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Summary

Background and aims: Oxysterols are assumed to play important roles in age-related macular degeneration (ARMD), a major cause of blindness. So, we characterized the cytotoxic, oxidative, inflammatory, and angiogenic activities of oxysterols (7β-hydroxycholesterol (7β-OH), 7-ketocholesterol (7KC), 25-hydroxycholesterol (25-OH)) in human retinal ARPE-19 cells, and evaluated the protective effects of resveratrol (Rsv: 1 µM), a polyphenol from red wine.

Methods: ARPE-19 cells were treated with 7β-OH, 7KC, or 25-OH (5–40 µg/mL; 24–48 h) without or with Rsv. Cell viability was determined using trypan blue and the MTT assay. Cell death was characterized by electron microscopy and in situ detection of activated caspases with fluorochrome-labeled inhibitors of caspases (FLICA). Reactive oxygen species (ROS) production was measured with hydroethidine. ELISA methods and a cytometric bead assay were used to quantify cytokines involved in inflammation (IL-8, IL-1β, IL-6, IL-10, IL-12p70, TNF-α, MCP-1) and VEGF.

Results: 7β-OH and 7KC triggered a caspase-independent cell death process associated with the presence of multilamellar cytoplasmic structures evocating phospholipidosis, increased ROS production, and IL-8 secretion. 7β-OH enhanced VEGF secretion. No cytotoxic effects were identified with 25-OH which highly stimulated ROS production, MCP-1, and VEGF secretion. With oxysterols, no IL-10, TNF-α, and IL-12p70 secretion were detected. 25-OH induced IL-8 secretion through the MEK/ERK½ signaling pathway, and Rsv showed cytoprotective activities and inhibited VEGF secretion.

Conclusion: 7β-OH, 7KC, and 25-OH have cytotoxic, oxidative, inflammatory, and/or angiogenic activities on ARPE-19 cells. As Rsv has some protective effects against oxysterol-induced cell death and VEGF secretion it could be valuable in ARMD treatment.

Introduction

Age-related macular degeneration (ARMD) is the leading cause of blindness in the elderly population in developed countries [1]. Since abnormal focal and diffuse lipid deposits containing high amounts of cholesterol are localized between retinal pigment epithelium (RPE) and Bruch's membrane (BrM), the pathogenesis of ARMD might have some similarities with atherosclerosis [2]. Indeed, lesions called drusens contain extracellular lipids involving esterified and unesterified cholesterol [3]. The origin of cholesterol in drusen remains unclear, and the study of lipid composition of the deposits in BrM have not shown exact similarity with plasma lipoprotein composition. As the retina is continuously exposed to different forms of physical and chemical oxidative stress, these pro-oxidative environmental conditions can favor the spontaneous cholesterol oxidation in oxysterols [4,5]. Consequently, by analogy with atherosclerosis, oxysterols could interfere with the genesis of cytotoxic, pro-inflammatory, pro-oxidative, and pro-angiogenic activities responsible for ARMD lesions [6,7].

This hypothesis is supported by clinical observations and in vitro investigations. Therefore, the involvement of reactive oxygen species (ROS) is widely suspected in ARMD [8], and the analysis of postmortem eyes of patients with ARMD suggested that retinal pigment epithelial cells, photoreceptors, and inner nuclear layer cells die by apoptosis [9]. In agreement with these observations, investigations conducted with various oxysterols, including 7β-hydroxycholesterol (7β-OH), 7-ketocholesterol (7KC), and 25-hydroxycholesterol (25-OH), on human retinal cell lines (R28, ARPE-19) [10, 11], PC-12 cells derived from rat neuroretina [12], as well as primary porcine retinal pigment epithelial cells [13] brought out the cytotoxic effects of 7KC and 7β-OH as well as a 25-OH pro-inflammatory activity. On 7KC-treated ARPE-19 cells, it has also been demonstrated that the mode of cell death observed had some of the characteristics of apoptosis [14]. However, on 7β-OH-treated ARPE-19 cells, a caspase-independent mode of cell death associated with lysosomal destabilization was observed [7]. Moreover, the contribution of oxysterols to the development of ARMD is suggested by the presence of 7KC in lipid deposits from primate retina [15] and by the ability of RPE cells to internalize low-density lipoproteins (LDL) and oxidized LDL (oxLDL) in large quantities [16]. The expression of LDL receptors and the scavenger receptor CD36 in pigmentary epithelial cells in rat eye histological sections has also been observed [16]. It was also reported that the cytotoxicity of oxLDL on cultured RPE cells was dependent on the formation of 7KC [11]. In addition, under treatment with oxLDL or in the presence of 7β-OH, 7KC, and 25-OH, severe side effects such as induction of cell death, stimulation of ROS production, enhanced secretion of inflammatory cytokines (IL-1β), chemokines (IL-8, MCP-1, MIP-1β, and/or TNF-α), and/or VEGF have been identified on different cell types [17-20]. Based on these different investigations, it is therefore important to determine the biological activities of

7β-OH, 7KC, and 25-OH on human retinal cells in order to precise whether these oxysterols might contribute to the development of ARMD.

Although recent advances have been made to treat the neovascular form of ARMD, there is still no effective and convenient treatment. Resveratrol, a polyphenol from red wine, has a wide range of pharmacological properties, with anti-oxidant, anti-inflammatory, and cytoprotective properties [21-23]. Moreover, this polyphenol induced significant reductions in the amount of VEGF secreted into the supernatant of cultured endometrial cancer cells [24]. So, we attempted to determine whether it was able to counteract some of oxysterol's noxious effects: oxysterol-induced cell death, pro-inflammatory cytokines, and VEGF-secretion.

Therefore, the present study consisted to determine the cytotoxic, oxidative, inflammatory, and angiogenic effects of 7β-OH, 7KC, and 25-OH in cultured human retinal pigment epithelial cells (ARPE-19) in order to give scientific evidence on the biological activities of these compounds, on the metabolic pathways activated in inflammation, and on the protective effects of Rsv on oxysterol-induced side effects.

