Effects of a "tall oil"-derived phytosterol mixture on the development of atherosclerotic lesions in apo E-deficient mice

by

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Abstract

Background: The effects of a phytosterol mixture (FCP-3PI) on the plasma cholesterol concentrations and development of atherogenic lesions have been evaluated in apo E-knockout (apo E-KO) mice using a number of biochemical and histological methods. In addition, the systemic effects and the tolerance to FCP-3PI have also been tested in this animal model. FCP-3PI, which is composed of β-sitosterol (69%), campesterol (15%) and sitostanol (16%), was extracted from “tall oil” soap, a by-product of the pulp and paper industry. The degree of purity of the final product was approximately 95% as assessed by gas chromatography.

Objectives: The objectives of this thesis were: 1) to determine the effects of dietary supplementation with FCP-3PI on plasma lipid concentrations in apo E-KO mice; 2) to evaluate the cholesterol-lowering properties of FCP-3PI in wild-type normolipidemic mice; 3) to evaluate the effects of FCP-3PI on the quality and extent of atherosclerotic lesions in apo E-KO mice; 4) to compare and contrast cholesterol-lowering and anti-atherogenic effects of FCP-3PI to those of probucol, a well-known lipid lowering agent with antioxidant properties; 5) to test systemic effects of FCP-3PI following its parenteral administration in apo E-KO mice; and finally, to investigate the tolerance of apo E-KO mice to FCP-3PI and its safety when administered over the long-term.

Results: Atherosclerosis progression experiments revealed that addition of 2% (w/w) FCP-3PI to a typical “Western” diet resulted in a significant reduction in average total plasma cholesterol concentrations in the treated animals compared to controls. This was accompanied by a significant (p<0.0001) reduction in the average lesion area in the treated animals. This reduced lesion area in the aortic sinuses was accompanied by a substantial reduction in all lesional components, reflecting a delay in the progression of atheromatous changes. The lesion size was strongly correlated with the average plasma total cholesterol concentrations (r=0.69).

Cholesterol lowering effects of FCP-3PI were tested in a number of male CD1 mice in the presence or absence of additional dietary cholesterol. FCP-3PI did not significantly reduce plasma total cholesterol in these normolipidemic mice. The lack of cholesterol-lowering effects of FCP-3PI may be due to limited cholesterol absorption in normolipidemic mice.

The next experiment was carried out to investigate possible mechanisms of FCP-3PI effects on cholesterol metabolism and atherosclerotic lesion development, and then to compare FCP-3PI effects to those of probucol in apo E-deficient mice. The cholesterol-lowering and anti-atherogenic activities of FCP-3PI were accompanied by significant alterations in several other features which may be directly/indirectly involved in atherogenesis. Thus, FCP-3PI treatment caused a significant increase in the activity of hepatic HMG-CoA reductase and to a lesser extent in the activity of hepatic cholesterol 7α-hydroxylase. These changes were associated with a significant decrease in hepatic cholesterol content and a 50% increase in fecal cholesterol excretion compared to controls. Hepatic lipase activity was also significantly reduced by FCP-3PI treatment. In addition, FCP-3PI caused a 20% decrease in plasma fibrinogen concentrations.
Unlike FCP-3PI, probucol caused a marked decrease in plasma total, VLDL-, LDL-, and HDL-cholesterol concentrations which was associated with a significant increase in atherosclerotic lesion size in the aortic roots of the mice. Probucol also caused a significant increase in plasma fibrinogen concentration compared to controls. These changes were associated with a significant increase in plasma antioxidant enzyme activities. In addition, probucol, unlike FCP-3PI, reduced hepatic LDL receptor binding to two-thirds of that in controls. The activity of both hepatic HMG-CoA reductase and cholesterol 7-hydroxylase was increased in the probucol-treated animals compared to controls.

The atherosclerosis regression study revealed no evidence for regression of pre-established atherosclerotic lesions in the aortic roots of the mice fed regular mouse chow supplemented with 2% (w/w) FCP-3PI for 25 weeks.

Intraperitoneal injection of FCP-3PI into apo E-KO mice resulted in a significant reduction in the activity of hepatic HMG-CoA reductase, a non-significant reduction in plasma total cholesterol concentration and a significant increase in the activity of plasma antioxidant enzymes as compared to controls.

Finally, parallel to the first experiment, tolerance of orally administered FCP-3PI was evaluated in apo E-deficient mice. Histological examination revealed no abnormality in tissues examined except for a certain degree of testicular atrophy. FCP-3PI treatment prevented the development of cutaneous xanthomatosis. Urinalysis and hematological data were comparable between the control and FCP-3PI-treated animals except for a significant decrease in platelet count in the treated group. The erythrocytes of the treated mice showed a decreased susceptibility to hypotonic lysis in vitro.

**Conclusions:** We have demonstrated that FCP-3PI has cholesterol-lowering and anti-atherogenic effects in apo E-KO mice. Moreover, we have demonstrated, for the first time, the effectiveness of FCP-3PI in preventing cutaneous xanthomatosis and retarding the development of atherosclerotic lesions in this animal model. Our data suggest that these effects of FCP-3PI may be mediated through a decrease in plasma VLDL-cholesterol, an increase in fecal cholesterol excretion (most likely due to decreasing cholesterol re-absorption and increasing biliary cholesterol excretion), a decrease in hepatic lipase activity and decrease in plasma fibrinogen concentrations. The lack of toxicity and its abundance in nature along with its low cost make potential use of FCP-3PI in prevention and treatment of human hypercholesterolemia attractive.
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<td>ACAT</td>
<td>acyl coenzyme A:cholesterol acyltransferase</td>
</tr>
<tr>
<td>ASL</td>
<td>atherosclerosis specialty laboratory</td>
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<tr>
<td>Apolipoprotein</td>
<td>apo</td>
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<tr>
<td>α</td>
<td>alpha</td>
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<tr>
<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>BW</td>
<td>body weight</td>
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<tr>
<td>CAD</td>
<td>coronary artery disease</td>
</tr>
<tr>
<td>CAT</td>
<td>catalase</td>
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<tr>
<td>CE</td>
<td>cholesteryl ester</td>
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<tr>
<td>CETP</td>
<td>cholesteryl ester transfer protein</td>
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<tr>
<td>DPPD</td>
<td>N,N'-diphenyl 1,4-phenylenediamine</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetra-acetic acid</td>
</tr>
<tr>
<td>e. g.</td>
<td>for example</td>
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<tr>
<td>FC</td>
<td>free cholesterol</td>
</tr>
<tr>
<td>FCP-3PI</td>
<td>the mixture of phytosterols used</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast protein liquid chromatography</td>
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<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>GPx</td>
<td>glutathione peroxidase</td>
</tr>
<tr>
<td>GRed</td>
<td>glutathione reductase</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
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<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N,-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>3-hydroxy-3-methylglutaryl-coenzyme A</td>
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<tr>
<td>IDL</td>
<td>intermediate density lipoprotein</td>
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<tr>
<td>i. p.</td>
<td>intraperitoneally</td>
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<tr>
<td>kg</td>
<td>kilogram</td>
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<tr>
<td>KO</td>
<td>knockout</td>
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<tr>
<td>LCAT</td>
<td>lecithin:cholesterol acyltransferase</td>
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<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
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<td>μ</td>
<td>micro</td>
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<tr>
<td>mg</td>
<td>milligram</td>
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<td>min</td>
<td>minute</td>
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<td>ml</td>
<td>milliliter</td>
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<td>mmol</td>
<td>millimole</td>
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<tr>
<td>μm</td>
<td>micrometer</td>
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<tr>
<td>n</td>
<td>nano</td>
</tr>
<tr>
<td>OCT</td>
<td>optimal cutting temperature</td>
</tr>
<tr>
<td>ORO</td>
<td>oil red O</td>
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<tr>
<td>POPC</td>
<td>1-palmitoy-2-oleoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>s. c.</td>
<td>subcutaneously</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
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<tr>
<td>TC</td>
<td>total cholesterol</td>
</tr>
<tr>
<td>TG</td>
<td>triglyceride</td>
</tr>
<tr>
<td>UBC</td>
<td>University of British Columbia</td>
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<tr>
<td>VLDL</td>
<td>very low density lipoprotein</td>
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DEDICATION

This thesis is dedicated to my son Paymahn.
1. **HYPOTHESIS AND OBJECTIVES OF THE THESIS**

1.1. *Hypothesis*

Administration of a mixture of plant sterols (FCP-3PI) reduces atherogenesis in apo E-knockout (apo E-KO) mice primarily by decreasing their plasma cholesterol concentrations. This treatment is well tolerated and may alter other predisposing factors for atherogenesis by mechanisms other than by decreasing the absorption of cholesterol.
1.2. Objectives

1.2.1. To determine the effects of dietary supplementation with FCP-3PI on plasma lipid concentrations in apo E-KO mice.

1.2.2. To evaluate the cholesterol-lowering properties of FCP-3PI in wild-type normolipidemic mice.

1.2.3. To evaluate the effects of FCP-3PI on the quality and extent of atherosclerotic lesions in apo E-KO mice.

1.2.4. To compare and contrast cholesterol-lowering and anti-atherogenic effects of FCP-3PI to those of probucol, a well-known lipid lowering agent with antioxidant properties.

1.2.5. To evaluate the effects of FCP-3PI on the regression of dietary-induced atherosclerotic lesions in apo E-KO mice.

1.2.6. To test systemic effects of FCP-3PI following its parenteral administration in apo E-KO mice.

1.2.7. To investigate the tolerance of apo E-KO mice to FCP-3PI and its safety when administered over the long-term.

In order to test the hypothesis and to accomplish the proposed objectives, the following experiments were designed:
1. Nineteen apo E-KO mice were fed with a “Western-type” diet (9% fat + 0.15% cholesterol, w/w) with or without 2% (w/w) FCP-3PI for 18 weeks. The cholesterol-lowering and anti-atherogenic activities of FCP-3PI treatment were examined.

2. In order to investigate the cholesterol-lowering effects of FCP-3PI in normolipidemic CD1 mice, the animals were fed with a diet supplemented with 2% (w/w) FCP-3PI in the presence or absence of additional dietary cholesterol for 15 weeks.

3. In order to investigate possible mechanism(s) of the anti-atherogenic effects of FCP-3PI and pro-atherogenic effects of probucol, we carried out a series of experiments using apo E-KO mice fed with either 1% (w/w) probucol or 2% (w/w) FCP-3PI for 20 weeks.

4. Effects of treatment with FCP-3PI on regression of atherogenesis in apo E-deficient mice were also investigated. In this experiment, the atherosclerotic plaques were developed by a “Western-type” diet (induction phase). The induction phase followed by a 25-week regression phase in which the mice fed regular chow with or without 2% (w/w) FCP-3PI.

5. Systemic effects of plant sterols were investigated by parenteral administration of FCP-3PI in apo E-deficient mice.

6. Finally, the tolerance of apo E-deficient mice to FCP-3PI over 18 weeks was assessed by a number of histological, hematological and biochemical assessments.
2. INTRODUCTION

2.1. Lipoprotein metabolism

Lipids of human or animal plasma are transported as complexes with different proteins; these particles are named lipoproteins. The digestion and absorption of lipids occurs in the small intestine. These processes are dependent on the presence of pancreatic juice and bile acids. Pancreatic juice contains several enzymes needed for hydrolysis of fats. Among them, glycerol ester hydrolase, cholesteryl ester hydrolase and phospholipase A\textsubscript{2} are important in the digestion of triglycerides, cholesteryl ester and lecithin, respectively. The products of fat digestion are combined with bile acids to form micelles. Micelles are multimolecular particles which have a hydrophobic pole (lipids) in their core and a hydrophilic one (polar ends of the bile acids) on their surface.

The micelles diffuse among the microvilli and reach the brush border and outer membranes of the enterocytes where the absorption of lipids takes place. Fatty acids, cholesterol and monoglycerides readily diffuse across the cell membranes and enter the enterocytes. Triglycerides, cholesteryl esters and phospholipids are re-formed inside the intestinal epithelial cells. The re-formed lipids along with de novo synthesized ones complex with apolipoproteins (apo) to form chylomicrons. Chylomicrons, which are the largest lipoproteins (75-120 nm in diameter) with the lowest density (0.94 g/ml), are secreted into the lymph by an exocytotic process and enter the systemic circulation via the thoracic duct. These particles are mainly composed of triglycerides and to a lesser extent cholesterol and cholesteryl ester. The main apolipoproteins in chylomicrons are apolipoproteins B\textsubscript{48}, A\textsubscript{I}, A\textsubscript{II}, A\textsubscript{IV}, C\textsubscript{I}, C\textsubscript{II}, C\textsubscript{III}.
and E. Lipoprotein lipase (LPL) which is present on the vascular endothelium rapidly hydrolyzes chylomicron TG into the free glycerol, monoglycerides and fatty acids. Chylomicron remnants are rapidly taken up by hepatic cells by receptor-mediated endocytosis and may undergo further metabolic processes which result in the formation and secretion of VLDL. VLDL particles are spherical, 30-70 nm in diameter, and have a density of 0.94-1.006 g/ml. They contain approximately 50-65% TG, 16-22% CE, 4-8% FC, 15-20% phospholipids and 6-10% protein. VLDL particles contain apo B₁₀₀, B₄₈, C₁, C₄, C₁₀₀, and E. They are also metabolized via LPL which leads to the formation of IDL particles. The formed IDL particles are converted to LDL mainly by the action of the hepatic lipase. LDL particles are cholesterol-rich and contain apo B₁₀₀. They have a density of 1.019-1.063 g/ml and a diameter of approximately 22 nm.

LDL particles are taken up by the cells (mainly hepatic, adrenal, testis and ovary) through LDL receptors. Apo B₁₀₀ and E are the ligands for LDL receptors. Once bound to LDL receptor, LDL particles are internalized and then packaged into endosomes which fuse with lysosomes. Lysosomal digestion of LDL results in the release of FC and amino acids. FC plays three important roles in cellular cholesterol metabolism: first, it down-regulates the production of HMG-CoA reductase, a rate-limiting enzyme in cholesterol synthesis. Secondly, it increases the activity of the enzyme ACAT which esterifies FC. Finally, it regulates the synthesis of LDL receptors. These mechanisms allow hepatic cells to maintain a constant level of intracellular cholesterol. Hepatic cells convert FC into the bile acids through the activities of cholesterol 7-hydroxylase or sterol 27 hydroxylase. Bile acids are
secreted into the small intestine to be used in lipid digestion processes (micelle formation). Bile acids can be reabsorbed by the small intestine and enter the liver via a process called “entero-hepatic circulation”.

