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Combination of dietary phytosterols plus niacin or fenofibrate: effects on lipid profile and atherosclerosis in apo E-KO mice

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Abstract

Patients with mixed dyslipidemias (increased LDL cholesterol and triglyceride as well as low HDL cholesterol levels) benefit from a combination of lipid-modifying drugs such as statins, niacin, fibrates and ezetemibe. However, safety, tolerability and cost are a concern in drug combination therapy. Dietary phytosterols reduce LDL cholesterol, and niacin or fenofibrate primarily reduces triglyceride and increases HDL-cholesterol levels. Thus, we hypothesized that a combination of phytosterols with niacin or fenofibrate will synergistically impact lipoprotein profile and atherogenesis in apo E-KO mice. Phytosterols alone significantly reduced plasma total cholesterol levels (14.1 vs. 16.9 mmol/L, $P<.05$) and the extent of atherosclerosis (0.42 vs. 0.15 mm^2, $P<.05$). The addition of fenofibrate to phytosterols increased plasma total cholesterol levels by >50% (14.1 vs. 21.6 mmol/L, $P<.05$) and decreased HDL-cholesterol concentrations by 50% (0.8 vs. 0.4 mmol/L). These changes were accompanied by slight reductions in the extent of atherosclerosis (0.42 vs. 0.34 mm^2, $P>.05$) as compared to controls, suggesting other potential anti-atherogenic effects of fenofibrate. Unlike fenofibrate, niacin caused an increase of 150% ($P<.05$) in HDL-cholesterol concentrations and a decrease of 22% ($P<.05$) in total cholesterol levels which were associated with significant reductions (65%, $P<.05$) in atherosclerotic lesion size as compared to controls. Neither the addition of niacin nor of fenofibrate reduced plasma triglyceride levels. In conclusion, the addition of niacin to phytosterols synergistically increases HDL-cholesterol levels, while a combination of phytosterols and fenofibrate results in no synergistic effects in apo E-KO mice. Further studies in other animal models are needed to establish synergetic effects between these lipid-modifying dietary and pharmacological agents.

Keywords: Apo E-KO mice; Atherosclerosis; Fenofibrate; Lipoproteins; Niacin; Phytosterols

1. Introduction

Several epidemiological, pathological and clinical studies have shown a significant correlation between increased levels of plasma lipids (cholesterol and triglycerides) and the incidence of coronary artery disease (CHD) in humans [1–3]. A number of animal models have been used to further study the mechanisms of such an association [4,5]. 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 structure to that of cholesterol. There are several types of phytosterols based on their chemical structure. The most abundant phytosterols are beta-sitosterol, campesterol, sitostanol and stigmasterol. Details of chemical structures, sources and biological activities of phytosterols are summarized elsewhere [6].

Humans and animals neither synthesize nor efficiently absorb phytosterols. Phytosterols interfere with cholesterol absorption from the intestine, leading to reductions in plasma cholesterol levels [7,8]. We have shown that phytosterols reduce both plasma cholesterol levels and the extent of atherosclerosis in apo E-KO mice [9–13]. Over the past several years, a number of phytosterol-enriched food

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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 [14]. In particular, cardiovascular patients are more likely to take these supplements than healthy individuals [14]. 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 nonstatin lipid-lowering agents have not been studied. Phytosterols reduce plasma total and low-density lipoprotein (LDL) cholesterol with no significant effect on high-density lipoprotein (HDL) cholesterol or plasma triglyceride levels [15]. On the other hand, nicotinic acid or fenofibrate significantly increases plasma HDL cholesterol and decreases plasma triglyceride concentrations [16,17]. Therefore, phytosterols plus nicotinic acid or fenofibrate may complementarily reduce plasma cholesterol and triglyceride 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 [18–20]. However, safety and cost-effectiveness are a concern for such a drug combination therapy. Regarding safety, four out of 29 hypercholesterolemic patients who received a combination of fenofibrate plus simvastatin developed myalgia [21]. Likewise, two patients from a cohort of 148 diabetic subjects treated with simvastatin plus bezafibrate developed myopathy [22]. 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 concentration is extremely low [12], 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 may be effective and safe. Thus, the aim of the present study was to investigate whether dietary phytosterols and triglyceride-lowering, HDL-cholesterol raising agents, such as nicotinic acid or fenofibrate, synergistically reduce both plasma cholesterol and triglyceride 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. Materials and methods

