and result in reduced ex-vivo platelet aggregation in patients with type IIa hypercholesterolemia (Notarbartolo et al., 1995). Moreover, HMG-CoA reductase inhibitors have been

suggested to decrease cholesterol absorption in animals (Hajri et al., 1995; 1997). Ishida et al. (1988; 1989) reported that the hypolipidemic effect of simvastatin in cholesterol-fed rabbits is related to a marked reduction of exogenous cholesterol absorption from the intestinal wall due to inhibition of acylcoenzyme A: cholesterol acyltransferase (ACAT) activity. This effect, however, has not been clearly demonstrated in humans (Miettinen, 1991; 1992; Vanhanen et al., 1992; Chisholm et al., 1994). Whether simvastatin affects cholesterol absorption in hypercholesterolemics is the subject of the current study.

One approach for determining cholesterol absorption is measurement of the content of the plant sterol campesterol in plasma. Campesterol is derived from the diet and found in small quantities in human plasma and is mainly transported in lipoprotein fractions (Connor and Lin, 1981; Boberg and Skrede, 1988; Robins and Fasulo, 1997). Like other plant sterols, it is not catabolized in vivo as has been shown in rat liver perfusate (Boberg et al., 1990). In addition, phytosterols are found in small concentrations in human bile (Ilias et al., 1980; Miettinen et al., 1986), suggesting that they are absorbed from the intestine, transported in plasma and secreted in part as neutral sterols in bile. Plasma campesterol levels have been shown to positively correlate with dietary cholesterol absorption efficiency in normal (Tilvis and Miettinen, 1986; Miettinen et al., 1989; 1990), hypercholesterolemic (Miettinen et al., 1995) and coeliac subjects (Vuoristo et al., 1988). Variation in campesterol levels in plasma is used as an index of cholesterol absorption.

The aim of the current study was to further explore the effect of 40 and 80 mg/day simvastatin on plasma campesterol and lipoprotein levels in hypercholesterolemic subjects over a 24-week treatment period.

8.3 Subjects and methods

8.3.1 Subjects

Eighteen subjects (8 women and 10 men) were recruited from patients participating in a simvastatin parallel clinical trial in the Atherosclerosis Speciality Laboratory, Lipid Clinic, St. Paul's Hospital, Vancouver, British Columbia. The subgroup patients had a mean age of 49 ± 11 (± SD) years and their plasma TC, low density lipoproteins (LDL), high-density lipoproteins (HDL) cholesterol and triglyceride (TG) had been measured four weeks before the trial (during the washout-period), at the baseline, every six weeks and at the end of the 24-week trial. Subjects were admitted into the study based on the following selection criteria: plasma low density lipoproteins (LDL) > 4.16 mmol/L, TG ≤ 3.95 mmol/L and age-range between 21 and 70 years. Patients with body mass index (BMI) (the weight in kg divided by the square of the height in meters) ≤ 31 were accepted in the study. Any previous lipid lowering medications were suspended during the washout-period. Subjects who volunteered for the study were checked for hypertension, diabetes and coronary heart problems. In addition, plasma chemical profile, hematology and urinalysis were performed. Patients suffering from renal failure, hypertension, myocardial infarction, diabetes type I or II, hypersensitivity to HMG-CoA, types I, III, IV, V hyperlipidemias, homozygous familial hypercholesterolemia (FH) or glandular problems were excluded from the study. The experimental protocol was approved by the Ethics Committee of the Lipid Clinic at St. Paul's Hospital and the Department of Pathology at the University of British Columbia.

8.3.2 Study design

The study was conducted for a 24-week period during which 7 (2 females and 5 males) and 11 (6 females and 5 males) subjects received either 40 or 80 mg/day simvastatin (Zocor, Merck Sharp and Dohme, Rahway, NJ). Blood samples were taken four weeks before the start of the study after subjects had fasted for 12 hr. During a one-month washout period, patients were put on a low cholesterol diet (Step One of American Heart Association: 30% total fat, 7-10% saturated fats, 200-300 mg/day cholesterol, 15-16% proteins and 54-55% total carbohydrates) (National Institute of Health National Cholesterol Education Program Step One Diet) (Anonymous, 1993; 1994a; Sempos et al., 1993) and all lipid lowering drugs taken previously by the subjects were discontinued. Four weeks later, subjects who fulfilled the selection criteria were admitted into the study and their plasma lipid concentrations, heights and weights were measured at baseline. After 24-week of daily oral administration of 40 or 80 mg simvastatin, blood samples were collected and body weights were measured. Dietary instructions were given to the patients with follow up by dietitians to monitor their food intake patterns and their adherence to the diet.