HDL particles play a key role in the reverse cholesterol transfer pathway. They are spherical with a diameter of 7-10 nm and have a density of 1.063-1.21 g/ml. They are rich in protein: apo A_i and A_ii are the main apolipoproteins of HDL particles. There are 4 different sources of HDL: synthesis in the intestine and liver and via catabolism of chylomicrons and VLDL. Nascent HDL particles combine with FC from cell membranes or other lipoproteins. FC is then esterified by plasma enzyme LCAT; the esterified cholesterol moves inside the particle to form larger particles (HDL-3). As further esterification of FC occurs, the size of HDL particles increases further (HDL-2). HDL-2 particles can be acted upon by CETP and converted to HDL-3. Hepatic lipase may also convert HDL-2 to HDL-3 particles. Although the catabolism of HDL particles has not been completely defined, the lipid component of them is preferentially removed by the liver and steroid hormone-producing tissues via class B scavenger receptor SR-BI (1).

2.2. Atherosclerosis

Atherosclerosis is characterized by intimal thickening and lipid deposition in the wall of blood vessels. This disorder accounts for more death and illness in the Western world than any other disease. For example, 56% of a total of 79,000 heart-related deaths in 1995 in Canada was attributed to ischemic heart disease (2). Hypercholesterolemia and other abnormalities of lipid metabolism are among the
most prevalent known risk factors which predispose to atherosclerosis and resultant ischemic heart disease.

Atherogenesis is a complex process in which the lumen of blood vessels becomes gradually narrowed by accumulation of cellular and extracellular substances to the final point of obstruction (Figure 1). Although the location of lesions varies among various species, they usually develop at branch points of the arteries. Development of atherogenesis occurs in several stages (3). The very first stage is the entrance of lipoproteins, particularly LDL, into the artery wall. This is not a receptor-mediated process; however, it may be a concentration-dependent process. In this regard, several studies (4-6) provide strong evidence that elevation of LDL concentration is associated with atherosclerotic lesion formation and vice versa. The lipoprotein particles in the artery wall become trapped in a network of extracellular matrix, including proteoglycans, elastin and collagen (3). The trapped lipoproteins may undergo oxidative processes by reactive substances which can be produced and secreted by the cells of the vessel wall. These oxidation processes can take place in two phases (3): the first one occurs before monocytes are recruited and results in the oxidation of lipoprotein lipids with little or no damage to apolipoproteins. The lipoproteins at this phase are called minimally modified lipoproteins. Further oxidation of lipoproteins occurs in the second phase. In this phase, apolipoproteins are also oxidized and thereafter called highly oxidized lipoproteins. Macrophages play a significant role in the second phase of lipoprotein
Figure 1: Development of atherosclerotic lesions in apo E-deficient mice: From fatty streak to complete obstruction. Arrows show: A, fatty streaks, H&E; B, foam cell accumulation, H&E; C&D, narrowing, ORO & H&E; E&F, complete obstruction, ORO (x25)
oxidation. Highly oxidized lipoproteins can be recognized by scavenger receptors and taken up by macrophages through these receptors. Unlike LDL receptors, scavenger receptors are not down-regulated by intracellular FC. Accumulation of lipoproteins and lipids inside the macrophages results in the formation of foam cells and fatty streaks (3). Oxidized lipoproteins are also potent inducers of inflammatory mediators which in turn accelerate recruitment of inflammatory cells into the artery wall and stimulate the formation of foam cells. Thus, oxidation of lipoproteins in particular of LDL, plays an important role in foam cell formation. The following mechanisms may potentially be involved in the pathogenesis of atherosclerosis as explained above: 1) enhanced rate of macrophage uptake and degradation of the oxidatively modified LDL through the scavenger receptors, 2) increased recruitment of monocytes into intima by the chemoattractant activity of oxidatively modified LDL for circulating monocytes, 3) retention of lipid-rich macrophages in the intima, and 4) cellular injury caused by peroxidized lipid components of oxidatively modified LDL.

The lipid-rich lesions can be relatively easily ruptured; therefore, they are called "vulnerable" plaques. A consequence of this event may be thrombus formation and transfer of emboli in the small arteries resulting in obstruction and in the case of coronary arteries myocardial infarction. The development of atherosclerotic lesions continues to progress via synthesis and secretion of extracellular matrix by cells within the atherosclerotic plaques (3). This extracellular matrix which makes the plaque's fibrous cap mainly contains interstitial collagen, elastin and proteoglycans. An atherosclerotic plaque with a well-defined fibrous cap is termed a "stable" plaque. Inflammatory cells such as activated T cells and
Macrophages can either inhibit extracellular matrix formation by vascular smooth muscle cells or increase degradation of extracellular matrix via secretion of proteases (3). These processes along with calcification of plaques can result in ulceration, fissuring, thrombus formation and plaque rupture.

A number of modifiable (cigarette smoking, hypertension, dyslipidemia, hyperhomocyst(e)inemia, diabetes, lack of physical activity, obesity, increased fibrinogen, etc.) and non-modifiable (age, sex, family history) risk factors play a significant role in the pathogenesis of atherosclerosis. Myocardial infarction, stroke and aortic aneurysms are the major consequences of this disease. The recognition of the involvement of various environmental (diet, cigarette smoking, exercise, etc.) and genetic (dyslipidemia, hypertension, hyperhomocyst(e)inemia, diabetes, etc.) factors which promote the lesion formation has led to the development of rational strategies for the prevention/treatment of atherosclerosis. In this regard, the beneficial effects of several pharmacological and dietary regimens have been tested. Thus, lipid-lowering agents have been extensively used in primary and secondary prevention trials in atherosclerosis (4-6). Recently, the use of plant sterols as a dietary approach against atherogenesis has attracted considerable interest (7,8).

Several experimental/clinical trials have shown that lowering plasma total and LDL cholesterol levels retards the formation of atherosclerotic plaques (4-6,9,10). Reduction of plasma LDL-cholesterol concentrations by a combination of dietary and pharmacological regimens resulted in a significant (p<0.05) decrease in atherosclerotic lesion size measured by computer-based quantitative angiography in
the major coronary arteries of patients with heterozygous familial hypercholesterolemia (11).

In addition to progression studies, several studies have been carried out to test the reversibility of atherosclerotic disease. For example, one study (12) showed frequent angiographically-documented regression in coronary atherosclerosis in subjects treated with a combination of cholesterol-lowering agents as compared to a conventional-therapy group. Moreover, non-human primates have been used in several regression studies (13-17). Although a low fat diet did not cause regression of atherosclerosis in rabbits, the combination of a low fat diet with cholestyramine and/or estrogen caused regression of lesions (18). HDL plasma fractions induced regression of aortic fatty streaks and lipid deposits in cholesterol-fed rabbits (19). Lovastatin significantly reduced the per cent of aortic lesions in cholesterol-fed rabbits (20).

Both pharmacological and non-pharmacological agents have been used extensively in an attempt to decrease mortality and morbidity from hypercholesterolemia-related disorders. A major limitation to the widespread use of lipid-lowering agents, particularly for long-term administration, is the low compliance which relates to their cost and also their associated side effects. For example, gastrointestinal disturbances, myopathy and liver dysfunction may occur in patients treated with lipid-lowering agents such as fibrates or HMG-CoA reductase inhibitors (21,22). Emerging candidates for therapy of dyslipidemias such as dietary phytosterols (7, 23-25) have not yet been extensively studied in regard to their side-effects at effective cholesterol-lowering doses but preliminary data suggest that both
their cost and side effect frequency may be lower than that of currently used hypolipidemic agents.

2.3. Apo E-deficient mice

Fast breeding, short generation time, availability of inbred strains and relatively low cost make the mouse one of the best mammalian animal models for investigation of a number of human diseases. However, mouse species are generally highly resistant to atherogenesis. This is due to their relatively low plasma total cholesterol concentrations (<4 mmol/L), most of which is in the form of a non-atherogenic HDL. However, if plasma cholesterol concentrations are raised by either environmental (e.g. dietary cholesterol supplementation) or genetic modifications, certain inbred strains of mice do develop atherogenesis. Therefore, attempts have been made to produce transgenic mice with high plasma lipid concentrations to make this animal species suitable for studies of atherogenesis, and also to investigate the etiological role of lipoproteins in atherogenesis.

Gene "knockout" technology has been used to produce mice lacking apo E. Apo E is made by numerous cells including hepatocytes, enterocytes and macrophages. This protein is a surface constituent of VLDL and HDL particles and serves as a ligand for lipoprotein remnant recognition at their receptor sites and remove them from systemic circulation. As a consequence, apo E-deficient mice have markedly delayed clearance of lipoproteins resulting in extremely high cholesterol concentrations of β-VLDL particles (VLDL particles which contain high cholesterol concentrations are named β-VLDL) and spontaneous atherogenesis at an early age (26-28). These cholesteryl ester-rich VLDL particles are found in
humans with type III hyperlipidemia and apo E deficiency. Unlike the homozygous animals, heterozygous E knockout mice have diminished plasma apo E concentrations, normal fasting lipoprotein profiles and slightly delayed postprandial lipoprotein clearance. These observations indicate that half-normal apo E expression in the mouse is nearly sufficient for normal lipoprotein metabolism.

Generally, apo E-KO mice develop atherosclerotic lesions in the aortic root, thoracic aorta, carotid, proximal coronary, femoral, subclavian and branchiocephalic arteries. By the age of 20 weeks, these mice have fibrous plaques which can further progress with time into calcification or aneurysm. Thus, a single gene deletion causes severe hypercholesterolemia and atherogenesis. This animal model has been widely used for the evaluation of anti-atherogenic diets and drugs for the treatment of atherogenesis. Since these animals resemble human type III dyslipidemia and develop atherosclerotic lesions similar to those in the human, we used them to test anti-atherogenic properties of plant sterols. Moreover, a human study showed that individuals with apo e_4 phenotype, known to have high cholesterol absorption, are more responsive to phytosterol treatment than hypercholesterolemic individuals with apo e_3 phenotype (29). We observed that apo E-KO mice are more responsive to dietary cholesterol supplementation than wild-type CD1 mice. This is another common feature between apo E-KO mice and human dyslipidemia making this animal model suitable for studying cholesterol-lowering effects of plant sterols.

2.4. *Phytosterols*

Several major clinical trials have demonstrated that treatment with cholesterol-lowering drugs reduces cardiovascular morbidity and mortality in patients
with both symptomatic and asymptomatic atherosclerosis (4-6). Among the many drugs and dietary regimens that have been employed, phytosterols have shown beneficial effects without any noteworthy side-effects. Since 1951, when Peterson (30) demonstrated the cholesterol-lowering effect of β-sitosterol in cholesterol-fed chickens, many investigators (7,8,23-25) have studied the effects of these natural substances on disorders of lipid metabolism and atherogenesis in both humans and laboratory animals.

Although the cholesterol-lowering effects of plant sterols have been demonstrated by several laboratories (7,8,23-25), a recent study (31) showed a lack of efficacy of dietary sitostanol (3 g/day) in reducing plasma total cholesterol, VLDL- and LDL-cholesterol concentrations in 33 men with moderate hypercholesterolemia who were consuming a diet restricted to <200 mg cholesterol per day. Furthermore, Bhattachary and Lopez (32) found that β-sitosterol given orally to rabbits resulted in a 60% increase in plasma cholesterol concentration but did not result in increased accumulation of cholesterol in the tissues. Attempts by other investigators (33) to reproduce the results of Bhattachary and Lopez, however, have not been successful. The rate of absorption of dietary phytosterols under physiological conditions is very low. Generally, it has been suggested that in human beings approximately 5% of ingested plant sterols is absorbed (34). However, their absorption rate increases
Figure 2: Chemical structures of cholesterol and certain plant sterols
markedly in sitosterolemia, a rare genetic disorder (35). Recently, abnormal regulation of cholesterol biosynthesis has been reported in patients with sitosterolemia, namely a decrease in the whole body cholesterol synthesis which may be related to a deficiency of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (36).

2.4.1. Chemistry, biochemistry and absorption

Phytosterols are synthesized in plant species but not in animals. Their chemical structures are very similar to that of cholesterol (Fig. 2). Addition of a methyl or ethyl group at carbon 24 of the cholesterol side chain leads to formation of campesterol or sitosterol, respectively. Dehydrogenation of carbon 24 of sitosterol leads to stigmasterol which is another widespread phytosterol. Chemical saturation of the delta 5 double bond of each of the aforementioned plant sterols leads to the formation of the 5-derivatives such as campestanol or sitostanol.

Since humans are not able to synthesize phytosterols, dietary consumption is the only source of plasma phytosterols which are less than 0.05 mmol/L in healthy individuals (37,38). The rates of absorption vary among the individual plant sterols. Heinemann et al. (39) compared the rate of intestinal absorption of cholesterol to that of several plant sterols in 10 healthy men who underwent intestinal perfusion over a 50 cm segment of the upper jejunum. They found the highest absorption rate for cholesterol (as much as 33.0%) followed by campestanol, campesterol, stigmasterol, sitosterol and sitostanol at 12.5%, 9.6%, 4.8%, 4.2% and 0.0%, respectively.
The mechanisms responsible for the different rates of absorption of plant sterols are not well understood. Micellar solubility is a major factor which affects the absorption rate (40,41). It has been suggested that the discrimination between absorbable and non-absorbable sterols occurs during the process of their uptake into intestinal mucosa (42,43). Other studies indicated that mucosal esterification could be a possible site of discrimination in sterol absorption (44).

Intragastric administration of radiolabeled cholesterol and sitosterol resulted in association of both sterols with the chylomicron fraction (45). While 90% of the total lymphatic cholesterol was detected as esterified cholesterol only 12% of sitosterol was esterified. Unlike cholesterol, which was esterified and located in the core of chylomicron particles, sitosterol was present mainly in the unesterified form on the surface of chylomicron particles.

2.4.2. Effects of plant sterols on lipid metabolism

Because plant sterols reduce cholesterol absorption, they have been used as anti-hypercholesterolemic agents. In three recent studies, a “tall-oil” derived phytosterol mixture significantly reduced plasma cholesterol concentrations in apo E-deficient mice in the presence or absence of dietary cholesterol supplementation and prevented the dietary cholesterol-induced increase in VLDL- and LDL-cholesterol in rats (7,8,23).