Materials and methods

Cell culture

ARPE-19 cells (American Type Culture Collection, Manassas, VA, USA) were grown in DMEM/F12 medium (Gibco) containing 10% heat-inactivated fetal calf serum, antibiotics (100 IU/mL penicillin, 100 µg/mL streptomycin (Gibco, Eragny, France)), and 1 mM sodium pyruvate (Gibco). The cells were seeded at $25–32 \times 10^3/cm^2$ in 75-cm² tissue culture flasks containing 13 mL of culture medium. They were incubated at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium was changed every 2 days. The cells were passaged once a week by trypsinization (0.05% trypsin, 0.02% EDTA) (Gibco). In all experiments, cells were used between passages 5 to 15.

Cell treatments

The purity of oxysterols (7β-hydroxycholesterol (7β-OH) (Steraloids, Newport, RI, USA), 7-ketocholesterol (7KC) (Sigma, Saint Louis, MO, USA), and 25-hydroxycholesterol (25-OH) (Sigma) was determined to be around 100% using gas chromatography coupled with mass spectrometry. Initial solutions of 7β-OH, 7KC, and 25-OH were prepared at 800 µg/mL in culture medium containing 2% ethanol as previously described [19, 20]. Resveratrol (Rsv) was from Sigma; the initial solution was prepared at 1 mM in absolute ethanol. All treatments were added on confluent cells previously cultured for 3–4 days. Oxysterol effects were assessed with 10, 20, 30,

and/or 40 µg/mL (25, 50, 75, and 100 µM, respectively). When ARPE-19 cells were simultaneously treated with 1 µM Rsv (highest concentration tested without toxicity), the oxysterols were used either at 20 or 30 µg/mL. In these conditions, Rsv and oxysterols were simultaneously added on confluent cells previously cultured for 3–4 days. In all conditions, the treatment times were 24 and 40 h, and the final concentration of ethanol was either 0.1 or 0.2% (At these concentrations, no effects on the different parameters studied were observed (our data not shown)). Di-phenylene iodonium (DPI), an inhibitor of NAD(P)H-oxidase [25], was from Sigma, and used at 10 µM. PD98059 and U0126 (Tebu-Biomol), two potent inhibitors of the MEK/ERK$^{1/2}$ signaling pathway [26] were used at 10 and 20 µM, respectively. These different compounds were added 30 min before oxysterol treatments.

Cell counting

The number of viable cells was determined with trypan blue. The cell suspension was mixed with trypan blue (volume to volume), and the number of cells was determined under a light microscope using a hematocytometer.

Transmission electron microscopy

Transmission electron microscopy was performed on ARPE-19 cells cultured for 48 h in the absence or presence of 7β-OH, and 7KC (20 µg/mL) as previously described [7]. The observations were realized with an H7500 electron microscope (Hitachi, Tokyo, Japan).

Measurement of mitochondrial activity by quantification of mitochondrial dehydrogenase activity with MTT

The mitochondrial activity was measured with an MTT-based assay on ARPE-19 cells seeded in 96-well plates at 5×10^3 cells/well. This colorimetric method, based on the ability of the cells to reduce a tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoilum bromide, and to form a blue formazan product by a mitochondrial enzyme, succinate dehydrogenase, was performed as previously described [7].

Detection of activated caspases with fluorochrome-labeled inhibitors of caspases (FLICA)

In situ detection of all active caspases was quantified on a GALAXY flow cytometer (Partec, Münster, Germany) with fam-VAD-fmk (Trevigen Inc, Gaithersburg, MD, USA) on ARPE-19 cells cultured in the absence or presence of 7β-OH or 7KC (20 µg/mL) for 48 h as previously described [7]. FAM-VAD-FMK is a carboxyfluorescein (FAM) derivative of benzyloxycarbonyl-valine-alanine-aspartic acid-fluoromethyl ketone (zVAD-FMK), which is a potent broad-spectrum inhibitor of caspases. FAM-VAD-FMK enters the cell and

irreversibly binds to activated caspases. Thus, cells containing bound FAM-VAD-FMK can be analyzed by flow cytometry,

Flow cytometric measurement of reactive oxygen species with hydroethidine

The production of reactive oxygen species (ROS) was determined by flow cytometry on a GALAXY flow cytometer (Partec) on confluent ARPE-19 cells cultured for 24 and 40 h in the absence or presence of 20 and 30 µg/mL of 7β-OH, 7KC, or 25-OH after staining with hydroethidine (HE) as previously described [19].

Measurement of IL-8, MCP-1, and VEGF secretion by ELISA

Interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), and vascular endothelial growth factor A (VEGF-A) secretion were measured in the culture medium of confluent ARPE-19 cells treated for 24 and 40 h in the absence or presence of 20 and 30 µg/mL of oxysterols associated or not with Rsv (1 µM). The samples were kept frozen at −80°C until analysis by ELISA according to the manufacturer's procedures (IL-8 and VEGF-A human module sets, Bender MedSystems™; human MCP-1 ELISA development kit, Peprotech).

Flow cytometric quantification of cytokine secretion with the Cytometric Bead Array (CBA)

The production of inflammatory cytokines was investigated in the culture medium of ARPE-19 cells untreated or treated for 24 and 48 h with the different oxysterols (30 µg/mL) using a flow cytometric bead-based assay. Culture medium of untreated or treated cells was collected by centrifugation and stored at −80° C. Samples were defrosted and centrifuged immediately before cytokine analysis. IL-8, IL-1β, IL-6, IL-10, TNF-α□ and IL-12P70 were quantified using the Cytometric Bead Array Human Inflammation kit (BD Biosciences, San Diego, CA, USA) according to the manufacturers' instructions as previously described [20].