2.1. Animals and diets

Thirty-one male 4-week-old apo E-KO mice were purchased from Jackson Laboratory and assigned to control ($n=7$), phytosterol-treated ($n=8$), phytosterols+niacin-treated ($n=8$) and phytosterols+fenofibrate-treated ($n=8$) groups matched with their mean body weight and plasma total cholesterol levels as previously published [9,10]. Pico Lab mouse diet was supplemented with 0.2% cholesterol ("base diet") for the control group; this "base diet" was further supplemented with 2% (w/w) soybean-derived phytosterol mixtures containing 58% β-sitosterol, 19% campesterol, 13% dihydrobrassicasterol and 10% stigmasterol for the phytosterol-treated group [9,10,23]. All of the agents (phytosterol mixture, fenofibrate, niacin and cholesterol) were purchased from Sigma-Aldrich Canada, Oakville, Ontario, Canada. 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" and used for combination-treatment groups. The doses of fenofibrate and niacin were estimated based on previous studies [24,25]. The experiments were carried out over 14 weeks. Body weights were recorded weekly and blood samples taken at baseline and 4-week intervals from the jugular vein of lightly anesthetized animals. At sacrifice, the hearts and aortas were removed and fixed in 10% buffered formalin for histological examination [9,10,23]. The Animal Care Committee at the University of Manitoba, Winnipeg, Canada, approved the experiments.

2.2. Lipid analyses

Total cholesterol (TC), triglycerides (TG) and HDL-cholesterol levels were measured at baseline, during and at the end of the study using standard enzymatic methods [9,10,23]. Non-HDL-cholesterol levels (in this animal model the non-HDL cholesterol comprises mainly of β-VLDL and, to a lesser extent, LDL) were calculated by subtraction of HDL-cholesterol levels from TC levels; standard precipitation method was used to prepare HDL fraction [23]. All of lipid measurements were performed in duplicates using internal standard solutions provided by the manufacturer (Thermo DMA, Arlington, TX, USA) as quality control. Our lipid measurements in standard

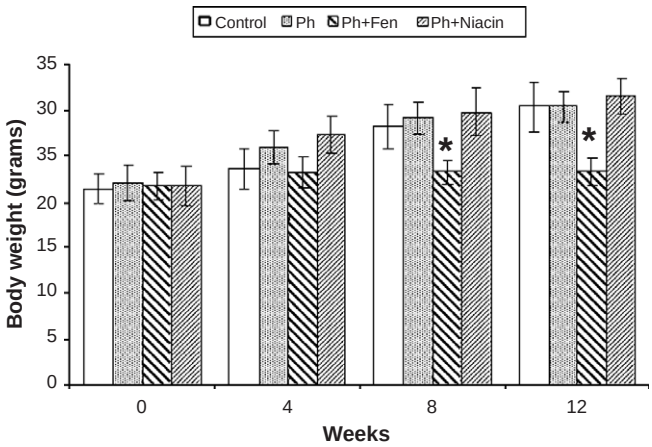

Fig. 1. Body weight measurements (mean±S.D., g) at baseline and during the experimental course in apo E-KO mice treated with phytosterols or a combination of phytosterols plus niacin or fenofibrate. Ph, phytosterol; Fen, fenofibrate.

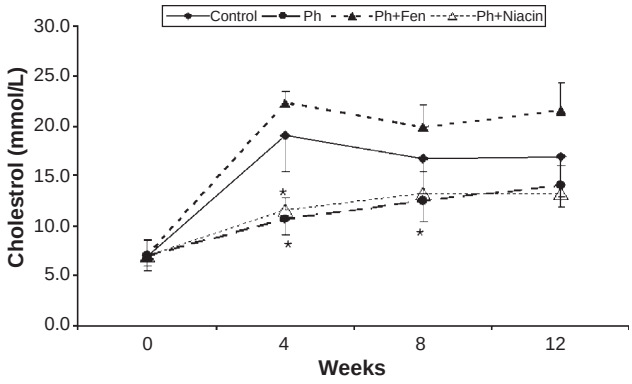

Fig. 2. Plasma total cholesterol (TC) concentrations (mean±S.D., mmol/L) at baseline and during the experimental course in apo E-KO mice treated with phytosterols or a combination of phytosterols plus niacin or fenofibrate. Ph, phytosterol; Fen, fenofibrate. *, $P<.05$ as compared to controls.

solutions always showed values within 94–98% of actual values reported by the manufacturer.

2.3. Histology and morphometry evaluations of atherosclerotic lesions

Sections at the aortic roots were cut and stained with hematoxylin and eosin (H&E) and oil red O (ORO) for histological and morphometrical examinations [9,10,23]. ORO-stained sections were used to estimate atherosclerotic lesion size and lesion-to-lumen ratio using Image Pro-Plus software [9,10,23].

