Benefits and Side Effects of Different Vegetable Oil Vectors on Apoptosis, Oxidative Stress, and P2X7 Cell Death Receptor Activation

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Purpose. Ocular side effects in patients using eye drops may be due to intolerance to the vector used in eye drops. Castor oil is the commonly used lipophilic vector but has been shown to be cytotoxic. Effects on cells of four oils (olive, camelina, *Aleurites moluccana*, maize) were compared with those of castor oil in human conjunctival cells.

Methods. Human conjunctival cells were incubated with the oils for 15 minutes. After a 24-hour recovery period, cells were tested for viability, proliferation, apoptosis (P2X7 cell death receptor and caspase 3 activation), intracellular redox potential, and reactive oxygen species production. Fatty acid incorporation in cell membranes was also analyzed. In vivo ocular irritation was assessed using the Draize test.

Results. Compared to the four other oils, castor oil was shown to induce significant necrosis and P2X7 cell death receptor and caspase 3 activation and to enhance intracellular reactive oxygen species production. *Aleurites moluccana* and camelina oils were not cytotoxic and increased cell membrane omega-3 fatty acid content. None of the five tested oils showed any in vivo ocular irritation.

Conclusions. The results demonstrated that castor oil exerts cytotoxic effects on conjunctival cells. This cytotoxicity could explain the side effects observed in some patients using eye drops containing castor oil as a vehicle. The lack of cytotoxic effects observed with the four other oils, *Aleurites*, camelina, maize, and olive, suggest that they could be chosen to replace castor oil in ophthalmic formulations. (*Invest Ophthalmol Vis Sci.* 2007;48:5000–5006) DOI:10.1167/iovs.07-0229

Topical drug administration is very often used to treat ocular surface and intraocular disease, providing higher local drug levels than systemic administration,[1] with minimal general side effects.[2] The therapeutic efficacy of a topical formulation depends on both its composition and the physicochemical properties of the vehicle. Use of an appropriate vehicle is critical to increase the optimal efficacy of the pharmacologically active drug.[3] Most commercialized eye drops are prepared in aqueous form, although most active components are lipophilic. Another drawback of the hydrophilic formulations is the fast elimination of the eye drop by tears, reducing contact duration between the drug and the ocular tissue.

The use of a lipophilic vehicle in eye drops increases solubility and pharmacologic effects of the drug. An improvement of the cell delivery of drugs using vector oil is based on the modulation of membrane fluidity that directly depends on its fatty acid composition. Castor oil, which mainly contains ricinoleic acid (90% of total fatty acid content),[4] is one of the lipophilic vehicles used in cyclosporine eye drops.[2,5,6] However, it presents both a low-stability and an epithelial and conjunctival toxicity[7] as well as systemic adverse effects such as purgative effects, hypersensitivity, nephrotoxicity, and neurotoxicity.[4,8] Since castor oil is presumed to be responsible for cytotoxic effects in the eye, its replacement by another lipophilic vector could result in better tolerance of the drops.

Omega-3 fatty acids are important in the structure and function of the visual system.[9] α-Linolenic acid (ALA) is the precursor of the long-chain omega-3 polyunsaturated fatty acids (n-3 PUFA)—mainly, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)[10]—found in fish oils and in nerve tissue cell membranes. Several vegetable oils exhibit a high content of omega-3 fatty acids. For instance, *Aleurites moluccana* and camelina oils are rich sources of linolenic acid (36%–40%).[11,12] Long-chain omega-3 fatty acids are rapidly incorporated into cell membrane phospholipids, thereby resulting in changes in receptor functions and alteration in cell signaling mechanisms and membrane-bound enzymes.[13] On the other hand, incorporation of α-linolenic acid or other n-3 series fatty acids into the diet results in marked changes in cell membrane composition as well as in arachidonic acid metabolism.[14] Arachidonic acid, a long-chain omega-6 polyunsaturated fatty acid (n-6 PUFA) abundant in membrane phospholipids, is the first step of the inflammatory process after metabolism by cyclooxygenases and lipoxygenases, which are present in the conjunctiva.[15,16] The omega-3 fatty acids contained in vegetable oils, such as camelina oil, could thus replace arachidonic acid in cell membranes and decrease inflammation. Therefore, it is of interest to develop lipophilic vectors that would be free of negative side effects and contain omega-3 fatty acids.