Statistical Analysis

Statistical analyses were performed on at least three independent experiments with Prism 5 software, version 5.01 (Prism Software Corporation, Irvine, CA, USA) with the Mann and Whitney test and the Kruskal-Wallis test when appropriate. Data were considered to be statistically different at a *p*-value of 0.05 or less.

Results

Effects of 7β-hydroxycholesterol, 7-ketocholesterol, and 25-hydroxycholesterol associated or not with resveratrol on cell growth and viability of ARPE-19 cells

The effects of 7β-OH, 7KC, and 25-OH used at various concentrations (10, 20, 30, and/or 40 μg/mL) on cell growth and viability of ARPE-19 cells was assessed at 24 and 40 h (Fig. 1). No cytotoxic effects were found under treatment with 25-OH (Fig. 1 A, B). In the presence of 7β-OH and 7KC, a marked inhibition of cell growth was observed (Fig. 1A). As determined from concentration-dependent curves, the mean concentrations required to reduce the number of living cells by 50% was as follows: >40 μg/mL at 24 and 40 h with 25-OH; 30 μg/mL at 24 h and 25 μg/mL at 40 h with 7KC; 25 μg/mL at 24 h and 20 μg/mL at 40 h with 7β-OH. When ARPE-19 cells were cultured (24-40 h) with 7β-OH and 7KC (20-30 μg/mL), which are within the range of concentrations associated with pronounced inhibition of cell growth, a significant decrease in mitochondrial activities was observed at all treatment times considered, especially at 30 μg/mL (Fig. 1B). Interestingly, when 7β-OH- and 7KC-treated ARPE-19 cells (30 μg/mL) were simultaneously cultured with Rsv (1 μM), the total number of viable cells was significantly higher than under treatment with 7β-OH and 7KC only (Fig. 1C); however, it was lower than the number of untreated cells.

Effects of 7β-hydroxycholesterol and 7-ketocholesterol on the induction of multilamellar cytoplasmic structure formation in ARPE-19 cells

Based on ultrastructural morphological criteria, around 10% of cells with condensed and/or fragmented nuclei, characteristic of apoptotic cells, were detected by transmission electron microscopy at 48 h of culture in 7β-OH- and 7KC (20 μg/mL)-treated cells (Fig. 2A-C). These observations were supported by FLICA analysis, which was chosen to quantify apoptotic cells by identifying the presence of activated caspases (untreated cells: 6 ± 2% FLICA+ cells; 7β-OH: 13 ± 3% FLICA+ cells; 7KC: 9 ± 2% FLICA+ cells). These data support that 7β-OH and 7KC induced on ARPE-19 cells a caspase-independent death style different from apoptosis. As we previously reported on several cell types that 7β-OH and 7KC were able to induce multilamellar cytoplasmic structure formation, considered to be ultrastructural features of phospholipidosis [27], some observations were made by electron microscopy on untreated as well as on 7β-OH- and 7KC-treated ARPE-19 cells taken at 48 h of culture to determine the absence or the presence of multilamellar structures (Fig. 2). Whereas these structures were not observed in untreated cells, these ultrastructural features, which have various sizes and shapes and can be connected with the endoplasmic reticulum, were frequently found in ARPE-19 cells treated with 7β-OH and 7KC (20 μg/mL) (Fig. 2D–G).

Effects of 7β-hydroxycholesterol, 7-ketocholesterol, and 25-hydroxycholesterol on the oxidative status of ARPE-19 cells

The production of ROS was measured with HE on ARPE-19 cells cultured for 24 and 40 h in the absence or presence of 7β-OH, 7KC, and 25-OH at the concentrations of 20 and 30 μg/mL (Fig. 3). The intracellular production of ROS was quantified by flow cytometry using the mean intensity of fluorescence (MIF). When compared to untreated cells, ROS production increased at 24-40 h with 7β-OH and 25-OH (20 μg/mL); 7KC had no effect at this concentration. However, when used at 30 μg/mL, the three oxysterols increased ROS production both at 24 and 40 h. Interestingly, when 25-OH-treated ARPE-19 cells (20-30 μg/mL) were simultaneously treated with DPI (10 μM), a potent inhibitor of NAD(P)H oxidase, which has no cytotoxic effects at this concentration, the overproduction of ROS in (25-OH + DPI)-treated cells evaluated by the MIF of HE positive cells was decreased by about 50% compared to 25-OH-treated cells, within the range of untreated cells (our data not shown).

Effects of 7β-hydroxycholesterol, 7-ketocholesterol, and 25-hydroxycholesterol associated or not with resveratrol on cytokines (IL-8, IL-1β, IL-6, IL-10, TNF-α, IL-12p70, MCP-1), and VEGF secretion

IL-8, MCP-1, and VEGF secretions were measured by ELISA in the culture media of ARPE-19 cells maintained for 24 and 40 h in the absence or presence of 7β-OH, 7KC, or 25-OH (20 and 30 μg/mL) (Fig. 4).

At 24 h (Fig. 4 A,C,E), with 7KC 20 μg/mL, when compared to untreated cells, no increase in IL-8 and VEGF secretion was identified. However, at 30 μg/mL, a significant decrease in IL-8 secretion was observed, and no effect on MCP-1 secretion was found, whereas a decrease in MCP-1 secretion was identified at 20 μg/mL. With 7β-OH, similar effects to those observed with 7KC were found concerning IL-8 secretion. MCP-1 secretion was either similar (20 μg/mL) or lower (30 μg/mL) than in untreated cells. With 25-OH, at both concentrations, IL-8 and MCP-1 secretions were sharply increased; an enhancement of VEGF secretion was only identified at 30 μg/mL.