Plasma TC, HDL cholesterol and TG concentrations were measured using commercial enzymatic kits (Abbott, Montréal, Canada). High-density lipoprotein cholesterol concentrations were measured in plasma after precipitation of apoB-lipoproteins with dextran sulfate/magnesium chloride (Warnick et al., 1982). The concentration of LDL cholesterol was calculated according to the methods of Friedewald et al. (1972). Plasma plant sterol concentrations were determined by gas liquid chromatography (GLC) from the non-saponifiable material of plasma lipid (Hamilton et

al., 1992). Briefly, 0.5 ml plasma samples were saponified with 0.5 M KOH for two hr and the non-saponifiable material extracted with petroleum ether. Five alpha cholestane was used as an internal standard. Samples were injected into a GLC equipped with a flame ionization detector (HP 5890 Series II, Hewlett Packard, CA, USA) using a 30-m capillary column, SAC-5 (Supelco, Bellefonte, PA, USA). Detector and injector temperatures were set at 320 and 300°C, respectively. Samples were run isothermically at 285°C. Plant sterol peaks were identified by comparison with standards (Supelco, Bellefonte, PA, USA).

8.3.3 Statistical analysis

All values are presented as mean ± SD. The effects of simvastatin on the measured variables, plasma lipid profile and plant sterol levels, were tested using paired Student's t-test. Student's t-test was applied to measure any difference in the variables between concentrations and males and females. The relationship between the variables, plant sterols and plasma TC levels, were determined using Pearson Product-Moment Correlation Coefficients.

8.4 Results

Body weights (mean 74.1 ± 13.63 kg) and BMI index (26.1 ± 2.8 kg/m^2) had not changed over the 24 weeks of the trial. Reported dietary cholesterol and fractions of energy derived from saturated, monounsaturated and polyunsaturated fats did not differ between males and females during the study period. In general, dietary cholesterol and total fat intakes were similar in the subjects pre- and post-treatment with simvastatin. No

adverse clinical side effects from simvastatin use were reported among patients. The only differences observed between males and females were in plasma HDL cholesterol levels. Females had consistently higher ($p = 0.016$ and $p = 0.049$, pre- and post-treatment, respectively) circulating HDL cholesterol concentrations than males. Male and female subjects were pooled into one group because there were no other gender differences for plasma lipid and plant sterols profiles (Table 1). Combined averages for both males and females were: height, 167.9 ± 9.8 cm (range: 152 - 184), weight, 74.1 ± 13.63 kg (range: 50 - 104), age, 48.5 ± 11.5 years (range: 22 - 66) and BMI, 26.1 ± 2.8 kg/m^2 (range: 22 - 31).

Plasma lipid profile during 24-week treatment is shown in Figure 1. At week 6 of simvastatin treatment, plasma TC, LDL and TG levels were significantly lowered ($p < 0.02$) but from week 6 to 24, there were no further changes in plasma lipid levels. The same pattern was seen for both doses: 40 and 80 mg/day simvastatin. Plasma lipid levels did not differ between groups given 40 or 80 mg/day simvastatin (Table 2). However, 80 mg/day simvastatin lowered ($p = 0.032$) plasma campesterol level more than 40 mg/day. Conversely, there were no differences in circulating stigmasterol and ß-sitosterol concentrations between the 40 and 80 mg/day simvastatin doses.

Table 3 shows the combined results of both subgroups of hypercholesterolemics. Simvastatin provided at dosages of both 40 and 80 mg/day lowered mean plasma TC level by an average of 40%. Mean plasma TC level was decreased ($p < 0.0001$) from 10.04 ± 1.90 mmol/L to 6.11 ± 1.24 mmol/L (95 percent confidence interval (95% CI), 3.18 - 4.68) by the simvastatin doses. Mean circulating LDL cholesterol level was lowered ($p < 0.0001$) by 47% (7.9 ± 2.0 mmol/L to 4.2 ± 1.2 mmol/L) (95% CI, 2.94 -

4.46). Similarly, simvastatin decreased ($p = 0.007$) mean plasma TG level by 32% (2.07 ± 1.02 mmol/L to 1.41 ± 0.60 mmol/L) (95% CI, 0.21 - 1.11). There was no effect of simvastatin on plasma HDL cholesterol concentrations. On the other hand, HDL/LDL ratio was increased ($p < 0.0001$) by 1.3-fold from 0.22 ± 0.04 to 0.30 ± 0.13 with the simvastatin doses.

