INTERACTIONS OF DIETARY PHYTOSTEROLS WITH LIPID-LOWERING DRUGS IN APOLIPOPROTEIN E DEFICIENT MICE

By

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A Thesis
Submitted to the Faculty of Graduate Studies in
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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES
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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

Of

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Behzad Khojir-Yeganehrad © 2005

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The Fable of the Elephant in the Darkness

An elephant had been brought
To a house in a remote place.
The house was too dark
To see the creature,
Many people had gathered in the darkness.
As it was not possible
To catch sight of the beast,
People tried to understand
The creature through touch.
One who had touched the trunk said
The creature was like a water pipe.
On touching the ears, another said
I envisage an elephant to be like a fan.
Another who felt the legs of the animal said
An elephant looks like a pole.
Another touched the back of the elephant and said
The creature is more like a bed.
So it continued,
Whosoever touched a part,
Built up his own picture of the animal
As their interpretations were all different,
One labelled the animal "D"
Another "A".
Had there been a candle
In each person's palm,
All differences would have parted
From their discussion.

Extract from the "Masnavi", a book of poems by Jalal Al-Din Rumi. (1207-1273 AD).

ABSTRACT

Phytosterols are chemicals naturally found in vegetable oils, seeds, nuts, and some tree products. It has been shown that plant sterols inhibit cholesterol absorption, thereby lowering plasma total and low-density lipoprotein (LDL) cholesterol levels without significantly altering high-density lipoprotein (HDL) cholesterol or triglycerides (TG). Cholesterol-lowering effects of phytosterols are associated with a significant reduction in atherosclerosis in apo E- knokout (apo E-KO) mice.

Monotherapy with either niacin or fibrates is an effective strategy in patients with predominantly elevated TG and low HDL-cholesterol levels. However, a combination drug therapy is necessary in patients who do not adequately respond to single therapy or in patients with mixed dyslipidemia (increased LDL-cholesterol and TG levels plus reduced HDL-cholesterol concentrations). Several clinical trials have studied the combination of statins plus niacin or fibrate in patients with mixed dyslypidemia. However, safety, tolerability and cost are major limiting factors in drug combination therapy.

It is well known that phytosterols reduce plasma total and LDL cholesterol concentrations with no significant effect on HDL-cholesterol or TG levels. On the other hand, niacin and fenofibrate increase plasma HDL-cholesterol levels and decrease TG concentrations. Therefore a combination of phytosterols with niacin or fenofibrate may reduce both plasma cholesterol and TG concentrations as well as increase HDL-cholesterol levels, resulting in a more anti-atherogenic lipoprotein profile as compared to single therapy.

The interactions between phytosterols and non-statin lipid-lowering agents have not been studied. Thus, it was hypothesized that a combination of phytosterols with niacin or fenofibrate will synergistically impact lipoprotein profile and atherogenesis in apolipoprotein E deficient (apo E-KO) mice.

Male apo E-KO mice were fed a cholesterol-enriched diet supplemented with phytosterols (2% w/w, n=8), fenofibrate (0.1% w/w, n=8), niacin (0.5% w/w, n=8) or a combination of 2% of the phytosterol mixture with either fenofibrate (0.1% w/w, n=8) or niacin (0.5% w/w, n=8) for 12 weeks. Body weights were recorded weekly and plasma lipid profiles were determined at four-week intervals. The hearts and aortas were collected and fixed for histological and morphometrical evaluations of atherosclerotic lesions.

Phytosterols alone significantly reduced plasma total cholesterol levels (11.7 vs 17.0 mmol/L, $p<0.05$) and the extent of atherosclerosis (0.15 vs 0.42 mm^2, $p<0.05$). In contrast, fenofibrate paradoxically increased total cholesterol and TG by 65% and 50%, respectively, and decreased HDL-cholesterol levels by 40% without significant changes on atherosclerosis as compared to controls. The addition of fenofibrate to phytosterols increased plasma total cholesterol levels by 40% (20.0 vs 14.1 mmol/L, $p<0.05$) and decreased HDL-cholesterol concentrations by 50% (0.4 vs 0.8 mmol/L). These changes were accompanied by slight reductions in the extent of atherosclerosis (0.34 vs 0.42 $mm^{2,}$ $p>0.05$) as compared to controls. Niacin alone had no significant effect on atherosclrotic lesions, and failed to decrease TG or to increase HDL-cholesterol levels. However, a combination

of niacin with phytosterols caused an increase of 150% ($p<0.05$) in HDL-cholesterol concentrations and a decrease of 22% ($p<0.05$) in total cholesterol levels which were associated with significant reductions (65%, $p<0.05$) in atherosclerotic lesion size as compared to controls. The results therefore suggest that: First, addition of plant sterols to niacin may beneficially alter plasma lipoprotein profile and consequently affect atherogenesis in apo E-KO mice. Second, addition of phytosterols to fenofibrate did not result in reductions in plasma cholesterol level and atherosclerotic lesion size in apo E-KO mice. Thirth, a combination of phytosterols plus fenofibrate or niacin do not appear to change the morphology of the liver and kidney tissues.

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Most of all my love, sincerest admiration, concern and apologies go to my wife who is also my best friend. I thank her for supporting me through all these years.

I dedicate this thesis to my son "Omid".

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ACAT	Acyl-Coenzyme A cholesterol acyltransferase
ALP	Alkaline phosphatase
ALT	Alanine transaminase
Apo	Apolipoprotein
AST	Aspartate transaminase
BAS	Bile acid sequestrants
CHD	Coronary heart disease
EDTA	Ethylenediamine tetra-acetic acid
GGT	Gama glutamyl transferase
H&E	Hematoxylin and eosin
HDL	High density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl Coenzyme A
i.p.	Intraperitoneal
IDL	Intermediate density lipoprotein
KO	Knockout
LDL	Low density lipoprotein
LP	lipoprotein lipase
Lp (a)	Lipoprotein (a)
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NPC_1L_1	Niemann-Pick C_1 $Like_1$ Protein

Abbreviations (count'd)

OCT	Optimal cutting temperature
ORO	Oil red O
PBS	Phosphosphate buffer solution
PPAR-α	Peroxisome proliferator activated receptor α
SD	Standard deviation
TG	Triglycerides
TRLs	Triglyceride-rich lipoproteins
VLDL	Very low density lipoprotein

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1. INTRODUCTION

Several epidemiological, pathological and clinical studies have shown a significant correlation between increased levels of plasma lipids (cholesterol and TG) and the incidence of coronary heart disease (CHD) in humans (Kannel et al. 1984; Sharrett et al. 1995; Natarajan et al. 2003). A number of animal models have been used to further study the mechanisms of such an association (Moghadasian et al. 2001; Moghadasian 2002). In particular, genetically modified animal models like the apolipoprotein E-knockout (apo E-KO) mice have been very useful in understanding the mechanisms of dyslipidemia and the pharmacological or dietary interventions to treat it. One example of a beneficial dietary intervention is the use of phytosterols. Phytosterols (plant sterols) are plant-derived lipids with similar chemical structures to that of cholesterol. There are several types of phytosterols based on their chemical structures. The most abundant phytosterols are beta-sitosterol, campesterol, sitostanol and stigmasterol.

Humans and animals neither synthesize nor efficiently absorb phytosterols. Phytosterols interfere with cholesterol absorption from the intestine, leading to reductions in plasma cholesterol levels (Plat and Mensink 2002; Ostlund et al. 2003). Phytosterols reduce both plasma cholesterol levels and the extent of atherosclerosis in apo E-KO mice (Moghadasian et al. 1997; Moghadasian et al. 1999; Moghadasian et al. 1999; Moghadasian et al. 2001; Lukic et al. 2003). Over the past several years, a number of phytosterol-enriched food products (margarine, snack bars, yogurt, etc.) have been developed and marketed in several Western countries. Recently, the American

Heart Association has recommended consumption of phytosterols to patients with moderately elevated plasma cholesterol levels (2001). In particular, cardiovascular patients are more likely to take these supplements than healthy individuals (2001). These patients are usually on other medications including lipid-lowering agents, such as nicotinic acid and fenofibrate.

To the best of our knowledge, the interactions between phytosterols and non-statin lipid-lowering agents have not been studied. Phytosterols reduce plasma total and LDL-cholesterol with no significant effect on HDL-cholesterol or plasma TG levels (Moghadasian and Frohlich 1999). On the other hand, niacin and fenofibrate significantly increase plasma HDL-cholesterol and decreases plasma TG concentrations (Capuzzi et al. 1998; Forcheron et al. 2002). Therefore, phytosterols plus niacin or fenofibrate may complementarily reduce plasma cholesterol and TG concentrations as well as increase HDL-cholesterol levels, resulting in more anti-atherogenic lipoprotein profile as compared to single therapy.

Several clinical trials have studied the combination of statins plus niacin or fibrate in patients with mixed dyslipidemias (Ballantyne 2001; Wink et al. 2002; Vega et al. 2003). However, safety and cost-effectiveness are a concern for such a drug combination therapy. For example, four out of 29 hypercholesterolemic patients who received a combination of fenofibrate plus simvastatin developed myalgia (muscle pain) (Wierzbicki et al. 1997). Likewise, two patients from a cohort of 148 diabetic subjects treated with simvastatin plus bezafibrate developed myopathy (Gavish et al. 2000). These

side effects are the outcome of interaction between the drugs at the level of hepatocytes.

Because phytosterols' intestinal absorption is very limited, their hepatic concentrations are extremely low (Moghadasian et al. 2001; Ostlund et al. 2003), and thereby, they will not interfere with fibrate or niacin hepatic metabolism. This lack of potential metabolic interaction and different modes of action and efficacy suggest that the combination of phytosterols with niacin or fenofibrate will be effective and safe. Thus, the aim of the present study was to investigate whether combination of dietary phytosterols with TG–lowering, HDL-cholesterol raising agents, such as niacin or fenofibrate synergistically reduce both plasma cholesterol and TG levels as well as increase HDL-cholesterol concentrations, resulting in a more profound anti-atherogenic lipoprotein profile and prevention of atherosclerosis in apo E-KO mice.

2. CURRENT STAGE OF KNOWLEDGE

2.1. Phytosterols

Phytosterols are triterpenes that are important structural components of plant membranes. Free phytosterols serve to stabilize phospholipid bilayers in plant cell membranes just as cholesterol does in animal cell membranes (Moreau et al. 2002). The most abundant phytosterol is beta-sitosterol; campesterol, sitostanol and stigmasterol are other most frequently found phytosterols (Moreau et al. 2002). Saturated forms of plant sterols are called stanols. For example, compestanol and sitostanol are saturated form of campesterol and sitosterol, respectively.

Phytosterols are structurally very similar to cholesterol except that they always contain some substitutions at the C_{24} position on the sterol side chain (Weihrauch and Gardner 1978; Ling and Jones 1995) [Fig. 1]. For instance, addition of a methyl or ethyl group to C_{24} of cholesterol molecule will result in formation of campesterol or sitosterol, respectivelye. Although phytosterols and cholesterol have similar chemical structures, they differ markedly in their synthesis, intestinal absorption, and metabolism. Phytosterols are naturally found in vegetable products, principally oils, pulses and dried fruits in free or esterified forms, or conjugated as glycosides (Pollack 1981; Piironen 2000). The total amount of phytosterols varies from about 8 g/kg in corn oil to 0.5 g/kg in palm oil, with intermediate levels being found in commonly used vegetables oils (Phillips 2002). Furthermore, genetic and environmental factors play a major role in sterol content of the plants (Abidi 1999).

The consumption of phytosterols varies greatly based on dietary habits, and can range between 170 mg/day in populations eating a Western diet and 360 mg/day in diets rich in vegetable products (De Vries 1997). In Mexico, Tarahumara Indians who consume a diet containing unusually high amounts of beans and corn, consume over 400 mg/day of phytosterols (Cerqueira 1979). In Japan, consumption of phytosterols is about 373 mg/day (Hirai et al. 1986). Western diets typically contain considerably lower levels of phytosterols than diets of many other parts of the world. The average intake of phytosterols by British and American has been estimated to be approximately 167 and 180 mg/day, respectively (Connor 1968; Morton 1995). Cooking oils and

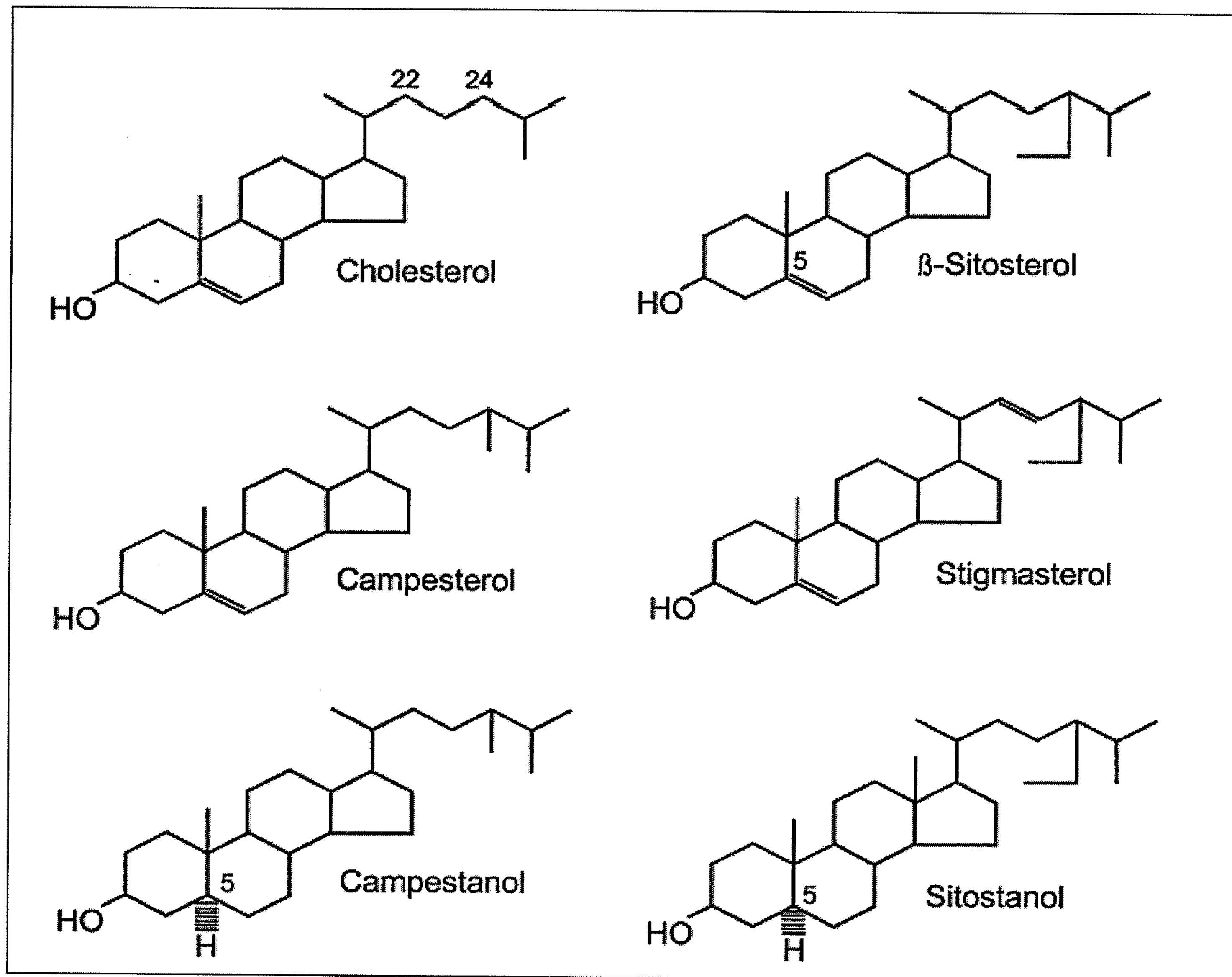

Figure 1. Chemical structure of cholesterol and common plant sterols.

margarines are the main sources of plant sterols in typical Western diets. In addition to legumes (contain up to 220mg/100g) and some seeds (sunflower and sesame 500 - 700mg/100g), breads and cereals are also important sources of plant sterols (Connor 1968; Morton 1995). East African Masai tribe have low serum cholesterol and low incidence of CHD despite high intake of animal products (Johns 1996). This is probably due to addition of plant sterols to the animal-derived food (Johns 1996). Nuts are one of the richest source of phytosterols, and it has been shown that diets rich in whole nuts are associated with a decreased incidence of cardiovascular disease. In numerous clinical studies, subjects who ate 40 to 100 g/day of almonds, hazelnuts, macadamia nuts, pecans, pistachios and walnuts experienced lowered serum TG, total cholesterol and LDL-cholesterol (Morgan and Clayshulte 2000).