At 40 h (Fig. 4 B,D,F), 25-OH at both concentrations induces IL-8, MCP-1 and VEGF secretion. 7β-OH at 30 μg/mL significantly decreased IL-8 and MCP-1 secretions whereas VEGF secretion was enhanced; at 20 μg/mL, an over-secretion of IL-8 was measured, MCP-1 and VEGF secretion were reduced. 7KC at 20 μg/mL enhanced the secretion of IL-8 and reduced those of MCP-1 and VEGF. At 30 μg/mL, 7KC reduced the secretion of VEGF and had no effect on IL-8 and MCP-1 secretion.

At oxysterol concentrations inducing the highest IL-8 secretion, the secretion of IL-1β, IL-6, IL-10, TNF-α, and IL-12p70 was investigated with a multiplexed Cytometric Bead Array (Fig. 5). Interestingly, IL-8 was the major cytokine identified. IL-12p70, TNF-α, and IL-10 made up very low proportions of the total cytokines analyzed.

So, the effect of Rsv (1 μM) on IL-8 secretion was only studied on 25-OH-treated cells, whereas the activity of Rsv on VEGF secretion was investigated on 7β-OH- and 25-OH-treated cells. In these conditions, Rsv and oxysterols were simultaneously added to the ARPE-cells. Both 7β-OH and 25-OH were used at 30 μg/mL, and the effects of Rsv were measured at 24 and 40 h of culture (Fig. 6). Rsv showed no effect on 25-OH-induced IL-8 secretion (Fig. 6A) whereas it decreased VEGF secretion on 25-OH-treated cells at 24 h (Fig. 6B) and on 7β-OH-treated cells at 40 h (Fig. 6C).

Effects of the MEK/ERK inhibitor signaling pathway, U0126 and PD98059, on 25-hydroxycholesterol-induced IL-8 secretion

As 25-OH was the most potent inducer of IL-8 secretion, we attempted to determine whether the MEK/ERK$^{1/2}$ signaling cascade playing key roles in inflammation [28], was a part of the metabolic pathway involved in 25-OH-induced IL-8 secretion. Therefore, two potent inhibitors of MEK, PD98059 (10 μM) and U0126 (20 μM), were used at concentrations that have no effects on cell growth and viability (data not shown). These inhibitors were added in the culture medium 30 min before 25-OH (10, 20, and 40 μg/mL). At 24 and 48 h of culture, in the presence of PD98059 and U0126, IL-8 secretion triggered by 25-OH was strongly inhibited, and the IL-8 levels were often similar than those observed in untreated cells (Fig. 7).

Discussion

Oxysterols come either from the diet or from cholesterol catabolism [27], and at the retinal level, RPE cells can internalize LDL and oxLDL containing elevated levels of lipid hydroperoxides, aldehydes, lysosphospholipids, and oxidized sterols (also called oxysterols) [16]. Thus, in ARMD, lipid deposits localized between RPE and BrM, called drusens, are made up of numerous types of lipids including esterified and unesterified cholesterol [3]. As retina is exposed to oxidative stresses, these environmental conditions can favor the spontaneous oxidation of cholesterol into oxysterols in RPE cells [4]. Thus, as in atherosclerosis lesions [9], oxysterols could have noxious effects on RPE cells [2,6]. Therefore, the cytotoxic, pro-oxidant, pro-inflammatory, and pro-angiogenic effects of oxysterols identified at high levels in oxLDL and at the retinal level (7β-OH, 7KC and 25-OH) [15,29] were investigated on ARPE-19 cells, which have the functional and structural properties of RPE cells in vivo [30].

Moreover, since Rsv has been shown to have cytoprotective [31] and anti-inflammatory activities [32], and as it has been described to down-regulate VEGF synthesis on tumor cells [24,33,34], the effects of this polyphenol on oxysterol-treated ARPE-19 cells was investigated, as well as the metabolic pathways activated in oxysterol-induced inflammation,

Interestingly, among the oxysterols studied, only 7β-OH and 7KC are toxic on ARPE-19 cells when the cytotoxicity was evaluated with the trypan blue exclusion test and the MTT assay. Considering the characteristics of these tests, this indicates that these oxysterols lead to mitochondrial dysfunctions associated with an increase in membrane permeability. These results are consistent with those described on the cells of the vascular wall [19,35] and on RPE cells [7,10,11]. As slight cytoprotective effects of Rsv were observed on 7β-OH- and 7KC-treated ARPE19 cells, these data are in agreement with increasing lines of evidence showing that Rsv can counteract different types of insults [31,36,37]. In addition, as previously described on 7β-OH-treated ARPE-19, only a few apoptotic cells with condensed and fragmented nuclei, characteristic of apoptotic cells, were observed by transmission electron microscopy, not only under treatment with 7β-OH, but also with 7KC [7]. Given that these observations were associated with low percentages of FLICA-positive cells, the absence of well-established biochemical hallmarks of apoptotic dying cells is consistent with a death style different from apoptosis, and independent of caspases. In addition, in agreement with our previous data collected on normal or tumoral cells cultured in the presence of 7β-OH and 7KC [38], multilamellar cytoplasmic structures were observed. The presence of these ultrastructural cytoplasmic features in 7β-OH- and 7KC-treated ARPE-19 cells supports the hypothesis that these oxysterols are also probably potent inducers of phospholipidosis on retinal cells, and consequently suggests that oxysterol-induced cell death is associated with lipid storage disorders since multilamellar structures are rich in cholesterol and phospholipids [39,40]. Therefore, 7β-OH and 7KC could also contribute to the lipid accumulation process observed in ARMD.

