

Absence of Oxysterol-like Side Effects in Human Monocytic Cells Treated with Phytosterols and Oxyphytosterols

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ABSTRACT: Oxysterols, found in some commonly consumed foods, can induce a wide range of cytotoxic effects, which have been extensively studied. On the other hand, the side effects of phytosterols and oxyphytosterols are less well-known. Over the past few years, different types of foods have been enriched with phytosterols on the basis of the properties of these compounds that reduce circulating cholesterol levels in certain experimental conditions. It is therefore important to gain better knowledge of the risks and benefits of this type of diet. In this study, conducted in human monocytic U937 cells, the ability of phytosterols (sitosterol, campesterol) and oxyphytosterols (7 β -hydroxysitosterol, 7-ketositosterol) to induce cell death, polar lipid accumulation, and pro-inflammatory cytokine (MCP-1; IL-8) secretion was determined and compared to that of oxysterols (7-ketocholesterol, 7 β -hydroxycholesterol). Phytosterols and oxyphytosterols had no significant effects on the parameters studied; only 7 β -hydroxysitosterol slightly increased cell death, whereas at the concentration used (20 μ g/mL), strong cytotoxic effects were observed with the oxysterols. With sitosterol, campesterol, and 7-ketositosterol, IL-8 secretion was decreased, and with campesterol the intracellular polar lipid level was reduced. The data show that phytosterols and oxyphytosterols have no oxysterol-like side effects, and they rather argue in favor of phytosterols' beneficial effects.

KEYWORDS: cell death, inflammation, oxysterols, phytosterols, polar lipids, U937 cells

■ INTRODUCTION

Cholesterol and its oxidized derivatives (oxysterols) are 27-carbon-atom cholesterol oxidation products.¹ They can be produced endogenously by enzymatic reactions or autoxidation.² In addition, oxysterols may be absorbed from the diet. Oxysterols are found in many commonly consumed foods: milk powder and infant formulas, clarified butter or *ghee*, red meat (beef, pork), dairy products, eggs and egg products, as well as seafood and seafood products.^{1,3} Some of these oxysterols can induce a wide range of cytotoxic effects: overproduction of reactive oxygen species (ROS) and of pro-inflammatory cytokines, cytoplasmic accumulation of polar lipids (phospholipidosis), and/or activation of cell death (apoptosis associated with a reticulum stress).^{4,5} These events might contribute to the initiation and progression of major age-related diseases: cardiovascular diseases, eye diseases (age-related macular degeneration, cataract, and diabetic retinopathy), neurodegenerative diseases (Alzheimer's disease and dementia of the Alzheimer's type, Parkinson's disease, multiple sclerosis), kidney failure, and osteoporosis.^{2,6–9} The different biological activities of toxic oxysterols, especially for inflammation and cell death, are triggered by metabolic pathways that are interconnected at the mitochondrial level via MEK/ERK1/2 and PI3-K/PDK1/Akt signaling pathways, respectively.^{2,3,10}

Phytosterols are structurally related to cholesterol and are mainly C-28 and C-29 carbon steroid alcohols.¹ The biological activities of phytosterols are less well-known than those of oxysterols. Therefore, a better understanding of the influence of these plant sterols and their oxides on consumer health is needed as the question of benefit versus side effects of these compounds remains highly controversial.^{11,12} Phytosterols, which are not synthesized in mammals, are present in various foodstuffs: oils, spreads, nuts, seeds, and, in lesser quantity, cereals, baked goods, vegetables, and fruits.¹³ Among these phytosterols, β -sitosterol, stigmasterol, and campesterol are the major phytosterols present in the diet.¹⁴ Depending on the storage conditions, phytosterols can be oxidized during heat treatments and long storage.^{15,16} These compounds have been identified in the plasma of healthy human subjects,¹⁷ and their level can be strongly enhanced in some pathological situations: phytosterolemia (characterized by a deficiency in ABCG5 and ABCG8 transporters) or Waldenström's macroglobulinemia.^{18,19} In humans, phytosterol absorption is considerably lower (5%) than that of dietary cholesterol (50%). Indeed, the