It has been documented that both oral and parenteral administration of plant sterols result in reduced concentrations of plasma cholesterol (7,8,23-25,46-47). This reduction in plasma cholesterol concentrations may be due to both inhibition of cholesterol absorption and alterations in hepatic/intestinal cholesterol metabolism.
Laraki et al. (48) reported a significant reduction in the activities of hepatic enzymes involved in lipid metabolism, such as acetyl-CoA carboxylase in rats fed a diet supplemented with a phytosterol mixture (0.5%-1% w/w) for 3 weeks as compared with those in control animals. These investigators also reported a significant increase in the content of liver plant sterol in the phytosterol-fed animals.

Sitosterol was also accumulated in mucosal cells of sitosterol-fed rats (both in whole homogenates and in the microsomal fraction); the sitosterol concentration reached twice that in control animals (49). This accumulation of sitosterol was not associated with a statistically significant alteration in ileac mucosal HMG-CoA reductase activity. In contrast, the activity of this rate-limiting enzyme of cholesterol synthesis in ileac mucosal cells and hepatocytes of sitosteroletic subjects was significantly lower than that in control individuals. This decrease in HMG-CoA reductase activity results in a reduction in cholesterol synthesis in sitosterolemia.

Supplementation of rats' diet with a phytosterol mixture (2% w/w) containing 92% sitosterol produces an up to 1.4-fold increase in the activity of hepatic cholesterol 7-hydroxylase compared to controls (50). Another important enzyme in cholesterol metabolism, lecithin:cholesterol acyltransferase (LCAT), was also affected by β-sitosterol consumption. The activity of this enzyme in the serum of hypercholesterolemic subjects significantly increased after 2 months of β-sitosterol supplementation (6 g/day) (51). Sitosterol ester reduced total, esterified and free cholesterol in LDL of hypercholesterolemic individuals who consumed sitostanol ester (3480 mg/day for 6 weeks) (52).
There is now also evidence that phytosterol treatment can lead to an increase in LDL sitosterol contents. For example, Aviram and Eias (53) showed that the sitosterol content of human LDL was elevated 2-fold when individuals consumed olive oil for 2 weeks. This alteration was associated with a marked reduction in LDL uptake by macrophages. Olive oil supplementation was also associated with a significant reduction in the propensity of LDL to \textit{in vitro} lipid peroxidation. Several studies showed a correlation between LDL lipid composition and its uptake by various cell lines (54,55).

A significant reduction in cellular cholesterol content by plant sterols was observed \textit{in vitro} when human skin fibroblasts or Hep G2 cells were incubated with liposomes containing sitosterol (56). This was accompanied by an increase in the concentration of sitosterol in the cells. Incubation of CaCo-2 cells (a colon tumor cell line) with \( \beta \)-sitosterol resulted in decreased uptake of cholesterol, cholesterol synthesis, HMG-CoA reductase activity and its mass and mRNA levels in CaCo-2 cells (57).

### 2.4.3. Other beneficial effects of phytosterols

In addition to their cholesterol-lowering effects, plant sterols have been shown to have a number of other metabolic effects in both humans and animals. For example, several epidemiological and animal studies suggest an anti-tumor activity of phytosterols against colonic neoplasia (58,59). It is unclear whether this activity is secondary to the cholesterol-lowering effects of phytosterols, because dietary cholesterol and its metabolites (such as coprostanol) have been reported to play a significant role in the epithelial cell proliferation in the colon. A three-month
randomized double-blind study showed significant therapeutic effects of plant sterols in alleviating symptoms of prostatic hyperplasia in 53 patients as compared to placebo-treated controls (60). In this regard, two other studies (61,62) suggested that plant sterols may have beneficial effects on prostate disorders.

**2.4.4. Adverse effects of plant sterols**

Several studies have indicated possible side-effects of phytosterols. For example, increased concentrations of phytosterols in erythrocyte membranes make the cells more rigid and result in an increased fragility. Indeed, episodes of hemolysis have been reported in patients with phytosterolemia (63,64). Increased membrane rigidity was also observed in rat liver microsomes enriched with B-sitosterol and campesterol (65). Furthermore, it has been shown that high B-sitosterol levels (up to 0.7 mmol/L) can cause contraction of human umbilical vein endothelial cells *in vitro* (66). These observations suggest that very high plasma concentrations of B-sitosterol may have potentially cytotoxic effects and may interfere with cellular functions.

High concentrations of phytosterols in the plasma of laboratory animals have adverse effects on their reproductive organs. In this regard, subcutaneous administration of 0.5 or 5 mg/kg body weight per day of B-sitosterol caused a significant reduction in both sperm count and the weight of testes in albino rats (67). Moreover, application of sitosteryl ester in the vagina of rabbits significantly lowered their pregnancy rate (68).
2.5. *Sitosterolemia*

Sitosterolemia (phytosterolemia) is a rare genetic disorder in which plasma concentrations of plant sterols, particularly sitosterol, are extremely high compared to those in normal individuals. Sitosterolemia is inherited as a recessive trait. This heritable disease was first discovered in two sisters with tendon xanthomas and normal plasma cholesterol concentrations in 1974 (35). Since then, a number of other patients have been reported by several investigators (63,64). A high percentage of dietary sitosterol absorption and a decrease in its elimination are believed to account for the plant sterol accumulation in the affected individuals.

Sitosterolemic patients develop tendon xanthomas, accelerated atherosclerosis (particularly in young males), hemolytic episodes, arthritis and arthralgias. Several young male subjects died of acute myocardial infarction associated with extensive coronary and aortic atherosclerosis. Among them, the youngest was an Amish boy who died at age of 13, and he had four other homozygous siblings. Another 17-year-old male subject who was followed by our collaborator, Dr. G. Salen, developed angina pectoris, showed an abnormal stress test with decreased coronary artery perfusion, and died suddenly of an acute myocardial infarction while exercising. Histological examination of his coronary arteries at post mortem showed 60% occlusion of the left anterior descending coronary artery. The multiple microinfarction profile of his myocardium provided evidence for a chronic atherosclerotic process. Moreover, sitosterolemic subjects show a decreased activity of hepatic HMG-CoA reductase and an increased hepatic LDL-receptor binding as measured by high affinity binding to radiolabeled LDL.
Presently, we have a 60-year-old female sitosterolemic subject registered in our Lipid Clinic. Her brother died suddenly before age 30 most likely due to sitosterolemia and atherosclerosis.

Bile acid resins such as cholestyramine or colestipole or ileal bypass surgery seem to be the most effective treatment of sitosterolemia. However, not all patients respond similarly to treatment, e.g., cholestyramine treatment does not result in a marked decrease in plasma plant sterol concentrations in Japanese subjects. In general, clinical improvements in appearance of xanthomas, aortic stenosis murmur, frequency of angina pectoris and arthritic attacks have been noted in several subjects treated with either cholestyramine or ileal bypass surgery. Unlike homozygotes, heterozygotes are clinically and biochemically normal, although plasma sitosterol concentrations may be slightly but significantly higher than those in controls.
3. MATERIALS AND METHODS

3.1. Animals

Seventy 5-week-old male C57BL/6J apo E-deficient mice engineered at the University of North Carolina, Chapel Hill, NC, were purchased from Jackson Laboratory, Bar Harbor, ME. After ten days of adaptation, animals were bled from the tail vein and their plasma cholesterol concentrations were measured. The mice were then divided into treated and control (untreated) groups with similar baseline mean plasma cholesterol concentrations and body weight. All mice were housed individually throughout the experimental period.

Sixty four 4-week-old male CD1 mice were purchased from the UBC animal unit and divided into three experimental groups each consisting of the FCP-3PI-treated and control groups. Each treated group was matched (as closely as possible) with its control counterpart regarding body weight and plasma cholesterol concentrations. The control mice were given a mouse chow containing 9% fat supplemented with either 0%, 0.15% (w/w) or 2% (w/w) cholesterol. The FCP-3PI-treated animals were given the same diet enriched with 2% (w/w) FCP-3PI. The animals were fed with the above mentioned diets for 14-16 weeks.

3.2. Preparation of FCP-3PI

The phytosterol mixture used in the experiments described in this thesis was extracted and purified in the laboratory of Dr. James Kutney, Department of Chemistry at UBC. Briefly, phytosterols were extracted from "tall oil" soap, a by-product of the B.C. Pulp and Paper Industry, with a mixture of hexane and acetone.
The extract was then refluxed with methanol and the crude phytosterol mixture were obtained as a precipitate. This product, which had a purity of about 40% was subjected to a precipitation by a hexane/methanol mixture. The precipitate was then extensively washed with hexane followed by evaporation of the organic solvent until the final product reached >95% plant sterols as analyzed by gas chromatography (GC). GC analysis revealed that the final product consisted of 69% β-sitosterol, 16% stigmastanol and 15% campesterol. The 5% impurity of this product was mainly long chain fatty alcohols, predominantly C_{20}OH, C_{22}OH and C_{24}OH.

3.3. Diets

PicoLab™ mouse diet 20 (9% fat w/w) or regular mouse chow (4.5% fat w/w) was purchased from Jamieson’s Pet Food Distributors Ltd., Delta, B.C., Canada. The chow was finely ground using a food processor. Cholesterol (Sigma Chemical Co., St. Louis, MO) was added at 0.15% (w/w) to 9% (w/w) fat chow and mixed well to make the “Western-type” diet. Similarly, 2% (w/w) FCP-3PI or 1% (w/w) probucol was added to either cholesterol-supplemented diet, PicoLab diet or regular chow and used for the treatment groups. The dietary mixture was repelleted and dried. Progression studies were performed using either Western-type diet for 18 weeks or with PicoLab diet for 20 weeks. Corresponding control animals were fed with either a “Western-type” diet, or PicoLab diet. Animals in regression study were fed with the “Western-type” diet during the induction phase for 18 weeks and with FCP-3PI-supplemented regular mouse chow during the regression phase for 25 weeks. Animals in the i.p. injection groups were fed with either a “Western-type” diet or regular mouse chow for 15 days. On day 16 of the experiment, treated groups were
given i.p. injections of 700 ul of a liposome solution containing 4.2 mg/ml FCP-3PI; the control groups received the same amount of liposome solution but without FCP-3PI. The injection procedure was repeated for an additional 8 days. One day after the last injection, the animals were anesthetized and blood was taken by cardiac puncture.

3.4. **Liposome preparation**

1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) has been used for incorporation of FCP-3PI. The vesicles were prepared by freeze-drying the lipid mixtures from benzene/methanol (95/5 v/v) followed by hydration in HEPES-buffered saline and extrusion through two stacked 100 nm filters using a device manufactured by Lipex Biomembranes (Vancouver, BC). The final concentration of POPC was 10 mg/ml and that of FCP-3PI was 4.2 mg/ml. The liposome solution was made by Northern Lipids Inc., Vancouver, BC.

3.5. **Blood sampling**

Each mouse was restrained briefly in a 50 ml plastic Falcon® tube, and blood was collected from tail veins into heparinized capillary tubes (Fisher Scientific, Pittsburgh, PA) and then transferred to 0.5 ml Eppendorf tubes. Blood samples were centrifuged for 3 minutes at 13,000 rpm using an ICMCentra centrifuge. Aliquots of the plasma were used for lipid analyses. At the end of each experiment, a final blood sample was obtained from the right ventricle of the pentobarbital-anesthetized animal. When needed, heparin was injected i.p. (1.5 g/kg) at the end of the study.
3.6. Lipid analyses

Plasma cholesterol and triglyceride concentrations were quantitated using enzymatic kits (Boehringer Mannheim, Germany) as described previously (7,69-71). Precinorm L Quality Control Serum (Boehringer Mannheim, Germany) has been used to ensure accurate measurements of plasma cholesterol concentrations. The method detects cholesterol concentrations as low as 0.325 mmol/L. HDL-cholesterol concentrations in plasma of CD1 mice were determined after removing apo B-containing particles by sodium phosphotungstate precipitation. Plasma lipoprotein fractions were separated using a fast protein liquid chromatography (FPLC). Aliquots of plasma were injected into the system which consisted of two columns (Superose 12 and 6) connected in series with a flow rate of 0.5 ml/min. Fractions corresponding to human VLDL and IDL, LDL, and HDL were collected. Cholesterol was extracted from the pooled fractions of VLDL/IDL, LDL and HDL and quantitated by the enzymatic method described above.

3.7. Histopathology

The hearts of pentobarbital-anesthetized animals were perfused slowly with 10 ml of 10% buffered formalin (Fisher Scientific) solution through the left ventricle. The heart and aorta were removed and placed in 10% buffered formalin (Fisher Scientific). Tissues surrounding the aorta including all fat were trimmed and the aorta was cut transversely at the aortic arch. The heart was sectioned transversely at the level of the atria as described previously (72). The atrial portion of the heart was placed in a 30% sucrose solution in 1% phosphate buffer pH 7.4 overnight and embedded in OCT compound and sectioned at the level of the aortic valve cusps.
When the aortic sinuses appeared on sections, alternate sections were mounted on 9 glass slides in such a way that, for example, slide #1 contained sections 1, 19, 37, 55, 73 and 91 and slide #6 sections 11, 29, 47, 65, 83 and 101. Slides were stained with Oil red O (ORO) and Movat's pentachrome. The formalin-fixed aortas were cut transversely into several short segments (each about 5 mm long) and used for both OCT and paraffin-embedding followed by 10µm (OCT) and 4µm (paraffin) sectioning. The sections were stained with ORO, hematoxylin-eosin (H&E) and Movat's stains. Normal appearing and affected skin specimens were also fixed, sectioned and stained with the aforementioned stains. Other organs, including the heart, lung, brain, kidney, skeletal muscle, esophagus, stomach, small and large intestines, liver, adrenal gland, spleen, pancreas, bladder and testis, were fixed in 10% neutral buffered-formalin. Four micrometer sections were cut from paraffin-embedded specimens of all the above organs and stained with H&E. ORO staining was performed on 10-µm frozen sections cut from OCT-embedded specimens as required.

