Comparative Effects of Phytosterol Oxides and Cholesterol Oxides in Cultured Macrophage-Derived Cell Lines

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The cytotoxicity of cholesterol and a mixture of β-sitosterol/campesterol (50%/40%) and their oxides was examined in a cultured-derived macrophage cell line, C57BL/6. Cell numbers, lactate dehydrogenase (LDH) leakage, protein content, lipid uptake, and mitochondria dehydrogenase activity were determined after exposure of cell mononlayers to sterols and sterol oxides at a concentration of 200 μg/mL for up to 120 h. Results indicate that the oxides of cholesterol, β-sitosterol, and campesterol exhibited similar patterns of toxicity as indicated by LDH leakage, cell viability, and mitochondria dehydrogenase activity. Greatest cell damage was associated with treatments containing 5α,6α-epoxide or cholesterol oxides, followed by β-sitosterol/campesterol oxides, cholesterol, and β-sitosterol. The oxides of β-sitosterol/campesterol caused less LDH leakage and less of an effect on protein content. Results of this study demonstrate that phytosterols contained in vegetable oils, when subjected to frying conditions, do oxidize and may cause cellular damage in an in vitro cell line similar to cholesterol oxides, although less severe.

Keywords: *Phytosterols; cholesterol; sterol oxides; macrophage*

INTRODUCTION

The tendency of cholesterol, the main sterol in animal cells, to oxidize in the presence of heat and air is well-defined (*1*). Several cholesterol oxidation products (COP) have recently been investigated for their possible harmful role in promoting the development of atherosclerosis and cancer along with inhibiting sterol metabolism (*2*−*5*). Some of the major COP identified include 25-hydroxycholesterol, cholestane-3β,5α,6β-triol, cholesterol 5,6-epoxide, 7-ketocholesterol, and 7α- and 7β-hydroxycholesterol (*6*). Several processed foods have been found to contain COP, including dried eggs and egg-containing products (*7*, *8*), milk and milk products (*9*−*11*), meat and meat products (*8*, *12*), and other foods (*7*, *13*).

The switch from animal fats to vegetable oil by many segments of the fast-food industry has led investigators to examine the potential of phytosterols to oxidize in a manner similar to cholesterol. Because many phytosterols contain structures analogous to cholesterol and are currently used in processed and stored foods, investigators have questioned the potential toxicity of phytosterol oxides and their presence in processed and stored foods. Little information is available on the potency of ingested phytosterol oxides.

Lipid-laden macrophage foam cells are an early component of atherosclerotic lesions. Macrophages accumulate cholesterol and triglyceride in cytoplasmic droplets, leading to the characteristic foam cells. Ingestion of low-density lipoproteins (LDL) and foreign particles (i.e., COP, etc.) by macrophages eventually results in the formation of foam cells (*14*, *15*). Any cholesterol and/or COP derived from foam cells can be deposited into the arterial wall, leading to atherosclerosis and subsequently heart disease (*14*). Human foam cells isolated from atherosclerotic plaque contain up to 5% of total sterols and oxysterols, such as 7-ketocholesterol, 7α-hydrocholesterol, and 7β-hydroxycholesterol (*16*). The sensitivity of macrophages to cholesterol accumulation and foam cell formation renders these cells useful for experiments dealing with the mechanisms of oxide cytotoxicity.

Therefore, the objectives of this study were to compare the relative toxicity of selected phytosterols and their derivatives to that of cholesterol and its derivatives using a cultured-derived macrophage cell line. As previous studies have shown that cell damage can be assessed by measuring changes in several cell parameters, including lactate dehydrogenase (LDH) leakage, cell viability, cell protein content, and lipid uptake, these parameters were used as standard indicators of cell damage and cell death (*17*).

MATERIALS AND METHODS

Cell Culture. Mouse macrophage (strain C57BL/6, American Type Culture Collection, Rockville, MD) were grown as monolayers in 100 cm^2 tissue culture flasks (Costar Corp., Cambridge, MA). The cells were grown in RPMI 1640 growth medium supplemented with 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO) in a 5% CO_2, water-saturated solution, and incubated at 37 °C. The medium was changed every second day. The cells were passaged with trypsin in a calcium−magnesium-free phosphate-buffered saline (PBS; Fisher Scientific, Pittsburgh, PA). The cells were plated on six-well (8 cm × 2 cm) tissue culture dishes (Corning Glass Works, Corning, NY) at 1×10^6 cells/mL for toxicity testing. At designated times, the cell cultures were examined by light microscopy. The viability of the macrophages was tested by counting live cells and dead or damaged cells using Trypan Blue exclusion. At 24 h intervals through 120 h, the medium was removed for analysis.