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majority of phytosterols are excreted into the intestinal lumen by sterol transporters such as ABCG5 and ABCG8,¹ and it has been reported that phytosterols decrease cholesterol absorption and thus reduce the circulating level of cholesterol.²⁰ Therefore, phytosterols might have some benefits in preventing cardiovascular diseases, and they could also contribute to preventing cancer and inflammatory diseases.^{21–23} These benefits have led to using phytosterols as nutraceuticals. As the functional food market has grown exponentially in recent years, the understanding of the potential health benefits of phytosterol-enriched foods and nutrients is continually evolving, and there is now some evidence that food supplementation with phytosterols may have detrimental health effects.¹² Indeed, regular consumption of foods enriched in phytosterols can affect the absorption of carotenoids and fat-soluble vitamins, and elevated levels of phytosterol can also increase cardiovascular risk.¹²

It is therefore necessary to detail the biological activities of phytosterols and their oxidized derivatives (oxyphytosterols) and to compare them with 7-ketocholesterol and 7 β -hydroxycholesterol, which are potent inducers of cell death, polar lipid accumulation, and cytokine secretion, respectively.²

In the present study, the effects of phytosterols (sitosterol, campesterol) and oxyphytosterols, especially sitosterol oxides (7 β -hydroxysitosterol, 7-ketositosterol), were evaluated on U937 cells, a well-referenced model, to determine the biological activities of these molecules^{2,3} (Table 1).

Table 1. Composition of the Phytosterol and Oxyphytosterol Solutions Used

sitosterol	campesterol	7 β -hydroxysitosterol	7-ketositosterol
97.63% sitosterol	12.64% sitosterol	88.35% 7 β -hydroxysitosterol	83.9% 7-ketositosterol
1.69% campesterol	86.82% campesterol	9.6% 7 β -hydroxycampesterol	10.04% 7-ketocampesterol
0.68% impurity	0.54% impurity	2% impurity	6.06% stigmasta-3,5-dien-7-one

MATERIALS AND METHODS

Chemicals. Propidium iodide, Hoechst 33342, Nile Red (NR), 7-ketocholesterol (7KC), 7 β -hydroxycholesterol, and cholesterol were provided by Sigma (L'Isle d'Abeau Chesnes, France). The purity of oxysterols and cholesterol was determined to be 100% by gaseous phase chromatography coupled with mass spectrometry. Phytosterols and oxyphytosterols were synthesized and provided by Dr. André Grandgirard (INRA, Dijon, France) (Table 1). The planar structures of these different compounds are given in Figure 1. The human monocytic U937 cells used were obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury, U.K.).

Synthesis and Analysis of Phytosterols and Oxyphytosterols. Phytosterols and oxyphytosterols were prepared as previously described and biochemically characterized using gas–liquid chromatography and gas–liquid chromatography coupled to mass spectrometry.²⁴ These compounds were 84–98% pure.

Cell Culture and Treatments. Human promonocytic U937 cells were grown in RPMI 1640 with GlutaMAX I (Invitrogen, Eragny, France) and antibiotics (100 U/mL penicillin, 100 μ g/mL streptomycin; Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Invitrogen). U937 cells were seeded at 500 000 cells/mL culture medium and passaged twice a week. U937 cells were chosen because they have been often used to evaluate the biological activities of oxysterols, phytosterols, and oxyphytosterols.^{8,25} For all experiments, a stock solution of sterols was prepared at a concentration of 800 μ g/mL, as previously described.⁴ In all experiments, phytosterols, oxyphytosterols, cholesterol, and oxysterols were added to the culture medium at the beginning of culture at a final concentration of 20 μ g/mL, and treatments were carried out for 24 h. We chose to work at 20 μ g/mL, for 24 h, with phytosterols and oxyphytosterols for the following reasons: with oxysterols used as positive control (7-ketocholesterol, 7 β -hydroxycholesterol) these conditions are optimal to induce cell death and trigger inflammation and phospholipidosis.^{2,4}