3.8. Quantitative analysis of atherosclerotic lesions

Six 10µm sections from different regions of the aortic sinuses of each mouse mounted on slide #6 were stained with ORO. The lesional area was quantitated using a digitizing morphometry image analysis system (Bioquant II, R&M Biometrics, Nashville, TN) with a calibration factor of X60 and in a blinded fashion repeated three times; the means of individual measurements were used for statistical analysis.
3.9. **Hepatic enzyme (HMG-CoA reductase, cholesterol 7-hydroxylase, sterol 27 hydroxylase) activities and cholesterol content**

Hepatic microsomes and mitochondria were prepared by differential ultracentrifugation; the various enzyme activities were measured using previously published methods (36,50,73). Briefly, for HMG-CoA reductase activity, an aliquot of the microsomal preparation was incubated for 15 min. at 37°C in a buffer (50 mmol/L K$_2$HPO$_4$, 30 mmol/L EDTA, 10 mmol/L dithiotreitol, 70 mmol/L KCl, pH 7.4) containing a NADPH-generating system and $^3$H-mevalonolactone as an internal recovery standard. The reaction was started with the addition of 30 nmol [3-$^{14}$C]HMG-CoA and stopped with the addition of 20 $\mu$l 6N HCl. The products were extracted and separated by thin-layer chromatography and quantified by liquid scintillation counting. Microsomal cholesterol 7-hydroxylase and mitochondrial sterol 27 hydroxylase activities were measured by the isotope method of Shefer et al. (73). Briefly, $^{14}$C-cholesterol was incubated with hepatic microsomes or mitochondria and the products were extracted and applied to silica-gel plates. The amount of 7-hydroxycholesterol or 27 hydroxycholesterol formed /mg protein/min was defined as the unit for the activity of the enzymes. Cholesterol content of the liver homogenates was determined as previously described (74). The hepatic enzyme analyses were performed in Drs. Shefer and Nguyen's laboratory, University of Medicine and Dentistry of New Jersey, Newark, NJ.

3.10. **Hepatic LDL-receptor binding**

Hepatic LDL receptor binding was determined by measuring high affinity binding of $^{125}$I-labeled mouse LDL (separated from cholesterol-fed apo E-KO mouse
plasma) to the liver membranes as previously described (75). The high affinity binding was determined as the difference between total and non-specific binding (measured in the absence and presence of 40-fold excess unlabeled LDL, respectively). This analysis was performed in Drs. Shefer and Nguyen's laboratory, University of Medicine and Dentistry of New Jersey, Newark, NJ.

3.11. Fecal sterol analysis

Fecal material was collected from each mouse cage and kept frozen prior to analysis. Lipids were extracted from fecal material and the sterol concentrations were determined using GC by a previously described method (76). The fecal sterol analysis was performed in Drs. Salen and Batta's laboratory, University of Medicine and Dentistry of New Jersey, East Orange, NJ.

3.12. Plasma lipase activity

Aliquots of plasma samples (pre- and post-heparin) were used for measurement of lipase (total, lipoprotein and hepatic) activity as previously described (77). Briefly, labeled triglyceride ([³H]-triolein) was incubated with plasma samples and the liberated fatty acids ([³H]-oleate) were quantitated by liquid scintillation counting. Hepatic lipase activity was measured after inhibition of lipoprotein lipase by 1M NaCl. This analysis was performed in Dr. Rodrigues' laboratory, Faculty of Pharmaceutical Sciences, UBC.
3.13. Plasma fibrinogen analysis

At the final sampling, heparin (1.5 unit/g) and pentobarbital (60 mg/kg) were administered i.p. Fibrinogen was measured by the Clauss method in the Hematology Laboratory at St. Paul’s Hospital, Vancouver, BC.

3.14. Red cell and plasma antioxidant enzyme analyses

Red blood cells were obtained from the final blood samples and washed twice with isotonic saline. Aliquots of the plasma samples and red cells were analyzed for the activity of glutathione peroxidase (Gpx), glutathione reductase (Gred), superoxide dismutase (SOD), and catalase (CAT) (78-81). Antioxidant analyses were performed in Dr. Godin’s laboratory, Department of Pharmacology and Therapeutics, UBC.

3.15. Hematology

Aliquots of whole blood in EDTA-coated tubes were sent to the Hematology Laboratory at British Columbia’s Children’s Hospital, Vancouver, BC, for blood smear and other hematological assessments in a blinded fashion, and assays were performed as quality-controlled standard diagnostic procedures. These measures included blood cell counts, platelet counts, hematocrit, hemoglobin, and morphological assessments.

3.16. Erythrocyte fragility

Erythrocyte fragility measurements were performed as previously described (82). Briefly, erythrocytes were isolated from whole blood by centrifugation. Cells were washed twice with isotonic saline and once with 0.15 M NaCl-15 mM Tris pH
7.0 and then resuspended in 0.15 M NaCl-15 mM Tris. Aliquots of this red cell suspension were added into tubes containing 0.05 M NaCl. After a 15-minute incubation at room temperature, samples were centrifuged and supernatant absorbance read at 540 nm using a Perkin-Elmer Lambda 3B UV/VIS spectrophotometer. Percent total hemolysis (in distilled water) was calculated for each sample.

3.17. Urinalysis

Urine was collected directly from the bladder of each anesthetized mouse (at the end of the study) into a tuberculin syringe. Urinalysis was performed by dripping each urine sample onto a reagent strip (Multistix, Miles Canada Inc., Etobicoke, ON).

3.18. Plasma glucose concentrations

Aliquots of plasma obtained at the end of the experiment were used for determination of glucose level as previously described (83).

3.19. Statistical analysis

Two-tailed Student’s t-test assuming equal variances was used to assess the significance of differences between results in the two experimental groups.
4. RESULTS

4.1. Atherosclerosis progression study

4.1.1. Body weight and lipid analyses

The average body weights in both groups of animals were not significantly different in the first 4 weeks of the study. However, by week 5 and throughout the rest of the study (except for weeks 7 and 9), animals in the FCP-3PI group showed a significant (p<0.05) increase in body weight as compared to the control (untreated) group (Figure 3).

Total cholesterol concentrations were determined at baseline, and at weeks 4, 11 and 18. Both groups of animals showed similar concentrations of plasma total cholesterol at week 0 (~15.5 mmol/L). Plasma total cholesterol increased markedly after the consumption of the 0.15% (w/w) cholesterol-enriched diets in both groups of mice. However, at four weeks, FCP-3PI-treated animals showed significantly (26.62 ± 2.86 vs 42.02 ± 1.89 mmol/L, p<0.0001) lower plasma cholesterol concentrations as compared to the control group. A significant difference was sustained until the end of the study (Figure 4).

4.1.2. Effect of FCP-3PI treatment on atherogenesis

Representative atherosclerotic lesions in the aortas and aortic sinuses of the treated and control groups of mice are illustrated in Figure 5 and Figures 6-8, respectively. Panel A of Figure 5 shows a section of normal-appearing aorta from a FCP-3PI-treated mouse with normal intima and medial elastic laminae and associated smooth muscle cells. Panels B and C show aortic sections from an
untreated control animal that developed prominent atherosclerotic lesions. Lipid-enriched foam cells are evident in the subendothelial space, but little increase in matrix is apparent. A similar lesion is present at the branch-point of a secondary vessel arising from the thoracic aorta (Figure 5, Panels D and E). Advanced atherosclerotic lesions in the aortic root of control mice are depicted in Figures 6C and D, 7B, and 8A and B. In these sections, in addition to foam cells, there is a significant extracellular matrix component. Corresponding sections from FCP-3PI-treated animals stained with ORO or Movat’s show not only a limited extent and severity of lesion involvement in the aortic sinuses (Figures 6A and B), but also that the origin of the epicardial coronary artery is free of lesions (Figure 7A, arrow). Marked differences between the two groups of animals in terms of the extent and maturity of the lesions are reflected in the extent of superficial foam cells, underlying extracellular glycosaminoglycans, sheaths of apparently proliferative smooth muscle cells and areas with many cholesterol clefts in the untreated animals. As noted, when a coronary ostium is visualized, the lesions in the most severely affected animals extend into the proximal coronary arteries (Figure 7B, curved arrow). In certain untreated animals, the coronary arteries showed substantial fat deposits and lesion formation (Figure 8A, arrow), such that certain small branches of the coronary arteries appeared to be completely blocked. On ORO stains, the lipid richness of the foam cells was dramatic, as was the rather clustered superficial pattern of foam cell accumulation. FCP-3PI treatment resulted in a more than 50% reduction (1.96 ± 0.8 vs 4.08 ± 0.3 mm², p<0.0001) in the average area of atheromata as compared to those in the control group (Figure 9).
4.1.3. Concordance of atherosclerotic lesion occurrence with plasma cholesterol concentrations

Regression analysis showed a linear correlation ($r=0.69$) between mean plasma cholesterol during the experimental period and the area of atherosclerotic lesions in the aortic sinuses as measured by image analysis. This analysis also demonstrated two clusters indicative of lesion size in the range of $1\text{--}2.5 \text{ mm}^2$ for treated and $3.7\text{--}4.5 \text{ mm}^2$ for control animals (Figure 10).

4.2. Cholesterol-lowering effects of FCP-3PI in CD1 mice

4.2.1. Plasma lipid concentrations

As is evident in Table 1, the control and treated mice had comparable plasma total cholesterol concentrations at baseline and at the end of the study. Approximately 70\% of total cholesterol in both control and treated animals was in the HDL fraction.

4.2.2. Histological examinations

Histological examinations showed no evidence of atherogenesis in any section of the aortae from either the control (fed 2\% w/w dietary cholesterol) or the treated mice.
Figure 3: Body weight (g) at baseline and at weekly intervals thereafter, throughout the experimental course. In this and all subsequent figures, values are expressed as mean ± SD. (* significant [p<0.05] difference between the two groups of mice.)
Figure 4: Plasma concentrations of total cholesterol in the control and FCP-3PI-treated mice at baseline and during the study period. The number of animals is shown in parentheses. p values are shown for the differences between the groups at the given time points.
Figure 5: Photomicrographs of transverse sections of aortae from FCP-3P1-treated (A) and untreated (B-E) animals. The section in Panel A has no visible intima with normal intact musculoelastic layers in the media. In panel B, a localized intimal lesion is evident. At higher power (Panel C), the lesion is seen to be composed of numerous foam cells. The overlying endothelium appears to be intact. There is disruption of the inner-most elastic lamina with projection of certain foam cells into the superficial media. In a similar fashion, at the bifurcation of a probable segmental (intercostal) branch of the aorta there is an atheromatous lesion (Panels D and E) which appears to virtually occlude the mouth of the small branch vessel. The nature of this lesion is more complex than that shown in Panels B and C. There are not only numerous foam cells, but also apparent intimal smooth muscle cells. As well, the media is variably, and focally very severely disrupted. (Movat's pentachrome stains, x 25-A, B and D, x 330-C, E).
Figure 6: Photomicrographs of serial transverse sections at the level of the aortic valve cusps taken from one FCP-3P1-treated (Panels A, B) and one untreated (Panels C, D) animal. As is evident, the volume of the lesions in the treated animal is markedly reduced and the lesions have much less complexity. The Movat's pentachrome stains (Panels A, C) depict the interstitial matrix as well as outlining cholesterol clefts and foam cells. Corresponding Oil red O-stained sections emphasize the prominence of neutral lipid, most dramatic in the untreated animal (Panel D). (Movat's stains, A, C; Oil red O stains B, D, x 25).
Figure 7: Photomicrographs of transverse sections at the level of the aortic valve cusps illustrating the severity of atheromatous lesions in FCP-3P1-treated (A) and control (B) animals. These images emphasize the lack of ostial narrowing of a major epicardial coronary artery in the treated group (arrow) with virtual occlusion by atheromatous change in the untreated animal (curved arrow) (Oil red O staining, x 25).
Figure 8: Photomicrographs of transverse sections of aortic root at the level of the aortic valve cusps illustrating the involvement of proximal epicardial coronary arteries (arrow, Panel A) and the severity of foam cell formation in underlying matrix and cellular components in an untreated mouse (Panels A, B) (Oil red O stains; A x 25, B x 330).
Figure 9: Average lesion area (mm$^2$) in the aortic sinuses measured in 6 (taken from six different levels representative of ~100-1,010 μm of the aortic root length) Oil red O stained sections from each aorta using an image analysis system with a calibration factor of X60. N=9 and 5 for treated and control animals, respectively. * $p<0.0001$, area of lesions in treated animals being less than in controls.
Figure 10: Relationship of total area ($\text{mm}^2$) of atherosclerotic lesions in the aortic sinuses to plasma total cholesterol (mmol/L). Triangles: treated animals, Circles: control animals.
Table 1: Plasma total cholesterol concentrations (mean±SD, mmol/L) in CD1 mice at baseline and the end of study.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control (8)</th>
<th>Treated (8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diets</td>
<td>Baseline</td>
<td>End</td>
</tr>
<tr>
<td>9% fat + No cholesterol</td>
<td>3.7±0.4</td>
<td>5.7±0.6</td>
</tr>
<tr>
<td>9% fat + 0.15% cholesterol</td>
<td>3.0±0.5</td>
<td>4.5±0.6</td>
</tr>
<tr>
<td>9% fat + 2% cholesterol</td>
<td>3.1±0.4</td>
<td>6.3±0.8</td>
</tr>
</tbody>
</table>
4.3. Comparison of the effects of FCP-3PI to those of probucol

4.3.1. Histological findings

Sections from aortic roots from all animals showed ORO-positive atherosclerotic lesions by light microscopic examination. However, the extent and severity of the lesions varied markedly among the three groups of mice. Probucol-treated animals had the worst lesions in terms of size, severity and lipid-enrichment. Sections from FCP-3PI-treated animals had lesions with the least volume and less complexity compared to either control or probucol-treated groups. Numerous cholesterol clefts were observed in sections from probucol-treated and control groups, but not from the FCP-3PI-treated group. The difference in the extent, severity and complexity of the lesions from probucol-treated and control groups was accentuated when sections were stained with Movat’s pentachrome. Figure 11 compares the lesions in representative sections stained with ORO and Movat’s staining of aortic root taken from a similar anatomical location of one mouse from either FCP-3PI-treated (Panels A&B), control (Panels C&D) or probucol-treated (Panels E&F) groups. Panel E shows an increased volume of extracellular matrix in the presence of sheaths of apparently proliferative smooth muscle cells, numerous foam cells and cholesterol crystals indicative of mature and complex lesions in the probucol-treated group. These components of the atherosclerotic lesions are present to a lesser extent in Panel C (control group) and are much less evident in Panel A (FCP-3PI-treated group). Panel E also presents evidence for the development of an aortic atherosclerotic aneurysm (arrow).
Similarly, as shown in Figure 12, thoracic aorta from probucol-treated animals showed severe atherosclerotic lesions by various histochemical staining methods. As evident in a Movat's-stained section (Panel H) from a representative aorta of the probucol-treated group, all elastic laminae are ruptured, making the aorta prone to the development of atherosclerotic aneurysm and, potentially, the rupture of the entire wall of the vessel. However, such prominent lesions in thoracic aorta were not observed frequently. In agreement with the findings in the aortic roots, the untreated control group also had advanced lesions in their thoracic aorta (Panels D, E&F). Normal-appearing aorta from a FCP-3PI-treated animal showing intact intima and medial elastic laminae and associated smooth muscle cells and endothelium is also shown (Panels A, B&C).