Sterols. Cholest-5-en-3β-ol (cholesterol) and a mixture of β-sitosterol/campesterol (50%/40%) were obtained from Aldrich

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Table 1. Identification and Quantitation of the Oxides Formed from Heated β-Sitosterol/Campesterol and Cholesterol Using GC-MS

compound	mg/mL[a]	retention time (min)
Cholesterol		
5α-cholestane-3α,5,6α-triol	648	19.72
cholest-5-ene-3α,4α-diol	735	20.8
7α-hydroxycholesterol	6471	20.32
25-hydroxycholesterol	5860	21.95
α-epoxide	2201	22.59
7-ketocholesterol	4988	23.75
β-Sitosterol/Campesterol		
7α- or 7β-hydroxycampesterol	576	18.86
7-ketocampesterol	963	19.24
7α-hydroxysitosterol	961	19.43
7β-hydroxysitosterol	961	19.52
5β,6β-epoxycampesterol	1292	19.85
5α,6α-epoxycampesterol	867	20.07
7α-hydroxycampesterol	596	20.30
5β,6β-epoxysitosterol	1233	20.60

[a] Amounts recovered and determined from original oxidized sterol standards using 5α-cholestane as an internal standard.

Chemical Co. (Milwaukee, WI). Additional sterol oxides including 5-cholesten-3β-ol-7-one (7-ketocholesterol) and cholesterol 5α,6α-epoxide were obtained from Sigma Chemical Co. Cholesterol and β-sitosterol were oxidized according to a modification of the Daly et al. procedure (*18*). Ten grams of sterols (cholesterol and β-sitosterol) was heated at 100 °C in separate test tubes on an oxidative stability instrument (Ominion, Rockland, MA) for 100 h using continuous air flow at 13 mL/min and a pressure of 5 psi. The sterols and their oxidation products were dissolved in boiling ethanol (Fischer Scientific, Atlanta, GA) and concentrated under vacuum at room temperature.

Sample Separation. Individual mixtures of oxides were purified following the method of Zubillaga and Maerker (*19*). Briefly, a column of 10 g of silicic acid, 9 g of Celite 545, and 1 g of $CaHPO_4 \cdot 2H_2O$ was prepared in 75 mL of hexane/ethyl acetate (9:1, v/v), and the sterols were eluted with 300 mL of the same solvent. Ethyl acetate (150 mL) was used to elute fraction 2. Fraction 1 was concentrated to 10 mL and reapplied to the column and a similar fraction 2 collected, combined with the first fraction 2, and concentrated to 5 mL. Confirmation of oxides in fraction 2 was conducted using thin-layer chromatography as described Daly et al. (*18*). Identification of phytosterol oxides and cholesterol oxides produced is shown in Table 1.

Parent sterols and their oxides were dissolved in ethanol and added to the culture medium to give a stock solution of 1000 μg/mL and 0.5% ethanol (*20*). The mixture of stock solution was sonicated for a period of 30 s to produce a fine dispersion of the sterols. The diluted sterol concentration of 200 μg/mL was obtained by diluting the stock solution with the medium and used in all experiments. The cells in each well were incubated with 2 mL of sterol-enriched or sterol oxide-enriched medium. Control samples were incubated with cells and ethanol (0.1%) and contained no sterols or oxides.