Flow Cytometric Evaluation of Cell Death with Propidium Iodide. Cell viability was determined with propidium iodide (PI) (1 μ g/mL), which stains dead cells only.²⁶ The red fluorescence was quantified by flow cytometry on 10 000 cells with a CyFlow Green flow cytometer (Partec, Münster, Germany) equipped with a green laser emitting at 532 nm. Data were acquired and analyzed using Flowmax software (Partec).

Morphological Characterization of Cell Death by Nuclear Staining with Hoechst 33342. Nuclear morphology was analyzed after nuclear staining with Hoechst 33342 (10 μ g/mL) to distinguish

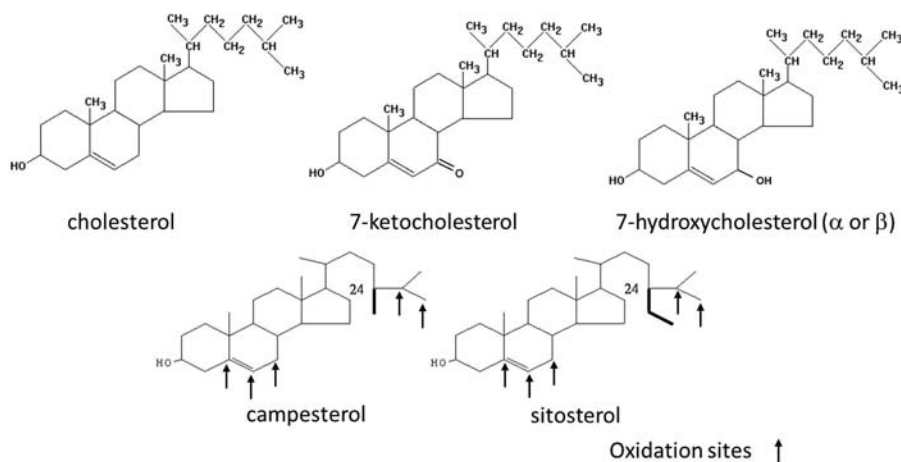


Figure 1. Structures of cholesterol, 7-ketocholesterol, 7-hydroxycholesterol (α or β), campesterol, and sitosterol. Campesterol and sitosterol are synthesized via the mevalonate pathway, leading to the production of the precursor squalene. In this synthesis, different sterol methyltransferases are involved and lead to the synthesis of different components: cholesterol-type side chain, campesterol-type side chain, and sitosterol-type side chain.⁴² The methyl group of the C24 position may exist as either of two epimers, 24 α -methylcholesterol (campesterol) or 24 β -methylcholesterol (dihydrobrassicasterol). (Oxidation sites are indicated by black arrows.)

between normal cells (cells with round and regular nuclei), necrotic cells (cells with disturbed nuclei of various shapes and sizes), apoptotic cells (cells with condensed and/or fragmented nuclei), and oncotic cells (cells with swollen nuclei).^{4,26} Cell deposits (40 000 cells/100 μ L of cell preparation) were observed under ultraviolet light by fluorescence microscopy with an Axioskop right microscope (Zeiss, Jena, Germany). For each sample, at least 300 cells were examined with $\times 63$ oil immersion objective.