4.3.2. Morphometric findings

Probucol caused an increase of 175% (relative to control) in the average lesion size in aortic roots, while FCP-3PI caused a decrease of 50% (relative to control). These changes paralleled other morphometric measurements such as perimeter of the aortic roots and lesion to lumen ratios. As summarized in Table 2, the greater the lesion area the higher the perimeter value and the higher the lesion to lumen ratio.

4.3.3. Plasma lipid concentrations

Table 3 demonstrates plasma concentrations of TC and FC at baseline and four intervals during the experimental course. It shows a significant reduction in TC concentrations in both probucol- and FCP-3PI-treated animals compared to controls.
Both treatments significantly reduced TC concentrations at week 6. Subsequently, probucol, but not FCP-3PI treatment, led to a progressive decrease in cholesterol concentrations during the remainder of the experimental course. Moreover, probucol-induced cholesterol reduction increased in a time-dependent manner (54%, 62% and 64% decreases at weeks 6, 13 and 20, respectively, compared to controls). In contrast, reductions in TC concentrations decreased with time in the FCP-3PI-treated group (37%, 26% and 15% decreases at weeks 6, 13 and 20, respectively, compared to controls). As can be seen in Table 3, a marked and sustained reduction in FC was also observed in the probucol-treated group. Lipoprotein profiles are shown in Table 4. Both probucol and FCP-3PI treatment significantly decreased VLDL/IDL-cholesterol. HDL-cholesterol concentration was significantly reduced only in the probucol-treated group (Table 4).

4.3.4. Fecal sterol composition

The most important findings from analyses of fecal sterols are summarized in Table 5. Compared to untreated controls, FCP-3PI treatment increased cholesterol and lanosterol fecal excretion by 43% and 100%, respectively. This was accompanied by a 40% reduction in coprostanol fecal content. Probucol treatment decreased fecal sitosterol, total sterol, and non-cholesterol sterol contents by 28%, 14% and 21%, respectively; a 40% increase in coprostanol excretion was also observed in probucol-treated mice compared to controls. As expected, dietary supplementation with phytosterols caused a marked increase in sitosterol and total sterol excretion.
4.3.5. Hepatic enzyme and LDL-receptor studies

The effects of probucol and FCP-3PI treatment on the hepatic enzymes involved in cholesterol biosynthesis (HMG-CoA reductase) and catabolism (cholesterol 7-hydroxylase, and sterol 27-hydroxylase) are shown in Table 6. Hepatic LDL receptor function/cholesterol contents are also presented in Table 6. Both probucol and FCP-3PI treatment markedly increased the activity of HMG-CoA reductase and cholesterol 7-hydroxylase [probucol by 115%, p<0.05 and 45%, p<0.05, respectively, and FCP-3PI by 184%, p<0.05 and 18% (not statistically significant), respectively]; the activity of sterol 27-hydroxylase was not affected by either treatment protocol. The capacity of LDL receptors to bind radiolabeled apo E-KO mouse LDL was not affected by FCP-3PI treatment; however, this binding of LDL receptors was markedly decreased (-64%, p<0.05) by probucol compared to controls. On the other hand, compared to controls, hepatic cholesterol content was significantly reduced (-54%, p<0.05) in the FCP-3PI-treated group, while it was not affected by probucol treatment.

4.3.6. Plasma lipolytic activity

Administration of heparin i.p. markedly increased total lipase activity in the plasma [30 vs 365 (control), 30 vs 248 (FCP-3PI-treated), 37 vs 250 (probucol-treated), mU, means). Table 7 shows significant reductions in post-heparin total, lipoprotein and hepatic (not in the probucol-treated group) lipase activities in response to both probucol and FCP-3PI treatments. Probucol and FCP-3PI treatments caused 43% and 31% decreases, respectively, in the average lipoprotein
lipase activity as compared to the control group (p<0.05). FCP-3PI, but not probucol, decreased significantly (p<0.05) the activity of hepatic lipase compared to controls.

4.3.7. Plasma fibrinogen concentrations

Fibrinogen concentrations were different in the two treatment groups as compared to the untreated (control) group. While treatment with probucol caused a significant increase (>42%) in fibrinogen concentrations (2.6 ± 0.7 vs 3.7 ± 0.7, g/L, mean±SD, n=8 p<0.02), FCP-3PI treatment reduced fibrinogen by 20% (2.6 ± 0.7 vs 2.1 ± 0.2, g/L, mean±SD, n=8, not statistically significant), compared to controls.

4.3.8. Antioxidant enzyme activities

Table 8 summarizes the effects of each treatment on the activity of various antioxidant enzymes in red blood cells and plasma. Both treatment regimens, particularly probucol, markedly influenced the activity of plasma antioxidant enzymes. Among all the erythrocyte enzymes examined, GRed activity increased most markedly in both treatment regimens. The extent of the increment in erythrocyte GRed due to probucol treatment was twice as high as that in the FCP-3PI-treated group (26% vs 13%). Similarly, the effect of probucol on increasing plasma GPx and SOD activity was 6 times greater than that of FCP-3PI. Probucol, but not FCP-3PI, caused a significant increase (12.5%, p<0.05) in erythrocyte catalase activity. Probucol and FCP-3PI affected the activity of plasma GRed in an opposite direction.
Figure 11: Photomicrographs of serial transverse sections at the level of the aortic valve cusps taken from one FCP-3PI-treated (A&B), one control (C&D) and one probucol-treated (E&F) animal. The volume and complexity of the atherosclerotic lesions are markedly reduced and increased in Panels A&B and Panels E&F, respectively, compared to Panels C&D. While ORO staining (Panels B,D&F) emphasizes the lipid accumulation in the lesions, Movat’s pentachrome staining (Panels A,C&E) highlights the components of the lesions including interstitial matrix, foam cells and cholesterol clefts. Panel E also presents evidence for an aortic aneurysm (arrow) (original magnification x25, B,D&F; x50 A,C&E).
Figure 12: Representative photomicrographs of transverse sections of thoracic aorta from one FCP-3PI-treated (A,B&C), one control (D,E&F) and one probucol-treated (G,H&I) animal stained with H&E, Movat’s pentachrome and ORO, respectively. Sections from the FCP-3PI-treated animal show no visible lesions and normal musculoelastic layers. Sections from both the control and probucol-treated mouse demonstrate advanced atherosclerotic lesions containing foam cells, cholesterol clefts and increased interstitial matrix. As is evident, the nature of the lesions is more complex in the probucol-treated animal. Panel H shows complete rupture of media (arrow) in the probucol-treated mouse (x25, A-F; X50 G-I).
Table 2: Calculated morphometric values from aortic roots (mean±SD per section).

<table>
<thead>
<tr>
<th>Group</th>
<th>Lesion area (mm$^2$)</th>
<th>Perimeter (mm)</th>
<th>Lesion/Lumen ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (7)</td>
<td>0.4±0.1</td>
<td>5.1±0.4</td>
<td>0.22±0.02</td>
</tr>
<tr>
<td>Probucol (8)</td>
<td>1.1±0.6*</td>
<td>6.4±1.0*</td>
<td>0.36±0.08*</td>
</tr>
<tr>
<td>FCP-3PI (8)</td>
<td>0.2±0.1*</td>
<td>4.3±0.3*</td>
<td>0.12±0.04*</td>
</tr>
</tbody>
</table>

*, p<0.01 compared to control, respectively; n values are indicated in parentheses.
Table 3: Plasma total (TC) and free cholesterol (FC) concentrations (mmol/L; mean±SD) at baseline and during the experimental period.

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Control (8)</th>
<th></th>
<th>Probucol (8)</th>
<th></th>
<th>FCP-3PI (8)</th>
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<tr>
<td>Lipids</td>
<td>TC</td>
<td>FC</td>
<td>TC</td>
<td>FC</td>
<td>TC</td>
<td>FC</td>
</tr>
<tr>
<td>0</td>
<td>14.7±1.4</td>
<td>6.8±1.4</td>
<td>15.8±2.7</td>
<td>6.5±1.3</td>
<td>14.7±1.8</td>
<td>6.7±1.2</td>
</tr>
<tr>
<td>6</td>
<td>27.7±3.6</td>
<td>8.2±1.2</td>
<td>12.6±2.6*</td>
<td>3.5±0.7*</td>
<td>17.5±3.3**</td>
<td>6.4±1.3**</td>
</tr>
<tr>
<td>13</td>
<td>31.5±3.0</td>
<td>10.4±1.5</td>
<td>11.9±2.6*</td>
<td>3.5±0.9*</td>
<td>23.3±2.8**</td>
<td>8.4±0.9**</td>
</tr>
<tr>
<td>20</td>
<td>27.3±2.3</td>
<td>11.2±2.6</td>
<td>9.8±1.6*</td>
<td>3.2±0.5*</td>
<td>23.2±3.2**</td>
<td>8.8±2.1</td>
</tr>
</tbody>
</table>

*, p<0.0001; **, p<0.05 compared to corresponding controls, respectively, n values are indicated in parentheses.
Table 4: Plasma lipoprotein cholesterol concentrations (mmol/L; mean±SD) recovered from pooled lipoprotein fractions isolated by FPLC.

<table>
<thead>
<tr>
<th>Lipoproteins</th>
<th>Controls</th>
<th>Probucol-treated</th>
<th>FCP-3PI-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLDL/IDL</td>
<td>29.6±6.4</td>
<td>6.5±4.7*</td>
<td>18.8±6.7*</td>
</tr>
<tr>
<td>LDL</td>
<td>20.0±3.3</td>
<td>10.4±5.3*</td>
<td>16.4±3.4</td>
</tr>
<tr>
<td>HDL</td>
<td>2.2±0.7</td>
<td>0.5±0.8*</td>
<td>2.5±1.8</td>
</tr>
</tbody>
</table>

*p<0.05 compared to controls
Table 5: Fecal sterol composition (µg/g dry feces; mean±SD).

<table>
<thead>
<tr>
<th>Sterols</th>
<th>Control (8)</th>
<th>Probucol (8)</th>
<th>FCP-3PI (8)</th>
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</thead>
<tbody>
<tr>
<td>cholesterol</td>
<td>1.6±0.4</td>
<td>1.7±0.2</td>
<td>2.3±0.8*</td>
</tr>
<tr>
<td>coprostanol</td>
<td>0.5±0.3</td>
<td>0.7±0.4</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>lanosterol</td>
<td>0.1±0.0</td>
<td>0.1±0.0</td>
<td>0.2±0.1*</td>
</tr>
<tr>
<td>sitosterol</td>
<td>1.8±0.3</td>
<td>1.3±0.2*</td>
<td>33.9±12.7*</td>
</tr>
<tr>
<td>total sterols</td>
<td>7.0±0.7</td>
<td>6.0±0.8*</td>
<td>58.3±21.2*</td>
</tr>
<tr>
<td>non-cholesterol sterols</td>
<td>5.4±0.5</td>
<td>4.2±0.8*</td>
<td>56.0±20.4*</td>
</tr>
</tbody>
</table>

*, p<0.05 compared to controls; n values are indicated in parentheses
Table 6: Hepatic cholesterol metabolism enzyme activities, LDL receptor binding and cholesterol content (mean±SD).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Probucol</th>
<th>FCP-3PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMG-CoA Red</td>
<td>68.4±39.6 (8)</td>
<td>147.0±89.4* (8)</td>
<td>194.2±116.3* (8)</td>
</tr>
<tr>
<td>Cholesterol 7-Hyd</td>
<td>36.5±4.6 (7)</td>
<td>52.9±17.5* (7)</td>
<td>43.1±4.7 (7)</td>
</tr>
<tr>
<td>Sterol 27-Hyd</td>
<td>18.4±4.1 (8)</td>
<td>23.8±8.1* (6)</td>
<td>19.0±7.0 (8)</td>
</tr>
<tr>
<td>LDL-R binding</td>
<td>962.6±335.8 (5)</td>
<td>342.0±193.6* (4)</td>
<td>819.2±192.9 (5)</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>30.0±10.3 (8)</td>
<td>28.6±7.0 (7)</td>
<td>13.9±3.6* (5)</td>
</tr>
</tbody>
</table>

Red, reductase; Hyd, hydroxylase; LDL-R, LDL receptor; , pmol/mg protein/min; , ng/mg protein (high affinity binding to labeled mouse LDL); , μmol/mg protein; *, p<0.05 compared to control; n values are indicated in parentheses.
Table 7: Plasma post-heparin lipase activity (mU; mean±SD).

<table>
<thead>
<tr>
<th>Group</th>
<th>Control (8)</th>
<th>Probucol (8)</th>
<th>FCP-3PI (8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total lipase</td>
<td>365.6±49.5</td>
<td>250.6±64.3*</td>
<td>248.4±28.8*</td>
</tr>
<tr>
<td>Lipoprotein lipase</td>
<td>244.0±64.4</td>
<td>139.1±68.5*</td>
<td>169.4±23.0*</td>
</tr>
<tr>
<td>Hepatic lipase</td>
<td>121.6±36.0</td>
<td>104.1±27.9</td>
<td>78.8±20.0*</td>
</tr>
</tbody>
</table>

*, p<0.05 compared to controls, respectively; n values are indicated in parentheses.
Table 8: Antioxidant enzyme activities in red blood cells and plasma (mean±SD)

<table>
<thead>
<tr>
<th>Group</th>
<th>Red Blood Cells</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GPx</td>
<td>GRed</td>
</tr>
<tr>
<td>Control (8)</td>
<td>40.1±10.8</td>
<td>2.1±0.1</td>
</tr>
<tr>
<td>Probucol (8)</td>
<td>44.8±12.4</td>
<td>2.6±0.2&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>FCP-3PI (8)</td>
<td>43.3±4.8</td>
<td>2.4±0.2&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

GPx, glutathione peroxidase; GRed, glutathione reductase; SOD, superoxide dismutase; CAT, catalase; , nmoles/min/mg protein/hemoglobin; , units/mg protein/hemoglobin; ¥, k/mg hemoglobin; n values are indicated in parentheses, *, p<0.05.
4.4. Atherosclerosis regression study

4.4.1. Body weights

The animals' body weights and food consumption were monitored at 2-week intervals during both the induction and regression phases. The animals gained weight during the induction period; however, their weight gain reached a plateau during the regression phase. There were no significant differences between the two groups of FCP-3PI-treated and controls (Table 9).