Simvastatin treatment significantly lowered ($p = 0.0172$) plasma campesterol levels at both dose levels by 24% from 0.0256 ± 0.0083 mmol/L to 0.0195 ± 0.0037 mmol/L (Table 4). The levels of stigmasterol and ß-sitosterol were not significantly affected by the simvastatin treatments. Moreover, the ratios of campesterol to cholesterol (2.65 ± 1.06 and 3.21 ± 0.98 mmol/mol) and campesterol to ß-sitosterol (0.66 ± 0.23 and 0.57 ± 0.15; $p = 0.08$) did not change significantly (Table 4). Plasma TC concentrations, however, were significantly correlated with campesterol levels ($r = 0.54$, $p = 0.027$ and $r = 0.63$, $p = 0.009$) in both pre- and post-treatment. Moreover, percent changes of circulating TC were positively correlated with campesterol/ß-sitosterol ratios in pre- ($r = 0.68$, $p = 0.003$) and post- ($r = 0.66$, $p = 0.005$) treatment. The correlation of percent changes in plasma TC and LDL concentrations with percent changes in circulating campesterol, stigmasterol and ß-sitosterol were not significant. In addition, the ratio of campesterol:LDL did not vary between starting and end of the trial.

8.5 Discussion

The main finding in this study is that plasma campesterol levels were reduced after 24-week on high dose simvastatin treatment. On the other hand, circulating ß-sitosterol and stigmasterol concentrations were unchanged by treatment. Plasma

campesterol levels were comparable with all those reported in healthy individuals (Gylling and Miettinen, 1988; Sutherland et al., 1991), those with hypercholesterolemia (Miettinen et al., 1992; 1995; Vanhanen, 1994;) and in subjects with non-insulin dependent diabetes mellitus (Sutherland et al., 1992).

Circulating plant sterol levels have been shown to be related to fractional cholesterol absorption in hypercholesterolemic subjects (Tilvis and Miettinen, 1986; 1990; 1995; Vuoristo et al., 1988; Gylling and Miettinen, 1988). Tilvis and Miettinen (1986) reported that plasma campesterol concentration, unlike ß-sitosterol, is indicative to changes in circulating TC in humans. Moreover, plasma plant sterol levels are unlikely to be modified by the Step One Diet (Anonymous, 1993; Sempos et al., 1993) which contains less cholesterol and fats but provides more vegetable and fruit servings than typical Western diets. Salen et al. (1970) showed that more than an eight-fold increase in dietary plant sterol load is needed in order to double the plasma ß-sitosterol levels in healthy subjects.

Accordingly, the lower plasma campesterol levels seen after simvastatin treatment suggest reduced cholesterol absorption. This finding is in agreement with work done in heterozygous FH subjects fed egg yolk and supplemented with lovastatin (Miettinen, 1991). Moreover, the ratio of plasma campesterol to cholesterol levels significantly increased in both non-FH and FH patients given 40 mg/day simvastatin (Miettinen et al., 1992) or FH subjects administered 80 mg/day pravastatin (Vanhanen, 1994) suggesting lowered cholesterol absorption.

Mechanisms involved in lowering intestinal cholesterol absorption by HMG-CoA reductase inhibitors are not fully understood. Vanhanen et al. (1992) suggested that the

entry of intestinal cholesterol into the liver as chylomicrons is reduced by pravastatin in FH subjects. In addition, they showed in the same subjects a reduction in esterified methyl sterol contents of the chylomicrons which may reflect a decrease in intestinal mucosal ACAT activity (Vanhanen et al., 1992). In more invasive animal experiments, Ishida et al. (1988) demonstrated that simvastatin reduced cholesterol absorption in cholesterol-fed rabbits. Furthermore, cholesterol absorption was lowered in hamsters (Hajri et al., 1997) and hypercholesterolemic rats (Hajri et al., 1995) given HMG-CoA reductase inhibitor. Ishida et al. (1989) reported that this effect was due to a reduction in microsomal ACAT activity in the intestinal mucosa. The ACAT enzyme has a major role in cholesterol absorption (Clark and Tercyak, 1984) by acting primarily on its esterification rate in the intestine (Spector et al., 1979; Clark, 1979; Field et al., 1982) and also by limiting cholesteryl ester incorporation into chylomicrons (Burrier et al., 1994; 1995). Agents that inhibit ACAT activity, and thus cholesterol absorption, have been shown to reduce plasma cholesterol levels in animal studies (Burrier et al., 1995). Though the precise mechanisms involved in cholesterol esterification by the intestinal mucosa are unclear, it has been shown that ACAT (Clark and Tercyak, 1984) and cholesterol esterase (Gallo et al., 1984) each play a role in the intestinal absorption of exogenous cholesterol in rats. Also, ACAT is suggested as the primary enzyme responsible for cholesterol esterification in the intestine of hamsters (Burrier et al., 1995). Results from this study show a reduced campesterol concentration in the plasma and suggest that intestinal cholesterol absorption was reduced but do not demonstrate direct simvastatin effect on ACAT activity.