2.4. Statistical analyses

All of the measurements were performed in a blinded fashion. One-way ANOVA analysis followed by the application of the Tukey test was used to test the intergroup differences for significance ($P<.05$). Data are presented as mean and standard deviation.

3. Results

3.1. Body weight

Fig. 1 demonstrates body weight gain in all of four experimental groups over the experimental course. All of the experimental animals gained body weight during the experimental course; however, the extent of body weight gain was lower in the phytosterol+fenofibrate-treated mice. By week 4 of the study, the phytosterol-niacin-treated animals had slightly higher mean body weight as compared to controls or phytosterol+fenofibrate-treated group. The group treated with a combination of phytosterols and fenofibrate had significantly lower body weight at week 8 and week 12 of the study as compared to the other three groups ($P<.005$). These observations indicate that addition of fenofibrate to the diet resulted in smaller body weight gain during the last half of the experimental course. Similar to our previous observations [9,10], the phytosterol-treated apo E-KO mice and the control group had comparable body weight throughout the study (Fig. 1), 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).

3.2. Plasma lipid levels

The levels of plasma total cholesterol (TC) at baseline and during the experimental course have been illustrated in Fig. 2. As is evident, "base diet" significantly increased the levels of total cholesterol in all of the experimental groups, but to a different extent, as compared to baseline data (week 0). By week 4 of the study, the control group had an increase of 172% in TC as compared to baseline value, while it was only 52% in the phytosterol-treated and 66% in phytosterol+niacin-treated groups. In contrast, phytosterol+fenofibrate-treated group had much larger increases (218%) in TC levels at week 4 as compared to baseline values. This suggests that addition of fenofibrate to phytosterol-enriched diet substantially increases plasma cholesterol levels and masks the cholesterol-lowering activity of phytosterols. On the other hand, addition of niacin to phytosterol-enriched diet had no significant effects on the cholesterol-lowering properties of phytosterols in this animal model. The degree of differences between the levels of TC among the four groups of mice slightly decreased during the rest of the experimental period (weeks 8–12). The significant effects of phytosterols and the combination of phytosterols+niacin on TC levels disappeared at week 12 (Fig. 2), most likely due to increases in cholesterol synthesis which develop after long-term phytos-

Table 1
The effects of either monotherapy or combination therapy on plasma triglyceride levels and high-density lipoprotein cholesterol concentrations during the experimental course in apo E-KO mice (data are presented as means and standard deviations)

Groups (n)	Triglycerides (mmol/L)			HDL cholesterol (mmol/L)		
	4 weeks	8 weeks	12 weeks	4 weeks	8 weeks	12 weeks
Control (7)	1.1±0.2	1.7±0.4	1.2±0.4	0.4±0.1	0.6±0.2	0.5±0.1
Phytosterol-treated (8)	1.1±0.2	1.6±0.3	1.4±0.3	0.8±0.1*	0.8±0.1*	0.9±0.1*
Phytosterol+niacin-treated (8)	0.9±0.2	1.4±0.3	1.2±0.3	1.0±0.2*	0.8±0.2*	0.8±0.2*
Phytosterol+fenofibrate-treated (8)	1.1±0.3	1.7±0.4	1.4±0.4	0.4±0.1**	0.4±0.1**	0.4±0.1**

Due to a lack of sufficient samples at baseline, plasma HDL cholesterol and triglyceride concentrations were not measured at week 0 of the study.

* $P<.05$ as compared to controls or to phytosterol+fenofibrate-treated group.