Flow Cytometric and Confocal Microscopic Evaluation of Polar Lipid Accumulation by Staining with Nile Red. NR is a phenoxazine dye used on living cells to localize and quantify neutral and polar lipids (Sigma). NR stains neutral lipids yellow (570–590 nm) and polar lipids orange-red (590 nm and above) when excited at 488 nm.²⁷ When excited at 532 nm, NR can identify polar lipids, which are colored orange-red.²⁸ In the present investigation, NR was prepared at 100 μ g/mL in DMSO and used as previously described at 0.1 μ g/mL final concentration.²⁸ Flow cytometric analyses were performed on a Galaxy flow cytometer. For each sample, 10 000 cells were acquired and analyzed with the Flowmax software. For confocal microscopical analyses, 30 μ L of a cellular suspension stained with NR and adjusted at 10^6 cells/mL was applied to glass slides, air-dried, mounted in a fluorescent mounting medium (Dako, Copenhagen, Denmark), coverslipped, and stored in the dark at 4 $^{\circ}$ C before examination.

Measurement of Interleukin-8 and Monocyte Chemoattractant Protein-1 Secretion by ELISA. To measure interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) secretion by ELISA, U937 cells were incubated for 24 h with phytosterols, oxyphytosterols, cholesterol, and oxysterols. At the end of the incubation time, the culture medium was collected by centrifugation and stored at -80° C. Samples were defrosted just before ELISA was carried out according to the manufacturer's procedure (Bender MedSystems, Vienna, Austria).²⁹ Human plasma containing or not IL-8 and MCP-1 was used as positive and negative control, respectively.

Statistical Analysis. Statistical analyses were performed using WinSTAT software (Microsoft). The Mann–Whitney *U* test was used to compare the different groups, and data were considered to be statistically different at a *P* value of ≤ 0.05 .

RESULTS AND DISCUSSION

The effects of phytosterols (sitosterol, campesterol) and oxyphytosterols (7 β -hydroxysitosterol, 7-ketositosterol) (20 μ g/mL) were measured and compared to those induced by 7-ketocholesterol, 7 β -hydroxycholesterol, and cholesterol used at a similar concentration (Table 1). At this concentration and in U937 cells, 7-ketocholesterol induced cell death and intracellular accumulation of polar lipids,²⁸ whereas 7 β -hydroxycholesterol triggered cell death and IL-8 secretion.²⁹ In the phytosterols and oxyphytosterols solutions used, as sitosterol, campesterol, 7 β -hydroxysitosterol, and 7-ketositosterol represent 84 and 98% (Table 1), we can consider that the major compound is responsible for the biological effects.

In the present experimental conditions after 24 h of incubation with phytosterols (sitosterol or campesterol) or oxyphytosterols (7 β -hydroxysitosterol or 7-ketositosterol), the impact of these compounds on cell death was determined compared to those of 7-ketocholesterol, 7 β -hydroxycholesterol, and cholesterol after staining with PI. Interestingly, among the compounds tested, no induction of cell death was observed with sitosterol, campesterol, 7-ketositosterol, or cholesterol, and a very slight but significant induction of cell death was found with 7 β -hydroxysitosterol, whereas 7-ketocholesterol and 7 β -hydroxycholesterol were able to strongly increase cellular permeability to PI (Figure 2). As apoptotic cells are not permeable to PI,²⁶ a morphological examination of the cell nuclei distinguishing between viable, apoptotic, necrotic, and oncotic cells was carried out after staining with Hoechst