Moreover, the data obtained on ARPE-19 cells underline that 7β-OH, 7KC, and 25-OH have strong pro-oxidant activities. With these different oxysterols, overproduction of ROS is found in a time- and dose-dependent manner, and these data are consistent with the results obtained on different cell types [19,41]. Furthermore, when U937 cells were cultured in the presence of 7β-OH and 7KC, apoptosis induced by these molecules was substantially decreased by vitamin E [41]. Moreover, on human smooth muscle cultivated in presence of 7KC, ROS overproduction was associated with NADPH oxidase (Nox-4) activation [25]. Since the use of DPI, a NADPH oxidases inhibitor, is responsible for a 50% decrease in ROS production in 25-OH-treated ARPE-19 cells, our results are consistent with those described on human aortic smooth muscle cells [25]. They also confirm that

depending on the oxysterol considered, overproduction of ROS was not necessarily associated with cell death, suggesting that efficient defense processes directed against oxidative stress could be simultaneously induced [42]. In ARMD, since various investigations have shown lipid deposits in Bruch's membrane to be related to inflammatory and immunologic reactions [2], the ability of 7β-OH, 7KC, and 25-OH to induce IL-8 and MCP-1 secretion has been studied as well. Only 25-OH strongly stimulated IL-8 and MCP-1 secretion by ARPE-19 cells. This 25-OH pro-inflammatory effect has already been reported on U937 and THP-1 cells [19,20,43]. Thus, this noncytotoxic oxysterol can be considered a potent inflammation promoter. Indeed, IL-8 is a chemokine able to promote immune system cells to a pathological site. We can therefore assume that the presence of 25-OH inside Bruch's membrane lipid deposits could facilitate the promotion of inflammatory cells potentially involved in ARMD pathogenesis [44]. As 25-OH increased ROS production in ARPE-19 cells and oxidative stress stimulated IL-8 synthesis [45], it could be speculated that ROS overproduction might play a role in IL-8 secretion. Consequently, given that 25-OH is a potential inducer of inflammatory reactions involved in the development of ARMD, it was important to detail the metabolic pathways activated by this oxysterol. Interestingly, PD98059 and U0126, two potent inhibitors of the MEK/ERK$^{1/2}$ pathway, were able to inhibit 25-OH induced-IL-8 secretion. On U937 and THP-1 cells, 7β-OH-induced IL-8 secretion was also inhibited by PD98059 [20]. Thus, these data underline that the activation of the MEK/ERK$^{1/2}$ signaling pathway does not depend on the oxysterol considered, and since oxysterol mixtures are generally present in lipid deposits, the development of drugs with activities that are similar to PD98059 and U0126 might be advantageous in the treatment of ARMD. As enhanced IL-6 secretion was reported on 7KC-treated vascular smooth muscle cells [46], IL-6 secretion was also studied on oxysterol-treated ARPE-19 cells, and an increase in IL-6 secretion was also observed under treatment with 7KC. In addition, in agreement with a previous investigation conducted on 7β-OH-treated U937 cells [17], 7β-OH also induced a slight increase in IL-1β secretion on ARPE-19 cells. Overall, these data suggest that the ability of 7KC and 7β-OH to induce IL-6 and IL-1β secretion, respectively, may not depend on the cell type considered.

Concerning the ability of oxysterols to induce VEGF secretion on human retinal cells, 25-OH induced an increase in VEGF secretion in a time- and dose-dependent manner, while 7β-OH led to VEGF secretion only at high concentrations and at the longest culture time. On the other hand, 7KC did not enhance VEGF secretion on ARPE-19 cells. Oxysterols could therefore have variable pro-angiogenic effects. Furthermore, IL-8 has been described as presenting pro-angiogenic activities [47]. The absence of a correlation between IL-8 and VEGF synthesis under treatment with 7β-OH and 7KC instead suggests that these two events are independent. However,

they could be linked under treatment with 25-OH since this oxysterol simultaneously induces the secretion of high levels of IL-8 and VEGF.

In addition, the present study also provides new evidence that Rsv used at 1 μM (a concentration found in human and mice plasma following oral supplementation of Rsv [48,49]) could prevent neovascularization, which is a major complication of ARMD. Indeed, compared to 7β-OH- and 25-OH-treated ARPE-19 cells, we found a marked decrease in VEGF secretion on 7β-OH- and 25-OH-treated cells simultaneously incubated with Rsv. Therefore, these anti-angiogenic properties of Rsv, which are consistent with the results found on U937 cells and endometrial tumoral cells [24,33], not only support the notion that Rsv may have potential interests as a neo-adjuvant in the treatment of vascularized tumors [21], but they also open interesting pharmacological perspectives in other diseases. Although Rsv is rapidly metabolized in vivo, some of its derivatives, which are more stable in vivo and have similar biological activities [50], could be used to develop new therapeutic approaches in order to counteract the formation of new vessels in ARMD.

In conclusion, on human retinal pigment epithelial ARPE-19 cells, 7β-OH, 7KC, and 25-OH have cytotoxic, pro-inflammatory, pro-oxidative, and/or pro-angiogenic activities, and favor intracellular lipid disorders (phospholipidosis). Therefore, based on their biological activities, these oxysterols can contribute to ARMD pathogenesis. Moreover, we show that the inhibtion of the MEK/ERK$^{1/2}$ signaling pathway strongly reduces oxysterol-induced IL-8 secretion, and that Rsv counteracts the pro-angiogenic activity of oxysterols. Thus, these observations open new therapeutic perspectives for the treatment of ARMD.

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Figures legends

- Fig. 1: **Effects of 7β-hydroxycholesterol (7β-OH), 7-ketocholesterol (7KC), and 25-hydroxycholesterol (25-OH) combined with resveratrol (Rsv) or alone on the cell growth and viability of ARPE-19 cells.** After 24 and 40 h of culture, the total number of viable cells per well of six-well plates was evaluated with trypan blue (A). Mitochondrial dysfunction was determined with the MTT test (B). The effects of Rsv on cell viability were determined at the highest concentration of oxysterols used (C). (*: $P<0.05$): comparison with control (untreated cells) and oxysterol-treated cells; (#: $P<0.05$): comparison with oxysterol-treated cells and (oxysterol + Rsv)-treated cells.