4.4.2. Plasma lipid concentrations

Table 10 shows TC concentrations during the induction period and regression phase. The plasma TC concentrations increased significantly with the feeding of the experimental induction diet (9% w/w fat and 0.15% w/w cholesterol) (Table 9). The concentrations of TC during the induction phase were comparable to those previously observed in similar experiments (7). Table 11 indicates that there was a significant reduction in the concentrations of plasma TC in both groups of FCP-3PI-treated and control animals at week 6 of the regression phase as compared to the beginning of the regression phase or during the induction phase. A similar reduction was also observed in FC but not in TG concentrations (Table 11). In contrast, no statistically significant changes were noted when a comparison was made between the plasma lipid concentrations of the two groups of mice during the regression phase.
4.4.3. Atherosclerotic lesions

4.4.3.1. Histology

Substantial deposits of lipids were observed in vascular lesions on sections stained with ORO. Movat's staining demonstrated that all lesions contained a significant amount of extracellular matrix as glycosaminoglycan. Cholesterol clefts, foam cells and apparently proliferative smooth muscle cells were other major components of the lesions.

4.4.3.2. Morphometry

Table 12 shows the lesion size in the aortic roots of the animals after the 18 week induction period and after the 43 week experimental course. The rate of lesion development during the induction phase was approximately 0.025 mm$^2$/week for each animal. This rate was reduced to 0.005 mm$^2$/week in treated animals and to 0.007 mm$^2$/week in the controls during the regression phase. This difference resulted in 28% and 40% increases in lesion size of treated and controls, respectively, as compared to the end of induction phase. Although no evidence of lesion regression was found in either control or treated animals, the lesion size in the treated group was 8% less compared to controls.

4.4.4. Cutaneous xanthomatosis

Three out of fifteen mice developed cutaneous lesions during the induction phase and these lesions resembled previous findings (7) both anatomically and pathologically. At the end of induction period, these three animals were allocated as follows: one animal was assigned to the treated group, one animal to the control
group and one animal was sacrificed to assess atherosclerotic and xanthomatous lesions at the end of the induction period. Similarly, one animal in the control group and three in the treatment group developed cutaneous lesions during the regression phase. Histological examination revealed substantial deposits of lipids in subcutaneous tissues which was accompanied by thickening of the skin and local alopecia. This observation is in contrast with our previous findings in which none of the FCP-3PI-treated mice showed any kind of skin lesion. Interestingly, treated animals with cutaneous xanthomatosis had lower concentrations of TC as compared to those with no skin lesions.
Table 9: Mouse body weights (mean±SD; g) during induction and regression phases of atherogenesis.

<table>
<thead>
<tr>
<th>group (n)</th>
<th>Induction Phase</th>
<th>Regression Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weeks</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>all mice (15)</td>
<td>24±1</td>
<td>30±1</td>
</tr>
<tr>
<td></td>
<td>31±2</td>
<td>32±2</td>
</tr>
<tr>
<td>FCP-3PI-treated (7)</td>
<td>33±2</td>
<td>32±2</td>
</tr>
<tr>
<td></td>
<td>33±2</td>
<td>33±2</td>
</tr>
<tr>
<td></td>
<td>33±1</td>
<td>33±2</td>
</tr>
<tr>
<td></td>
<td>33±2</td>
<td></td>
</tr>
<tr>
<td>controls (6)</td>
<td>31±2</td>
<td>31±2</td>
</tr>
<tr>
<td></td>
<td>33±2</td>
<td>33±3</td>
</tr>
<tr>
<td></td>
<td>34±2</td>
<td></td>
</tr>
</tbody>
</table>
Table 10: Mouse plasma total cholesterol concentrations (mean±SD; mmol/L) during induction and regression phases of atherogenesis.

<table>
<thead>
<tr>
<th>group (n)</th>
<th>Induction Phase</th>
<th>Regression Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weeks</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td>all mice (15)</td>
<td>22±2</td>
<td>42±2</td>
</tr>
<tr>
<td></td>
<td>42±2</td>
<td>35±5</td>
</tr>
<tr>
<td></td>
<td>23±4</td>
<td>20±5</td>
</tr>
<tr>
<td></td>
<td>19±5</td>
<td>13±1</td>
</tr>
<tr>
<td></td>
<td>37±3</td>
<td>26±4</td>
</tr>
<tr>
<td></td>
<td>25±4</td>
<td>25±7</td>
</tr>
<tr>
<td></td>
<td>19±6</td>
<td></td>
</tr>
</tbody>
</table>

FCP-3PI-
treated (7)
Table 11: Mouse plasma lipid concentrations (mean±SD, mmol/L) at the beginning and at week 6 of the regression phase.

<table>
<thead>
<tr>
<th>Lipids</th>
<th>Groups</th>
<th>base line</th>
<th>At week 6</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC</td>
<td>FCP-3PI-treated</td>
<td>34.79±5.39 (7)</td>
<td>22.60±4.10 (7)</td>
<td>0.00046</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>36.74±3.23 (6)</td>
<td>25.70±3.61 (5)</td>
<td>0.00046</td>
</tr>
<tr>
<td>FC</td>
<td>FCP-3PI-treated</td>
<td>11.20±3.09 (7)</td>
<td>7.09±1.01 (7)</td>
<td>0.0058</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>12.27±2.37 (6)</td>
<td>7.85±1.27 (5)</td>
<td>0.0048</td>
</tr>
<tr>
<td>TG</td>
<td>FCP-3PI-treated</td>
<td>1.46±1.17 (7)</td>
<td>1.46±1.24 (7)</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>1.54±0.87 (6)</td>
<td>1.58±0.60 (5)</td>
<td>0.933</td>
</tr>
</tbody>
</table>
Table 12: Atheromatous lesion size (mean±SD, mm²) per section of aortic root in different phases of the study.

<table>
<thead>
<tr>
<th>groups (n)</th>
<th>lesion size</th>
<th>% increase in the mean lesion size during the regression phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>induction (2)</td>
<td>0.45±0.10</td>
<td>NA</td>
</tr>
<tr>
<td>FCP-3PI-treated (7)</td>
<td>0.58±0.19</td>
<td>28.9</td>
</tr>
<tr>
<td>controls (6)</td>
<td>0.63±0.12</td>
<td>40.0</td>
</tr>
</tbody>
</table>

NA, not applicable
4.5. Systemic effects of FCP-3PI

4.5.1. Plasma lipid concentrations

Table 13 shows the level of plasma cholesterol in all groups of mice. As is evident, both vehicle and liposome-encapsulated FCP-3PI reduced the concentrations of total cholesterol in plasma; however, this reduction was greater in FCP-3PI-injected animals. Diet-induced hypercholesterolemia did not significantly influence the cholesterol-lowering effects of FCP-3PI.

4.5.2. Hepatic enzyme activities

The activities of hepatic enzymes of cholesterol metabolism are summarized in Table 14. Diet-induced hypercholesterolemia significantly suppressed the activity of HMG-CoA reductase such that the FCP-3PI treatment regimen no longer modified the activity of this enzyme, although, FCP-3PI treatment did significantly reduce the activity of this enzyme in mice fed regular (i.e. non-cholesterol supplemented) chow. No significant changes were observed in the activity of the other enzymes measured (cholesterol 7-hydroxylase and sterol 27-hydroxylase).

4.5.3. Plasma antioxidant enzyme activities

Plasma antioxidant enzyme activities are shown in Table 15. Dietary cholesterol supplementation had no effect on the activity of the enzymes measured. However, the activity of enzymes was increased in both groups of FCP-3PI-injected mice as compared to corresponding controls.
Table 13: Total cholesterol concentrations (mmol/L, n=3, mean±SD) at baseline, one day before injection and one day after the last injection.

<table>
<thead>
<tr>
<th>Diets</th>
<th>Low Fat Diet</th>
<th>High Fat Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups</td>
<td>Control</td>
<td>Treat</td>
</tr>
<tr>
<td>Baseline</td>
<td>15.5±0.9</td>
<td>15.6±0.3</td>
</tr>
<tr>
<td>Before injection</td>
<td>15.2±1.6</td>
<td>15.4±2.1</td>
</tr>
<tr>
<td>After injection</td>
<td>11.7±4.6</td>
<td>8.9±1.1</td>
</tr>
<tr>
<td>% change</td>
<td>-22</td>
<td>-42</td>
</tr>
</tbody>
</table>
Table 14: Hepatic cholesterol metabolism enzyme activities (pmol/mg protein/min, mean±SD) after the last injection.

<table>
<thead>
<tr>
<th>Diets</th>
<th>Low Fat Diet</th>
<th>High Fat Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (3)</td>
<td>Treated (3)</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>143±3</td>
<td>84±27*</td>
</tr>
<tr>
<td>Cholesterol 7-hydroxylase</td>
<td>48±19</td>
<td>29±10</td>
</tr>
</tbody>
</table>

*p<0.05 compared to control
Table 15: Plasma antioxidant enzyme activities (mean±SD) after the last injection.

<table>
<thead>
<tr>
<th>Diets</th>
<th>Low Fat Diet</th>
<th>High Fat Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (2)</td>
<td>Treated (3)</td>
</tr>
<tr>
<td>Groups</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GPX$^1$</td>
<td>GRed$^1$</td>
</tr>
<tr>
<td></td>
<td>227±7</td>
<td>275±11*</td>
</tr>
<tr>
<td></td>
<td>89±4</td>
<td>121±10*</td>
</tr>
<tr>
<td></td>
<td>11±1</td>
<td>15±2</td>
</tr>
</tbody>
</table>

* *p<0.05 compared to control; 1, nmol/min/mg protein; 2, units/mg protein.
4.6. Tolerance of apo E-KO mice to FCP-3PI

4.6.1. Body weights and plasma glucose concentrations

Both control and FCP-3PI-treated groups of mice had a similar mean body weights at baseline [21.6 ± 1.1 g (treated) vs 21.1 ± 0.9 g (control)]. However, by the end of the experiment, the treated group had significantly higher body weights than the control group (34.2 ± 2.0 g vs 30.8 ± 1.9 g; p<0.05).

Treatment with phytosterols did not alter plasma glucose concentrations [14.6±2.3 mmol/L (treated) vs 13.9±1.9 mmol/L (control)].

4.6.2. Plasma lipid concentrations

Table 16 displays the concentrations of plasma lipids in mice at the outset and at end of the study. Both groups of mice had similar plasma lipid concentrations at baseline. As previously shown, after 18 weeks on the experimental diet, the treated group showed a significantly lower plasma total cholesterol concentrations as compared to controls (30.4±2.5 mmol/L vs 39.2±2.1 mmol/L, p<0.05). Concentrations of plasma FC and TG were comparable between the two groups.

4.6.3. Hematological measurements

Table 17 summarizes the hematological data obtained in treated and control animals. Hemoglobin concentration, red cell counts and hematocrits were comparable in the two groups; however, a statistically significant difference was noted in platelet counts [681.6 ± 118.9 (treated) vs 857.1 ± 185.4 (controls), x10^9/L, p<0.04]. Leukocyte counts showed large but non-significant variations between the two groups. This variability may be related to the stress of animal
handling for blood sampling with a resultant release of leukocytes from peripheral pools. Blood smears revealed no abnormalities or differences between the two groups.

4.6.4. Osmotic fragility

The percent hemolysis in red blood cells from both groups when exposed to 0.05 M NaCl for 15 minutes at room temperature was calculated. Erythrocytes from treated animals were significantly less fragile at the experimental concentration of NaCl as compared to controls (83.3 ± 6.7 per cent vs 95.5 ± 2.3; per cent; p<0.001).

4.6.5. Urinalysis

No significant changes were observed in the treated mice insofar as urinary analytes are concerned, including specific gravity, glucose, erythrocytes, hemoglobin, leukocytes, nitrites, protein, bilirubin, urobilinogen, pH and ketone concentrations.

4.6.6. Gross and microscopic tissue examination

Macroscopic examination of organs from both groups of animals revealed no abnormalities except for localized skin lesions (induration, rubor, alopecia) in two control mice (Figure 13, Panel A). Histological examination of the affected skin revealed numerous cholesterol crystals, with a granulomatous cellular reaction including eosinophils and histiocytes (Figure 13, Panels B & C). Routine histochemical staining (H & E) revealed no histological abnormalities in other tissues of either animal group examined, apart from changes in certain kidneys, livers and testes.
Sections from a number of kidneys of both treated and control animals showed focal and non-specific interstitial inflammation; occasionally, the inflammation extended to the pelvic area of the affected kidneys. However, tissue injury was not apparent. Sections taken from the kidneys of five controls and one treated animal had a vacuolated appearance in tubular cells when stained with H&E (Figure 14). Similar hepatocyte vacuolization was observed in livers of control animals (Figure 15). ORO staining of frozen sections obtained from kidneys and livers did not reveal any lipid deposits as the cause of cellular vacuolization. Testicular sections from almost all treated animals revealed mild atrophy of seminiferous tubules. In some tubules, the effect was more substantial, with a lack of active spermatogenesis (Figure 16). The overall findings of the histological evaluation are shown in Table 18.
Table 16: Plasma lipid concentrations (mmol/L) at the beginning and at the end of the experiment (mean±SD).