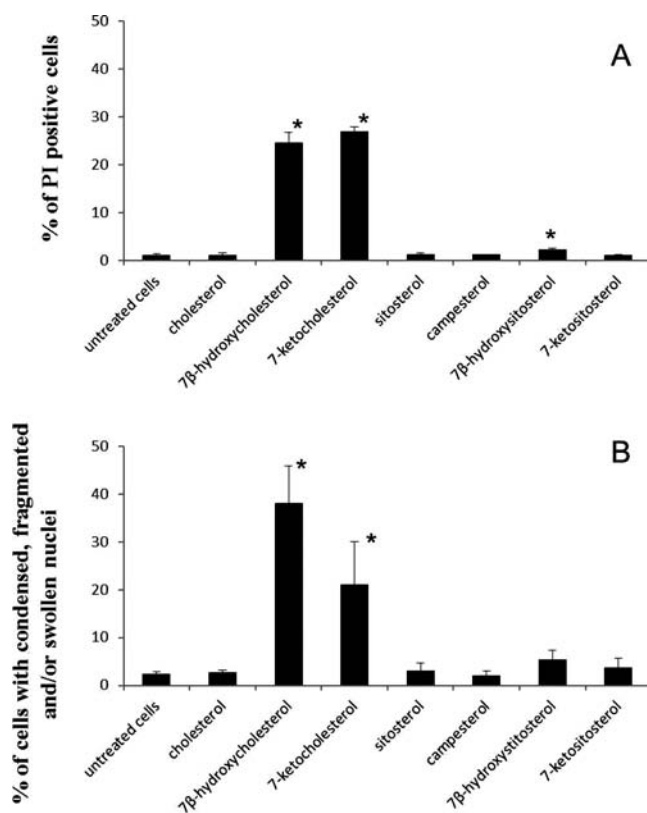


Figure 2. Effects of different sterols (cholesterol, 7 β -hydroxycholesterol, 7-ketocholesterol, sitosterol, campesterol, 7 β -hydroxysitosterol, and 7-ketositosterol) (20 μ g/mL, 24 h) on cell viability of U937 cells: (A) evaluation of the percentage of dead cells after staining with propidium iodide (PI); (B) characterization of cell death after staining with Hoechst 33342. Data represent three individual experiments (mean \pm SE). Mann–Whitney test: comparison of treated versus untreated cells. *, *P* ≤ 0.05 .

33342.²⁶ With phytosterols, oxyphytosterols, and cholesterol, mainly cells with nuclei with regular sizes and shapes, characteristic of living cells, were observed (Figure 2), whereas in agreement with previous data, 7-ketocholesterol and 7 β -hydroxycholesterol induced an apoptotic mode of cell death associated with the presence of oncotic cells.⁸ Thus, no or minor incidence on cell viability was observed under treatment with phytosterols (sitosterol or campesterol) and oxyphytosterols (7 β -hydroxysitosterol or 7-ketositosterol). In agreement with our observations, Maguire et al. reported in U937 cells that β -sitosterol (30 and 60 μ M), α -epoxysitosterol (60 μ M), and β -sitosterol oxides (30 μ M) were unable to induce cell death.³⁰ With β -sitosterol oxides, 60 and 120 μ M concentrations were required to reduce cell viability to 35 and 66%, respectively, and to induce an apoptotic mode of cell death.³⁰ With 7-ketositosterol and 7 β -hydroxysitosterol, concentrations as high as 60 and 120 μ M were also required to induce high percentages of dead cells in U937, Caco-2, and HepG2 cells.³¹ O'Callaghan et al. also demonstrated that stigmasterol oxides were mainly toxic in the highest concentrations tested (60–120 μ M) and were able to induce apoptosis involving glutathione depletion, caspase-3 activation, and Bcl-2 down-regulation.²⁵ In HepG2 cells, Koschutnig et al. demonstrated that 7-ketositosterol was more cytotoxic than 7 α -hydroxysitosterol, whereas 7 β -hydroxysitosterol had no effect.³² Moreover, when the cytotoxicity of cholesterol, of a mixture of β -sitosterol and