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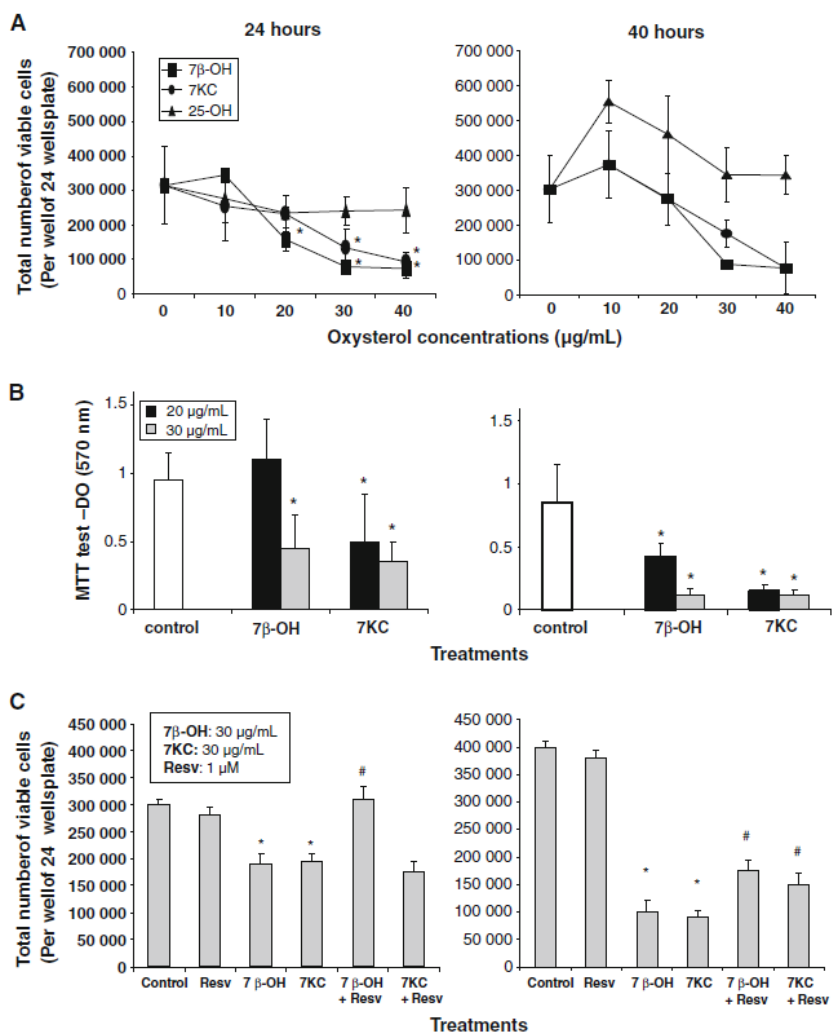
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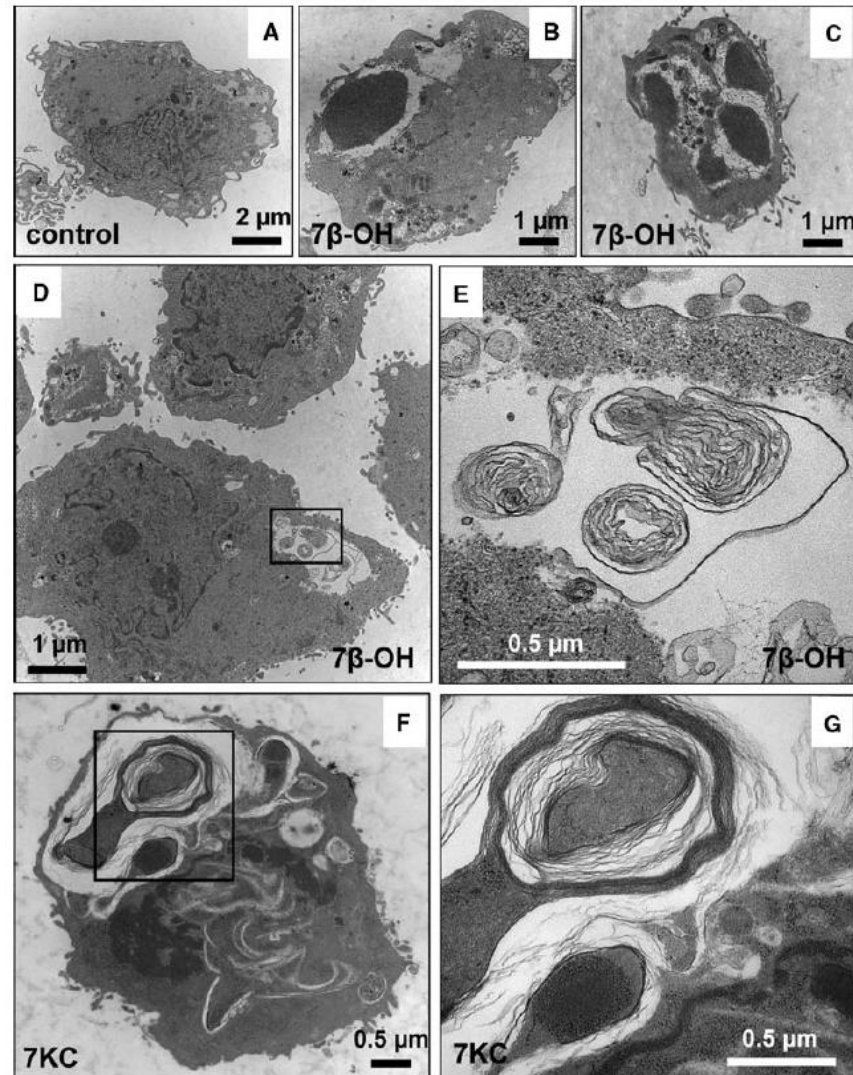