A dose of 80 mg/day was more effective than 40 mg/day simvastatin in lowering circulating campesterol levels. This superior effect of 80 over 40 mg/day suggests that simvastatin has a dose-dependent factor in lowering plasma campesterol levels probably by inhibiting more its absorption. On the other hand, Chisholm et al. (1994) failed to show any effect of ≈30 mg/day simvastatin on plasma campesterol levels in patients with familial hypercholesterolemia put on low and then high fat diets. The discrepancy seen between their results and those in this study may be due to the lower simvastatin dosage. The difference between the high-fat/cholesterol diets used by Chisholm et al. (1994) and the low-fat/cholesterol diets used in this study may also have confounded the results due to the fact that dietary plant sterol absorption are affected by dietary fat intakes (Miettinen and Siurala, 1971; Vanhanen and Miettinen, 1992).

Another possible explanation for simvastatin effect on plasma campesterol and cholesterol levels could be due to the marked decrease in cholesterol synthesis. Chang and Chang (1986a) and Chang et al. (1986b) showed that providing 20 mM DL-mevalonate to Chinese hamsters ovary cells grown in sterol-free medium increases the activity of ACAT enzyme 6-fold. Such activation of ACAT by adding mevalonate was inhibited by adding squalene oxide cyclase inhibitor to the cells. These results suggest that active endogenous sterol synthesis is required in order to manifest the effect of mevalonate (Chang and Chang, 1986a; Chang et al., 1986b). Simvastatin inhibits significantly endogenous cholesterol synthesis. Consequently and as suggested by Ishida et al. (1989), such reduction in endogenous cholesterol levels may inhibit ACAT activity and thus reduce campesterol and cholesterol absorption.

Finally, several investigators have demonstrated that simvastatin affected cellular membrane functions by decreasing their cholesterol content (Martinez et al , 1996; Morita et al., 1997). Such changes in the content of intestinal mucosal cells might alter membrane fluidity and subsequently, the functions of membranes associated proteins such as ACAT. Hence, cholesterol absorption in the intestinal tract might be impaired due to simvastatin effect on endothelial membranes.

In conclusion, our findings show that 24-week use of simvastatin lowers plasma TC, LDL-cholesterol, TG levels, increases HDL/LDL ratio and reduces circulating campesterol concentrations. This suggest that the simvastatin may have an inhibitory role on cholesterol absorption in hypercholesterolemic subjects.

8.6 Acknowledgments

We would like to thank François Bertrand, M.D., Debra DeAngelis, RT, CCRC, Frances Johnson, RDN and Shauna Ratner, RDN for their excellent assistance and contribution to the study.

8.7 Figure Legend

Figure 1.

Plasma lipid profile in hypercholesterolemic subjects (n=18) given 40 or 80 mg simvastatin for 24 weeks. Time effect within the same group in contrast to week 1.

Table 1. Comparison of plasma lipid and phytosterol levels (mmol/L) in hypercholesterolemic females versus males pre- and post-treatment.

		Females (n=8)		Males (n=10)		p value	95% CI
		Mean	SD	Mean	SD		
Total cholesterol	Pre:	10.34	2.05	9.82	1.87	0.6	-1.52 , 2.56
	Post:	5.74	1.09	6.36	1.32	0.33	-1.91 , 0.68
Low density lipoproteins	Pre:	8.61	1.98	8.33	1.88	0.77	-1.74 , 2.29
	Post:	3.7	0.84	4.62	1.31	0.13	-2.12 , 0.29
Triglycerides	Pre:	1.73	0.77	2.31	1.13	0.26	-1.63 , 0.48
	Post:	1.28	0.52	1.5	0.67	0.47	-0.87 , 0.42
High density lipoproteins	Pre:	1.39	0.37	1.03	0.17	0.02	0.0767 , 0.6360
	Post:	1.44	0.41	1.14	0.15	0.05	0.0045 , 0.6020
Campesterol	Pre:	0.02	0.01	0.03	0.0141	0.11	-0.0223 , 0.0025
	Post:	0.02	0.008	0.02	0.0073	0.39	-0.0111 , 0.0046
Stigmasterol	Pre:	0.01	0.007	0.01	0.0058	0.61	-0.0047 , 0.0078
	Post:	0	0.003	0.01	0.0042	0.59	-0.0050 , 0.0030
ß-sitosterol	Pre:	0.03	0.016	0.05	0.0206	0.12	-0.0332 , 0.0044
	Post:	0.03	0.011	0.04	0.0111	0.26	-0.0140 , 0.0053

Subjects were administered a dosage of 40 or 80 mg/day simvastatin. The 95 percent confidence interval (CI) is for difference of means.

Table 2. Effect of 40 versus 80 mg/day simvastatin on plasma lipid and phytosterol levels (mmol/L) in hypercholesterolemic subjects.