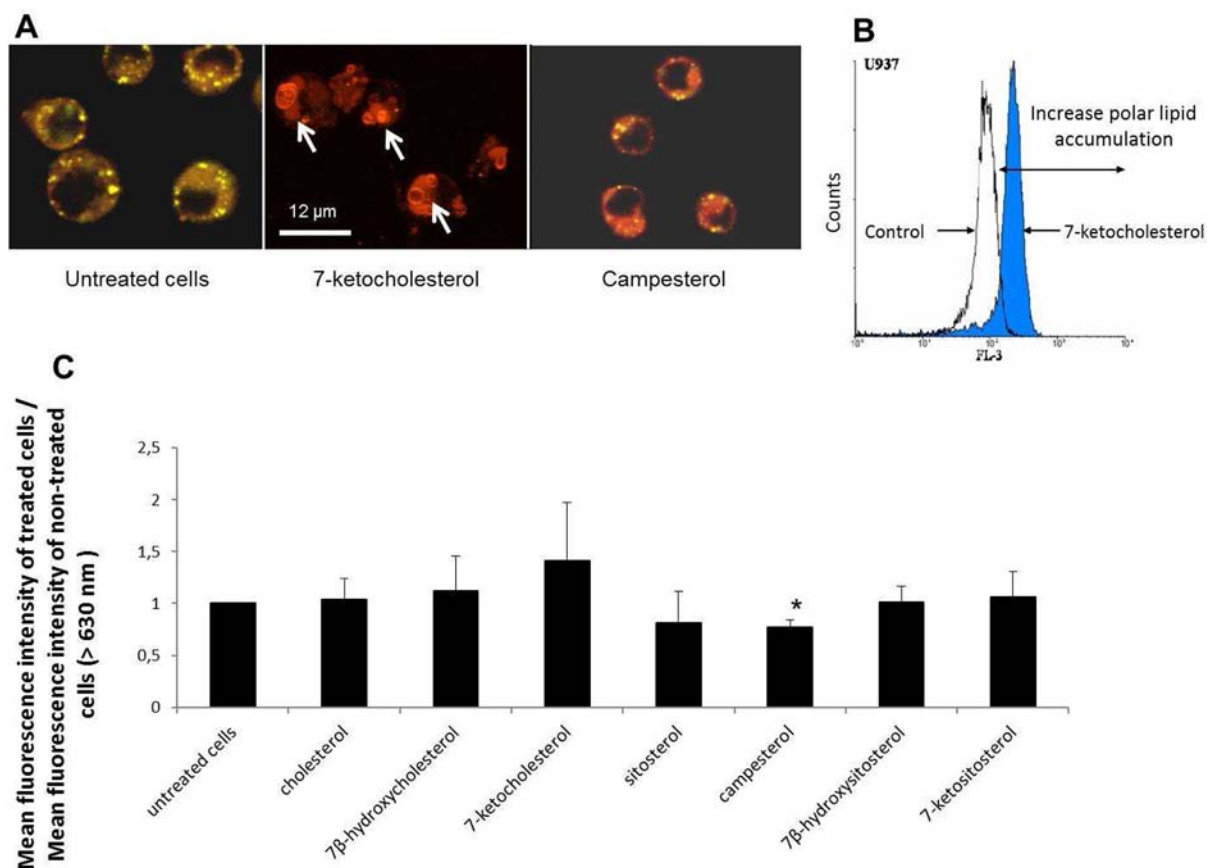


Figure 3. Quantification of polar lipids with Nile Red. U937 cells were treated with cholesterol, 7 β -hydroxycholesterol, 7-ketocholesterol, sitosterol, campesterol, 7 β -hydroxysitosterol, or 7-ketositosterol (20 μ g/mL, 24 h) and then stained with NR. Under a blue light, NR gives a yellow fluorescence with neutral lipids and an orange-red fluorescence with polar lipids. (A) Confocal microscopy of NR stained cells: (left) untreated cells (some yellow spots can be observed); cells with similar aspects were visualized under treatments with phytosterols and oxyphytosterols (data not shown); (right) 7-ketocholesterol-treated cells (large orange-red cytoplasmic structures (white arrows), suggesting phospholipidosis was detected,² cells with similar aspects were observed with 7 β -hydroxycholesterol (data not shown). (B, C) Flow cytometric analysis of polar lipid content after staining with NR. Similar data were obtained when cholesterol, sitosterol, campesterol, 7 β -hydroxysitosterol, and 7-ketositosterol were used at 20 μ g/mL for 24 h. Data represent three individual experiments (mean \pm SE). Mann–Whitney test: comparison of treated versus untreated-cells. *, $P \leq 0.05$.