On the basis of the studies undertaken to date, no obvious side effects of phytosterols have been reported. Plant sterols are generally poorly absorbed in human (Salens 1970; Heinemann 1991), except in individuals with phytosterolemia (sitosterolemia). Sitosterolemia is a rare genetic disorder in which plant sterols can get absorbed, thus concentrations of plant sterols particularly sitosterol in plasma, are extremely high (Bhattacharyya and Connor 1974; Miettinen 1980; Salen 1992; Ling and Jones 1995).

Animal studies have shown that plant sterols may accumulate in the liver, adrenal gland, ovary and testis (Subbiah 1973; Sugano et al. 1978), therefore may interfere with steroid hormone synthesis. These observations

suggest that long-term effects of dietary plant sterols on endocrine function should be studied further (Moghadasian and Frohlich 1999).

2.2. The absorption of dietary sterols

Body cholesterol is composed of two sources: dietary cholesterol which account for ~25% of body cholesterol pool, and endogenous cholesterol (Shepherd 2001). Dietary cholesterol absorption, endogenous cholesterol synthesis and biliary cholesterol metabolism excretion regulate whole body cholesterol balance. The average North American diet provides approximately 300–500 mg of cholesterol per day, while endogenous sources of cholesterol, which originate from bile (enterohepatic cycling), newly synthesized cholesterol and the intestinal mucosal epithelium, is estimated to be between 1000–1600 mg per day (Wilson and Rudel 1994). Unlike cholesterol, plant sterols are poorly absorbed in the intestine (<5%), while intestinal cholesterol absorption ranges between 35%-70% (Kuksis et al. 1976; Ostlund et al. 2002).

The intestinal absorption of cholesterol and plant sterols is a complex process that is regulated at multiple levels. Recent studies have significantly advanced our understanding of intestinal sterol absorption at molecular level. In the intestinal tract, dietary sterols including both cholesterol and plant sterols are first esterified by acyl-coenzyme A cholesterol acyltransferase (ACAT) and then hydrolyzed to form the mixed micelles. One explanation for the low absorption of plant sterols and stanols might be related to poor estrification of plant sterols and stanols by ACAT. This is possibly due to the low affinity of ACAT for these components (Ntanios et al. 1998a; Ntanios et al.

1998b). The produced micelle is a mixture of free sterols (both cholesterol and plant sterol), mono- and di-acylglycerols, fatty acids, phospholipids, and bile salts. The transfer of free sterols from the mixed micelles through the apical brush border membrane into the enterocyte is not fully understood (Eric and Patel 2004). This process was thought to be driven by passive diffusion (Grundy 1983), however recent findings suggest the existence of a specific, saturatable cholesterol transporter in the intestinal mucosa (Hernandez et al. 2000; Eric and Patel 2004). It has been suggested that Niemann-Pick C_1 $Like_1$ Protein (NPC_1L_1), expressed at the apical surface of enterocytes, may be the transporter that selectively absorbs dietary cholesterol and non-cholesterol sterols from micelles in the lumen of the small intestine into vesicles that then move through a subapical endosomal sorting compartment (Eric and Patel 2004).

The majority of plant sterols absorbed through NPC_1L_1, are transported out of enterocytes into the gut lumen by two members of the ATP-binding cassette (ABC) transporter family located in the membranes of enterocytes, namely $ABCG_5$ and $ABCG_8$ (Berge 2000; Lee 2001). Within the enterocyte, absorbed sterols are esterified by ACAT and incorporated into chylomicrons. Chylomicrons subsequently excrete into the circulation and convert into a chylomicron-remnant by the action of lipoprotein lipase (LP). The liver then takes up these chylomicron remnants and forms very low density lipoproteins (VLDLs) (Grundy 1983).

It has been also suggested that the extent and rate of plant sterol or stanol absorption depends on the side chain length and the presence of the Δ5 double bond (saturation) (Bhattacharyya 1981; Heinemann et al. 1993; Ling and Jones 1995). One study has investigated the intestinal absorption of cholesterol, campesterol, campestanol, stigmasterol and sitosterol in 10 healthy subjects (Heinemann et al. 1993). Cholesterol absorption was highest and averaged 33%, whereas the absorption rate of sitosterol averaged 4.2% and of stigmasterol 4.8%. Higher absorption rates were found for campesterol (9.6%). These results showed that increasing the length of the side-chain of cholesterol decreases the absorbability of the sterol (Heinemann et al. 1993).

Intestinal absorption of stanols is markedly lower than absorption of plant sterols. Only one study has shown that campestanol, the 5 alpha saturated derivate of campesterol, has higher absorbability compared with its unsaturated compound (Heinemann et al. 1993). The difference in absorption between plant sterols and stanols is reflected by their serum concentrations. On regular diets as well as on plant sterol or stanol-enriched diets, serum plant stanol concentrations are 10-30 times lower than plant sterol concentrations (Ostlund et al. 2002).

Sitosterolemia was first described in two sisters with increased intestinal absorption and high plasma levels of plant sterols (Bhattacharyya and Connor 1974). These authors concluded that: the occurrence of β-sitosterolemia in these two sisters with unaffected parents suggests an inherited recessive trait (Bhattacharyya and Connor 1974). This description

suggested that the process of sterols absorption from the intestines might be subject to genetic control and provoked researchers to focus on the genetics of sterols absorption. A recent study has shown that sitosterolemia is caused by mutations in either the $ABCG_5$ or $ABCG_8$ transporter, resulting in hyperabsorption of plant sterols from the small intestine (Berge 2000). As mentioned above, these ABC transporters preferentially pump plant sterols out of intestinal cells into the gut lumen and also out of liver cells into the bile ducts, thereby decreasing sterol absorption. Much of the variation in plasma concentrations of plant sterols may be due to $ABCG_5$ and $ABCG_8$ gene polymorphisms (Berge et al. 2002).

Total plasma campesterol and β-sitosterol in healthy adults are typically maintained within a range of 7.14 and 4.8 μmol/L, respectively, while sitostanol is virtually nondetectable (Heinemann et al. 1993; Vanhanen et al. 1993). However in sitosterolemic patients a 30-100 fold increase in plasma plant sterol levels has been reported (Miettinen 1980; Lutjohann et al. 1996). Most sitosterolemic patients show tendon xanthomas with normal plasma cholesterol concentrations with a strong susceptibility toward premature coronary atherosclerosis (Moghadasian and Frohlich 1999).

2.3. The effects of plant sterols and stanols on intestinal cholesterol absorption

The cholesterol lowering effects of plant sterols have been recognized for almost half a century (Pollack 1953). It has been shown that the reduction

of cholesterol absorption by plant sterols in the intestine not only reduce total serum cholesterol level, but also increase the ratio of HDL-cholesterol to LDL-cholesterol in blood (Sierksma et al. 1999; Andersson et al. 2004).

Several studies have shown that plant sterols and stanols reduce cholesterol absorption through several mechanisms, thereby lowering plasma total and LDL-cholesterol levels, without significantly altering HDL-cholesterol or TG (Howell et al. 1998; Moghadasian and Frohlich 1999; Sato 2001; Amundsen 2002; De Graaf et al. 2002; Judd 2002; Matvienko et al. 2002; Mensink 2002; Mussner 2002; Temme 2002; Vanstone 2002). At low intake levels, plant sterols and stanols have little effect on cholesterol absorption. However, higher levels of consumption can interfere with cholesterol absorption (Pollack 1953). This effect is greatest in individuals with high cholesterol absorption rates and low rates of hepatic *de novo* cholesterol synthesis. Cholesterol lowering effects of phytosterols may also vary depending on type and dose of phytosterols and subjects. Early studies used very large amounts, 10 to 15 g/d, of unesterfied sitosterol to reduce serum cholesterol by 10% to 20% (Pollack 1953; Best et al. 1955; Farquhar and Sokolow 1958; Lees et al. 1977). Further studies showed that 3 g/d of phytosterols can reduce serum cholesterols levels as much as 9 g/d can (Lees et al. 1977).

Sitostanols, which are found in much smaller quantities in plant foods, are believed to be more effective in reducing plasma cholesterol levels than other phytosterols. In one study, giving 3 grams per day of tall oil derived

phytosterols in hypercholesterolemic subjects, resulted in reduction in plasma cholesterol levels by 12% (Lees et al. 1977), whereas ingestion of 1.5 g/day sitostanol reduced low density lipoprotein cholesterol levels by 33% (Becker et al. 1993). Most recently the advantage of stanols over sterols has been blurred with the demonstration of similar LDL-cholesterol reduction using esterified soybean oil sterols (Weststrate and Meijer 1998)

Given the link between plasma cholesterol levels and heart disease, the cholesterol-lowering effects of dietary plant sterols and stanols are of great interest. The mechanisms of cholesterol-lowering activity of plant sterols and stanols are still unclear. Different mechanisms have been suggested. Firstly, plant sterols or stanols may compete with cholesterol during micelle formation (Child and Kuksis 1986; Mel'nikov et al. 2004). This can result in the reduction of micellar cholesterol concentrations and consequently lowers cholesterol absorption. Vanhanen et al. (1993) and Ostlund et al. (1999) reported that the administration of plant sterols in a soluble form might be important for obtaining sufficient levels in the micellar phase for the inhibition of cholesterol absorption in the intestine. However, Mattson et al. (Mattson et al. 1982) showed that β-sitosterol is more efficient than β-sitosterol esters in decreasing cholesterol absorption in humans. β-Sitosterol esters reportedly showed better dissolution in dietary oils and hydrolysis to β-sitosterol during lipolysis of dietary fats (Miettinen and Siurala 1971). Also, there were no significant increases in the hypocholesterolemic activity due to differences in oil solubility (Miettinen and Vanhanen 1994). These results suggested that factors other

than the solubility of palnt sterols in oil might be responsible for the competition of cholesterol for incorporation into micelles in the intestine.

Secondly, it has been suggested that plant sterols or stanols may reduce the esterification rate of cholesterol in the enterocyte (Child and Kuksis 1983) and as a result, the amount of cholesterol excreted via the chylomicrons. Plat and Mensink (2002) have recently shown in caco-2 cells (a colon tumor cell line) that mixed micelles enriched with sitostanol or with cholesterol plus sitostanol upregulate the expression of $ABCA_1$ transporters in intestinal cells, which may result in an increased excretion of cholesterol by the enterocyte back into the intestinal lumen (Plat and Mensink 2002).

The effects of plant stanols on cholesterol absorption may continue for at least several hours after ingestion (Plat et al. 2000). It has been shown that the decrease in serum cholesterol in subjects consuming margarines enriched with plant stanol esters once a day is similar to that in subjects who consumed the same amount of plant stanol esters divided over three servings a day (Plat et al. 2000). The potential explanations for this effect are that the plant stanols may remain present in the intestine for a while, or they may directly affect intestinal lipoprotein metabolism at the level of enterocyte.

Lymphatic absorption of sitosterol, stigmasterol, and fucosterol administered intragastrically with radiolabeled cholesterol has been studied in rats (Vahouny et al. 1983). The results demonstrated that plant sterols were poorly absorbed to the extent of only 3 to 4% of the administered dose of 50 mg. In contrast, cholesterol absorption under similar conditions was about

42% of the same administered dose. All sterols incorporated within the chylomicron fraction, but to a different extent. Esterified cholesterol was located in the core of chylomicron particles, whereas sitosterol was present mainly in the unesterified form on the surface of chylomicron particles. Cholesterol ester contained 90% of the total lymphatic cholesterol, while only 12% of sitosterol was esterified (Vahouny et al. 1983).

2.4. Effects on lipid and lipoprotein metabolism

The hepatic availability of cholesterol is determined by intestinal cholesterol absorption and by synthesis and degradation of cholesterol in the liver. The liver also contains the highest numbers of LDL receptors of all organs in the body. Hepatic cholesterol content and the number of LDL receptors are the major regulators of the plasma cholesterol levels. As mentioned before, most animal and human studies have shown that plant sterols and stanols reduce intestinal cholesterol absorption resulting in redued cholesterol flux through chylomicrons to the liver. In response to the decreased cholesterol absorption, endogenous cholesterol synthesis (Gylling and Miettinen 1994) and LDL receptor expression in the liver are upregulated (Plat and Mensink 2001).

Gylling et al. (1999) investigated the changes of cholesterol and non-cholesterol sterol metabolism during plant stanol ester margarine feeding (2 or 3 g/day) in 153 hypercholesterolemic subjects. Using radiolabeled LDL-cholesterol, they demonstrated that increasing in the number of LDL receptors

increases clearance of LDL and intermediate density lipoprotein (IDL) particles. The overall effect of these metabolic changes is a reduction in serum LDL-cholesterol concentrations.

Several studies have reported no effects of phytosterols on HDL-cholesterol (Vanhanen et al. 1993; Pelletier et al. 1995; Miettinen 1996) and TG (Heinemann 1991; Vanhanen et al. 1993; Miettinen 1996) levels. In a recent study, Vaskonen et al. (2002) have looked at the effects of plant sterols (1 g/100 g) in obese Zucker rats while feeding a high cholesterol diet. Plant sterols effectively prevented the diet-induced increases in total and LDL cholesterols, and markedly improved endothelial function. However they did not look at the effect of plant sterols on development of atherosclerosis.

Gylling et al. have also looked at the kinetics of lipoprotein metabolism in hypercholesterolemic non-insulin-dependent diabetic patients treated with plant sterols (Gylling and Miettinen 1996). The results demonstrated that the kinetics of HDL apoAI were not affected by sterol supplementation, consistent with the lack of effect on HDL-cholesterol in these patients. The authors, however, did not examine whether plant sterols alter the turnover, or metabolic transport rate of VLDL or IDL apo B in plasma (Gylling and Miettinen 1996).

At the cellular level, Bhadra and Subbiah studied incorporation of liposomal phytosterols into human skin fibroblasts and HepG2 cells in culture (Bhadra and Subbiah 1991). A significant reduction in cellular cholesterol content was observed which was accompanied by a simultaneous increase in the cellular concentrations of sitosterol. Ho and Pal (2004) have recently

shown that in HepG2 and caco-2 cell lines, plant sterols significantly decreased $apoB_{100}$ and cholesterol ester concentrations. These finding suggest that plant sterols may reduce production and secretion of VLDL and chylomycron

2.5. Effects on development of atherosclerosis

Several lines of evidence support the concept that there is a direct relationship between increasing plasma LDL-cholesterol levels and the progression of atherosclerosis in humans (Pollak 1985; Goldstein 2000; Whittaker 2000) and animals (Moghadasian and Frohlich 1999; Moghadasian et al. 1999; Meguro et al. 2003). Anti-atherogenic properties of phytosterols have been studied in different animal models (Moghadasian et al. 1997; Meguro, Hase et al. 2003). In apo E-KO mice, cholesterol-lowering effects of phytosterols are associated with significant reductions in atherosclerosis (Moghadasian et al. 1997; Moghadasian et al. 1999). In one study (Moghadasian et al. 1997) oral administration of 2% (wt/wt) "tall oil"-derived phytosterol in apo E-KO mice resulted in 20% decrease in plasma total cholesterol and >50% reduction in the average of atherosclerotic lesion size as compared with the control group.

Meguro et al. (Meguro et al. 2003) have investigated the effects of phytosterols in combination with diacylglycerol on serum lipids and atherosclerosis in cholesterol-fed New Zealand white rabbits. The authors reported that, serum total cholesterol level and the ratio of the atherosclerotic lesion area and the mean thickness of the intima in the aortas in the

phytosterols+diacylglycerol group were statistically lower than those in the control group. Serum HDL and TG levels were not statistically different between these two groups.

Bocan et al. (1993) reported that aortic atherosclerosis formation is strongly correlated with the exposure of the arterial wall to cholesterol in cholesterol-fed rabbits. These may suggest that phytosterols reduce the exposure of the aorta to the total cholesterol thereby reducing development of atherosclerosis. It has also been suggested that antiatherogenic effect of phytosterols in apo E-KO mice may be due not only to its cholesterol-lowering properties alone but also to other mechanisms, such as their antioxidant properties (Moghadasian et al. 1997).

2.6. Coronary heart diseases and dyslipidemia

Along with high blood pressure, inactivity, smoking, and diabetes, dyslipidemia and disorders of lipoprotein metabolism are important modifiable risk factors for CHD, strokes, and peripheral vascular disease. CHD is one of the leading causes of death in North America. Several epidemiological studies have clearly shown that plasma cholesterol, TG, and LDL-cholesterol are positively correlated to the development of atherosclerotic lesion (Kannel et al. 1984; Sharrett et al. 1995; Natarajan et al. 2003). It has been estimated that in men, a 10% decrease in blood cholesterol levels reduces the risk of CHD by 54% at age 40 years, 39% at age 50, 27% at 60, 20% at 70, and 19% at 80 (Law 1994).