** $P<.05$ as compared to either phytosterol-treated or phytosterol+niacin-treated group.

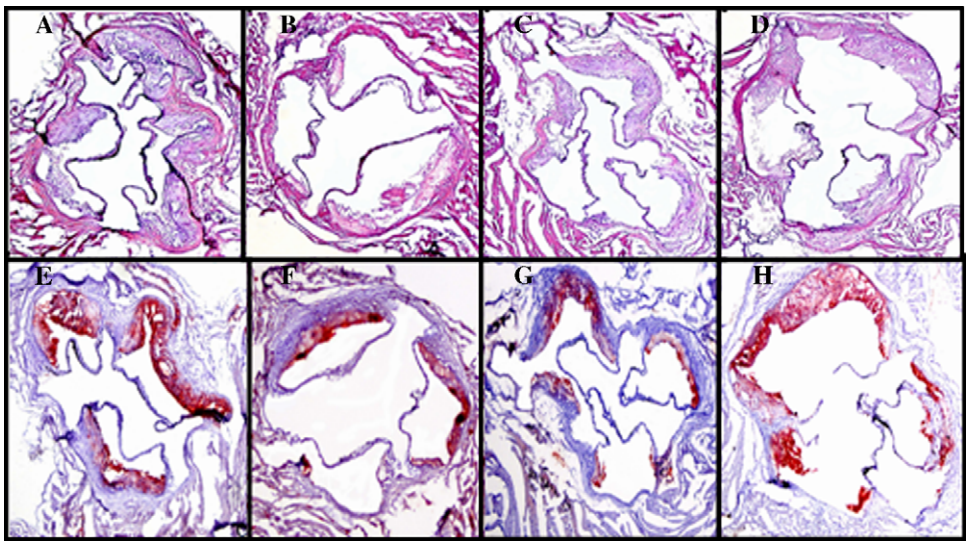

Fig. 3. Representative photomicrographs taken at aortic roots of control (panels A and E), phytosterol-treated (panels B and F), phytosterol+niacin-treated (panels C and G) and phytosterol+fenofibrate-treated (panels D and H) apo E-KO mice. Panels A–D show the cellular and morphological features of the atherosclerotic lesions, while panels E–H demonstrate the extent of lipid deposits in the atherosclerotic lesions. Atherosclerotic lesions from both phytosterol-treated (panels B and F) and phytosteosterol+niacin-treated (panels C and G) are less advanced and less lipid-rich as compared to the atherosclerotic lesions in control (panels A and E) or phytosterol+fenofibrate-treated (panels D and H) mice. (A–D) Hematoxylin and eosin staining; (E–H) oil red O staining.

terol administration [13]. Unlike TC, at week 4 of the study, the HDL-cholesterol levels were significantly 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% ($P<.05$) more HDL cholesterol, while this increase was 150% ($P<.05$) in phytosterol+niacin-treated mice, suggesting a synergistic effect (Table 1). This higher HDL-cholesterol level in phytosterol-treated or phytosterol+niacin-treated animals was also observed during week 8 and week 12 of the study. In contrast, the HDL-cholesterol levels in phytosterol+fenofibrate-treated mice were slightly and consistently lower than those in controls (Table 1). Table 1 also presents the triglyceride levels in all of the experimental groups over the 12 weeks of the study. 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 mice. Data in Fig. 2 and Table 1 suggest that neither niacin nor fenofibrate can further improve the lipid-lowering effects of dietary phytosterols in apo E-KO mice, except for the HDL-cholesterol levels in phytosterol+niacin-treated mice.

3.3. Atherosclerotic lesion development

Representative photomicrographs of aortic roots from the four groups of experimental animals are illustrated in Fig. 3. Oil red O stained sections show the presence of lipid-rich atherosclerotic lesions in the control group as well as treated groups. However, the lesions in the phytosterol-treated (Fig. 3, panel F) and phytosterol+niacin-treated (Fig. 3, panel G) mice are much less lipid-rich as compared with the other two groups of mice (Fig. 3, panels E and H). Hematoxylin and eosin staining (Fig. 3, panels A–D) shows that the atherosclerotic lesions contain a well-developed fibrous cap and sheets of apparently proliferating smooth muscle cells which are more abundant in the control (Fig. 3, panel A) and phytosterol+fenofibrate-treated (Fig. 3, 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 (Fig. 3, panels A, E, D, H). Table 2 summarizes the morphometrical analysis of the atherosclerotic lesions in the aortic roots of the mice. Table 2 demonstrates statistically significantly smaller lesion size in the phytosterol-treated and phytosterol+niacin treated group (0.42 vs. 0.15 mm^2), while atherosclerotic lesion size in the phytosterol+fenofibrate-treated animals was comparable with that in the control group (0.42 vs. 0.34 mm^2) and slightly higher than that in phytosterol-treated mice. This indicates that the addition of fenofibrate

Table 2
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 at the end of the study (data are presented as means and standard deviations)

Groups	Lesion size (mm^2)	Lumen area (mm^2)	Lesion/lumen ratio
Control	0.42±0.14	1.26±0.22	0.32±0.07
Phytosterol	0.15±0.08*	1.19±0.12*	0.13±0.06*
Phytosterol+niacin	0.15±0.06*	0.96±0.27*	0.16±0.05*
Phytosterol+fenofibrate	0.34±0.08	1.16±0.32	0.31±0.09

* $P<.05$ as compared to controls.

to the phytosterol-enriched diet slightly diminished the anti-atherogenic properties of phytosterols in apo E-KO mice.