campesterol (50 and 40%, respectively), and of their oxides (200 μ g/mL for up to 120 h) was examined in a cultured-derived macrophage cell line from C57BL/6 mice, it was demonstrated that these phytosterols and oxyphytosterols may cause cellular damages similar to oxysterols, although less severe.³³ Whereas the toxicity of phytosterols and oxyphytosterols must be considered with caution,^{34–36} results from a previous study and those obtained in the present investigation show that phytosterols and oxyphytosterols are less active than oxysterols and that elevated concentrations of phytosterols or oxyphytosterols, 30–60 times higher than those observed in the plasma of healthy subjects receiving a conventional diet,¹⁹ are required to induce cell death.

Next, we studied the effects of phytosterols, oxyphytosterols, oxysterols, and cholesterol on cellular lipid content. To this end, NR staining was used to visualize both polar and neutral lipids because this dye stains polar lipids orange-red (590–620 nm) and neutral lipids yellow (570–590 nm) when excited at 488 nm²⁸ (Figure 3). Until now, some studies have focused on the effects of phytosterols on circulating cholesterol,^{37,38} but no investigation has been conducted to determine the effect of phytosterols on polar lipids. With sitosterol, campesterol, 7 β -hydroxysitosterol, and 7-ketositosterol as well as under

treatment with cholesterol (used at 20 μ g/mL), the staining pattern obtained by microscopy with NR was similar to untreated cells (Figure 3A). However, under treatment with 7-ketocholesterol and 7 β -hydroxycholesterol used at the same concentration, only a few yellow spots were observed, whereas large orange-red cytoplasmic structures, evoking phospholipidosis, were detected^{2,28} (Figure 3A). This increase of red cytoplasmic structures was confirmed by flow cytometry (Figure 3B). The ratios [orange-red mean fluorescence intensity of treated cells/orange-red mean fluorescence intensity of untreated cells] were also determined by flow cytometry for the different compounds used (Figure 3C): with 7 β -hydroxysitosterol and 7-ketositosterol, this ratio was similar to that in untreated cells; with sitosterol, although not statistically significant, this ratio was decreased; with campesterol, we observed a significant decrease of this ratio, suggesting a reduction of polar lipids. In agreement with previous data, with 7-ketocholesterol and 7 β -hydroxycholesterol a marked increase of the ratio was found.²⁸ Thus, in contrast to 7-ketocholesterol and 7 β -hydroxycholesterol, our data establish that sitosterol, campesterol, 7 β -hydroxysitosterol, and 7-ketositosterol are unable to induce phospholipidosis. Moreover, as it has been proposed that phospholipids might

favor stress response in the cells of the vascular wall, the ability of campesterol and sitosterol to reduce polar lipids may prevent vascular damage.^{39,40}

Because inflammation plays crucial roles in the initiation and development of some diseases, and as oxysterols are known to activate the secretion of pro-inflammatory cytokines,² we also studied the capacity of phytosterols and oxyphytosterols to induce pro-inflammatory cytokine secretion (IL-8, MCP-1) on U937 cells. When compared to 7 β -hydroxycholesterol, which is a strong inducer of IL-8 secretion in U937 cells,^{4,29} treatment with sitosterol, campesterol, and 7-ketositosterol induced either a slight nonsignificant or significant decrease in IL-8 secretion (Table 2; *P* values for sitosterol, campesterol, and 7-

Table 2. IL-8 and MCP-1 Secretion in Untreated U937 Cells and in U937 Cells Treated with Cholesterol, 7 β -Hydroxycholesterol, 7-Ketocholesterol, Sitosterol, Campesterol, 7 β -Hydroxysitosterol, or 7-Ketositosterol^a

treatment	MCP-1 (pg/mL)	IL-8 (pg/mL)
control	1100.8 \pm 122	87.7 \pm 11
cholesterol	1065 \pm 107	82.7 \pm 1
7 β -hydroxycholesterol	351.2 \pm 23*	491.8 \pm 1*
7-ketocholesterol	607.4 \pm 19*	91.9 \pm 1
sitosterol	1068.3 \pm 92	76.9 \pm 1
campesterol	1099.9 \pm 6	77.7 \pm 1
7 β -hydroxysitosterol	945.3 \pm 193	95.3 \pm 1
7-ketositosterol	1091.2 \pm 80	74.4 \pm 1*