Unlike LDL-cholesterol levels HDL-cholesterol concentrations have an inverse relationship to atherosclerotic lesion development. Numerous epidemiological studies have also demonstrated that the decreased levels of HDL-cholesterol are correlated with progression of CHD (Kashyap 1998; Wong 2000). It is also established that the amount of HDL-cholesterol as a fraction of total cholesterol is an important inverse determinant of CHD risk (Kashyap 1998). Increased HDL-cholesterol without reductions in LDL-cholesterol in patients treated with gemfibrozil resulted in significant reductions in the risk of major cardiovascular events (Rubins et al. 1999; Robins et al. 2001). Increasing the HDL-cholesterol level by 1 mg/dl may reduce the risk of cardiovascular disease by 2 to 3 percent.

The beneficial effect of lowering plasma LDL-cholesterol to decrease or prevent CHD progression in hyperlipidemic patients has been also clearly demonstrated in primary and secondary prevention trials (Kwiterovich 1998; Zambon and Hokanson 1998; Wong 2000). In this respect, patients with mixed dyslipidemias are at higher risk for CHD (Sharrett 1995; Natarajan 2003). This higher risk is further augmented if it is accompanied by a cluster of other risk factors including family history, male gender, postmenopausal status, obesity, hypertension, and smoking. Improvements in plasma lipoprotein profile by lifestyle modification, dietary and/or pharmacological agents have been shown to reduce both cardiovascular mortality and morbidity (1994).

2.7. Lipid lowering drugs

2.7.1. Fibrates

Fibrates are a class of lipid lowering drugs that have been used for the treatment of hypertriglyceridemia or mixed dyslipidemias for many years. They are well known in lowering elevated plasma triglyceride-rich lipoproteins (TRLs), and increase HDL-cholesterol levels (Despres 2001; Forcheron et al. 2002; Le Roux et al. 2002; Sharpe, Ormrod et al. 2002; Jove et al. 2003). This reflects a substantial decrease in the plasma concentrations of apolipoprotein B-containing, TRLs and their remnants, to a lesser extent LDL particles, as well as a significant elevation in apolipoprotein A-containing lipoproteins.

At the molecular level, fibrates are ligand and activator of peroxisome proliferator–activated receptor (PPAR-α) (Brisson et al. 2002; Le Roux et al. 2002; Le Jossic-Corcos et al. 2004), a member of nuclear hormone receptor superfamily, which regulates the transcription of a number of genes encoding proteins such as lipoprotein lipase (LP) and apo CIII, resulting in the clearance of triglyceride-rich lipoproteins and reduction of circulating TG levels (Forcheron 2002; Kaletha et al. 2003). PPAR-α is expressed by many organs such as the liver, kidney, heart, and muscles, as well as in cells of the arterial wall, monocytes, macrophages and lymphocytes (Fruchart et al. 2001; Brisson et al. 2002). The following transcription-related changes have been associated with fibrate adminstration:

1. Reduction in apolipoprotein C-III in liver (Genest et al. 2000). This apolipoprotein inhibits LP activity (lipolysis), thereby treatment with fibrates

leads to increasing the clearance of VLDL and remnant lipoproteins (Genest et al. 2000; Vega et al. 2003).

2. Increase LP in adipose tissue or muscle (Desager et al. 1996; Genest et al. 2000), which accelerates the rate of hydrolyses of VLDL and chylomicron-triglyceride peripherally to IDL and LDL-cholesterol. LP also plays a role in the binding and clearance of remnant lipoproteins in the liver through LDL receptors (Schoonjans et al. 1996).

3. Increase in acyl-coenzyme A synthetase and fatty acid transport protein (Schoonjans et al. 1995). This stimulates cellular fatty acid uptake and leads to increasing catabolism of fatty acids by β-oxidation resulting in decreased availability of fatty acids for TG synthesis and a decrease in VLDL production.

4. Increase apolipoprotein AI and AII levels (apoAI and apoAII) (Vu-Dac et al. 1995; Vu-Dac et al. 1998) through their gene expression resulting in increases in HDL-cholesterol levels. Moreover, it has recently been shown that fibrates can affect HDL metabolism in mouse by significantly decreasing hepatic levels of the HDL receptor Scavenger Receptor B-I (SR-BI) (Lan and Silver 2005).

In addition to direct effects of fibrates at the level of gene transcription, fenofibrate also increases LDL size and enhances LDL resistance to oxidation (Badiou et al. 2004). Fibrates also quantitatively normalize the atherogenic LDL-cholesterol profile by an elevation in LDL subspecies of intermediate density (LDL-3) which possess optimal binding affinity for the cellular LDL

receptor (Guerin et al. 1996). One study (Ikewaki et al. 2004) has looked at the effect of fenofibrate on HDL distribution. Treatment with fenofibrate resulted in an increase in small HDL and a decrease in large HDL particles, leading to a significant decrease in HDL particle size, from 9.1 to 8.9 nm, as well as a 27% increase in the number of HDL particle.

Fibrates are frequently used in combination with bile acid sequestering agents. Fibrate therapy is associated with a number of adverse effects, including liver enzyme elevations, gastrointestinal symptoms and rhabdomyolysis (Gholami et al. 1998; Rabasa-Lhoret et al. 2001; Najib 2002). The combination of statins with fibrates may cause serious complications and should be avoided when possible. In order to prevent or minimize adverse clinical outcomes, patients should be closely monitored and informed of the most common symptoms (Najib 2002).

2.7.2. Niacin

Niacin, or Vitamin B_3, is a water-soluble B-complex vitamin with a metabolic role as a precursor for two essential coenzymes, nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). Both NAD and NADP can be reduced to NADH and NADPH, respectively. NADH and NADPH are important coenzymes that participate in oxidation-reduction reactions catalyzed by dehydrogenase and oxidoreductase enzymes involved in virtually every aspect of metabolic processes. Clinically, niacin deficiency causes pellagra characterized by

dermatitis, diarrhea and dementia. Pharmacological doses of niacin have been introduced as a lipid-lowering agent as early as 1955 (Altschul et al. 1955) and is currently used as an agent in the treatment of dyslipidemia (Tavintharan and Kashyap 2001). However, the use of niacin may be associated with adverse effects such as flushing and hepatic toxicity (Capuzzi 1998; Capuzzi et al. 2000).

The cellular and molecular mechanisms by which niacin exerts its lipid lowering effects are still not fully understood. In pharmacological doses (1-3 g/day), niacin has been shown to lower total plasma cholesterol, apolipoprotein (apo) B, TG, VLDL, LDL, and lipoprotein (a) [Lp (a)], while raising HDL-cholesterol in patients with type II, III, IV, and V hyperlipoproteinemia (Hutchinson 1990; Drood et al. 1991; Kiseleva 1994). Because of these diverse effects on lipid profile, niacin is considered as a broad-spectrum lipid-regulating agent. The beneficial effect of niacin to reduce TG and apolipoprotein-B containing lipoproteins (e.g., VLDL and LDL) are mainly through:

a) Decreasing the mobilization of free fatty acids from adipose tissue and TG stores to the liver resulting in a decrease in the hepatic production of VLDL, and lowering circulating TG concentrations (Carlson et al. 1968; Grundy et al. 1981).

b) Inhibiting hepatocyte diacylglycerol acyltransferase and TG synthesis reducing synthesis of apolipoprotein B-100, leading to impaired assembly of VLDL particles (Capuzzi et al. 2000). Niacin can also enhance VLDL

catabolism by inducing lipoprotein lipase (Grundy et al. 1981; Drood et al. 1991).

Niacin is the only currently approved lipid-lowering drug in the market that effectively lowers serum levels of Lp (a) (Carlson et al. 1989; 2002). Lp (a), an emerging risk factor for CHD (Berg et al. 1997; Stein and Rosenson 1997), is a specialized form of LDL that contains an apolipoprotein (a) chain bound to the apolipoprotein B-100 chain by a polypeptide linkage. Lp (a) increases cholesterol deposition within the arterial wall by impairing endothelium-dependent vasodilation and enhancing oxidation of LDL-cholesterol. In addition, because apolipoprotein (a) is structurally similar to plasminogen and competitively inhibits plasminogen activity, Lp (a) promotes thrombosis in advanced atherosclerosis by reducing fibrinolysis. LDL-cholesterol reducing effects of niacin is association with a shift in the subclass distribution of LDL from small particles which are more atherogenic to larger particles that may be several folds less atherogenic (Zambon et al. 1999; McKenney et al. 2001; Rosenson et al. 2002).

The mechanisms of action of niacin to raise plasma HDL-cholesterol are mainly through:

a) Increasing the synthesis of apo AI, a cardioprotective subfraction of HDL, which in turn increases circulating levels of HDL-cholesterol and promotes reverse cholesterol transport, resulting in lipid efflux from peripheral tissues (Jin et al. 1997; Kamanna and Kashyap 2000; Sakai et al. 2001; Ganji et al. 2003).

b) Decreasing fractional catabolic rate of HDL-apo AI. Furthermore, study on $HepG_2$ cells (Sakai et al. 2001), has shown that niacin selectively inhibits the uptake/removal of HDL-apo AI by hepatocytes. This also increases the capacity of retained HDL-apo AI to augment cholesterol efflux through reverse cholesterol transport pathway. Nicotinic acid may also improve endothelial function, reduce inflammation, increase plaque stability and diminish thrombosis (Blankenhorn et al. 1992; Blankenhorn et al. 1993).

2.7.3. Other lipid lowering drugs

2.7.3.1. Statins

Statins are the most powerful drugs affecting lipid and lipoprotein levels in plasma by reducing cholesterol biosynthesis and enhancing LDL-cholesterol catabolism. Statins inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which is the rate-limiting enzyme in the biosynthesis of cholesterol in the liver (Abookire et al. 2001; Shepherd 2001; Ambrosi et al. 2003). At the molecular level, the lactone moiety of statins has a conformation similar to the substrate HMG-CoA resulting in binding with high affinity to the active site of the enzyme, inhibiting its activity (Stark 2003; Thilagavathi et al. 2005). The affinity of statins for the enzyme HMG-CoA reductase is approximately three orders of magnitude greater than that of the substrate HMG-CoA (Moghadasian 1999). All statins are highly protein bound (95-98%) except for pravastatin (50%, due to carboxylate moiety) (Stark 2003). Most statins have a

short half-life of about 1-3 hours except for atorvastatin which has a half-life of about 14 hours (Motti et al. 2000).

An estimated 25% to 50% of high-risk patients will not achieve their LDL-cholesterol goal, even at the highest statin doses. Additionally, some patients cannot tolerate statins. Between 2.2% and 13.6% of subjects discontinued statin therapy because of adverse effects such as myopathy and rhabdomyolysis (1994; Sacks et al. 1996; Downs et al. 1998). Statins are highly lipophylic; they are readily reabsorbed across the renal tubule walls (Borortoff 1999). Statins are metabolised to hydrophilic derivatives before they can be excreted from the kidney. Majority of statins are metabolized via the cytochrome P_{450} (CYP) enzyme system and its 3A4, 2C9 or 2C19 paths. All compounds interfering with the same cytochrome system may either impair or enhance the elimination of statins.

2.7.3.2. Bile acid sequestrants (BSA)

Intestinal absorption of dietary cholesterol from diet and bile, which accounts for approximately 75% of the total body cholesterol (Shepherd 2001), and intestinal reabsorption of bile salts, are important targets for cholesterol-lowering therapy. BSA's are water insoluble anion exchange resins which bind to bile acids in the small intestine and thereby facilitate their excretion through stool. The interruption of the enterohepatic recirculation of bile acids stimulates hepatic bile acid formation, resulting in reduced plasma cholesterol levels. BSA's have modest LDL-cholesterol lowering effects and are not as effective

as statins (LaRosa 1989; Schectman et al. 1993). They are not absorbed into the body and therefore they do not have systemic side effects. However, BSA's may decrease the absorption of folic acid and the fat-soluble vitamins D, E, K and A (Threlkeld 1997; Probstfield et al. 1985). Animal studies suggest that cholestyramine may also reduce the absorption of calcium and zinc (Watkins et al. 1983).

Schectman et al, have evaluated efficacy of several lipid-lowering drugs including BSA, niacin, and lovastatin in 297 patients with type IIa hyperlipidemia. Their results showed that BSA (4 packets/day) was associated with a high rate of adverse effects (constipation, abdominal pain and vomiting) (Schectman et al. 1993).

2.7.3.3. Ezetimibe

The chemical name of ezetimibe (EZE) is 1-(4-fluorophenyl)-3(R)-[3-(4-fluorophenyl)-3(S)-hydroxypropyl]-4(S)-(4-hydroxyphenyl)-2-azetidinone with a molecular weight of 409.4. Ezetimibe selectively inhibits absorption of dietary and biliary cholesterol across the intestinal wall without affecting intestinal absorption of bile acids, fatty acids, triglycerides and lipid-soluble vitamins (Masana et al. 2005). Davis et al, have reported >90% inhibition in cholesterol absorption by administration of ezetimibe (>3 mg/kg) in apo E-KO mice (Davis et al. 2001). At the molecular level, ezetimibe binds to a protein namely Niemann-Pick C_1 $Like_1$ Protein (NPC_1L_1, also known as NPC_3), which is expressed on the apical surface of enterocytes and required for cholesterol

absorption (Seedorf et al. 2004; Wang et al. 2005). In rodents, NPC_1L_1 is expressed predominantly in the intestine, whereas in humans it is expressed almost equally in the small intestine and the liver (Eric and Patel 2004). Ezetimibe is also effective in the treatment of sitosterolemia because it is able to block the uptake of plant sterols and other non-cholesterol sterols as well as cholesterol from the small intestine (Morris and Tiller 2003). With regard to toxicity, no major side effect with administration of ezetimibe has been reported (Lindberg 2005; Wierzbicki et al. 2005).

2.7.4. Combination therapy

Monotherapy is an effective strategy in patients with predominantly elevated LDL-cholesterol or TG and low HDL-cholesterol levels (2000; Belalcazar and Ballantyne 1998; Isusi et al. 2000). However, a combination drug therapy must be considered in patients who do not adequately respond to single therapy or in patients with mixed dyslipidemias. The rising frequency of diabetes mellitus and metabolic syndromes, along with a growing elderly population, increases the number of people with mixed dyslipidemias. Additive effects in reducing LDL-cholesterol levels and increasing HDL-cholesterol concentrations may be achieved by combining statins with fibrates and/or niacin or other classes of lipid-lowering agents including ezetimibe.

The efficacy of fibrate-statin combinations is well documented in clinical studies showing significant reductions in LDL-cholesterol and TG and increases in HDL-cholesterol levels (Ellen and McPherson 1998; Papadakis,

Ganotakis et al. 1999). Ellen and McPherson (1998) have investigated the efficacy of fibrate-statin combinations in eighty patients with combined hyperlipidemia and CHD. The subjects received fenofibrate (300 mg/day) or micronized fenofibrate (200 mg/day) plus pravastatin (20 mg/day) or simvastatin (10 mg/day) over 2 years. LDL-cholesterol was decreased by 28% and TG by 41%, and HDL-cholesterol was increased by 22%.

Although the combination of statins plus fibrate or niacin may further reduce LDL-cholesterol and TG levels as well as increase HDL-cholesterol levels (Wierzbicki et al. 1997; Gavish et al. 2000; Brown et al. 2001), tolerability and adverse effects of such a combination are major limiting factors. Moreover, cost effectiveness is another concern in drug combination therapy (Cook et al. 2004; Perkerson et al. 2005). In one study, four out of 29 hypercholesterolemic patient who received a combination of fenofibrate plus simvastatin developed myalgia (Wierzbicki et al. 1997). Likewise, two patients from a cohort of 148 diabetic subjects treated with simvastatin plus bezafibrate developed myopathy (Gavish et al. 2000). These side effects are the outcome of interactions between the drugs at the level of hepatocytes.

Safety of combination therapy between statin and fibrate or niacin has been reviewed by Taher et al. (2002). This study reported that of 106 fibrate-statin-treated patients, four discontinued therapy due to muscle pain accompanied by a rise in creatine kinase, three due to muscle pain without a rise in creatine kinase, and two due to gastrointestinal upset (Taher et al. 2002). A review of 36 published clinical trials and 29 case reports from a

combination of fibrate-statin revealed a 0.12% overall frequency of muscle damage (Shek and Ferrill 2001).

It is now well known that gemfibrozil (fibrate) interferes with glucuronidation of statins, resulting in severe adverse effects (Prueksaritanont et al. 2002). In addition, the interaction between drugs at the level of P_{450} system may also result in fatal consequences such as rhabdomyolysis, requiring close monitoring of patients (Moghadasian 2002). A recent American College of cardiology/American Heart Association/National Heart, Lung and Blood Institute clinical advisory publication has outlined guidelines for safe management of statin/fibrate combination therapy (Pasternak et al. 2002).