In this study, we evaluated the in vitro and in vivo toxicity of four vegetable oils to validate their use as vehicles of lipophilic drugs in eye drops. They were chosen because of their biological properties and fatty acid composition[11,17,18]: Two of them are rich in omega-3 fatty acids (Aleurites and camelina) and two are free of omega-3 (maize and olive). The olive oil was highly refined (neutral, denatured, and free of antioxidants) and was chosen as the negative control; castor oil was tested to confirm its cytotoxic effects in our cell model. Cell assays for necrosis, apoptosis, intracellular redox status,

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and antioxidant properties were performed on human conjunctival cells. In addition, we analyzed the incorporation of oils (i.e., of their constitutive fatty acids) in cultured conjunctival epithelial cell membranes. In vivo ocular irritation was evaluated by using the Draize test.

Materials and Methods

Reagents

Castor, maize, and olive oils were purchased from Sigma-Aldrich (St. Louis, MO) at the highest purity grade available. Camelina oil was purchased from Phytocos (Poitiers, France); and *Aleurites moluccana* oil was purchased from Plant Extract Laboratory (Antanarivo, Madagascar).

Ocular Cytotoxicity Assessment on a Conjunctival Cell Line

Wong Kilbourne–derived human conjunctival epithelial cells (WKD, ECACC 93120839) were cultured under standard conditions (moist atmosphere of 5% CO2 at 37°C) in Dulbecco's minimum essential medium (DMEM; Eurobio, Les Ulis, France) supplemented with 10% fetal bovine serum (FBS, Eurobio), 2 mM l-glutamine, 50 IU/mL penicillin, and 50 IU/mL streptomycin (Eurobio). The culture medium was changed every 3 days; confluent cultures were removed by trypsin incubation. The cells were counted, seeded into 96-well microplates (Costar; VWR, Fontenay sous Bois, France), and kept at 37°C for 24 hours. The different undiluted oils, including olive oil as the control, were incubated for 15 minutes (30 minutes for the fatty acid incorporation test), and tests were performed after a 24-hour recovery period in culture medium. Olive oil was chosen as the control.

Fluorometry was performed with a microplate cold light cytofluorometer[19] (Fluorolite 1000; Dynex, Cergy Pontoise, France). All fluorescent dyes were added to living cells, since this method allows detection of the fluorescent signal directly from the microplate.

Cell Viability

Membrane Integrity. Membrane integrity, which is closely correlated with cell viability, was evaluated with neutral red (Fluka, Buchs, Switzerland), by fluorometric detection (λexcitation [ex.] = 535 nm; λemission [em.] = 600 nm). Neutral red was used at a concentration of 50 (g/mL, according to Borenfreund and Puerner[20]; 200 μL per well of medium containing neutral red was added to living cells, and the microplate was incubated for 3 hours at 37°C in moist atmosphere with 5% CO2. The neutral red fluorescence was then measured.

Redox Status. The Alamar blue assay was used to measure redox status variations.[21,22] Alamar blue penetrates cells passively and thereby is reduced. The oxidized nonfluorescent dye becomes a reduced fluorescent dye. A solution at 0.1 mg/mL in PBS is diluted (1/11) in culture medium containing 2.5% FBS. Cells were incubated at 37°C for 7 hours, and fluorescence detection (λex. = 535 nm; λem. = 600 nm) was then measured.

Cell Proliferation. Cell proliferation was evaluated by the incorporation of BrdU in DNA during replication. The BrdU kit (Cell Proliferation ELISA, BrdU) was provided by Roche (Meylan, France).