	40 mg/day (n=7)		80 mg/day (n=11)		p value	95% CI
	Mean	SD	Mean	SD		
Total cholesterol	6.68	0.51	5.71	1.45	0.11	-2.12 , 0.21
Low density lipoproteins	4.63	0.73	3.77	1.4	0.16	-1.92 , 0.40
High density lipoproteins	1.35	0.36	1.21	0.29	0.41	-0.50 , 0.21
Triglyceride	1.43	0.71	1.39	0.56	0.91	-0.65 , 0.59
Campesterol	0.025	0.06	0.017	0.007	0.032	-0.0145 , 0.0008
Stigmasterol	0.011	0.01	0.01	0.003	0.45	-0.0054 , 0.0025
ß-sitosterol	0.04	0.013	0.034	0.011	0.075	-0.0205 , 0.0011
Campesterol/ß-sitosterol	0.63	0.16	0.55	0.16	0.32	-0.244 , 0.086

Subjects were administered a dosage of 40 or 80 mg/day simvastatin. The 95 percent confidence interval (CI) is for difference of means.

Table 3. Plasma lipid levels (mmol/L) in the hypercholesterolemic subjects (n = 18).

	Pre-treatment		Post-treatment		p value	95 % CI
	Mean	SD	Mean	SD		
Total cholesterol	10.04	1.9	6.11	1.24	< 0.0001	3.18 , 4.68
High density lipoproteins	1.77	0.32	1.25	0.35	0.12	-0.16 , 0.02
Low density lipoproteins	7.91	1.96	4.22	1.21	< 0.0001	2.94 , 4.46
Triglycerides	2.07	1.02	1.41	0.6	0.007	0.21 , 1.11
HDL/LDL	0.22	0.04	0.3	0.13	< 0.0001	-0.22 , -0.12
Pearson Product Moment Correlation between plasma TC and campesterol	r = 0.54 p = 0.003		r = 0.63 p = 0.009			

Subjects were administered a dosage of 40 or 80 mg/day simvastatin. The 95 percent confidence interval (CI) is for difference of means.

Table 4. Plasma phytosterol levels (mmol/L) in hypercholesterolemic subjects (n = 18).

	Pre-treatment		Post-treatment		p value	95% CI
	Mean	SD	Mean	SD		
Campesterol	0.026	0.01	0.02	0.0088	0.02	0.0013 , 0.0110
Stigmasterol	0.012	0.01	0.01	0.0037	0.39	-0.0017 , 0.0034
ß-sitosterol	0.041	0.02	0.034	0.0111	0.26	-0.0051 , 0.0175
Campesterol/ß-sitosterol	0.66	0.229	0.569	0.154	0.08	-0.016 , 0.245

Subjects were administered a dosage of 40 or 80 mg/day simvastatin. The 95 percent confidence interval (CI) is for difference of means.

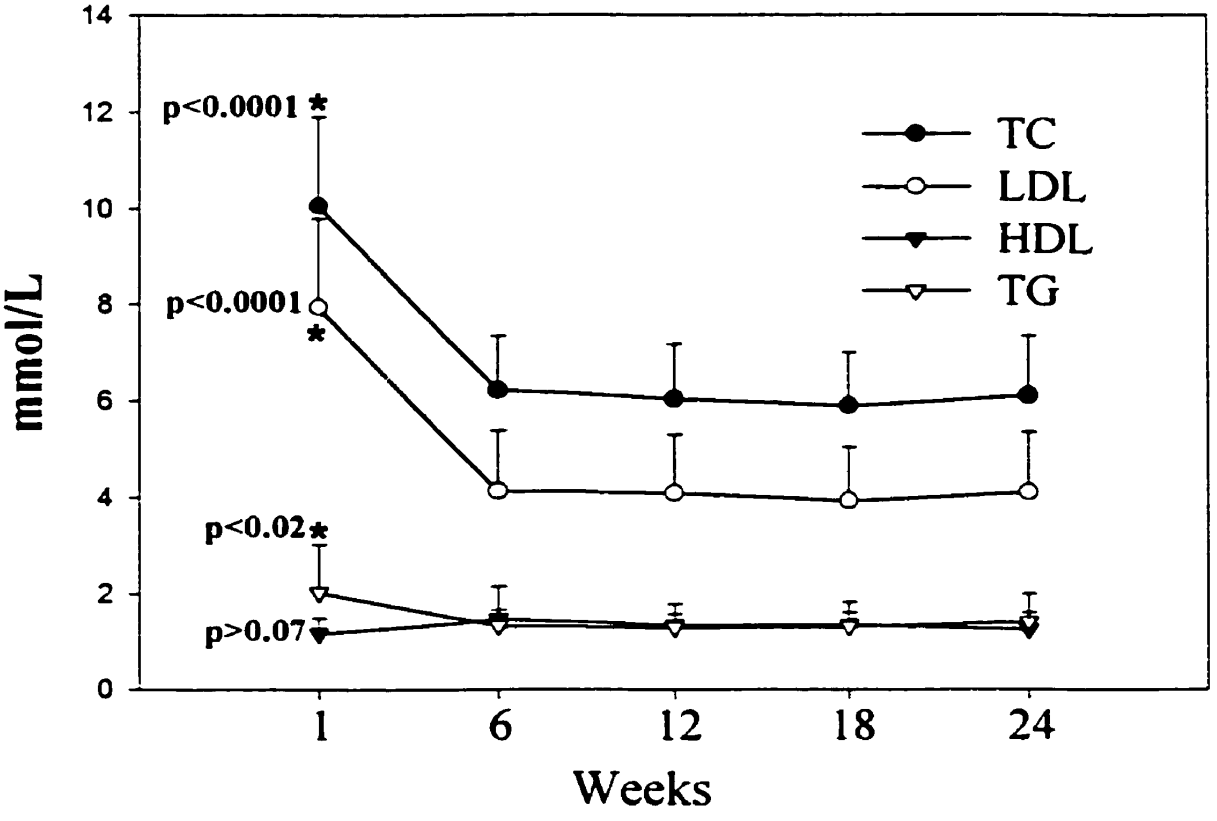
14
12
10
8
6
4
2
0
mmol/L
p<0.0001 *
p<0.0001 *
p<0.02*
p>0.07
TC
LDL
HDL
TG
1
6
12
18
24
Weeks