<table>
<thead>
<tr>
<th>Lipids</th>
<th>Baseline</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n=6)</td>
<td>FCP-3PI-treated (9)</td>
</tr>
<tr>
<td>TC</td>
<td>14.8±1.6</td>
<td>15.5±2.2</td>
</tr>
<tr>
<td>FC</td>
<td>NM</td>
<td>NM</td>
</tr>
<tr>
<td>TG</td>
<td>1.6±0.8</td>
<td>1.1±0.5</td>
</tr>
</tbody>
</table>

TC, total cholesterol; TG, triglyceride; FC, free cholesterol; NM, not measured; *, p<0.05
Table 17: Effects of FCP-3PI on hematological properties (mean±SD).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n=6)</th>
<th>FCP-3PI-treated (9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>leukocytes (x10^9/L)</td>
<td>14.5 ± 5.2</td>
<td>12.7 ± 4.3</td>
</tr>
<tr>
<td>erythrocytes (x10^{12}/L)</td>
<td>10.6 ± 0.7</td>
<td>10.1 ± 0.7</td>
</tr>
<tr>
<td>platelets (x10^9/L)</td>
<td>857.1 ± 185.4</td>
<td>681.6 ± 118.9*</td>
</tr>
<tr>
<td>hemoglobin (g/L)</td>
<td>153.0 ± 10.3</td>
<td>140.0 ± 13.7</td>
</tr>
<tr>
<td>hematocrit</td>
<td>0.53 ± 0.03</td>
<td>0.49 ± 0.04</td>
</tr>
</tbody>
</table>

*p<0.04
Figure 13: Skin lesion on the shoulder area of a control mouse (A). Photomicrographs of the nuchal skin illustrate marked infiltration by lipids and presence of cholesterol "granulomas" and cholesterol clefting (B) and prominence of ORO staining (C) (H & E stain, B, x50; ORO stain, C, x25).
Figure 14: Representative photomicrographs of kidney samples from one control (A) and one treated (B) mouse. As is evident, non-specific vacuolization (arrow) is seen in the kidney of the control mouse (A), but not in the treated mouse (B). (H & E stain, A & B, x40).
Figure 15: Representative photomicrographs of liver samples from one control mouse (A & C) and one treated mouse (B & D). As is evident, non-specific vacuolation (arrow) is seen only in the liver of the control mouse (A & C), but not in the treated mouse (B & D). (H & E stain, A & B, x16; C & D, x40).
Figure 16: Representative photomicrographs of a testis samples from one control (A) and one treated (B) mouse. Atrophy of a seminiferous tubule and lack of active spermatogenesis are illustrated on Panel B. (H & E stain, A, x100).
Table 18: Histopathological evaluation of tissues from control and FCP-3PI-treated mice.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Control</th>
<th>FCP-3PI-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>kidney</td>
<td>severe non-specific vacuolization in proximal tubular cells, mild inflammatory process either in interstitial space or in the pelvic area.</td>
<td>Only one mouse showed vacuolization in proximal tubular cells. Some mice had a small degree of inflammation.</td>
</tr>
<tr>
<td>lung</td>
<td>appearance normal</td>
<td>appearance normal</td>
</tr>
<tr>
<td>liver</td>
<td>vacuolation and appearance of clumping of cytoplasmic proteins</td>
<td>appearance normal</td>
</tr>
<tr>
<td>gastrointestinal tract and pancreas</td>
<td>appearance normal</td>
<td>appearance normal</td>
</tr>
<tr>
<td>Heart</td>
<td>appearance normal with some foam cells in the small vessels</td>
<td>appearance normal with some foam cells in the small vessels</td>
</tr>
</tbody>
</table>
Table 18 (cont’d): Histopathological evaluation of tissues from control and FCP-3PI-treated mice.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Control</th>
<th>FCP-3PI-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>testis</td>
<td>appearance normal</td>
<td>two thirds of mice showed</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mild atrophy of</td>
</tr>
<tr>
<td></td>
<td></td>
<td>seminiferous tubules. No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>evidence for active</td>
</tr>
<tr>
<td></td>
<td></td>
<td>spermatogenesis was</td>
</tr>
<tr>
<td></td>
<td></td>
<td>observed in the severely</td>
</tr>
<tr>
<td></td>
<td></td>
<td>affected seminiferous</td>
</tr>
<tr>
<td></td>
<td></td>
<td>tubules.</td>
</tr>
<tr>
<td>brain</td>
<td>appearance normal</td>
<td>appearance normal</td>
</tr>
<tr>
<td>adrenal gland</td>
<td>appearance normal</td>
<td>appearance normal</td>
</tr>
<tr>
<td>skeletal muscle</td>
<td>appearance normal</td>
<td>appearance normal</td>
</tr>
<tr>
<td>bladder</td>
<td>appearance normal</td>
<td>appearance normal</td>
</tr>
</tbody>
</table>
5. DISCUSSION

Similar to previous observations (84), apo E-deficient mice had very high plasma cholesterol concentrations (~15.5 mmol/L) when fed mouse chow (9% w/w fat). After consuming a 0.15% cholesterol-enriched diet, the level of plasma cholesterol increased markedly, as also observed by others (85,86). This further rise in plasma cholesterol was significantly inhibited by the addition of FCP-3PI (2% w/w) to the diet (Figure 4). Similar effects of FCP-3PI were observed by Ling and Jones (23) in cholesterol-fed (1% w/w) rats. Specifically, rats fed FCP-3PI (1% w/w) showed a significant decrease in LDL-cholesterol accompanied by an increase in high density lipoprotein (HDL)-cholesterol.

The mechanism of the cholesterol-lowering effect of plant sterols is not fully understood. However, most investigators conclude that the major mechanism of action of phytosterols is decreasing cholesterol absorption (25,41,44,87). We and other investigators (85,86) observed that apo E-KO mice are very responsive to dietary cholesterol. This finding supports inhibition of cholesterol absorption by FCP-3PI leading to a significant decrease in plasma TC observed in our studies. On the other hand, plasma TC concentrations in CD1 mice did not significantly increase after dietary cholesterol supplementation. Thus, the lack of cholesterol-lowering effects of FCP-3PI can be explained by low cholesterol absorption in this strain of mouse.

Decreased plasma cholesterol concentrations were accompanied by a significant decrease in hepatic cholesterol concentration and a significant increase in hepatic HMG-CoA reductase activity. This increase in HMG-CoA reductase activity
may be related to decreased plasma and hepatic cholesterol concentrations which, in turn depend, at least in part, on intestinal absorption. Fecal sterol analyses showed that FCP-3PI treatment increased fecal cholesterol excretion by approximately 50% (p<0.05) compared to controls. Decreased absorption/re-absorption of cholesterol by FCP-3PI resulted in: a) decreased hepatic cholesterol content (to less than half of that in either of the other groups), most likely due to increased biliary secretion; and b) a further increase in HMG-CoA reductase activity. Our data show that hepatic cholesterol concentration in FCP-3PI-treated apo E-KO mice was reduced to almost two thirds of that in the control wild-type counterparts. Therefore, this observation suggests that FCP-3PI stimulates biliary secretion of cholesterol in apo E-KO mice. These changes occurred without any detectable alteration in LDL-receptor binding. Our own observations as well as a report by Kuipers et al. (88) show that, compared to their wild-type counterparts, apo E-KO mice have several times higher plasma TC, 50-60% higher hepatic cholesterol concentrations and approximately one half of the activity of HMG-CoA reductase.

Advanced atherosclerotic lesions and cutaneous xanthomatosis were observed in control (untreated) animals. FCP-3PI treatment significantly reduced the development of atherosclerotic lesions, and also effectively prevented the occurrence of cutaneous xanthomatosis. Inhibition of intestinal cholesterol absorption may be one of the major mechanisms of action of FCP-3PI in reducing lesion area in aortic sinuses and in the prevention of cutaneous xanthomatosis in treated animals. The reduction of plasma cholesterol in the treated animals most likely resulted in the absence of cholesterol clefts in the attenuated atherosclerotic lesions of the treated
animals. Thus, at the end of our study, the mean plasma total cholesterol concentration of treated mice was decreased by about 20% as compared to the control group, while the mean atherosclerotic lesion area was reduced by more than 50% in the aortic sinuses of treated mice as compared to the control group. Regression analysis of plasma cholesterol levels and lesion areas showed a significant positive correlation \( r=0.69 \).

The observed anti-atherogenic effect of FCP-3PI may not be due to its cholesterol-lowering properties alone, but also to other mechanisms such as its antioxidant properties. In this regard, it is of interest that dietary olive oil (50 g/day) significantly \( p<0.01 \) increased LDL sitosterol content in ten healthy men; this alteration was associated with a corresponding significant \( p<0.01 \) decrease in the sensitivity of their LDL to \textit{in vitro} oxidation and a significant \( p<0.01 \) reduction in macrophage uptake by LDL (53). It was also reported that the LDL of apo E-deficient mice was 360 times more susceptible to \textit{in vitro} oxidation as compared to controls (89). Therefore, it may be suggested that at least some plant sterols are also absorbed and incorporated into lipoproteins, especially LDL and very low-density lipoprotein (VLDL), which may significantly alter their sensitivity to oxidation. Thus, if LDL is resistant to oxidation, it becomes less atherogenic with a resultant reduction in the area and complexity of lesions.

Another study was conducted to test the effects of the antioxidant N,N'-diphenyl 1,4-phenylenediamine (DPPD) on the development of atherosclerosis in apo E-deficient mice (90). The overall anti-atherogenic effects of this antioxidant were quantitatively similar to our observations. However, DPPD, unlike FCP-3PI, has
no cholesterol-lowering properties and may have toxic effects causing a significant decrease in the animals' body weights.

To test the role of antioxidants on the atherogenesis in apo E-KO mice, we used a well-known antioxidant agent with cholesterol lowering properties, namely probucol. Surprisingly, in contrast to FCP-3PI (7,8) or DPPD (90), probucol paradoxically promotes atherosclerosis in mice lacking the apo E gene (91,92). While both probucol and FCP-3PI reduced plasma TC concentrations, this effect was more profound and persistent in probucol-treated mice. The mechanism of the cholesterol-lowering effect of probucol is not well defined (93-95). Phytosterols, on the other hand, are well known for their inhibition of cholesterol absorption (40,44,45,87). The cholesterol-lowering activities of both probucol and phytosterols were associated with decreased atherosclerosis in hypercholesterolemic mice and rabbits (7,96). Although the causes of probucol's marked pro-atherogenic effects in apo E-KO mice are unknown (92), one possible explanation is the well-known HDL-cholesterol lowering effect of probucol in various species, including apo E-deficient mice (92, 97,98). As plasma HDL-cholesterol concentration is inversely related to the risk of atherosclerosis (99), the increased atherogenesis observed in this and another study (92) may be due, at least in part, to this effect. Furthermore, the decreased hepatic lipase activity in FCP-3PI-treated mice might be associated with an increase in HDL production as was reported in hepatic lipase-deficient mice (100). Decreased hepatic lipase activity may also prevent formation of small \( \beta \)-VLDL particles and halt their entry to the arterial wall leading to decreased atherogenecity (100).
A more likely cause of the pro-atherogenic effect of probucol is the increase in plasma fibrinogen observed in our study. Fibrinogen concentrations have been significantly correlated with the likelihood of coronary artery disease in epidemiological studies (101-104). It has been reported that probucol treatment causes a significant reduction in plasma fibrinogen concentration which is associated with decreased atherosclerosis in rabbits (105). These changes occurred without any significant reduction in plasma cholesterol concentrations. In the present study, we observed the opposite effects of probucol, namely a marked decrease in plasma cholesterol concentration, accelerated atherogenesis and an increase in fibrinogen concentration. The increased plasma fibrinogen concentration may, at least in part, be responsible for the accelerated development of atherosclerotic lesions. In contrast to probucol-treated mice, FCP-3PI-treated animals had lower fibrinogen concentrations and fewer (and less mature) atherosclerotic lesions compared to either the control or the probucol-treated groups. The mechanism by which either probucol or FCP-3PI modifies fibrinogen concentrations is unclear. Probucol may stimulate hepatic protein synthesis including that of fibrinogen and antioxidant enzymes. However, such an effect was not observed for other proteins such as LDL-receptors or hepatic lipase.

Probucol's antioxidant effects, manifested as a significant reduction in LDL oxidation, have been well documented (106). Decreased oxidation of LDL is accompanied by decreased foam cell formation and atherosclerotic plaque development. In this study, it has been shown that treatment with probucol is accompanied by an increase in the activity of several endogenous antioxidant
enzymes. However, despite the probucol-induced increases in the activity of these enzymes in plasma, foam cell formation was also increased. We have documented an increase in foam cell formation in probucol-treated mice while it was significantly reduced in FCP-3PI-treated animals. As apo E-deficient mice are a "β-VLDL model" of atherosclerosis, it may be speculated that β-VLDL is beneficially modified by phytosterols in such a way that they were not recognized by macrophages leading to a delay in foam cell formation and plaque development. The opposite may happen in probucol-treated mice.

Based on a previous report (92) and our own data, it can be concluded that the atherogenicity of probucol is dose- and time-dependent. Probucol's half-life in plasma depends on the rate of clearance of lipoproteins. Mice lacking apo E have a substantial delay in lipoprotein metabolism, particularly that of VLDL. This may significantly increase the biological half-life of probucol leading to an increased occurrence of toxic effects. Thus, in addition to accelerated atherogenesis, it is of interest that probucol treatment resulted in a loss of virtually all abdominal fat and the occurrence of non-xanthomatous skin lesions, these changes being absent in the two other groups (control and FCP-3PI).

Our studies demonstrate that plant sterols have a number of effects which may be independent of inhibition of cholesterol absorption. To investigate the effects of FCP-3PI beyond intestinal cholesterol absorption, we injected mice i.p. with liposome-incorporated-FCP-3PI. Although plasma cholesterol concentrations in FCP-3PI-injected mice in both high fat and low fat diet groups decreased, the activity of HMG-CoA reductase did not correlate with changes in plasma cholesterol
concentrations. The high fat diet per se markedly depressed the activity of HMG-CoA reductase in such a way that this enzyme did not respond to the injection regimens. On the other hand, mice fed a low fat diet and injected with FCP-3PI had significantly lower HMG-CoA reductase activity as compared to corresponding controls. At present, it is not clear whether this reflects a direct effect of FCP-3PI on hepatic cholesterol biosynthesis.

Results of the i.p. injection study clearly showed that injected FCP-3PI causes a significant (except for SOD of low fat diet mice) increase in the activity of all plasma antioxidant enzymes measured regardless of the amount of fat in the diet. This could be the result of a stimulatory effect of plant sterols on the synthesis of antioxidant enzymes. Another possible mechanism may be detoxification of free radicals by FCP-3PI which may result in a sparing of endogenous antioxidants.

Effects of FCP-3PI on the regression of atherosclerotic lesions in apo E-KO have been also tested. A lack of cholesterol-lowering activity of FCP-3PI in this setting was observed. This may indicate a possible interaction between dietary fat and phytosterols. As we observed in our previous studies (7,8), FCP-3PI had a significant cholesterol-lowering effect when it was added to a mouse diet containing 9% (w/w) fat while in the regression study the diet had lower amount of fat (4.5% w/w).