Given the superior efficacies of niacin and statins with regard to HDL-cholesterol and LDL-cholesterol, respectively, their combination seems highly effective in patients with mixed dyslipidemia (Vacek et al. 1995; Wolfe et al. 2001). However, similar to fibrate-statin combinations, safety is a concern. A potential drug-drug interaction may result in skeletal muscle myopathy (Jacobson et al. 1994; Taher et al. 2002; Ballantyne et al. 2003). The incidence of myopathy associated with statin therapy is dose related (Ballantyne et al. 2003) and is increased when statins are used in combination with agents such as fibrates and niacin that share common metabolic pathways.

Combination of BAS with a statin or niacin is an effective way and most useful to achieve large reductions in LDL-cholesterol (Hunninghake et al. 2001). The statin-BAS combination can lower LDL-cholesterol levels by

approximately 50%. However, this approach has been limited by gastrointestinal side effects. Ezetimibe can be administered in combination with a statin in patients who are unable to tolerate large dosages of statins or require further reductions in LDL-cholesterol. Ezetimibe-statin therapy is well tolerated, with a safety profile similar to that of the statin alone (Gagne et al. 2002). A combination of low dose atorvastatin with ezetimibe produced clinical benefits that were similar to those achieved with high doses of atorvastatin in hypercholesterolemic patients (Ballantyne et al. 2003).

Similar to ezetemibe, phytosterols interfere with cholesterol absorption from the intestine, thereby reducing plasma cholesterol levels. Thus, a combination of statins with plant sterols resulted in further reductions in plasma cholesterol levels as compared to single statin therapy in post-menopausal women (Gylling et al. 1997).

To the best of our knowledge, the interactions between phytosterols and non-statin lipid-lowering agents have not been studied. It is believed that phytosterols reduce plasma total and LDL-cholesterol with no significant effect on HDL-cholesterol or plasma TG levels (Moghadasian and Frohlich 1999). On the other hand, niacin and fenofibrate increase plasma HDL-cholesterol levels and decrease plasma TG concentrations (Capuzzi 1998; Forcheron 2002). Therefore, a combination of phytosterols with TG-lowering drugs such as niacin or fenofibrate may reduce both plasma cholesterol and TG concentrations as well as increase HDL-cholesterol levels, resulting in a more anti-atherogenic lipoprotein profile as compared to single therapy. Because

phytosterols' intestinal absorption is very limited, their hepatic concentration is extremely low (Moghadasian et al. 2001; Ostlund et al. 2003), and thereby, unlike statin/fibrate combination, they will not interfere with fibrate or niacin hepatic metabolism. This lack of potential metabolic interaction and different modes of action and efficacy suggest that the combination of phytosterols with niacin or fenofibrate will be effective and safe. Therefore it was hypothesized that a combination of phytosterols with niacin or fenofibrate will synergistically/or additively impact lipoprotein profile and atherogenesis in apo E-KO mice

3. OBJECTIVES

3.1. Whether simultaneous administration of phytosterols with niacin or fenofibrate results in a significant reduction in both plasma TCH and TG levels as well as a significant increase in HDL-C concentrations in apo E-KO mice.

3.2. Whether these changes in lipoprotein profile lead to a better anti-atherogenic outcome in this animal model.

3.3. To test the general safety and tolerability of such combination in apo E-KO mice.

4. APO E-KO MOUSE MODEL

Studies in animal models, including rabbits, pigs, non-human primates and rodents have greatly enhanced our understanding of atherosclerosis

(Wojcicki et al. 1985). This study was conducted using a well-known animal model of hypercholesterolemia and atherosclerosis, namely the apo E-KO mice. Apo E-KO mice develop hyperlipidemia and are highly prone to diet-induced atherosclerosis.

Human apolipoprotein E (apoE) is a two-domain plasma protein that plays important roles in health and disease. Apo E is a glycoprotein with 299 amino acids and has a molecular weight of approximately 34,000. This protein can bind to lipids through its carboxyl terminal portion, from residues 202-209. The five arginine and three lysine residues between residues 140 and 160 are essential for its binding to the LDL receptor in the liver (Mahley 1988). In humans, the apo-E gene is found on chromosome 19 with 3.7 kb in length (Mahley 1988).

The three common isoforms of apo E differ at two positions in the protein and are the products of 3 alleles, ε_2, ε_3 and ε_4, which give rise to three homozygous and three heterozygous genotypes (Schneider et al. 1981; Yadong et al. 1998). The apo E2 isoform (Cys^{112} and Cys^{158}) is the least common and is associated with type III hyperlipoproteinemia. The most common isoform, apo E3 (Cys^{112} and Arg^{158}), is considered the wild-type protein since it is associated with normolipidemia; whilst the apo E4 isoform (Arg^{112} and Arg^{158}) is independently linked to an increased risk of heart disease and late-onset Alzheimer's disease (Roses 1996; Yadong et al. 1998). Furthermore, each isoform of human apo E has different affinity for the LDL receptor (Schneider et al. 1981; Knouff et al. 2004).

Plasma total apo E level is about 5 mg/dl in normal individuals (Mahley 1988). Apo-E is synthesized in several organs, mainly in the liver (three-fourths of the total plasma apo E). In the liver apo E is produced primarily by hepatic parenchymal cells, and it becomes a major component of VLDL. The brain also produces a large amount of apo E by astrocytes (the star-shaped branching neuroglial cells). Apo E is also synthesized in small amount in many organs such as the spleen, lungs, adrenals, ovaries, kidneys, muscle cells, and in macrophages (Mahley 1988). The apo E synthesized from macrophages is involved in reverse cholesterol transport, local redistribution of cholesterol, and protection against the development of atherosclerotic lesions (Linton, Atkinson et al. 1995). Apo E is a major component of intestinally synthesized chylomicrons.

One of the most important roles of apo E is to mediate high-affinity binding of chylomicrons and VLDL particles, as well as HDL. Apo E is a ligand for uptake of these particles by the liver. The homozygous inactivation of the apo E gene results in animals that are devoid of apo E in their plasma. The mice appear to develop normally however they exhibit hyper-cholesterolemia and spontaneous atherosclerotic lesions. These features are similar to those reported in patients with type III dyslipidemia.

Apo E-KO mice can be used to study the role of apo E in lipid metabolism, atherogenesis, and nerve injury and to investigate intervention therapies that modify the atherogenic processes. Apo E-KO mice develop advanced lesions and is commonly used to understand the mechanisms of

dyslipidemia and the pharmacological or dietary interventions to treat it (Tamminen 1999; Moghadasian 2001; Moghadasian 2002). It has been shown that phytosterols reduce both plasma cholesterol levels and the extent of atherosclerosis in apo E-KO mice (Moghadasian et al. 1999; Moghadasian 2001). This animal model responds very well to dietary cholesterol. Based on its well-known features and our previous experiences, we used this model to test our hypothesis.

5. MATERIALS AND METHODS

5.1. Animals and diets

Forty-seven 4-week old male apo E-KO mice were purchased from Jackson Laboratories. Because male gender is a risk factor for CHD, we used male animals. Animals were acclimatized to the animal facility for 10 days, during which time they were fed a standard cholesterol free mouse chow diet. After acclimatization to the animal facility, animals were divided and assigned to control (n=7); phytosterol-treated (n=8); niacin-treated (n=8); fenofibrate-treated (n=8); phytosterol+niacin-treated (n=8) and phytosterol+fenofibrate-treated (n=8) groups matched with their mean body weight and plasma total cholesterol levels as previously published (Moghadasian et al. 2001; Plat and Mensink 2002; Ostlund et al. 2003).

Pico Lab mouse diet (Table 1) were supplemented with 0.2% (w/w) cholesterol ("base diet") for the control group; this atherogenic "based diet" was further supplemented with 2% (w/w) soybean-derived phytosterol

Table 1. Composition of Pico Lab mouse diet

Component	%
Ash	6.0%
Carbohydrate	53%
Fat	9%
Fiber	6.0%
Fiber	4.7
Minerals	2.5%
Protein	20%

mixtures containing 58% β-sitosterol, 19% campesterol, 13% dihydrobrassicasterol and 10% stigmasterol (Sigma-Aldrich Canada Ltd. Oakville, Ontario) for the phytosterol-treated group (Moghadasian et al. 2001; Plat and Mensink 2002; Ostlund et al. 2003). For the fenofibrate and niacin groups, base diet was supplemented with 0.1% (w/w) fenofibrate (Haubenwallner 1995; Kon Koh 2004; Kuwabara 2004) and 0.5% (w/w) niacin (Elam 2000; Poynten AM 2003; Rosenson 2003), respectively. In addition to these diets, a combination of 2% (w/w) of the phytosterol mixture with either 0.1% (w/w) fenofibrate or 0.5% (w/w) niacin was added to the "base diet". Ground mouse chow was supplemented with above mentioned agents and mixed with distilled water. Prepared diets were dried in the oven at 60$^{\circ C}$. According to the Sigma-Aldrich MSDS sheet and previous study (Deliang 2002) both drugs, fenofibrate and niacin, are stable to heat during preparation of the diet.

The experimental groups were fed these diets for 14 weeks. This period is an appropriate time to induce advanced atheroscleroic lesions in apo E-KO mice (Moghadasian 1999; Moghadasian 2001). The experimental groups and their diets are summarized in Table 2. During the experimental course, animals' body weights were recorded weekly. Blood samples were taken from jugular vein (under light anesthesia induced by 1-2% isoflurane) in tubes containing EDTA (Becton Dickinson) at baseline and 4-week intervals during the study period, and at the end of the study through cardiac puncture. Plasma were separated from blood samples by centrifugation at 4°C and were used

Table 2. The summary of experimental groups and their diet

Experimental groups	Diet
Control (n=8)	0.2% (w/w) Cholesterol (Control diet)
Niacin-treated (n=8)	Control diet + 0.5% (w/w) Niacin
Fenofibrate-treated (n=8)	Control diet + 0.1% (w/w) Fenofibrate
Phytosterols-treated (n=8)	Control diet + 2% (w/w) Phytosterols
Phytosterols + Niacin-treated (n=8)	Control diet + 2% Ph + 0.5% (w/w) Niacin
Phytosterols + Fenofibrate-treated (n=8)	Control diet + 2% Ph + 0.1% (w/w) Fenofibrate

for biochemical analyses including lipid profiles. The Animal Care Committee on the use of Animals in Research at the University of Manitoba, Winnipeg, Canada, approved the experiments.

All animals looked healthy and were active during the experimental period. At the end of the study the animals were euthanized with a high dose of pentobarbital (60 mg/kg i.p). Blood samples were taken through cardiac puncture. The heart and aorta were collected and fixed for histological examination.

5.2. Plasma Lipid Analyses

5.2.1. Total cholesterol

Plasma total cholesterol levels were measured using a standard enzymatic kit, (Thermo DMA, Arlington, TX, USA). Solutions with known concentrations of cholesterol (Roche Diagnostic Corporation, Indianapolis, IN, USA) were used to generate standard curve with an R^2 value >0.995. Accordingly, cholesterol concentrations in the experimental samples were calculated.

Because of high plasma levels of cholesterol in apo E-KO mice, samples were diluted 5 times by mixing 10 μL of sample plasma with 40 μL of saline, 4 μL of diluted samples and 300 μL of reagent were added to each well and the plate was incubated at room temperature for 15 minutes. During incubation a number of steps took place: cholesterol esters were enzymatically hydrolyzed by cholesterol esterase that is found in the reagent to form cholesterol and free fatty acids; free cholesterol was then oxidized by

cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide; hydrogen peroxide combined with hydrobenzoic acid and 4-aminoantipyrine in the presence of peroxidase to form chromophore, a dye that can be quantified at 500-550 nm. After incubation absorbance was read at by a spectrophotometer, PerkinElmer, Lambda 3B (PerkinElmer Life And Analytical Sciences, Inc. Boston, MA, USA). Cholesterol concentrations were calculated considering the dilution factor. Quality control assurance was established using "control samples" from the manufacturer (Roche Diagnostic Corporation, Indianapolis, IN, USA). All experimental samples were analyzed in duplicate.

5.2.2. Triglycerides

Plasma TG was measured using a standard enzymatic kit (Thermo DMA, Arlington, TX, USA). Standard solutions (200 mg/dL) provided by the manufacturer were used to produce a linear response curve. Four μL of samples plus 300 μL of reagent were added to each well and the plate was incubated at room temperature for 15 minutes. During this time, triglycerides were hydrolyzed by lipoprotein lipase to give a glycerol and free fatty acids. The glycerol was phosphorylated in the presence of ATP and glycerolkinase to form glycerol-1-phosphate and ADP. The glycerol-1-phosphate was oxidized by glycerophosphate oxidase to form dihydroxyacetone phpsphate and hydrogen peroxide. The hydrogen peroxide and 4-aminoantipyrine and N-ethyl-N(3-sulphopropyl)m-anisidine were catalyzed by peroxidase to form a quinoneimine dye that is quantified at 540 nm. Quality control assurance was

established using "control samples" from the manufacturer (Roche Diagnostic Corporation, Indianapolis, IN, USA). All experimental samples were analyzed in duplicate.

5.2.3. HDL-cholesterol

Plasma total HDL-cholesterol levels were measured using a standard precipitation method (Moghadasian et al. 2001), based on precipitation of VLDL and LDL particles followed by quantification of HDL-cholesterol in the supernatant. Standards were made using standard solution (200 mg/dL of cholesterol) provided by manufacture (Thermo DMA, Arlington, TX, USA) to produce a linear response curve and the "control samples" were prepared using materials from Roche Company (Roche Diagnostic Corporation, Indianapolis, IN, USA). Into each tube 20 µL of the samples plus 30 µL precipitation reagent were placed, mixed vigorously and incubated for 15 minutes at room temperature. After 15 minutes, tubes were centrifuged at 2666 g force (5000 rpm, Ependorf centrifuge 5804 R) and 4° C for 20 minutes. Thirty µL of the supernatant plus 300 µL of reagent were added and the plate was incubated at room temperature for 15 minutes. The cholesterol values in supernatants were measured using a standard kit, (Thermo DMA, Arlington, TX, USA) as described above. Quality control assurance was established using "control samples" from the manufacturer (Roche Diagnostic Corporation, Indianapolis, IN, USA). All experimental samples were analyzed in duplicate.

The lipid measurements in "control solutions" always showed values within 94-98% of actual values reported by the manufacturer.

5.3. Histology and morphometry evaluations

5.3.1. Assessment of atherosclerotic lesions in the aortic root

At sacrifice, the mice were perfused with cold isotonic saline. Hearts were taken and placed in fresh paraformaldehyde for 48 hours prior to embedding in optimal cutting temperature (OCT) for sectioning. Sections of the aortic roots were cut and stained with hematoxylin and eosin (H&E) and oil red O (ORO) for histological and morphometrical examinations (Moghadasian 1999; Moghadasian 2001). The complete details of the procedures of tissue preparation and sectioning can be found in the Appendix section.

Quality of atherosclerotic lesions was examined using light microscopy techniques. To examine the involvement of extracellular matrix and cellular components of the atherosclerotic lesions, sections were also stained with H&E. Briefly, 6-μm-thick tissue sections were deparaffinized with xylene and dehydrated with graded alcohols. (complete detail of staining protocol can be found in the Appendix section). This stain illuminates cellular wall and nuclear proteins allowing for cell identification.

ORO-stained sections were used to estimate atherosclerotic lesion size and lesion-to-lumen ratio (Moghadasian et al. 2001; Plat and Mensink 2002; Ostlund et al. 2003). Image Pro Plus® software was used to determine the atherosclerotic lesion size and lesion to lumen ratios. We measured total

lesion area, external circumference of the aortic root, and total lumen area of the aortic root sections. We also calculated lesion to lumen ratio by dividing the average lesion area by the average lumen area.

5.3.2. Histology of the liver and kidney

To investigate potential toxicity of the combination therapy, we also examined the internal organs such as the liver, kidney, testis, spleen and the lungs at autopsy. Specimens of liver and kidney were taken for histological examinations. The tissues were fixed in 10% neutral buffered formalin, dehydrated and then embedded in paraffin wax. The samples were cut by microtome (Thermo Shandon company). Multiple sections from each block (5 micron thick) were prepared and all sections were stained with hematoxylin and eosin. Slides were examined using light microscope (Olympus DP70) at 40x, 100x and 400x magnifications.

5.4. Sample size and statistical analysis

For sample size, we estimated that 8 mice per group provide sufficient statistical power (>85%) for detecting difference of ½ standard deviation as significant at the level of $p<0.05$. This applies to both biochemical parameters and the morphometrical measurements. We previously showed that the intra and inter-observer variations for morphometrical analyses of atherosclerosis lesions in the aorta using Image Pro Plus® were less than 5%. The coefficient of variations (CV) for plasma cholesterol, TG and HDL-cholesterol, using quality control, were <1%, and <2% and <1% respectively.