Lipid Uptake. The ability of sterols and sterol oxides to induce lipid droplet formation was determined with Oil Red O staining. Two days after plating, cells were washed with PBS and incubated with sterols and oxidized sterols. Every 24 through 120 h of exposure of the culture to the treatments, the macrophages were washed with PBS and fixed on slides at room temperature for 1 h with 1 mL of 1% glutaraldehyde (Sigma Chemical Co.). The cells were washed twice with PBS and stained (*21*). A stock solution of Oil Red O was prepared by dissolving 0.7 g of the dye in 200 mL of absolute 2-propanol. The solution was filtered through Whatman No. 1 filter paper, diluted with 180 mL of the staining solution and 120 mL of distilled water, and left overnight at 4 °C. The solution was filtered, allowed to stand for 30 min, and filtered once again to obtain the final dye solution. The fixed cells were stained for 20 min with 2 mL of final dye solution, washed with five 1 mL portions of distilled water, coverslipped, and viewed with a 1000× oil immersion lens under a light microscope (Optiphot 2, Nikon Inc., Melville, NY) equipped with a Cohu 8210 RGB Color CCD camera (San Diego, CA). Assessment of lipid formation was accomplished by observing a random >50 cells/culture dish and examining the proportion of the cytoplasm of each cell for lipid droplets. Two observers assessed each cell, one of whom was not associated with the experiment. Sterol and sterol oxide enriched cells were compared to the control, and all cells were graded on a five-point scale with 1 showing low steatosis and 5 severe droplet formation.

LDH. Changes in LDH activity were quantified by measuring enzymatic activity spectrophotometrically (Sigma Diagnostics, St. Louis, MO) at 24 h intervals through 120 h. Release of intracellular LDH to the medium was expressed in units per liter, with absorbance measured at 340 nm.

Protein Determination. Cellular protein content was determined using the microBCA protein assay (Pierce, Rockford, IL). In this assay, the cells were washed with PBS and lysed with 200 μL of 0.1% Triton X-100 for 30 min at 37 °C. In addition, 200 μL of Bio-Rad reagent was mixed with 10 μL cell samples, plated in 96-well plates, incubated for 1 h, and read at 550 nm on an automated spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH). Protein concentration was calculated by a standard curve of bovine serum albumin (BSA) using a range of 200−1200 μg/mL.

Enzyme Assay. Cell proliferation was measured by the sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) assay. In this assay, XTT is metabolized by mitochondrial dehydrogenase to form a formazan product. The intensity of the yellow color developed is a measure of cell proliferation. Mouse macrophage cells (1×10^4 cell/well) were seeded with 10 μg/mL of parent sterol and oxysterols in a 96-well plate in a total volume of 100 μL of medium. At intervals of 24 h through 120 h, 50 μL of XTT labeling mixture/well was incubated for 4 h at 37 °C and the adsorption measured at 450 nm.

Statistical Analysis. Data were analyzed with a repeated measure analysis of variance. Analysis was performed using a general linear model procedure. The dependent variables measured were cell numbers, protein, LDH, and XTT. Duplicate samples were measured and averaged. The analysis of each dependent variable included two replicate measures for each of the six oxide treatments over six time periods. Tests for significant differences in oxide treatments, changes over time, and interactions with time and oxide treatments were performed using Duncan's multiple-range test for separation of means showing significant differences (*22*).

RESULTS AND DISCUSSION

Figure 1 shows the viability of a cultured-derived macrophage cell line expressed as the number of live or viable cells per milliliter initially and after treatment with sterols and sterol oxides. When observed over the 120 h time, all treatments reduced cell viability, some to a greater degree than others. The control also declined in cell numbers after 72 h. Treatments containing sterol oxides of cholesterol and β-sitosterol/campesterol had the lowest cell numbers at 120 h (<10000/mL). 7-Ketocholesterol, β-sitosterol/campesterol, cholesterol oxides, and 5α,6α-expoxide all demonstrated similar patterns of reduction in numbers and thus reductions in cell viability at 120 h ranging from 20000 to 30000 cells/mL.

The low cell numbers found with treatments containing COP and β-sitosterol/campesterol oxides may be due to the hydroxy compounds (Table 1) identified in the mixtures of oxidized sterols. One hydroxy compound in particular, 5α,6α-dihydroxycholesterol, has been shown to decrease the barrier function of cultured porcine pulmonary artery endothelial cells (*23*). Another oxide,

Table 2. Mean Protein Concentration (Micrograms per Milliliter) of Mouse Macrophages at 0 h (before) to 120 h after Addition of Treatments[a]

treatment	time					
	0 h	24 h	48 h	72 h	96 h	120 h
epoxide	920 bBC	802 bC	1257 bAB	1423 bcA	821 bC	540 cC
β-sitosterol	585 cB	674 bB	817 dB	3663 aA	561 bB	894 bB
β-sitosterol/campesterol oxides	109 0aB	750 bCD	956 cdBC	1997 bA	561 bD	1178 abB
cholesterol	849 bD	1424 aB	1879 aA	1593 bcAB	1034 aCD	1302 aBC
cholesterol oxides	1177 dE	786 bC	1156 bcA	973 cB	525 bD	61 4cD
7-ketocholesterol	981 abC	1370 aB	2059 aA	1506 bcB	1034 aC	975 abC
control	1034 aC	1396 aB	2001 aA	1658 bcB	859 abC	995 abC

[a] Means within a column with common lower case letters are not significantly different at $p < 0.05$. Means within rows with common capital letters are not significantly different at $p < 0.05$.