Both control and treated groups, to varying degrees, continued to develop lesions during the regression phase. The control group had a 40% increase in lesion size while the treated group had a 28% increase in lesion size compared to lesion size at the end of the induction phase. This finding suggests that FCP-3PI may slow
down atheromatous lesion formation during the regression phase. However, the
difference between the lesion size in the control and treated groups was not
statistically significant. The lack of effectiveness in inducing regression of the lesions
may be related to the small cholesterol-lowering effect of FCP-3PI during the
regression phase.

Thus, there was no evidence of regression of pre-established atherogenic
lesions in apo E-deficient mice treated with FCP-3PI. However, the treatment
decreased the rate of lesions' progression despite its lack of significant effects on
plasma cholesterol concentrations as compared to controls. In addition to a decrease
in plasma cholesterol concentrations, several other features such as endothelial
function and vascular smooth muscle cells also play a role in the regression of
atherosclerotic plaques. The fact that apo E-deficient mice develop severe
atherogenic lesions even when fed a low fat diet, suggests that: a) this animal model
may not be a good one for studying regression of atherosclerotic plaques, b) for
lesion regression, a longer period of time may be required, and c) a more intensive
lipid-lowering treatment may be needed.

Components of FCP-3PI have been shown to be absorbed and reduce
plasma cholesterol concentration rats (23). Thus, we have tested the tolerance of
apo E-KO mice to FCP-3PI over an 18-week period. Preceding work by others
suggests a high tolerance to dietary plant sterols. Consumption of \( \beta \)-sitosterol for
four years by humans resulted in no adverse effects as determined by kidney and
liver function tests, hematology, urinalysis, electrocardiographic records and gall
bladder visualization (107). In addition, long-term (22 months) oral administration of
β-sitosterol to rats, rabbits and dogs was claimed to be safe (107). Chronic administration of β-sitosterol subcutaneously to either male or female rats was well tolerated without any evidence of tissue damage (108).

The concentration of plant sterols in the plasma may play a significant role in their physiological action in tissues; however, the nature of the sterol is also a major determinant. Accelerated atherosclerosis and increased fragility of red cells have been reported in sitosterolemic patients (63,64). In contrast, we showed that in apo E-deficient mice, phytosterols, 2% (w/w), are anti-atherogenic (7,8); this beneficial effect of plant sterols was accompanied by significantly less fragile red cells (the difference between the groups was very small and likely functionally not significant), unlike the situation in sitosterolemia. Absorbed components of FCP-3PI may have been incorporated into red cell membranes and made them more resistant to hemolysis.

Hematological tests revealed a small but significant decrease in the number of platelets in FCP-3PI-treated animals as compared to controls. It is unclear if these effects reflect the direct impact of FCP-3PI on hematopoietic tissues because: a) other hematological measurements were comparable in the two groups, and b) blood smear examination revealed no cellular abnormalities in the blood of either group. Control animals did have platelet counts comparable to those in C57BL mice (109), the wild-type counterparts for apo E-deficient mice used in the present study. In agreement with our observations, Clayton et al. (110) reported thrombocytopenia in five children with parenteral nutrition-induced phytosterolemia, a condition which was improved by reducing the intake of lipid emulsion-containing phytosterols.
Although the reason is unclear, we observed a significant decrease in plasma fibrinogen in male apo E-deficient mice fed 2% (w/w) of FCP-3PI. Moreover, other studies (111,112) have reported that phytosterols increased the release of tissue plasminogen activator from cultured bovine carotid artery endothelial cells, but not from human umbilical vein endothelial cells (66). Altogether, our observations and those of others suggest an anticoagulant activity for plant sterols. As such, $\beta$-sitosterol has been proposed as an agent for prevention and treatment of thrombosis (111).

Gross inspection of organs failed to detect any damage attributable to phytosterol treatment per se. In particular, histological examination revealed no abnormalities in treated animals except for a slight atrophy of testicular tissues. The latter observation is in accordance with previous findings (67,108) in which the administration of $\beta$-sitosterol resulted in a reduction in testicular weight and impaired spermatogenesis in albino rats and young rabbits. These observations may be explained by the estrogenic effects of $\beta$-sitosterol (113-115). Plant sterols may also affect female reproductive organs in animals. For example, Bruck and colleagues (68) showed that application of $\beta$-sitosterol to the reproductive tube of female rabbits before coitus significantly decreased the number of implantation sites and pregnancy rate.

The observed cellular vacuolization in the kidneys of control animals does not appear to have functional significance, since urinalysis failed to demonstrate any abnormalities. Although we did not perform liver function tests, similar conclusions may apply to the observed hepatocyte vacuolization.
The cholesterol-lowering effects of FCP-3PI were accompanied by a small but significant increase in body weights of treated mice which may be due to an increased food intake observed. This was associated a non-significant increase in the plasma glucose levels of the treated animals as compared to the control group (14.6 ± 2.3 mmol/L vs 13.9 ± 1.9 mmol/L). It is of interest that there was a marked difference between plasma glucose levels in apo E-deficient mice and those in their wild-type C57BL/6J counterparts (13.9 ± 1.9 mmol/L vs 6.5 ± 0.1 mmol/L) (116). A high fat diet (58% fat on an energy basis) significantly increased the levels of insulin, glucose and total cholesterol in the plasma of male C57BL/6J mice (116). Therefore, our observations in regard to plasma glucose levels suggest that deletion of the apo E gene, which leads to a very high level of plasma cholesterol, may also alter the metabolism of glucose, both in turn contributing to accelerated atherogenesis. Further investigation of the relationship between lipid metabolism, apo E, insulin and glucose metabolism is necessary for better understanding of the mechanism(s) of accelerated atherosclerosis in apo E-deficient mice.
6. SUMMARY AND COMMENTS

A phytosterol mixture, namely FCP-3PI, was extracted and purified from "tall oil" soap, a by-product of the BC Pulp and Paper Industry. The extraction of plant sterols was performed using acetone and hexane. The product was purified after precipitation with methanol and several washing procedures using hexane. GC analysis showed that the final product was approximately 95% pure in phytosterols and contained 69% sitosterol, 16% sitostanol and 15% campesterol.

The effects of FCP-3PI on lipid metabolism and atherosclerotic plaque formation and also its tolerance by mice were tested using a variety of criteria in apo E-deficient mice. Apo E-deficient mice are a well-studied model of atherogenesis. They have extremely high plasma cholesterol concentrations and they develop atherogenesis. These transgenic mice also develop cutaneous xanthomatosiis if fed additional dietary cholesterol. We first assessed the cholesterol-lowering effects and anti-atherogenic properties of FCP-3PI in male apo E-deficient mice fed a "Western-type" diet containing 9% (w/w) fat and 0.15% (w/w) cholesterol for 18 weeks. Addition of 2% (w/w) FCP-3PI to the above-mentioned diet significantly reduced plasma total cholesterol concentration over the experimental course (42 vs 26 mmol/L, p<0.0001). The decrease in plasma cholesterol was accompanied by a marked decrease in the size (4 vs 2 mm², p<0.0001) and complexity of atherosclerotic lesions in the aortic valves as assessed by a number of histochemical techniques. Similarly, the atherosclerotic lesions in the thoracic aortae of the FCP-3PI-treated animals were less mature and complex as compared to those in untreated controls. The results suggest that FCP-3PI treatment reduces plasma
cholesterol concentration in cholesterol-fed mice probably by inhibition of cholesterol absorption. This reduction in plasma cholesterol significantly and positively correlated (r=0.69) with the size of atherosclerotic lesions in the aortic roots. Therefore, this experiment demonstrated the cholesterol-lowering and anti-atherogenic properties of FCP-3PI in apo E-deficient mice fed dietary cholesterol.

In order to further explore the cholesterol-lowering and anti-atherogenic properties of FCP-3PI, another experiment was carried out using the same animal model. The mice were fed a diet containing 9% (w/w) fat with or without 2% (w/w) FCP-3PI for 20 weeks. Similar to the results from the previous experiment, we observed marked cholesterol-lowering and anti-atherogenic effects of FCP-3PI.

Among those, hepatic cholesterol metabolism was significantly modified by the FCP-3PI treatment. The activity of the rate limiting enzyme HMG-CoA reductase was markedly increased compared to the control group. This may indicate a positive feedback effect of lower cholesterol absorption/re-absorption. There was also a trend towards increasing the activity of the bile acid synthetic enzyme, cholesterol 7-hydroxylase with no changes in the activity of sterol 27-hydroxylase as compared to controls. FCP-3PI-treated animals had significantly lower hepatic cholesterol concentrations (less than half) compared to controls. Hepatic cholesterol content in the FCP-3PI-treated mice was even lower than that of their wild-type C57BL/6J counterparts. The reduction in the hepatic cholesterol concentration was not accompanied by changes in the binding of hepatic LDL receptors. Fecal cholesterol excretion was significantly increased to 150% of that observed in controls. Altogether, these observations suggest a stimulatory effect of FCP-3PI on biliary
secretion of cholesterol and/or inhibition of its re-absorption. Another potentially important finding was a significant decrease in hepatic lipase activity by FCP-3PI. Reduction in the activity of hepatic lipase may be associated with larger VLDL particles and increased HDL-cholesterol, which in turn may be associated with a decrease in atherogenicity.

In addition to the effects on cholesterol metabolism, FCP-3PI also modified other factors such as plasma fibrinogen concentrations and antioxidant enzyme activities. We observed that mice treated with FCP-3PI had a 20% reduction in plasma fibrinogen concentration compared to controls. As increased plasma fibrinogen concentrations are associated with an increased risk of cardiovascular disease, this effect of FCP-3PI may also contribute to its anti-atherogenic effects. FCP-3PI treatment resulted in a significant increase in erythrocyte glutathione reductase and plasma glutathione peroxidase activities compared to controls. Oxygen-derived free radicals and oxidation of LDL play a significant role in foam cell formation and development of atherosclerotic plaques. Increased antioxidant activity could result in a decreased foam cell formation and slow down the plaque development. The results of this experiment suggest that systemic effects of FCP-3PI also play a significant role in its cholesterol-lowering and anti-atherogenic activities.

Systemic effects of FCP-3PI have been tested in apo E-deficient mice by i.p. injection of liposome-incorporation FCP-3PI. A pilot study was carried out in apo E-deficient mice fed either a low fat diet or a Western-type diet. The results showed a significant decrease in the activity of hepatic HMG-CoA reductase in treated mice fed
low fat diet only. Plasma cholesterol concentrations were 40-50% lower in the treated animals compared to controls. The plasma antioxidant enzymes glutathione peroxidase, glutathione reductase and superoxide dismutase had significantly higher activities in the treated animals compared to controls. Therefore it is likely that the effects of FCP-3PI are not limited to the intestinal lumen.

Finally, the tolerance of male apo E-deficient mice to FCP-3PI has been tested over a period of 18 weeks. Hematological, biochemical and histological tests were used to evaluate the safety of FCP-3PI. By the end of the study, the treated animals had significantly higher body weights compared to controls. This might happen because of sporadic higher food consumption as observed during the experimental course. FCP-3PI treatment also made erythrocytes significantly more resistant to osmotic fragility compared to controls. The treated animals had a significantly lower platelet count and mild seminiferous atrophy. Histological examination revealed cutaneous xanthomatosis along with non-specific vacuolization in the kidneys and livers in the untreated control group only. It appears that, except for a mild testicular atrophy, FCP-3PI dietary supplementation at 2% (w/w) is safe over 18 weeks, at least in this animal species.

No cholesterol-lowering effect of FCP-3PI was seen in CD1 normolipidemic mice. Neither controls nor treated CD1 mice developed any atherosclerotic lesion in their thoracic aortae as examined by light microscopy. The lack of efficacy of FCP-3PI in lowering plasma cholesterol could be related to: a) strain-related variation in intestinal cholesterol metabolism (it is of interest that we observed apo E-KO are
much more responsive to dietary cholesterol than CD1 mice); b) a longer period of time may be needed to achieve a significant decrease in plasma TC.

In conclusion, FCP-3PI has anti-atherogenic and cholesterol-lowering properties in male apo E-deficient mice. FCP-3PI can also prevent cutaneous xanthomatosis in this animal model. These effects of FCP-3PI are probably mediated through intestinal and/or systemic mechanisms as follows: a) inhibition of intestinal cholesterol absorption/re-absorption; b) possible increase in biliary cholesterol secretion; c) decreasing hepatic lipase activity; d) decreasing plasma fibrinogen concentrations; and e) increasing the activity of antioxidant enzymes. FCP-3PI is well tolerated over 18 weeks in this animal model. Further experiments are needed to better understand the mechanism of the anti-atherogenic effects of FCP-3PI and also to document that this effect is not limited to apo E-KO mice. In this regard, hormonal effects of FCP-3PI and its effects on the quality of lipoproteins remain to be answered. Effects of FCP-3PI on antioxidant enzyme activities, plasma fibrinogen concentrations and plasma lipolytic activity remain to be investigated as to whether or not they are dependent on cholesterol absorption inhibition. It also needs to be answered if increased plasma fibrinogen concentrations by probucol leads to deposition of this coagulation factor in the atherosclerotic lesions. Finally, combination therapy of FCP-3PI and conventional lipid-lowering agents warrants further investigation.
7. ORIGINAL ASPECTS OF THE WORK

- the demonstration of the anti-atherogenic effects of plant sterols including FCP-3PI in apo E-KO mice.
- the first report on atherogenicity of probucol in apo E-KO mice (April 1997).
- the prevention of cutaneous xanthomatosis in high fat diet-fed apo E-deficient mice by FCP-3PI.
- testing the ability of phytosterols to induce regression of atherosclerosis.
- the demonstration of effects of probucol and FCP-3PI on antioxidant enzyme activities.
- the determination of the long-term (18 weeks) tolerance and safety of FCP-3PI by using histological assessment and hematology and biochemical tests.
- the findings regarding plasma fibrinogen level, post-heparin lipase activity, plasma glucose level, LDL-receptor function, hematological values, osmotic fragility, urinalysis, liver and kidney vacuolization and fecal sterol composition in apo E-deficient mice.
- the determination of dose of oral administration of FCP-3PI, the length of the experiments, and the comparison of its effects both in oral and i.p. injection studies.
8. REFERENCES


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