Data were analyzed using SPSS software performing TWO-WAY ANOVA followed by the TuKey test to determine significant differences among various experimental groups.

6. RESULTS

6.1. Body weight

Figure 2 demonstrates body weight gain in all the experimental groups over the study period. All experimental animals gained body weight during the experimental course; however, the extent of body weight gain was lower in the fenofibrate and phytosterol+fenofibrate-treated mice.

At four weeks, the phytosterol and niacin treated animals had slightly higher mean body weight as compared to other groups. However, body weight gain in phytosterol+niacin-treated animals was significantly greater as compared to control ($p<0.05$) or to fenofibrate ($p<0.005$) and phytosterol-fenofibrate-treated groups ($p<0.005$). Fenofibrate treated group had significantly lower body weight as compared to phytosterol and phytosterol-niacin-treated groups ($p<0.05$ and $p<0.005$ respectively) but not the control group. Phytosterol-fenofibrate-treated animals had slightly lower body weight, as compared to controls.

At eight weeks both fenofibrate and phytosterol+fenofibrate-treated animals had lower mean body weight as compared to other groups ($p<0.005$); no further weight gain was observed in this group of mice till the end of the study. These observations indicate that addition of fenofibrate to the diet

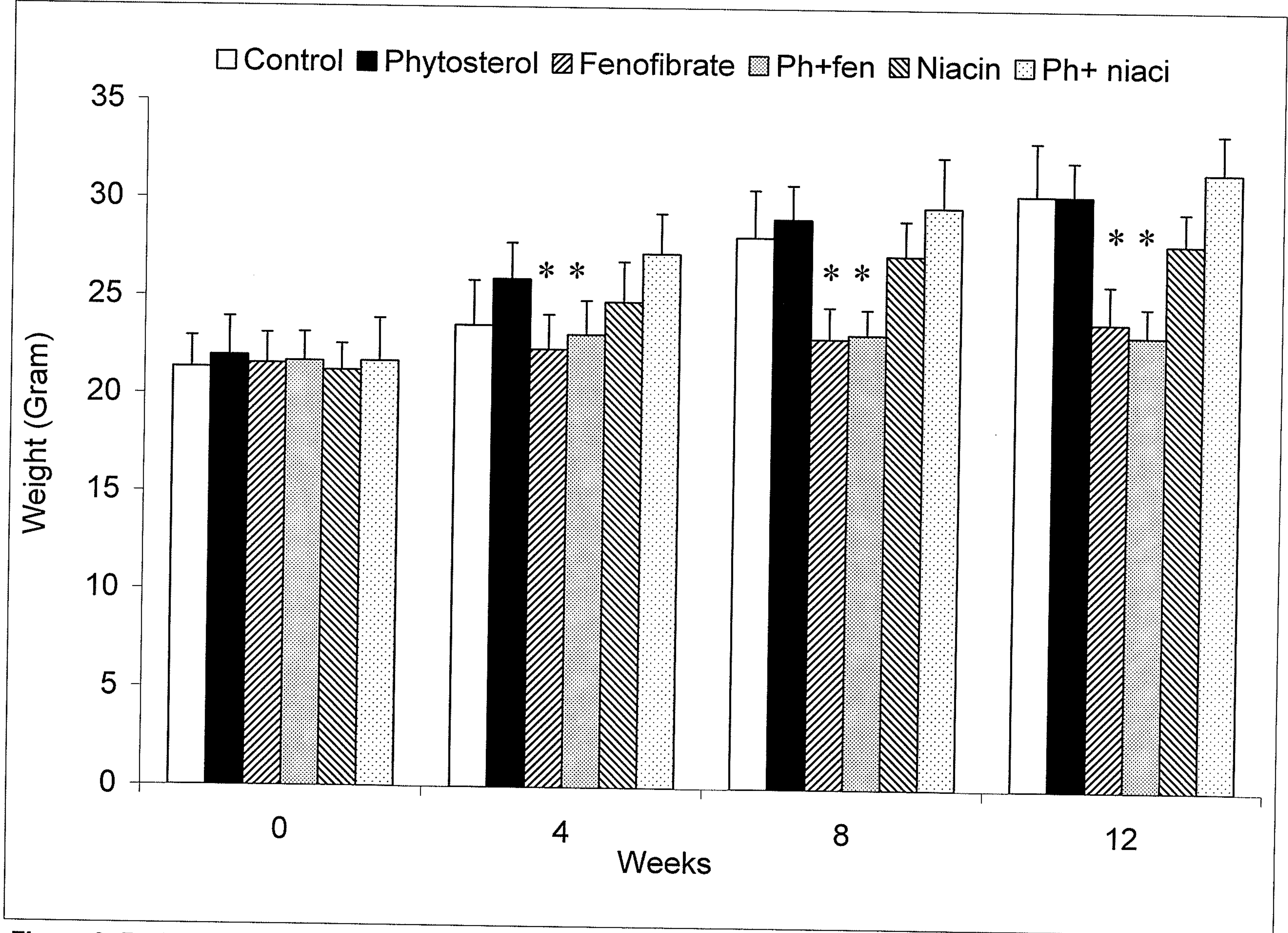

Figure 2: Body weights of mice in the different experimental groups. *, p<0.05 as compared to controls Ph+fen; Phytosterol+fenofibrate, Ph+niaci; Phytosterol+niacin

resulted in smaller body weight gain during the experimental course. However addition of phytosterol did not prevent this effect of fenofibrate in apo E-KO mice.

Similar to the previous observations (Moghadasian et al. 1997; Moghadasian 1999), the phytosterol-treated apo E-KO mice and the control group had comparable body weight throughout the study (Figure 2), indicating tolerability and safety of dietary phytosterol mixtures regardless of their origin (wood or vegetable) and composition (percentage of each plant sterol/stanol in the mixture).

6.2. Plasma lipid levels

6.2.1. Total cholesterol

The levels of plasma total cholesterol at baseline and during the experimental course have been illustrated in Figure 3. The "base diet" significantly increased the levels of total cholesterol in all experimental groups, but to a different extent. By week 4 of the study, the control group had an increase of 172% in total cholesterol as compared to baseline values, while there was only a 52% increae in the phytosterol-treated and a 66% increase in phytosterol+niacin-treated groups. Total cholesterol increased by 276% ($p<0.001$, compared to week 0) in fenofibrate treated mice, while cholesterol increased by 172% ($p<0.05$, compared to week 0) in niacin-treated groups. This suggests that addition of fenofibrate to the diet substantially increases plasma total cholesterol levels in apo E-KO mice. There was a significant

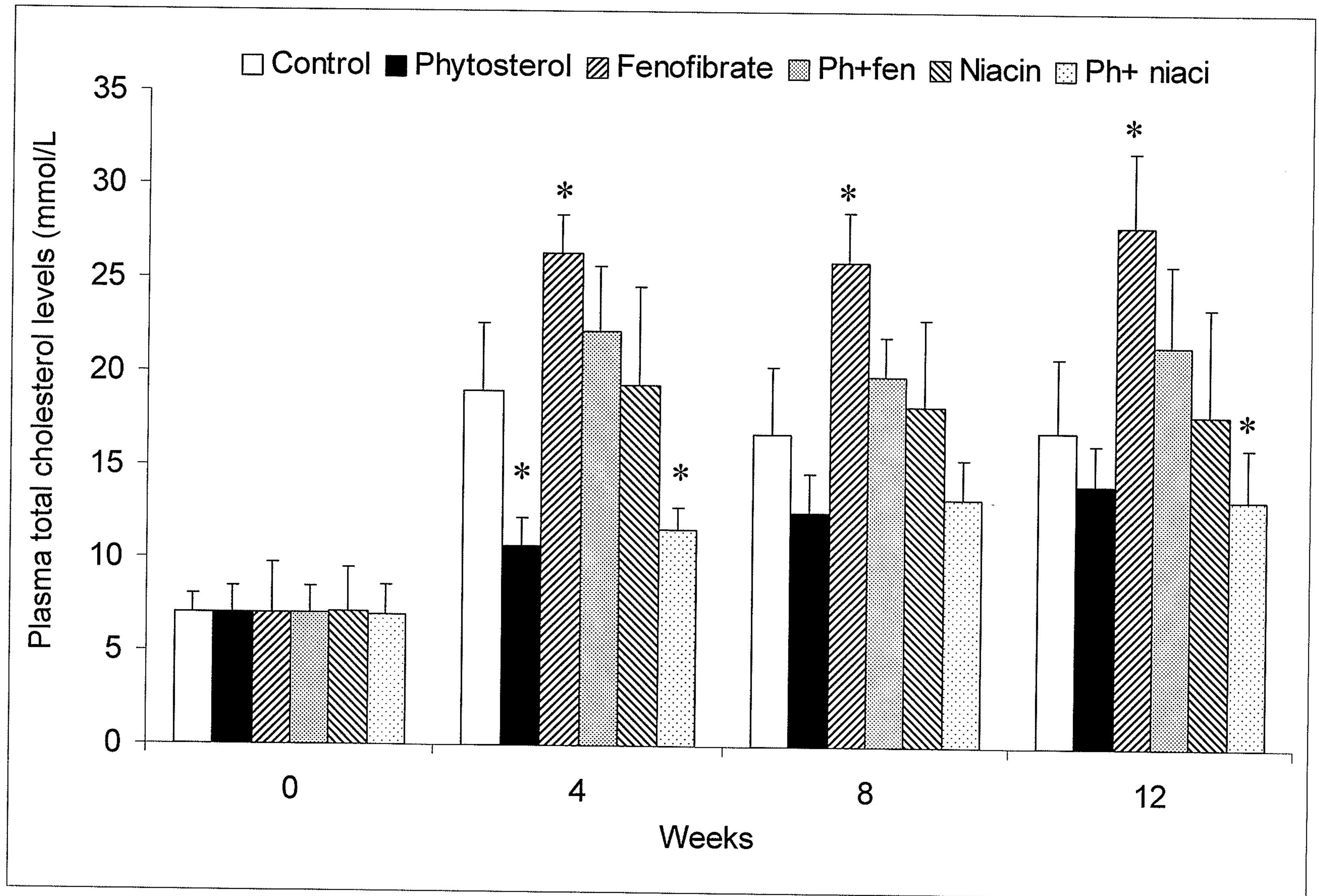

Figure 3: Plasma cholesterol concentrations in different groups of mice. *, p<0.05 as compared to controls Ph+fen; Phytosterol+fenofibrate, Ph+niaci; Phytosterol+niacin

decrease ($p<0.005$) in total cholesterol levels of phytosterol-trated animals as compared to the control group at week four of the study. However, addition of niacin to the "base diet" had no significant effects on circulating total cholesterol levels as compared to controls. In contrast, a combination of phytosterol+niacin significantly reduced total cholesterol levels ($p<0.05$) as compared to the control group. The extent of reduction in phytosterol+niacin-treated group was almost the same as the phytosterol-treated group alone.

The phytosterol+fenofibrate-treated group had large increases (218%) in total cholesterol levels at week four as compared to baseline values. This suggests that addition of fenofibrate to a phytosterol-enriched diet substantially increases plasma cholesterol levels and masks cholesterol-lowering activity of phytosterols. On the other hand, addition of niacin to the phytosterol-enriched diet had no significant effects on cholesterol-lowering properties of phytosterols in this animal model. The degree of differences between the levels of total cholesterol among the six groups of mice slightly reduced during the rest of experimental period (weeks 8-12).

6.2.2. HDL-Cholesterol

Figure 4 shows the levels of plasma HDL-cholesterol in experimental groups during the study period. Due to the small size of the animals at the beginning of the study and limitation of blood samples, it was not possible to measure HDL-cholesterol and TG in the experimental groups at base line.

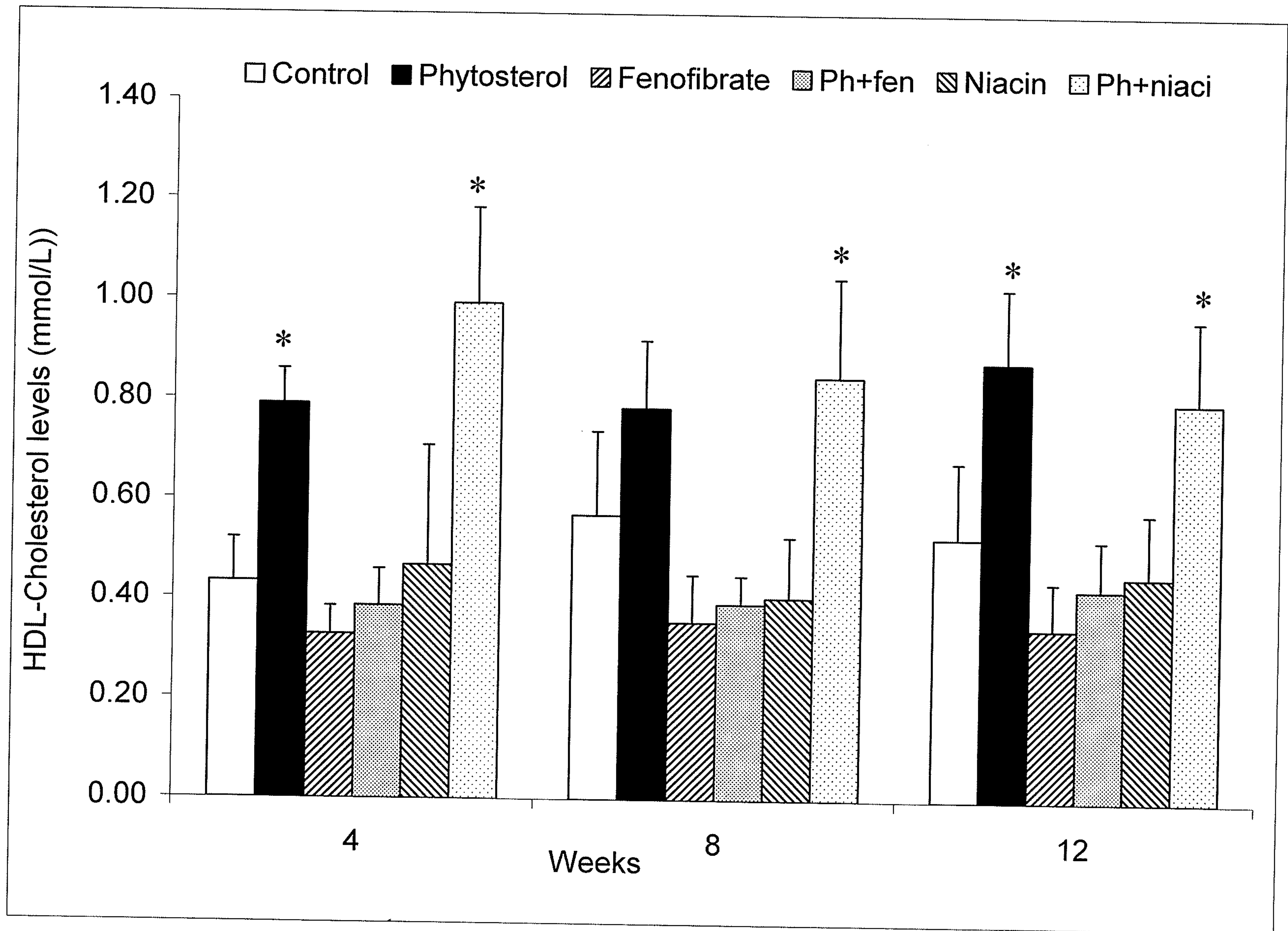

Figure 4: Plasma HDL-cholesterol concentrations in different experimental groups. *, p<0.05 as compared to controls
Ph+fen; Phytosterol+fenofibrate, Ph+niaci; Phytosterol+niacin

Unlike total cholesterol, at the end of four weeks, the HDL-cholesterol levels were higher in phytosterol-treated and phytosterol+niacin-treated mice as compared to the control group. For example, as compared to controls phytosterol-treated mice had 100% ($p<0.05$) more HDL-cholesterol.

At eight weeks, in the niacin-treated group there was a marginal decrease ($p>0.05$) in HDL-cholesterol level as compared to controls. The effects of niacin on HDL-cholesterol disappeared by the end of 12 weeks study. In contrast, in phytosterol+niacin-treated mice the HDL-cholesterol levels was increased by 41% ($p<0.05$). This higher HDL-cholesterol level in phytosterol-treated or phytosterol-niacin-treated animals was also observed during the week 8 and week 12 of the study.