^aData represent three individual experiments (mean \pm SE). Mann–Whitney test: comparison of treated versus untreated cells. *, *P* \leq 0.05. U937 (500 000 cells/mL) treatments: cholesterol, oxysterols, or phytosterols (20 μ g/mL; 24 h).

ketositosterol = 0.121, 0.376, and 0.049, respectively). IL-8 concentration in the culture medium of cholesterol-, 7-ketocholesterol-, and 7 β -hydroxysitosterol-treated cells was in the same range as in the culture medium of untreated cells (Table 2). When the effects of these different compounds were evaluated on the secretion of MCP-1, no effects were observed with cholesterol, sitosterol, campesterol, 7 β -hydroxysitosterol, and 7-ketositosterol, whereas marked decreases were observed with 7 β -hydroxycholesterol and 7-ketocholesterol. As these oxysterols are potent inducers of cell death, it can be supposed that this inhibition of MCP-1 secretion is related to cell death induction. In agreement with our data, potential anti-inflammatory activities of phytosterols and oxyphytosterols have been reported. In previous studies, the effect of phytosterols and oxyphytosterols on cytokine secretion was determined on a mouse model of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation. In this model, the oxygenated stigmastane-type sterols stigmastane-3 β ,6 α -diol, stigmastane-3 β ,6 β -diol, and their diacyl derivatives were able to inhibit TPA-induced inflammation in mice.⁴¹ More recently, Calpe-Berdiel et al. demonstrated that phytosterol intake was able to modulate immune response.⁴² A study conducted by Kurano et al. describes lower levels of pro-inflammatory cytokine secretion (TNF- α , IL-6) in macrophages treated with phytosterols (sitosterol, campesterol).⁴³ Using experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis (MS), Valerio et al. demonstrated reduced inflammatory activities of immune cells (increased IL-10 level and increased CCL2, TNF- α , IFN- γ , and IL-6 levels) in the central nervous system of the mice receiving a daily phytosterol

emulsion (β -sitosterol (60%), campesterol (25%), and stigmasterol (15%)) via gavage.²³ Altogether, our results support that certain phytosterols and oxyphytosterols can modulate immune response.

In conclusion, in U937 cells, phytosterols (sitosterol, campesterol) and oxyphytosterols (7-ketositosterol, 7 β -hydroxysitosterol) do not induce cell death, do not favor polar lipid accumulation, and do not stimulate IL-8 secretion (a slight decrease was even observed), which are major oxysterol side effects.^{2,3} They also do not modulate MCP-1 secretion. Although the in vivo toxicity of phytosterols (sitosterol, campesterol) and oxyphytosterols (7-ketositosterol, 7 β -hydroxysitosterol) studied is not excluded (under conditions of regular consumption of processed food enriched with these compounds¹²), the present investigation shows the absence of toxicity of these compounds at a concentration (20 μ g/mL) known to induce strong cytotoxic effects with 7-ketocholesterol and 7 β -hydroxycholesterol. This study suggests that it would be important to reconsider the analysis of cholesterol derivatives (oxysterols) and cholesterol structurally related products (such as phytosterols) in foods. Indeed, as 7-ketocholesterol and 7 β -hydroxycholesterol,^{9,44,45} can be present in large quantities in the diet, it is important to take care of not only phytosterol and oxyphytosterol content but also oxysterol content. This could be significant in terms of public health given the involvement of oxysterols in numerous widespread age-related diseases such as cardiovascular and neurodegenerative diseases.

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Notes

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