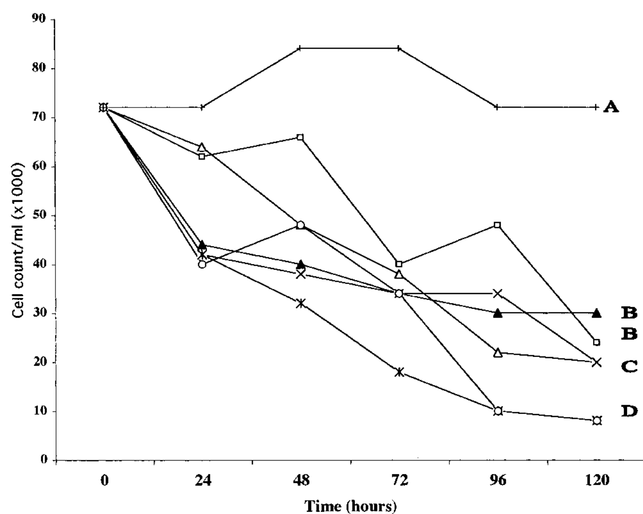

Figure 1. Cell viability expressed as the number of live cells per milliliter (×1000) over a 120 h observation period: control (+); cholesterol (□); cholesterol oxide (∗); 7-ketocholesterol (△); 5α,6α-epoxide (▲); β-sitosterol (×); β-sitosterol oxides (○). Lines followed by A−D were significantly different at $p < 0.05$.

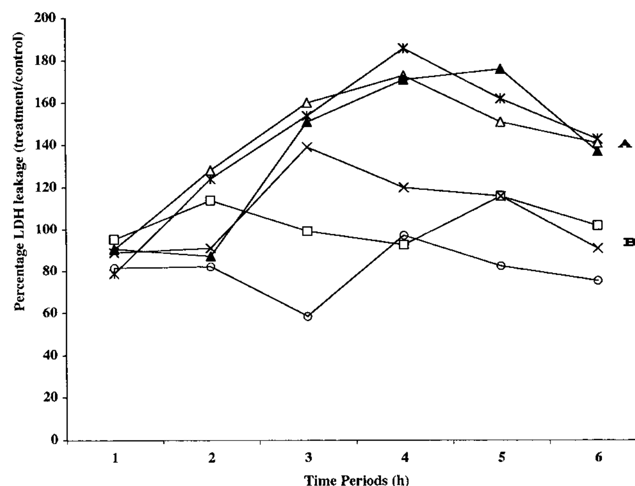

Figure 2. LDH leakage expressed as a percent of control over a 120 h observation period: cholesterol (□); cholesterol oxide (∗); 7-ketocholesterol (△); 5α,6α-epoxide (▲); β-sitosterol (×); β-sitosterol oxides (○). Lines followed by A−D were significantly different at $p < 0.05$.

25-hydroxycholesterol, inhibited the growth of human umbilical vein endothelial cells (*24*). Both of these oxysterols were cytotoxic to cultured rabbit aortic smooth muscle cells (*5*).

Table 2 shows the data of culture-derived macrophage cell protein content after treatment with parent sterols and their oxides (200 μg/mL) for up to 120 h. Protein concentration increased with treatments of epoxide and β-sitosterol up to 72 h and then declined through 120 h. All treatments including the control showed increases in protein content, which reached a peak at 48 or 72 h, followed by subsequent decreases up to 120 h. With the exception of 7-ketocholesterol, all oxides showed significantly lower mean protein content than the parent sterols. Greatest reductions in protein content occurred in treatments containing cholesterol oxide, epoxide, and β-sitosterol oxides. Treatments containing 7-ketocholesterol, cholesterol, and β-sitosterol were not significantly different from the control ($p < 0.05$). The addition of all treatments initially through 48 h resulted in decreased cell numbers or viability and reduced rate of protein production, which might indicate all external insults to the cells negatively impacted cell growth and viability. However, the slower recovery observed by some treatments (e.g., oxides) compared to parent sterols appears to be more than just an adaptation response.