The HDL-cholesterol levels in fenofibrate treated mice were consistently lower than those in controls (0.3 vs 0.4 mmol/L), while addition of phytosterols could not improve the HDL-cholesterol levels in apo E-KO mice (0.4 vs 0.4 mmol/L). Both fenofibrate and niacin paradoxically reduced the levels of HDL-cholesterol in apo E-deficient mice by 34% and 14%, respectively, as compared to controls.

6.2.3. Triglycerides

The levels of plasma TG in all of the apo E-KO groups during the experimental course are illustrated in Figure 5. None of the treatment protocols resulted in statistically significant reductions in plasma TG levels, indicating a lack of TG-lowering effects for niacin and fenofibrate in apo E-KO

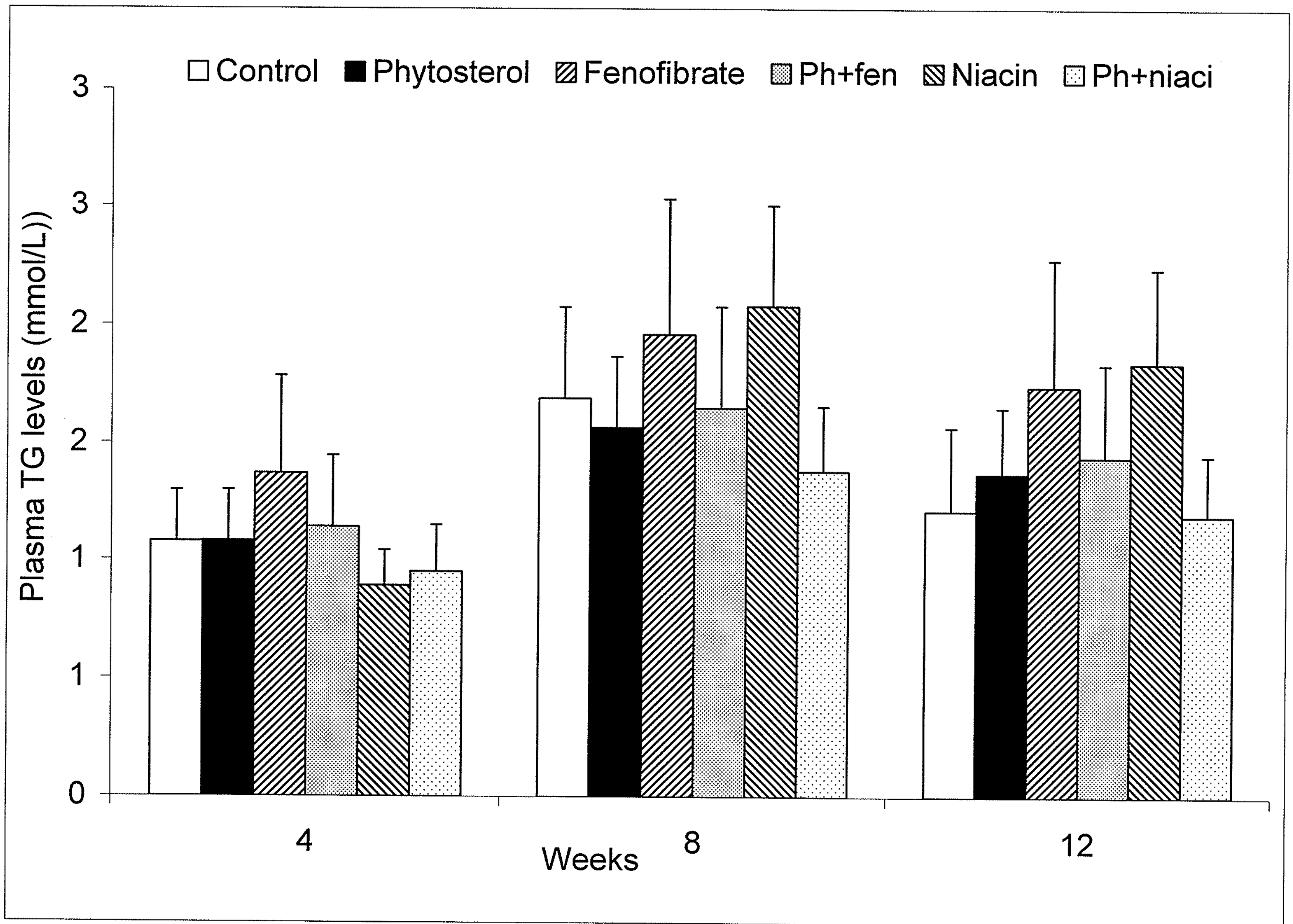

Figure 5: Plasma triglyceride concentrations in different experimental groups. Ph+fen; Phytosterol+fenofibrate, Ph+niaci; Phytosterol+niacin

mice (Figure 5). For example, at week 8 of the study the plasma TG levels in niacin and fenofibrate-treated mice were higher than those in the control group (2.1 and 2.0 respectively vs 1.7 mmol/L, $p<0.05$). Pharmacological doses of fenofibrate and niacin significantly reduce plasma TG levels in humans.

6.3. Atherosclerotic lesion development

Representative photomicrographs of aortic roots from all of the experimental animals are shown in Figures 6 and 7.

Oil red O stained sections present the presence of lipid-rich atherosclerotic lesions in the aortic roots of control group as well as treated groups. Sections from the phytosterol-treated (Figure 6, Panel B) and phytosterol+niacin-treated (Figure 6, Panel F) mice show much less extracellular matrix component with lipid deposits in the aortic sinuses as compared with the other groups of mice (Figure 6). Hematoxyline and eosin staining (Figure 7, Panels A-F) shows that the aortic root atherosclerotic lesions contain a well-developed fibrous cap and sheets of apparently proliferating smooth muscle cells which are more abundant in the control (Figure 7, Panel A) and both groups of fenofibrate and phytosterol+fenofibrate-treated mice (Figure 7, Panel D) groups. No noticeable difference was observed in the quality and morphological characteristics of the atherosclerotic lesions between the control group and phytosterol+fenofibrate-treated group (Figure 7, Panels A, E, D, H).

Table 3 summarizes the morphometrical analysis of the atherosclerotic lesions in the aortic roots of the mice. Evaluation of the lesion size formed in

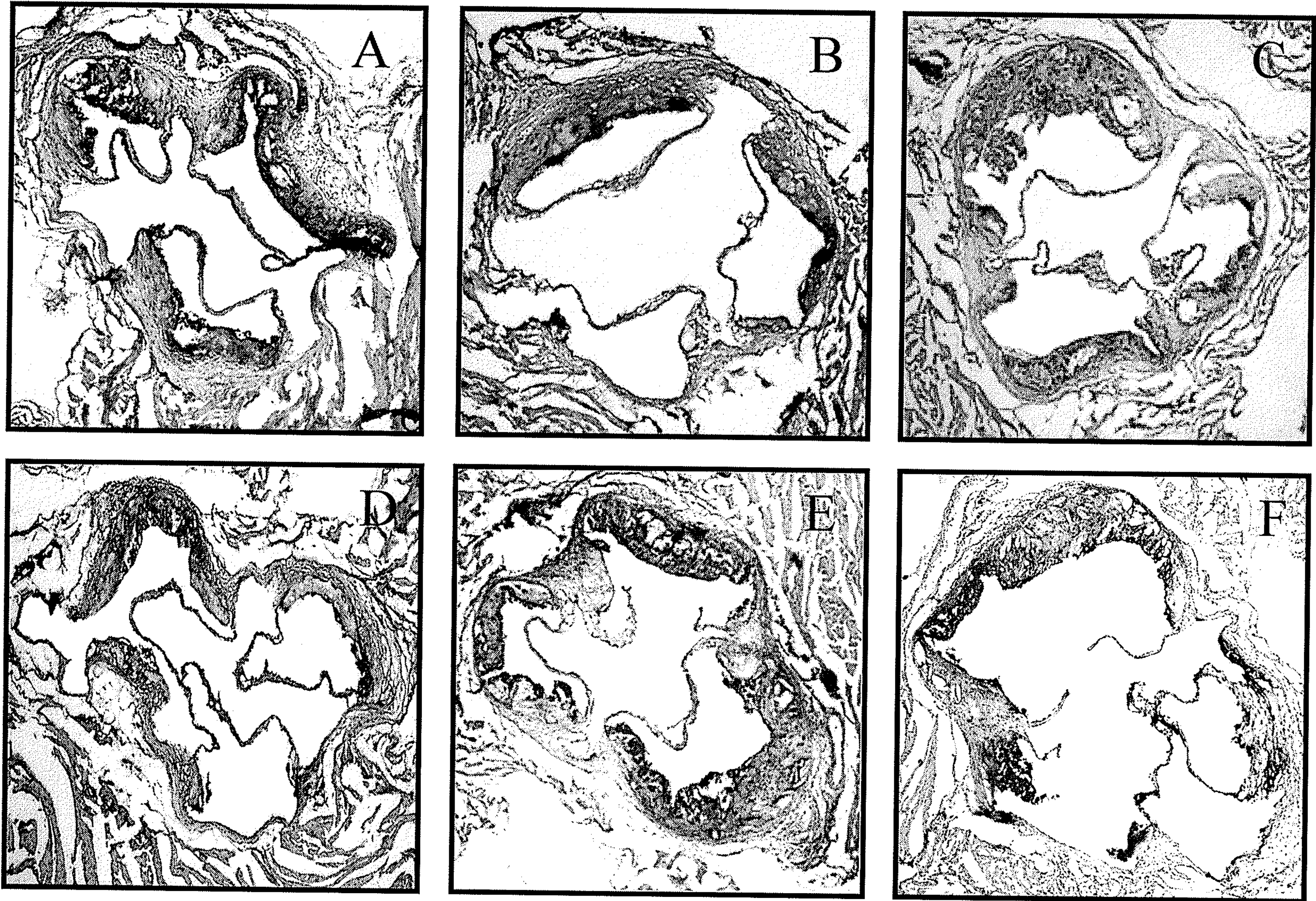

Figure 6: Representative photomicrographs illustrating atheroscerotic lesions in the aortic roots of experimental mice. ORO staining, A; Control, B; Phytosterol, C; Fenofibrate, D; Phytosterol+fenofibrate; E: Niacin, F: Phytosterol+niacin

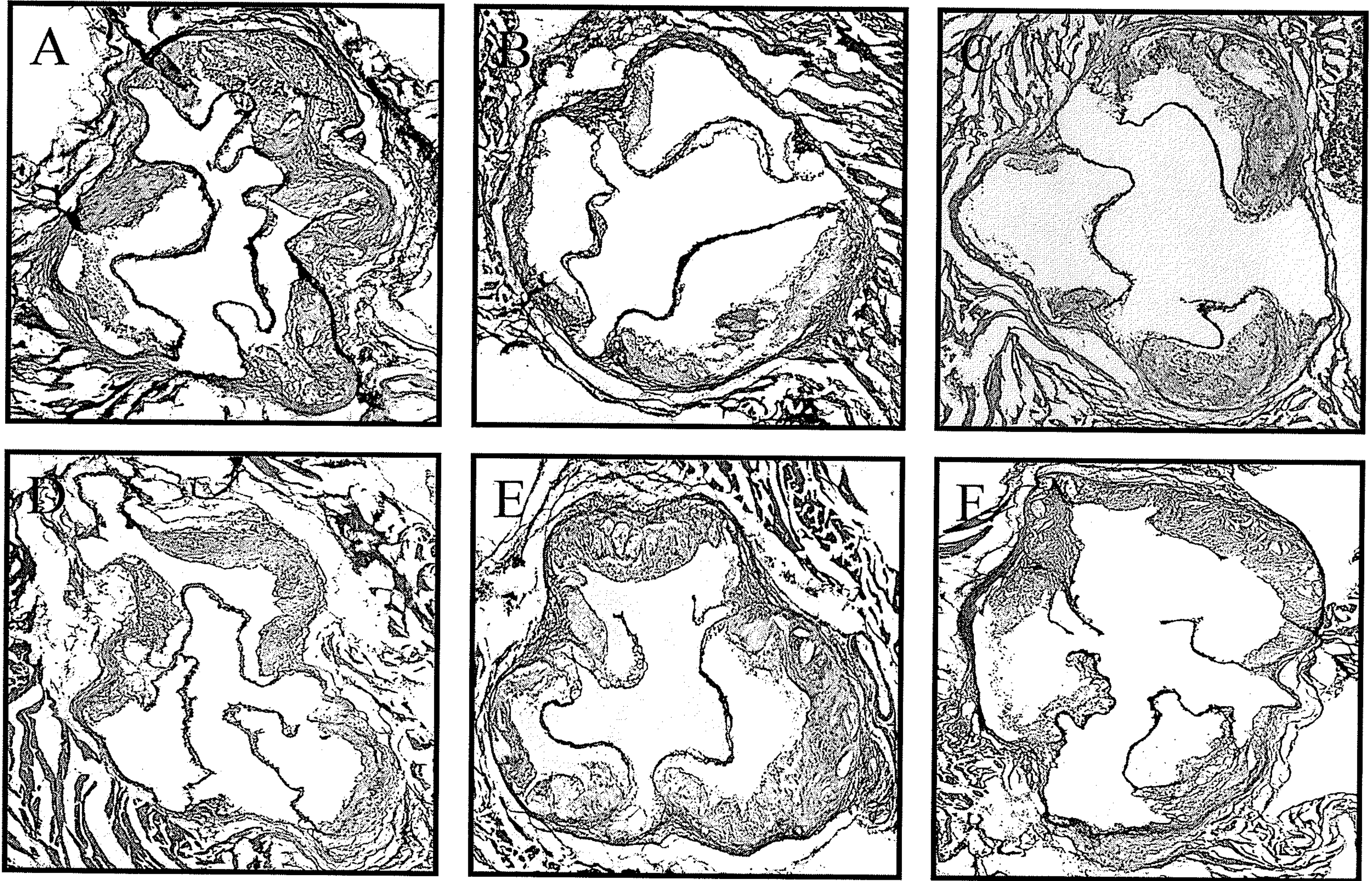

Figure 7: Representative photomicrographs illustrating atheroscerotic lesions in the aortic roots of experimental mice. H& E staining, A; Control, B; Phytosterol, C; Fenofibrate, D; Phytosterol+fenofibrate; E: Niacin, F: Phytosterol+niacin

the aortic roots of apo E-KO mice during 12 weeks of experimental protocol was statistically comparable among fenofibrate (0.40 ± 0.07mm2), niacin (0.48±0.08 mm2), and control groups (0.42 ± 0.14 mm2) (Table 3).

The data in table 3 demonstrate statistically significantly smaller lesion size in the phytosterol-treated (0.15 vs 0.42 mm^2) and phytosterol+niacin-treated group (0.15 vs 0.42 mm^2) as compared to control group. Although atherosclerotic lesion size in the niacin-treated mice was trivially larger than in the control group (0.48 vs 0.42 mm^2), the phytosterol+niacin-treated group had significantly smaller atherosclerotic lesion size compared to niacin-treated alone (0.15 vs 0.48 mm^2). The fenofibrate-treated group had slightly lower atherosclerotic lesion size than the control and niacin-treated groups, despite significant decreases in their circulating HDL-cholesterol and increases in total cholesterol concentrations. In the phytosterol+fenofibrate-treated animals, atherosclerotic lesion size was comparable with that in the control group (0.34 vs 0.42 mm^2) and slightly higher than that in phytosterol-treated mice. This indicates that addition of fenofibrate to the phytosterol-enriched diet slightly diminished antiatherogenic properties of phytosterols in apo E-KO mice.

Table 3: The effects of either monotherapy or combination therapy on atherosclerotic lesion size, lumen area and lesion/lumen ratio in the aortic roots of apo E-KO mice (data are presented as mean and standard deviation).

Groups	Lesion size (mm^2)	Lumen Area (mm^2)	Lesion/Lumen Ratio
Control (7)	0.42 ± 0.14	1.26 ± 0.22	0.32 ± 0.07
Phytosterol-treated (8)	0.15 ± 0.08*	1.19 ± 0.12*	0.13 ± 0.06*
Fenofibrate-treated (7)	0.40 ± 0.1	1.32 ± 0.1	0.30 ± 0.1
Phytosterol + fenofibrate-treated (8)	0.34 ± 0.08	1.16 ± 0.32	0.31± 0.09
Niacin-treated (8)	0.48 ± 0.1	1.34 ± 0.1	0.36 ± 0.1
Phytosterol + niacin-treated (8)	0.15 ± 0.06*	0.96 ± 0.27*	0.16 ± 0.05*

*, $p<0.05$ as compared to controls

6.4. Evidence of safety

Representative photomicrographs of liver and kidney sections from all of the experimental animals are shown in Figures 8 and 9, respectively. The liver plays a central role in detoxification of drugs from circulation through the effects of several hepatic enzymes. Histology examination of liver and kidney show that none of the agents used alone or in combination with phytosterols caused toxicological defects at the cellular levels as compared with controls. Photomicrographs of livers from different groups of mice show normal hepatocytes. Hepatical and renal sections revealed no abnormal features in the morphology of hepatocytes or renal tubes. Glomerulus within the renal cortex appeared normal with a few red blood cells inside.