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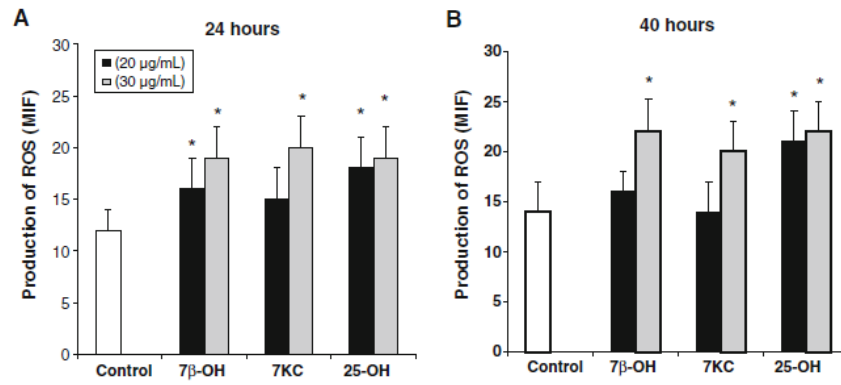
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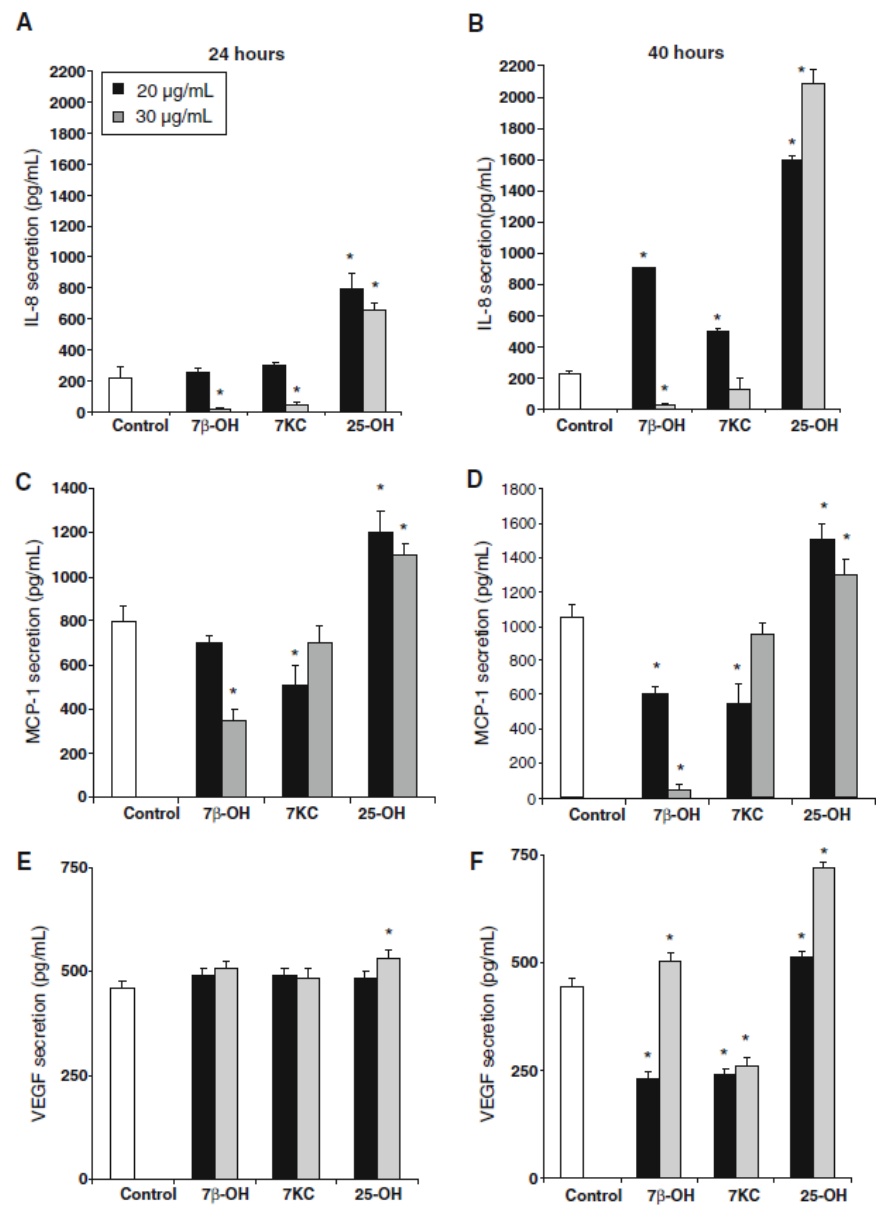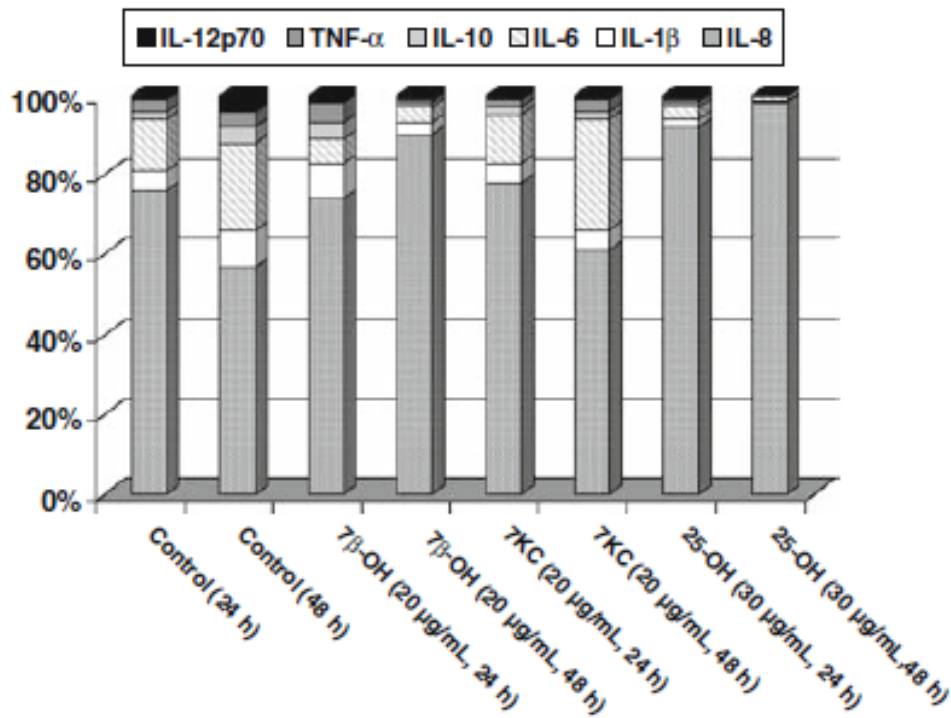