Figure 1

FINAL CONCLUSION AND SUMMARY

The 90-day hamster study in Chapter 3 demonstrated gender differences in cholesterol metabolism in tall oil (0.2% (w/w) SI)- and soybean (0.01% (w/w) SI)-phytosterols. These findings suggest that phytosterol extracts with high sitostanol concentrations are more effective cholesterol-lowering agents in males than sitostanol-free phytosterols. However, this is not the case in female hamsters. Cholesterol biosynthesis increased by two-fold in groups fed tall oil (0.2% (w/w) SI)- as compared to soybean- (0.01% (w/w) SI) phytosterols. An intrinsic hormonal difference between male and female hamsters was speculated to be behind the discrepancy in the effects of tall oil versus soybean on their plasma lipid levels. To limit confounding variables such as gender, female hamsters were not included in the subsequent studies. The effective dosage and components of phytosterol mixtures responsible for altering lipid profiles and cholesterol metabolisms in animals and humans were determined in the studies that followed.

To investigate further the impact of variable sitostanol concentrations on circulatory lipoprotein cholesterol and phytosterol levels, 50 hamsters were used in a 45-day study. This work showed that dietary sitostanol's effect is proportional to its fractional concentration in phytosterol mixtures and in the diet. Dietary sitostanol lowered plasma cholesterol levels at concentrations higher than 0.2% (w/w) SI. Independent of phytosterol concentrations, pure dietary sitostanol was superior to both tall oil (0.2% (w/w) SI)- and soybean (0.01% (w/w) SI)-derived phytosterols and

resulted in a more effective decrease in circulating total cholesterol levels in hamsters and rabbits. No synergistic effect was observed between ß-sitosterol and sitostanol. Although the efficacy of tall oil-phytosterols in lowering plasma cholesterol levels did not reach that of sitostanol, data had shown that a phytosterol mix containing high concentrations of sitostanol was effective in lowering blood lipid profiles when compared to control. Thus, a reasonable balance between the cost and the benefit could be determined which will permit the utilization of phytosterol mixtures as food additives for most people at much lower costs than pure sitostanol.

To extend the hamster studies, further sub-chronic feeding investigations were done on rabbits. Rabbits were used to assess the cardiovascular complications and phytosterol effects on plasma LCAT activity and the fractional esterification rate of cholesterol. This study concluded that dietary sitostanol at a concentration of 0.8% (w/w) SI, or 0.64 g/day, lowered plasma cholesterol levels and significantly delayed plaque formation in the aorta of hyperlipidemic rabbits. High concentrations of sitostanol increased the cholesterol fractional esterification rate but did not affect LCAT activity in this species. The rabbit study confirmed that sitostanol is not absorbed and that LCAT enzyme cannot be altered by any absorbable phytosterols from the mixture. This study led to the next question of how do phytosterol mixtures work in lowering lipid levels either in hamsters or rabbits?

In agreement with the previous data obtained for lipid profiles in hamsters and rabbits, dietary sitostanol at 1% (w/w) SI was more effective than 0.01 and 0.2% (w/w) SI mixtures in inhibiting cholesterol absorption and increasing cholesterol excretion and synthesis rates in hamsters with potentially similar effects in rabbits.

This study was one of the first to apply isotope mass spectrometry technology to measure simultaneously cholesterol absorption and biosynthesis in hamsters and rabbits. It provided further evidence that phytosterols exhibit their action in the intestine by inhibiting cholesterol absorption and increasing its excretion in the fecal material. These factors led to an up-regulation in cholesterogenesis. Using this methodology, we determined rates of cholesterol absorption and absolute values of cholesterogenesis in animals. However, the impact of tall oil-phytosterols on plasma lipid and phytosterol levels, as well as on cholesterogenesis, still warranted further investigation in humans.