7. DISCUSSION

7.1. Summary

Hypertriglyceridemia, low HDL-cholesterol and small, dense LDL particles are among major risk factors for CHD. Patients with mixed dyslipidemias (increased LDL-cholesterol and TG levels plus reduced HDL-cholesterol concentrations) are at higher risk for CAD. This higher risk is further augmented, if it is accompanied by a cluster of other risk factors, including diabetes, a family history, male gender, postmenopausal status, obesity, hypertension, and smoking. Improvements in plasma lipoprotein profiles by lifestyle modification, dietary and/or pharmacological agents have been shown to reduce cardiovascular mortality and morbidity.

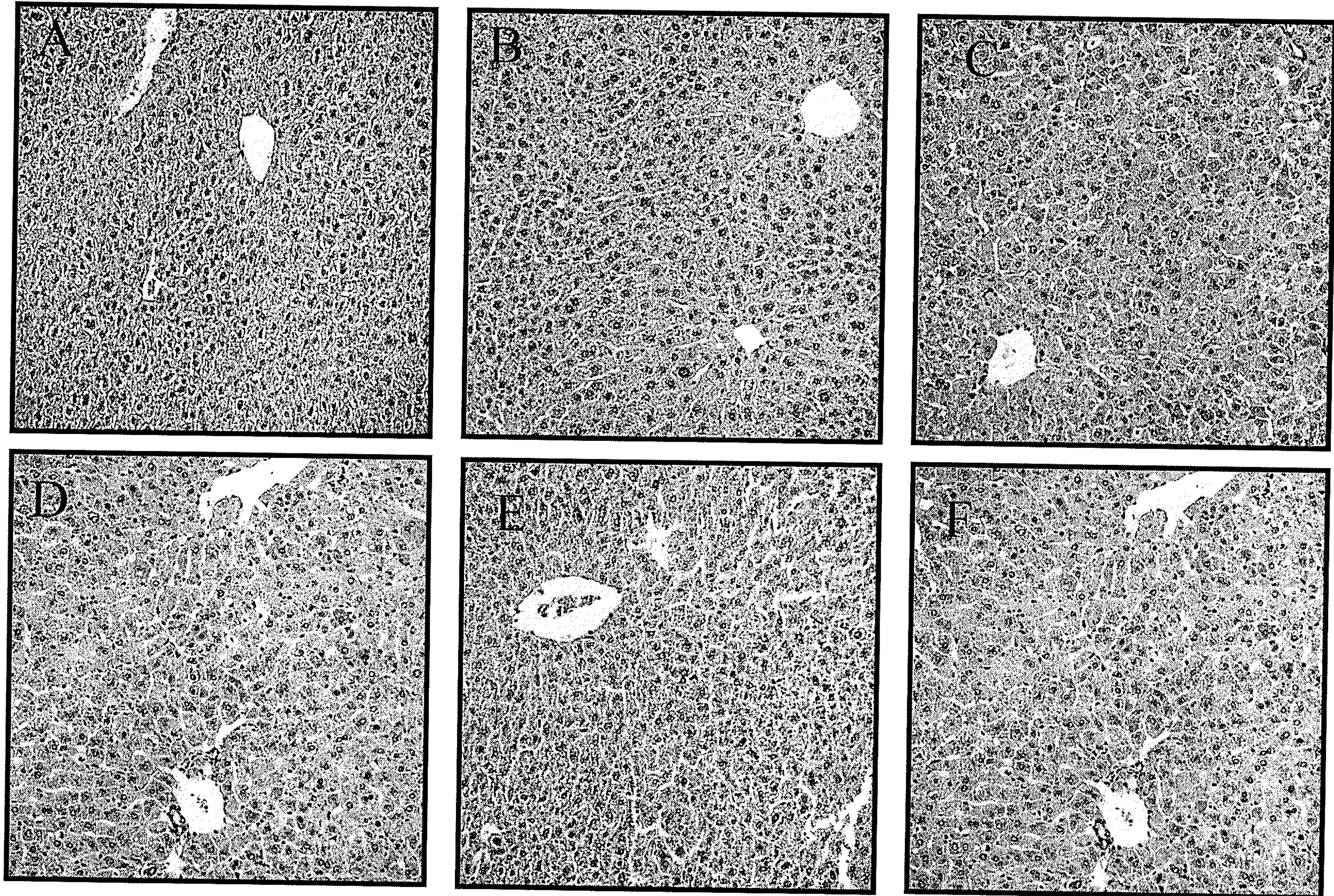

Figure 8: Photomicrograph of liver sections of different experimental groups (Magnification is 400 X) A; Control, B; Phytosterol, C; Fenofibrate, D; Phytosterol+fenofibrate; E; Niacin, F; Phytosterol+niacin

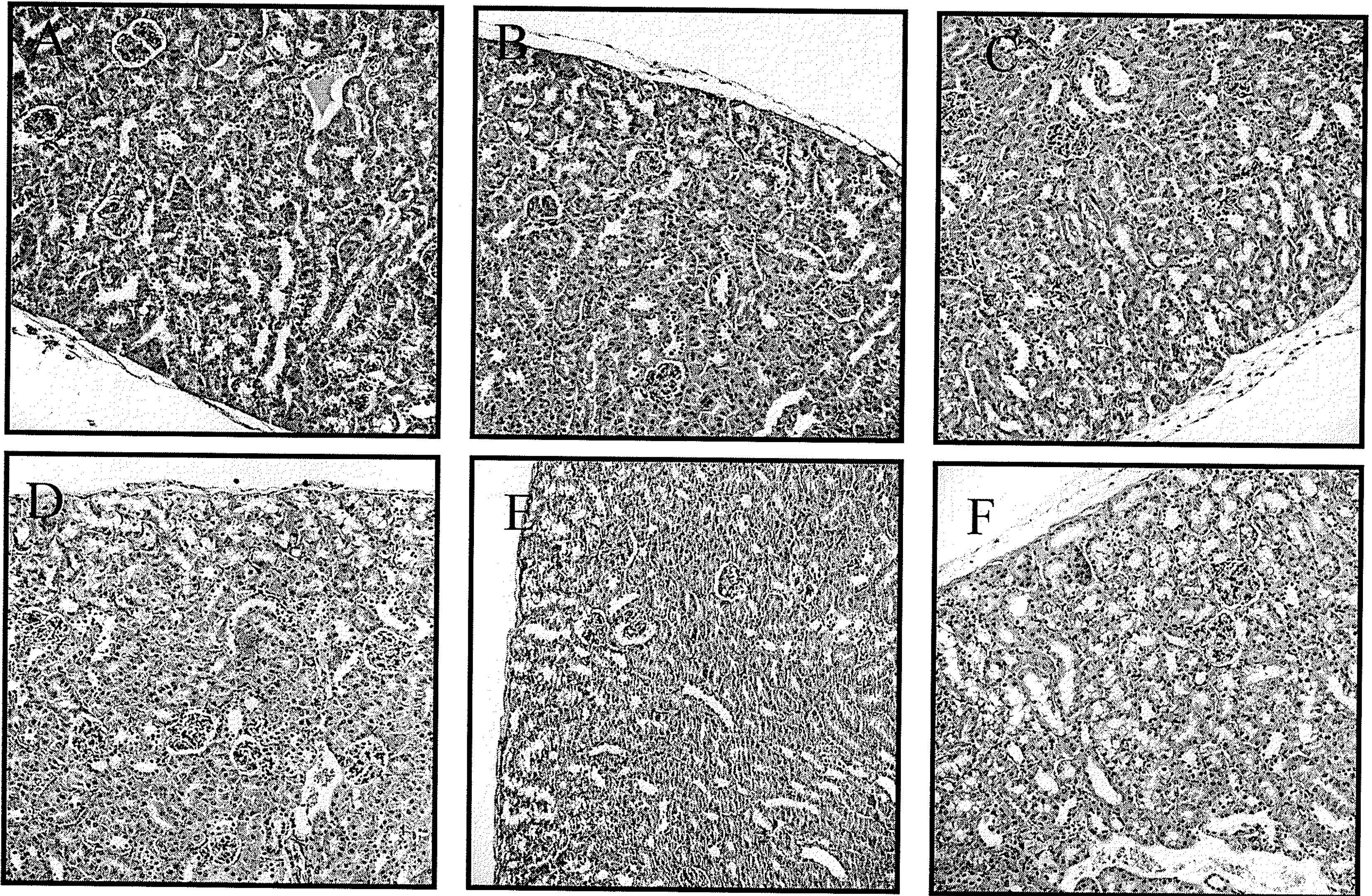

Figure 9: Photomicrograph of kidney sections of different experimental groups (Magnification is 400 X) A; Control, B; Phytosterol, C; Fenofibrate, D; Phytosterol+fenofibrate; E; Niacin, F; Phytosterol+niacin

The drugs of choice for the treatment of hyperlipidemia are either fibrates, in the case of hypertriglyceridemia, or statins, in the case of hypercholesterolemia. Statins significantly reduce LDL-cholesterol levels, and remain a popular treatment for reducing LDL-cholesterol (1994; Shepherd et al. 1995; Sacks et al. 1996; Pfeffer et al. 1999). Although these drugs are effective in preventing morbidity and mortality from cardiovascular events, their adverse effects may be a limiting factor in some patients. Drug-induced myopathy and rhabdomyolysis are among serious adverse drug reactions, which can be induced in particular by fibrates and satins. Hepatic toxicity is another important side effect of fibrate and statins treatment. Myopathy may start as early as a few days after treatment. Muscular necrosis can lead to kidney failure, and eventually to death.

For the class of statins, myopathy is more often seen, and it is their most feared adverse effect, especially if statins are combined with other lipid lowering drugs that share common metabolic pathways. While strong epidemiological data are lacking, the incidence of myopathy is probably similar for all lipid-lowering drugs and is in the range of 0.1-0.5% with monotherapy (Gaist et al. 2001), increasing to 0.5-2.5% with combination therapy (Carvalho et al. 2004).

Although monotherapy with either niacin or fibrates is a mainstay of lipid treatment in patients with predominantly elevated TG and low HDL-cholesterol levels (Belalcazar and Ballantyne 1998; 2000), a co-administration of other cholesterol-lowering therapies is necessary in patients who do not adequately

respond to single therapy or in patients with mixed dyslipidemias. Additive effects in reducing LDL-cholesterol levels and increasing HDL-cholesterol concentrations may be achieved by combining statins with fibrates and/or niacin or other classes of lipid-lowering agents including ezetimibe. On the other hand, in patients with very elevated TG levels a combination of fibrate plus niacin may be an optimal approach. Although the combination of statins plus fibrate or niacin may further reduce LDL-cholesterol and TG levels as well as increase HDL-cholesterol levels (Wierzbicki et al. 1997; Gavish et al. 2000; Brown et al. 2001), tolerability and adverse effects of such a combination are major limiting factors (Taher et al. 2002).

In general, ezetimibe which selectively inhibits absorption of sterols across the intestinal wall can be administered in combination with a statin in patients who are unable to tolerate large dosages of statins or require further reductions in LDL-cholesterol. It has been shown that a combination of low dose atorvastatin with ezetimibe can improve the lipoprotein profile of hypercholesterolemic patients similar to those achieved with high doses of atorvastatin (Ballantyne et al. 2003).

Similar to ezetemibe, plant sterols reduce plasma cholesterol levels by interfering with cholesterol absorption. Therefore the addition of phytosterols to statin therapy is an effective way to achieve large reductions in plasma cholesterol levels as compared to single statin therapy in post-menopausal women (Gylling et al. 1997). Dietary phytosterols reduce plasma cholesterol levels by 10%, thus, they may be of value in patients with moderately elevated

plasma cholesterol levels. On the other hand, phytosterols do not reduce plasma TG levels. Therefore, a combination of phytosterols with TG-lowering drugs such as niacin or fenofibrate may result in better prevention of coronary heart disease.

It is believed that plant sterols are poorly absorbed from the small intestine, thus the levels of plant sterols in circulation are very low. As a result, unlike statin/fibrate combination, a co-administration of phytosterols with fibrate or niacin is very unlikely to result in serious adverse effects because of drug- drug interaction. Accordingly, it was hypothesized that a combination of dietary phytosterols with niacin or fenofibrate will result in a greater reduction in plasma total and LDL-cholesterol, TG levels and increased HDL-cholesterol levels without major side effects in apo E-KO mice.

7.2. Effects of fenofibrate and combination of phytosterols with fenofibrate

Results of this study show that administration of 0.1% (w/w) fenofibrate in apo E-KO mice not only failed to reduce plasma TG levels, but paradoxically increased total cholesterol and decreased HDL-cholesterol levels, as compared with those in the control group. This finding is in agreement with a previous study (Duez et al. 2002) in which oral adminstration of 0.05% (w/w) fenofibrate in apo E-KO mice increased plasma total cholesterol and TG levels and reduced HDL-cholesterol concentrations over eight weeks. These paradoxical effects on plasma lipid levels are dose dependent since in Duez's

study (Duez et al. 2002) total cholesterol was increased by 31% with 0.05% (w/w) dose of fenofibrate while in the present study adminstration of 0.1% (w/w) fenofibrate increased the total cholesterol by 53% as compared with control mice. Furthermore the effects of fenofibrate on lipoprotein profile seem to be independent of the dietary fat, as the fat content of the diet in Duez's study was 20% (w/w) and in our study was 9% (w/w).

Unlike in humans, fenofibrate does not increase HDL-cholesterol levels in hamsters (Fruchart 2001). This may be related to species specific variations in lipoprotein metabolism (Green and Moghadasian 2004) as well as tissue expression and activity of PPAR receptors. Moreover Guo et al. (2001) have reported that treatment of hamsters with fenofibrate led to reduction in hepatic enzyme activities and mRNAs for HMG CoA synthase and HMG CoA reductase. However, the results of this study show that fenofibrate paradoxically increased the levels of plasma total cholesterol in apo E-KO mice. It is possible that fenofibrate may positively influence dietary cholesterol absorption and/or *de novo* cholesterol synthesis. Further investigations on intestinal absorption of cholesterol using labeled cholesterol may elucidate the mechanisms of these findings. Moreover, studying the effects of fenofibrate on cholesterol biosynthesis using radioactive hydrogen (H3), examining hepatic enzyme activities and mRNAs for HMG CoA synthase and HMG CoA reductase may help to understand the mechanisms of effects of fenofibrate.

It is also possible that fenofibrate may negatively affect hepatic cholesterol metabolism, including reductions in receptor-mediated uptake of

lipoproteins and/or reductions in bile acid synthesis in apo E-KO mice. Our data may suggest that apo E is needed for beneficial lipid-modifying activities of fenofibrate. If so, patients with hyperlipidemia type III (lack of apoprotein E) who are taking fenofibrate may not benefit from fenofibrate treatment. More investigations are needed to understand the role of apo E in lipid-modifying effects of fenofibrate.

The data of this study also show that an addition of phytosterols to fenofibrate improved plasma total cholesterol levels as compared to fenofibrate treated mice. Our data do not allow us to draw definitive conclusions on a mechanism of interaction. Phytosterols reduce plasma total cholesterol mainly by competing with cholesterol absorption from the intestine. No direct effects of phytosterols on *de novo* biosynthesis of cholesterol in the liver have been reported.

Another observation from our study is that both experimental groups of fenofibrate and phytosterol+fenofibrate-treated mice had lower body weight gain throughout the study as compared to controls. This finding is in agreement with previous observations (Yoon et al. 2003; Jeong et al. 2004; Jeong et al. 2004) in which administration of fenofibrate reduced body weight and plasma lipid levels without affecting food intake in mice.

The effects of fenofibrate on lipoprotein metabolism and body weight have been investigated in different animals such as rhesus monkeys (Winegar et al. 2001) and hamsters (Rizvi et al. 2003). In rhesus monkeys, fenofibrate reduced body weight with no effect on food intake and plasma total cholesterol

levels (Winegar et al. 2001). Thus, it is reasonable to consider a relationship between plasma lipid levels and body weight gain. However, it has been shown that body weight gain in apo E-KO mice is independent of plasma cholesterol levels (Zhang et al. 1994)

It is well known that fenofibrate activates PPAR-α, and that hepatic PPAR-α activation is involved in regulation of energy balance and body weight gain (Fruchart 2001; Fruchart et al. 2001). Therefore, the mechanism underlying body weight lowering effects of fenofibrate observed in our study and others (Yoon et al. 2003; Jeong et al. 2004; Jeong et al. 2004) may be related to PPAR-α activation. This concept is further supported by Costet's study (Costet et al. 1998) in which deletion of PPAR-α gene in mice caused abnormalities in TG and cholesterol metabolism and eventually led to obesity.