Loss of protein content gives an indicator of reduced protein synthesis. Inhibition of protein synthesis can occur without other signs of toxicity being evident (*25*). Our results indicate that toxicity may be evident from loss of protein content at 120 h as observed in treatments containing β-sitosterol oxide and epoxide as well as decreased rate of protein synthesis observed in other treatments.

LDH leakage is a typical marker of membrane integrity and cell viability in that damaged cells tend to lose LDH more quickly than healthy cells. Treatments containing epoxide, 7-ketocholesterol, and cholesterol oxides increased the release of intracellular LDH by >140% over the control containing only the culture medium (Figure 2). Treatments containing cholesterol, β-sitosterol, and β-sitosterol oxide did not increase LDH leakage significantly over that observed for the control. When cell viability and LDH leakage for parent sterols and their oxides are compared, it appears that the cells adapted and recovered more readily from the insults caused by the parent sterols than their oxides, as noted by both cholesterol and β-sitosterol oxides. Other studies using endothelial cells have shown a similar pattern (*20*). When endothelial cells were incubated with 200 μmol/L cholesterol, no increase in LDH release appeared. However, an oxide of cholesterol, 7α-hydroxycholesterol (75−200 μmol/L), increased the release of LDH into the culture medium by 50−70% compared to their controls.

Other investigators have shown that cytotoxic compounds, such as oxidized sterols, might affect membrane fluidity, stability, and overall functionality (*26*). The present study results indicate that cultured macrophage-derived cell lines were most sensitive to 7-keto-

Table 3. Mean Mitochondrial Dehydrogenase Activity from 24 to 120 h after Addition of Treatments[a]

treatment	time				
	24 h	48 h	72 h	96 h	120 h
5α,6α-epoxide	0.75 bA	0.62 cB	0.52 dC	0.27 cD	0.39 cC
β-sitosterol	0.77 bB	1.32 bA	1.00 cB	0.93 bB1	0.41 bA
β-sitosterol/campesterol oxides	0.27 cBC	0.24 dC	29 eB	0.24 cC	0.37 cA
cholesterol	1.04 aC	1.55 aA	1.25 bB	0.99 bC	1.61 aA
cholesterol oxides	0.22 cC	0.18 dD	0.25 eB	0.18 cB	0.30 cA
7-ketocholesterol	0.35 cA	0.21 dC	0.24 eB	0.25 cB	0.29 cA
control	0.97 aD	1.65 aB	1.72 aA	1.40 aC	1.65 aB

[a] Means within a column with common lower case letters are not significantly different at $p < 0.05$. Means within rows with common capital letters are not significantly different at $p < 0.05$.

cholesterol, cholesterol oxides, and epoxide. Another study conducted in a murine epidermal cell line found that after exposure of cells to 0.1−200 μmol/L of 25-hydroxycholesterol and 26-hydroxycholesterol for 2 and 6 h, LDH leakage was not affected (*17*). They concluded that 25-hydroxycholesterol and 26-hydroxycholesterol appeared to exert protective or stabilizing effects on the cell membrane, resulting in reduced leakage of LDH. In our study, the lowest level of LDH leakage was observed in treatments containing the oxides of β-sitosterol/campesterol and the parent sterol, cholesterol. The β-sitosterol/campesterol oxide treatment appeared to have a stabilizing effect on the cells as no additional LDH leakage occurred and the mean LDH leakage was significantly less than with the control or parent sterols. Cholesterol exhibited a slight protective effect by not promoting any additional increase of LDH leakage. However, as a native constituent of membranes, one would anticipate that the addition of cholesterol would have no effect or a positive effect as observed in the present study. LDH is a classical marker of cell viability and would seem to be an appropriate choice for measuring the effects of selected compounds (i.e., oxides, etc.) that may affect stability and functionality of membranes (*17*).

To confirm results from the viability assay in which the Trypan Blue exclusion assay was used, the XTT assay was performed. The XTT assay is based on the principle that viable cells have a relatively constant amount of mitochondria dehydrogenase. In the presence of the enzyme, a yellow tetrazolium salt is converted to the orange product, formazan, the concentration of which can be measured spectrophotometrically.