For this reason, hypercholesterolemic subjects were fed a low saturated fat/cholesterol North American diet with or without tall oil-phytosterols for 30 days. Pure sitostanol was not used due to its unavailability in sufficient quantities to be fed to 32 subjects, and since the usage of pure sitostanol in humans would have required additional purification steps that were beyond our capabilities. This human study demonstrated for the first time that tall oil-phytosterols fed to hypercholesterolemic subjects on a controlled prudent North American diet do not alter endogenous cholesterol synthesis. The difference in total cholesterol levels between control and treatment groups was not large enough to detect any differences in cholesterogenesis in hypercholesterolemic subjects. Subjects were fed phytosterol mixtures that contain campesterol. Since campesterol is absorbed, its circulatory concentrations cannot be used as an index of cholesterol absorption. Consequently, another group of hypercholesterolemic subjects were recruited and were not fed phytosterols. Hence, the variation in circulating campesterol levels would indicate changes in fractional

cholesterol absorption in these patients. In the next human trial, hypercholesterolemic subjects were given treatment that has been shown to inhibit HMG-CoA reductase activity and lower cholesterol absorption rate in animals.

Our findings showed that a 24-week use of simvastatin lowered plasma campesterol level, a marker of cholesterol absorption rate, as well as total-, LDL-cholesterol, triglyceride concentrations and increased HDL/LDL ratio in humans. This observation suggests that simvastatin might have an inhibitory role on cholesterol absorption in hypercholesterolemic subjects. The inhibition of cholesterol absorption would contribute to the mechanisms by which simvastatin lowers circulating cholesterol levels. In this study, the changes in campesterol concentrations would reflect cholesterol absorption rates since patients did not consume any additional plant sterols in their regular diets.

In conclusion, data from this thesis demonstrated the followings:

(i) the efficacy of phytosterols in hamsters is influenced by gender.

(ii) sitostanol is more effective than soybean- and tall oil-derived phytosterols in lowering plasma total cholesterol levels, inhibiting cholesterol absorption, increasing cholesterol excretion in feces and stimulating cholesterogenesis in hamsters.

(iii) sitostanol reduces circulating lipid levels, delays plaque development and does not affect LCAT activity in rabbits.

(iv) tall oil-phytosterols lower plasma cholesterol levels in human

hypercholesterolemic subjects fed prudent North American diet but does not alter significantly their cholesterogenesis.

(v) simvastatin lowers plasma campesterol levels in hypercholesterolemic patients which suggests that simvastatin might also inhibit cholesterol absorption.

LIMITATIONS OF THE THESIS

The investigations in these studies were not without limitations. Animal models have their own inherent disadvantages. In addition, the sample size, the duration and the phytosterols utilized contributed to some technical and procedural constraints.

1. Animal models in lipid research

Choosing the appropriate animal model for an experimental design can be as important as the study itself. The best animal models for any study are the ones that show major physiological and functional similarities with human beings in whatever metabolic system is under study. For lipids, certain animal species are resistant to exogenous dietary cholesterol (pigs, rats), while on the other hand, some animal species show a high increase in their plasma cholesterol due to intakes from exogenous sources such as rabbits (Daley et al., 1994a; 1994b).

1.1 Hamsters

Golden Syrian hamsters are used largely in studies concerning the mechanisms by which FAs, sterols, and other dietary components exert their effect on plasma cholesterol levels (Wade et al., 1983; Spady and Dietschy, 1988). Although hamsters have been unsusceptible to atherosclerotic development when fed 15 different diets with a range of cholesterol levels, they are considered as a more

appropriate animal species to be used in lipid profile studies than rats (Goldman, 1950). The plasma cholesterol concentration of hamsters responds to changes in dietary lipids in the same fashion to that seen in man. Additionally, their bile closely resembles that of man. The major constituents of this bile are glycine and taurine conjugates of cholic and deoxycholic acids (Goldman, 1950).

The hamsters' biological rhythms are more equally spaced between the dark and light period than those of rats (Zucker and Stephan, 1973) suggesting a much lower variation in their metabolic rates. On the other hand, the development of hypercholesterolemia in hamsters might change the composition of their lipoprotein particularly in the lipoprotein fraction of $d < 1.006$ g/ml which becomes enriched with cholesterol esters (Tsai and Gong, 1987).