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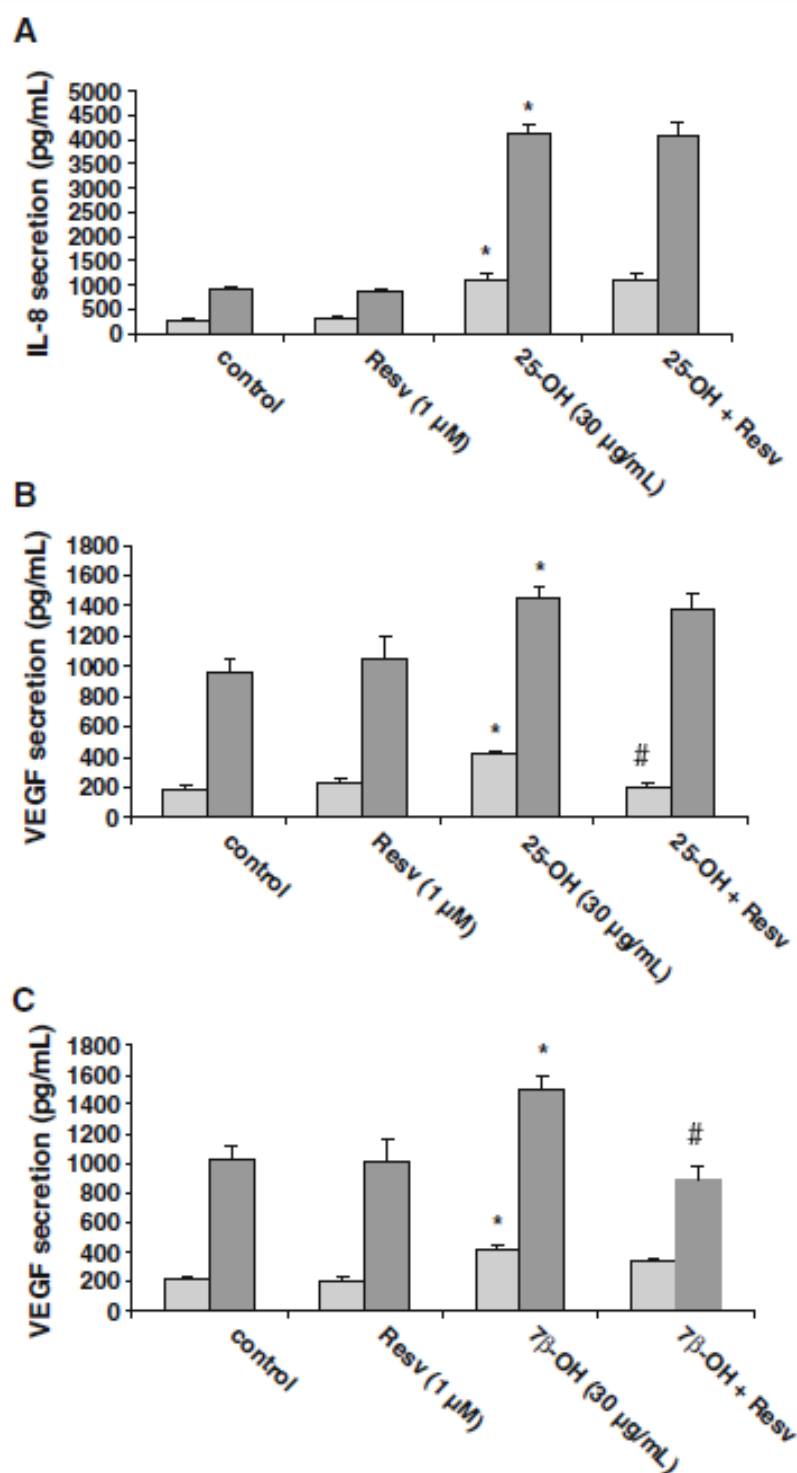
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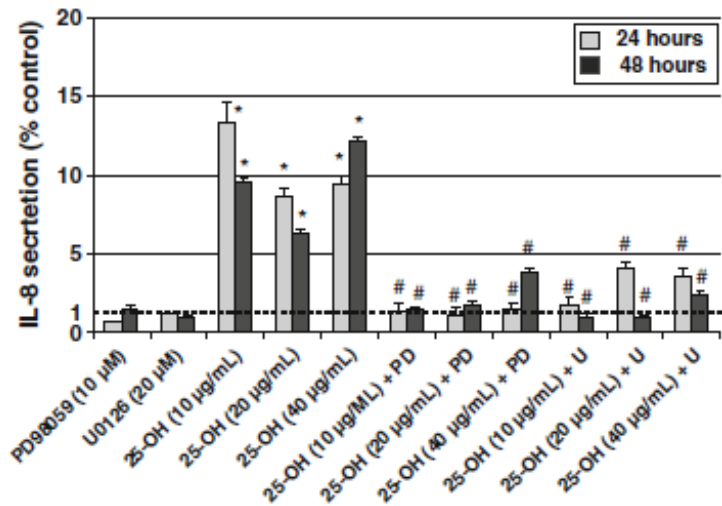
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