4. Discussion

Patients with mixed dyslipidemias (increased LDL cholesterol and triglyceride levels plus reduced HDL-cholesterol concentrations) are at higher risk for coronary artery disease. 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 profiles by lifestyle modification, dietary and/or pharmacological agents have been shown to reduce cardiovascular mortality and morbidity. Among several lipid-lowering agents, statins have been shown to significantly reduce LDL-cholesterol levels and still remain at the center of treatment of most dyslipidemias [26–29]. Monotherapy with either niacin or fibrates is an effective strategy in patients with predominantly elevated triglycerides and low HDL-cholesterol levels [30,31]. However, a combination drug therapy 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 triglyceride 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 triglyceride levels as well as increase HDL-cholesterol levels [21,22,32], tolerability and adverse effects of such a combination are major limiting factors. It is now well known that gemfibrozil (fibrate) interferes with the glucuronidation of statins, resulting in severe adverse effects [33]. In addition, the interaction between drugs at the level of cytochrome P450 system may also result in fatal consequences such as rhabdomyolysis, requiring close monitoring of patients on combination therapy [34]. A recent American College of Cardiology/American Heart Association/National Heart, Lung and Blood Institute clinical advisory publication has outlined guidelines for safety managements of statin/fibrate combination therapy [35]. Cost-effectiveness is another concern in drug combination therapy.

A combination of low-dose atorvastatin with ezetimibe (an inhibitor of cholesterol absorption) produced clinical benefits that were similar to those achieved with high doses of atorvastatin in hypercholesterolemic patients [36]. 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 [37]. Dietary phytosterols reduce plasma cholesterol levels by 10%; therefore, they may be of value in patients with moderately elevated plasma cholesterol levels. Because phytosterols do not reduce plasma triglyceride levels, a combination of phytosterols with triglyceride-lowering drugs such as nicotinic acid (niacin) or fenofibrate may result in better prevention of coronary heart disease. Unlike statin–fibrate combination, phytosterol–fibrate or phytosterol–niacin combination is very unlikely to result in serious adverse effects, because the absorption of phytosterols is very limited in humans and animals. Accordingly, we hypothesized that a combination of dietary phytosterols with nicotinic acid or fenofibrate will result in a greater reduction in plasma total and LDL-cholesterol, triglyceride levels and increased HDL-cholesterol levels without major side effects in apo E-KO mice. Our data show that addition of niacin to plant sterols does not augment the 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. Such effects are expected because numerous animal and human studies have shown the anti-atherogenic effects of HDL. On the other hand, addition of fenofibrate to the diet paradoxically increased TC and reduced HDL-cholesterol levels. These effects were associated with slight increases in atherosclerotic lesion size as compared to that in phytosterol-treated animals; these observations again are in agreement with clinical data supporting the pro-atherogenic effects of low HDL and high TC levels. It is of interest that neither niacin nor fenofibrate affected plasma TG levels in this animal model, suggesting that the TG-lowering properties of niacin or fenofibrate may be mediated through apolipoprotein E. 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 TC levels with the atherosclerotic lesion size, we observed an indirect association between these parameters in controls and phytosterol-treated and phytosterol+niacin-treated mice, but not in phytosterol+fenofibrate-treated animals. The latter group of animals had the highest plasma TC concentrations with an intermediate atherosclerotic lesion size. This suggests that fenofibrate may have other properties that prevent atherosclerosis despite increasing plasma TC levels.

In conclusion, this study shows that a combination of niacin with plant sterols reduce plasma total cholesterol and increases HDL-cholesterol concentrations in apo E-KO mice as compared to controls. These changes are associated with significant reductions in atherogenesis. On the other hand, a combination of phytosterols plus fenofibrate paradoxically increases plasma total cholesterol and decreases HDL-cholesterol levels in this animal model. These changes in plasma lipid levels do not significantly increase atherogenesis, suggesting that fenofibrate may have other anti-atherogenic properties independent of its effects on lipid metabolism. Our data failed to show significant synergistic effects for phytosterols and TG

lowering drugs on atherosclerosis and plasma lipids in apo E-KO mice. The reason for this failure may be the animal model used, as these animals are not severely hypertriglyceridemic. Further studies in other animal models that are responding to both fenofibrate and phytosterols may provide conclusive data whether these agents have synergetic effects on lipids and atherosclerosis. This study may suggest that, at least until further documentation, patients who are taking fenofibrate may not additionally benefit from phytosterol-enriched food products.

Acknowledgments

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