As can be seen from Table 3, with the exceptions of 72 and 96 h, the cholesterol treatment was not significantly different ($p < 0.05$) from the control. The β-sitosterol oxide, ketocholesterol, and cholesterol oxide treatments all resulted in statistically significant reductions in cell viability greater than those with the control and the cholesterol treatment (Figure 2). This corresponds to measurement of cell viability using cell numbers as the indicator of viability as observed in Figure 1.

Exposure of cells to sterols and oxides resulted in differences in the uptake of Oil Red O as indicated by the degree of clumping and lipid uptake (Table 4). When compared to the control, β-sitosterol and epoxides took up the least amount of stain and showed the least amount of clumping. Oxides of cholesterol and 7-ketocholeserol exhibited the greatest amount of clumping and lipid uptake. The oxides of β-sitosterol appeared intermediate in lipid uptake, being less than cholesterol and its oxides but greater than controls containing no sterols or sterol oxides.

Our experiments do not allow for elucidation about the actual mechanism of action of the oxidized sterols.

Table 4. Light Microscopically Detected Lipid Uptake of Cultured-Derived Macrophage Cell Line Incubated with Parent Sterols and Their Oxides

treatment	uptake value[a]
5α,6α-epoxide	++
cholesterol	+++
β-sitosterol/campesterol	++
β-sitosterol/campesterol oxides	+++
cholesterol oxides	++++
7-ketocholesterol	++++
control	+

[a] +, no change; ++, light staining; +++, moderate staining; ++++, dark staining.

On the basis of other investigations using different cells, it has been proposed that cell growth and division are necessary for an inhibitory effect to be expressed. However, due to the small growth fraction of macrophage cells in confluent cultures, effects may be seen in <24 h. One such direct effect may be replacement of the parent sterols in cellular membranes by their oxides, resulting in altered membrane functions.

Numerous oxidation products have been isolated from cholesterol and cholesterol-containing foods (*27*−*29*). During frying, fats undergo considerable oxidative changes especially when cholesterol, a constituent of lard and tallow, is used as a frying medium (*27*). Growing interest in utilizing vegetable oils as the frying medium to avoid the presence of cholesterol has occurred. This shift has led to concern over the production of oxides formed during the use of vegetable oils as a heating medium. French fries prepared in a rapeseed oil/palm oil blend, sunflower oil, and high-oleic acid sunflower oil contained total sterol oxides at levels of 2.4, 2.8, and 4.0 ppm, respectively (*28*). Infant milk formulas were investigated for their sterol oxidation products, and 7-ketositosterol was found at <4 μg/g of extracted lipid (*30*, *31*). Although the levels of sterol oxides added in this experiment were higher than levels found in some natural products exposed to processing and storage, they compare very well with other in vitro studies (*15*, *17*, *20*). The present study demonstrates that under frying conditions, phytosterols can oxidize, and when added to an in vitro cell line model system, they cause effects similar to the more toxic cholesterol oxides.

Once oxidized compounds are consumed and absorbed, they may be transported in LDL. In oxidized LDL, a considerable enrichment of oxidized sterol derivatives may occur, leading to a concentration of oxidized sterols in macrophages. The concentration macrophages may be subjected to after ingestion of a meal containing oxidized cholesterol or phytosterol oxides is unknown. However, studies have shown that oxidized materials, including sterol oxides, do accumu-

late as part the plaque-forming material found in arteries and capillaries (*14*, *15*).

In conclusion, these in vitro data demonstrate that oxidized phytosterols inhibit growth in cultured-derived cells. The in vitro approach is able to provide rapid information on the potential toxic action of different compounds that could aid in designing more appropriate in vivo toxicity studies. There are, however, some limitations to the approach used in our study in that only one level of treatments was used and a mixture of β-sitosterol/campesterol oxides was used rather than a single phytosterol oxide. These data show that under frying conditions, phytosterols can oxidize, and when added to a cultured-derived cell model system, they exhibit toxic effects similar to those of cholesterol, although less severe. Results from this study and similar studies show that phytosterols oxidize under food-processing conditions. The results would also suggest that foods high in phytosterols should be monitored for their potential contribution to dietary risk factors associated with the overconsumption of oil-containing foods and their oxidation products.

ABBREVIATIONS USED

COP, cholesterol oxidation products; LDH, lactate dehydrogenase; LDL, low-density lipoprotein; GC-MS, gas chromatography−mass spectrometry.

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