1.2 Rabbits

Rabbits have been used in laboratories for almost 100 years as a major experimental model for arterial lesion development. Rabbits fed a high cholesterol diet exhibited elevated levels of total plasma cholesterol with atherosclerotic lesions. Serum cholesterol concentrations of these animals could reach 2000-3000 mg/dl in 6 months, but remains leveled from 9 to 11 months of the feeding period (Chao et al., 1994). Such values are not normally seen in humans. Further investigations have suggested (Daley et al., 1994a; 1994b) that a cholesterol diet of <1% (w/w) cholesterol can produce human-like atherosclerosis in rabbits. It has been reported that casein-fed rabbits develop human like fibrous lesions (Gresham and Howard,

1962) while other reports identified only fatty streaks (Ross et al., 1978). Recently, Daley et al. (1994a; 1994b) have shown that rabbits fed a semi-purified casein-based diet with a 0.2-2% cholesterol content show atherosclerotic lesions with close morphological resemblance to those observed in humans. After a six-month period of low-cholesterol casein-based diet, rabbits showed 77% of advanced plaque lesions (fibrous and atheromatous types combined), with only 12% consisting of foam cells (Daley et al., 1994b). Casein, alone without cholesterol, produced hypercholesterolemia in rabbits fed a semi-purified diet, while soy protein did not induce such a disorder. It is strongly believed that casein effects are due to the amino acid composition (Roberts et al., 1981). Overall, arterial lesions in rabbits are dissimilar to those seen in humans. Rabbit lesions consist primarily of foam cells derived from macrophages. While in humans, atherosclerotic plaque is constituted of smooth muscle cells, fibromuscle caps and fatty streaks with high cholesterol content.

2. Sample size, duration and phytosterols

Another restriction was the rabbit sample size. The lipid profile data and cholesterol excretion rates were significant and provided strong evidence that sitostanol is effective in rabbits as it is in hamsters. However, we did not expect such variations in the absorption and synthesis values. This factor contributed to lower statistical power and led to no significance being generated in the statistical analysis. However, a similar trend to hamsters was noticed in rabbits. Therefore, we decided to include the rabbit data of cholesterol kinetics with those of the hamsters. Future

investigations should take into consideration the large variability in the values of cholesterogenesis obtained for rabbits.

The second limitation was in the phytosterol mixtures fed to hamsters, rabbits and humans. Pure sitostanol was available only from a commercial company (Sigma) which provided limited amount of pure sitostanol. The available sitostanol was sufficient to conduct the hamsters 45-day study only. For the rabbits, we obtained sitostanol from Professor von Bergmann (Germany) as a courtesy. However, we were unable to obtain sufficient amounts of pure sitostanol for use in the human study.

Miettinen et al. (1995) showed that phytosterols start to lower plasma lipid levels as early as two weeks following administration. We explored the effect of tall oil on total cholesterol concentrations in hypercholesterolemic subjects over a 1 month period. A longer feeding period of three to 6 months would have been preferable. The Ethics Committee at McGill University did not allow longer feeding period of tall oil-phytosterols. Therefore, we were unable to compare precisely our results with those of other investigators who used sitostanol-ester in their studies for a duration of 6 to 12 months. Another limitation in the human study was in the subjects admitted. Subjects were admitted based on their total cholesterol levels. Although all subjects were randomized to either control or treatment groups, by chance 5 subjects with high triglyceride levels were placed in the treatment group. Consequently, the 30-day period of the trial and the abnormal triglyceride levels in some subjects contributed to a lower difference in lipid levels between control and treatment. This factor probably contributed to the fact that no significance was observed in the data

obtained for *de novo* cholesterogenesis in the human trial. An alternative design for the current human study would be to implement a free-living style prospective approach in which another group is added where subjects would consume phytosterols without changing their regular diet.

The simvastatin study covered an important aspect of phytosterol metabolism in the context of its medical implications. Subjects involved in the simvastatin study were part of a major multi-center parallel trial. This trial was designed to measure the impact of different simvastatin concentrations on plasma lipid profiles in hypercholesterolemic patients. We decided to perform additional analysis to examine whether simvastatin affects circulating phytosterol levels and to assess its possible implications on cholesterol absorption. The only limitation was that we did not have much control over the study design, although all the measurements we needed were available to us.

Despite the limitations, results obtained from the different studies conducted in this thesis contributed to increasing the scientific knowledge in the field of plant sterols and their implications on cholesterol metabolism in animals and humans. In addition, in future experimentations, the limitations of the current studies will be taken into account and more advanced analyses can be applied to probe what was not explored in this thesis. For example, the newly developed method for measurement of cholesterol absorption rate and cholesterogenesis can be applied in humans. Stable isotopes are safe to use in human subjects. Moreover, the new development in the technology of isotope ratio mass spectrometry will result in less cumbersome work

conducted to separate and measure isotopes enrichments in biological samples. It is true that the cost of employing such a method is high but the data obtained regarding cholesterol metabolism in humans will be more precise. Furthermore, the effects of variable concentrations of sitostanol on cholesterol metabolism can be investigated in both normal and hypercholesterolemic subjects in a free-living study design without biopsy or feces collections.

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