In Duez's study (Duez et al. 2002), the authors reported that administration of 0.05% (w/w) fenofibrate in male apo E-KO mice not only does not reduce aortic atherosclerosis, but it also increases atherosclerotic lesion size in the aortic roots by up to 20%. Higher doses of fenofibarte in our study (0.1% w/w) also failed to prevent atherogenesis in the aortic roots of the apo E-KO mice. It is well documented that there is a direct relation between plasma homocysteine levels and atherosclerosis development, and that administration of fenofibrate increases plasma homocysteine in humans (Dierkes et al. 1999; Bostom 2001) and mice (Legendre et al. 2002). Therefore, lack of anti-atherogenic effects of fenofibrate in the present study or even proatherogenic effects of fenofibrate in other studies (Duez et al. 2002)

may be mediated through increases in plasma homocysteine levels. Further investigations are needed to identify the mechanisms of these findings.

The pathological features of the atherosclerotic lesions formed in the aortic roots were also similar in both control and fenofibrate groups, in spite of substantial increases in total cholesterol concentrations in fenofibrate-treated mice. A combination of phytosterols and fenofibrate results in no significant reduction in atherosclerotic lesion size (20%, $p>0.05$) as compared with control groups. This observation is in agreement with the effects of a combination of phytosterols and fenofibrate on the lipoprotein profile as the addition of phytosterols to fenofibrate failed to synergistically increase HDL-cholesterol levels in apo E-KO mice. Altogether, the results of this study demonstrated that the protective effects of phytosterols have been impaired in combination with fenofibrate. A combination of phytosterols plus fenofibrate paradoxically increases plasma total cholesterol and decreases HDL-cholesterol levels in this animal model. These changes in plasma lipids levels do not significantly increase atherogenesis, suggesting that fenofibrate may have other anti-atherogenic properties independent of its effects on lipid metabolism.

In this study a lack of beneficial lipid-modifying effects and anti-atherogenic properties of fenofibarte in apo E-KO mice was also observed. Fenofibrate substantially increased plasma total cholesterol levels and reduced plasma HDL-cholesterol levels in apo E-KO mice. Interestingly, these changes in plasma lipid profile were not associated with increased

atherogenesis in apo E-KO mice, indicating other potential anti-atherogenic effects of fenofibrate. Taken together, the finding oh this thesis may indicate that lipid-modifying effects of fenofibrate and niacin are species-dependent which may be related to the variations in lipid metabolism (Green and Moghadasian 2004). Further studies warrant the establishment of the mechanisms by which fenofibrate paradoxically change plasma lipoprotein profile in apo E-KO mice.

Another observation from this study is that plasma TG levels seem to have limited effects on atherogenesis in apo E-KO mice. When we correlated plasma total cholesterol levels with the atherosclerotic lesion size, we observed an indirect association between these parameters in phytosterol-treated but not in fenofibrate-treated animals.

This finding is in agreement with previous observations (Moghadasian et al. 1997) in which the authors reported a linear correlation between atherosclerotic lesion size and plasma total cholesterol in phytosterol-treated apo E-KO mice. In the present study, fenofibrate-treated animals had the highest plasma total cholesterol concentrations with an intermediate atherosclerotic lesion size. This suggests that fenofibrate may have other properties that prevent atherosclerosis despite increasing plasma total cholesterol levels.

7.3. Effects of niacin and combination of phytosterols with niacin

Niacin or nicotinic acid is a water soluble vitamin of group B (vitamin B_3) that is synthesized through several enzymatic steps from tryptophan (Ragazzi et al. 2002). It has been widely used in clinical trials either as monotherapy or in combination with other lipid-lowering agents to regulate abnormalities in plasma lipid and lipoprotein metabolism. The use of niacin in the treatment of hyperlipidemia is unique since it improves all lipoprotein abnormalities. Large doses of niacin (1-3 gram/day) have the ability to decrease TG, total cholesterol, and LDL-cholesterol concentrations, while it profoundly increases circulating HDL-cholesterol levels in humans (Rosenson 2003; Chapman et al. 2004).

The effects of niacin on lipoprotein metabolism indicate that niacin has potential anti-atherogenic properties. Despite the reported beneficial effects, our studies in apo E-KO mice showed no benefits of niacin therapy on atherosclerotic lesion development. One reason for this may be a lack of increases in HDL-cholesterol concentrations as these animals are depleted in HDL. An investigation of the effects of niacin on atherogenesis in apo E-KO mice over-expressing human apo AI will address this speculation. It is also possible that niacin failed to reduce apolipoprotein-B containing lipoproteins like LDL and VLDL and/or shift the size of LDL particles from small and dense to larger particles. Further investigation on the effects of niacin on apoprotein B100 and the size of LDL particles will address the questions.

The present data show that addition of niacin to plant sterols does not augment cholesterol-lowering effects of plant sterols, however, it significantly increases HDL-cholesterol concentrations. This increase in HDL-cholesterol levels was associated with significant reductions in atherosclerotic lesion size in phytosterol treared animals. Such effects are expected because numerous animal and human studies have shown anti-atherogenic effects of HDL-cholesterol. It is of interest that the effects of combination of phytosterols and niacin on lipiprotein profile and the size of atherosclerotic lesion in apo E-KO mice are almost similar to effects of phytosterol treatment alone; indicating no synergistic effects between these agents in in apo E-KO mice. Comparison of atherosclerotic lesion size (table 3) indicates that fenofibrate but not niacin may mask anti-atherogenic effects of phytosterols.

7.4. Conclusion

Based on data demonstrated in this study it can be concluded that:

7.4.1. Addition of plant sterols to niacin may beneficially alter plasma lipoprotein profile and consequently affect atherogenesis in apo E-KO mice.

7.4.2. Addition of phytosterols to fenofibrate did not result in reductions in plasma cholesterol level and atherosclerotic lesion size in apo E-KO mice.

7.4.3. A combination of phytosterols plus fenofibrate or niacin do not appear to change the morphology of the liver and kidney tissues.

7.5. Strengths and limitations of the present study

The major strength of the present study is that this research thesis is the first to investigate the interactions between phytosterols and non-statin lipid-lowering agents. Study design, length of the study, appropriate controls and number of samples are among strengths of this study. Another strength of this study is that it is a study related to human health and nutraceuticals. It is belived that research on the effects of nutraceuticals (like phytosterols) in human health and understanding the biochemical and molecular mechanisms by which food affects health in order to validate new food products are important area for research.

One of the limitations of this study is that a histological examination of the liver and kidney was the only test used to examine possible toxicity of combination therapy. Although histology of the liver and kidney provide valuable information through morphology of cells and tissue, it does not give insight into the potential metabolic interactions between phytosterol and niacin or fenofibrate. Measuring activity of enzymes related to liver functions such as alanine and aspartate transaminase (ALT and AST), alkaline phosphatase (ALP) and γ-glutamyl transferase (GGT) would have provided more insight in to the possible toxicity of combination therapy. The fact is that because of limited samples evaluation of activity of these enzymes in the blood was not possible.

Another limitation of this study is that we did not measure animal food intake during the experimental period. The animals in all of the experimental

groups were housed in a group of 3-4 mice per cage. Thus, it was not feasible to measure each animal food intake; however, based on previous observations (Moghadasian et al. 1997, Moghadasian et al. 1999) we estimate that each mouse ingests approximately 5 g of chow per day.

The animal model used may be another limitation of the present study. Apo E-KO mouse is a well-known animal model of hypercholesterolemia and atherosclerosis. Furthermore, anti-atherogenic and cholesterol lowering effects of phytosterols in apo E-KO mice are well documented. In the present study however, apo E-KO mice failed to show significant synergistic effects for phytosterols and TG-lowering drugs both in atherosclerotic lesion and lipoprotein profiles. Further studies in other animal models that are responding to both phytosterols and TG-lowering drugs may provide conclusive data. Despite this limitation, the results of this study led to another study (Declercq et al. 2005) that was conducted in wild type mice to investigate whether apoprotein E is needed for lipid-modifying and anti-atherosclerotic effects of niacin and fenofibrate. This study revealed that fenofibrate does not have cholesterol lowering effects in wild type mice.

7.6. Future studies

This study has demonstrated that no synergistic effects of combination therapy of phytosterols and TG-lowering drugs in apo E-KO mice, however, it could be important to elucidate the potential mechanisms of this effect. Further studies in other animal models that are responding to both fenofibrate and phytosterols may provide conclusive data. To understand the mechanisms by

which fenofibrate paradoxically worsen lipoprotein profiles in apo E-KO mice, it may be beneficial that in future study analysis of the following parameters are considered:

- apoprotein B100 synthesis and secretion.
- the size of LDL particles.
- hepatic enzyme activities and mRNAs for HMG CoA synthase and HMG CoA reductase.
- apoprotein AI senthesis and secretion.
- intestinal cholesterol absorption using labeled cholesterol

This information will produce a new understanding of the mechanism of paradoxical effects of fenofibrate and may lead to the development of a better therapy for the treatment/prevention of atherosclerosis.

Moreover, possible synergistic effects between phytosterols and ezetemibe may be considered.

8. APPENDICES

In the following section the procedures for collecting, fixing, sectioning and staining of the aortic roots for histological and morphometrical evaluations of atherosclerotic lesions have been explained:

8.1. Appendix A: Preparation of hearts and sectioning in aortic roots

1. Prepare a 10 ml syringe and fill the syringe with normal saline. Remove all the air from the syringe. Cap with a 30g needle. The syringe will be used to perfuse the heart.
2. Anesthetize the mouse with a high dose of pentobarbital using 1 ml syringe (60 mg/kg i.p).
3. Determine weight of the mouse.
4. Tape the mouse's arms to 3-4 layers of paper towels and place under a light source. Use the pedal reflex to ensure the mouse is in deep surgical anesthesia.
5. Cut the skin of the mouse from the abdomen to the top of the thorax.
6. Open the abdominal wall below the ribcage.
7. Lift the sternum with tweezers and cut the diaphragm. Then cut away the lower part of the ribcage to partially expose the heart. It is very important to work quickly to have the heart beating while collecting the blood and perfusing.
8. Draw blood from the heart by sticking the needle of the 1 ml syringe into the apex of the right ventricle. Draw blood by slowly pulling the plunger,

twisting the needle. Do not draw for over a minute to avoid blood clots. Transfer the blood into a tube containing EDTA (Becton Dickinson). Mix the tube by inverting it a few times. Keep the sample on ice and centrifuge at 5000 rpm for 15 minutes, transfer the plasma into a fresh tube.

9. Cut the vena cava and then stick the 10 ml syringe into the apex of the left ventricle and flush the blood from the mouse gently and constantly with cold normal saline to clean out residual blood in the arteries.
10. Perform Autopsy and take samples if need (the liver and kidney), put them in 10% buffered formalin.
11. Dry the mouse using paper towels.
12. Carefully cut the right clavicle leaving the brachiocephalic artery and its branches intact. Cut out all of the fat around the ascending aorta and brachiocephalic artery using microdissection scissors. (You can place the mouse under a dissecting microscope for this prosedure)
13. Cut all the fat and tissue surrounding the heart, including the pulmonary artery, and veins.
14. Cut the aorta proximal to the branching site of the brachiocephalic artery.
15. Take out the heart with the aorta and put it in 10% buffered formalin. The atria must remain intact as they serve as landmarks when sectioning aortic roots.

16. Take a cleaned piece of the brachiocephalic artery and place it in a base mold, fill it up to the first lip of the base mold with OCT compound and make sure it fills with OCT. Gently dip mold in cold 2-methylbutane and store in -80°.

Note: All the tissue in formalin will be incubated overnight and then the fluid will be exchanged by PBS.

Histology and morphometry evaluations of atherosclerotic lesions:

Reagents and Materials:

- Buffered Formalin
- Embedding capsules
- OCT
- Razor blades
- Cryosection microtome
- Microscope slides
- Isopropanol
- Oil red-O stain
- Harris Hematoxylin
- Glycerol gelatin
- Image Pro Plus® software
- Microscope with video imaging system

Embedding and sectioning Hearts in OCT for Aortic Root Analysis:

1. Locate the left and right atria. Using a sharp scalpel cut the bottom half of the heart off in a plane parallel to the atria. NOTE: It is essential that

the gross cut is parallel to the atria so that a cross section of all three aortic valves is in the same geometric plane. Discard lower half of the heart.

2. Place the upper cardiac portion into a tissue mold and covered with OCT.
3. The mold is then ready to be placed on sample pedestal of cryostat.

Note: Each mold should be individually wrapped in parafilm and aluminum foil and stored in freezer at -20°.

4. Mount the trimmed heart on a sample pedestal in the cryostat using OCT compound, the apex side of the block facing the pedestal and the aortic side of the block facing you.
5. Set section thickness to 30μm and start sectioning until the part of the block that contains the aorta and atria tissue is visible.
6. Place section on a slide and to check the anatomic location of the section (initially you will see the rounded aorta in the middle surrounded by right and left atrium). (Note: use dissection microscope for better viewing).
7. If interested adjust the thickness to 8 μm and save the sections from aorta.
8. Continue cutting and save every other section until aortic sinus approaches.
9. Prepare 10 slides and label slide and mouse number in order.

10. Start saving the sections when the valve leaflets become clearly visible. (Reposition the pedestal if necessary to have all 3 leaflets of the valve in one plane)

11. Put 6 to 8 sections on one slide in order. (First section on slide no.1 and second section on slide no.2 and third on slide no.3 and so on)

12. Cut and save sections until all of the valve leaflets are gone.

Place slides in a box and store slides at 4°C for at least 12 hours prior to staining.

Quantification of Aortic Root Lesion Area by Image Pro Plus® Software:

1. Use a microscope equipped with a video imaging system attached to a computer with Image Pro software
2. Quantify all slides obtained from aortic root of one group
3. Preferentially use section number 1 on each slide. If section number 1 is not readable, go to the second section of the slide.
4. Measure only the area of oil red-O stained lesions on top of the internal elastic lamina; do not quantify any oil red-O stained material underneath the internal elastic lamina. In some cases, lesions may be sheared off the internal elastic lamina and are found in the lumen. Those lesions should also be counted.
5. The atherosclerotic lesion area is expressed as the mean of the lesion areas of at least three sections per slide from the different parts of aortic root (first, middle and the end).

8.2. Appendix B: Oil red O staining protocol

This procedure was based on the protocols described in Preece A. 1997 and Boston et al. 1991.

Propylene Glycol:
Place in two coplin jars, label #1 and #2, can be reused.

85% Propylene Glycol:

1. Propylene glycol 85.0 ml
2. Distilled water 15.0 ml

Hematoxylin (Harris)

Ready to use

Oil Red O Solution:

1. Oil red O 0.7 gm
2. Propylene glycol 100.0 ml

Dissolve oil red O in propyleneglycol, slowly, while stirring. Heat to 100°C, but not over 110°C, for a few minutes, stirring constantly. Filter through Whatman #2 filter paper. Cool, and filter again through a frittered glass filter of medium porosity with suction. Store at room temperature. Solution is stable for 1 year.

Procedure:

1. Pick-up frozen sections on clean glass slides.
2. Fix slides in 10% formalin if fresh.
3. Wash well with tap water, rinse in distilled, drain off excess water.
4. Propylene glycol, two changes, 5 minutes each.
5. Oil red O, 12 hours, agitate.
6. 85% Propylene glycol, 3 minutes.

7. Rinse in distilled water.

8. Hematoxylin, 20 seconds.

9. Wash in water.

10. Bluing solution, 20 dips, or running tap water.

11. Wash in tap water, rinse in distilled water.

12. Mount with aqueous mounting media, Glycerin Jelly.

8.3. Appendix C: Hematoxylin and Eosin staining protocol

Procedure:

1. Deparaffinize sections, 2 changes of xylene, 10 minutes each.
2. Re-hydrate in 2 changes of absolute alcohol, 5 minutes each.
3. 95% alcohol for 2 minutes and 70% alcohol for 2 miuntes.
4. Wash briefly in distilled water.
5. Stain in Harris hematoxylin solution for 8 minutes.
6. Wash in running tap water for 5 minutes.
7. Differentiate in 1% acid alcohol for 30 seconds.
8. Wash running tap water for 1 minute.
9. Bluing in 0.2% in saturated lithium carbonate solution for 30 seconds to 1 minute.
10. Wash in running tap water for 5 minutes.
11. Rinse in 95% alcohol, 10 dips.
12. Counterstain in eosin-phloxine solution for 30 seconds to 1 minute.

13. Dehydrate through 95% alcohol, 2 changes of absolute alcohol, 5 minutes each.
14. Clear in 2 changes of xylene, 5 minutes each.
15. Mount with xylene based mounting medium.

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