Apoptosis

P2X7 Cell Death Receptor Activation: the YO-PRO-1 Test. YO-PRO-1, a DNA probe (Invitrogen-Molecular Probes, PoortGebouw, The Netherlands), penetrates apoptotic cells only through the P2X7 receptor. After incubation with the different oils, a 2-μM YO-PRO-1 solution in phosphate-buffered saline was applied (200 μL per well), and the microplate was placed for 10 minutes in the dark at room temperature. The fluorescence signal was scanned by using a cytofluorometer with a small band pass and precise wave lengths for YO-PRO-1 fluorescence detection (λex. = 491 nm; λem. = 509 nm). This test was simultaneously performed with the neutral red test.

Caspase 3 Activation. To assess apoptosis, caspase 3 activity was evaluated by using a caspase 3 kit assay with rhodamine 110-DEVD fluorogenic substrate (Invitrogen-Molecular Probes).

Reactive Oxygen Species Production

Intracellular reactive oxygen species (ROS) production (mainly H_2O_2) was detected with the 2′,7′-dichlorofluorescein diacetate fluoroprobe (DCFH-DA; Invitrogen-Molecular Probes).[23] This probe is a nonfluorescent compound currently used in flow cytometry and adapted for use in the microplate assay.[24] Once incorporated into the cells, the probe is cleaved by endogenous esterases and can no longer exit the cell. The de-esterified product becomes a fluorescent compound 2′,7′-dichlorofluorescein after its oxidation by intracellular ROS. Fluorescent signal detected (λex.: 490 nm; λem.: 535 nm) is proportional to ROS production.

Fatty Acid Incorporation in Membrane

The cells were centrifuged for 5 minutes at 1000*g*. The cell pellets were resuspended in 1 mL of 0.1 M sucrose-10 mM Tris buffer (pH 7.4)

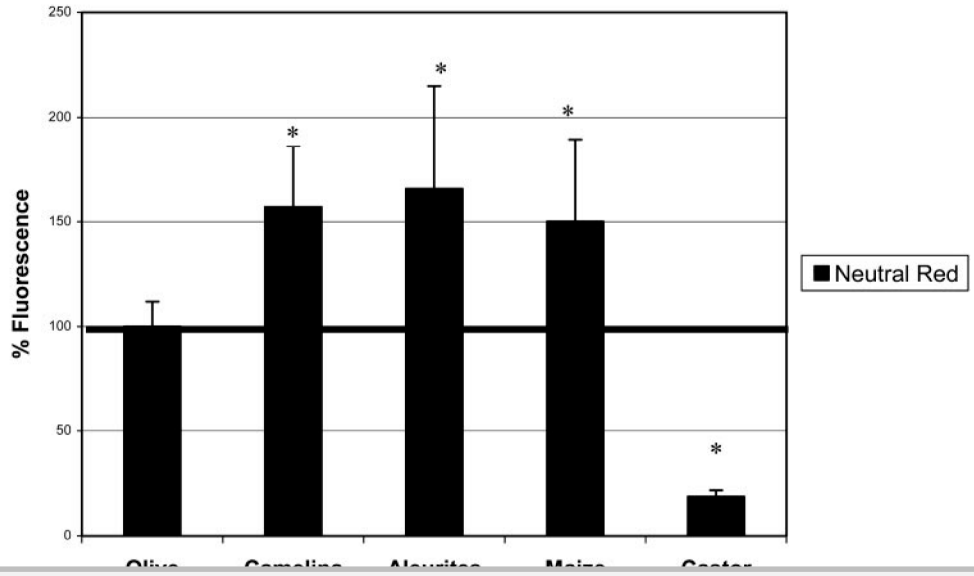

Figure 1. Membrane integrity evaluation using the neutral red test after incubation with different oils for 15 minutes followed by a 24-hour recovery period. Results are expressed as a percentage of control. Castor oil de-

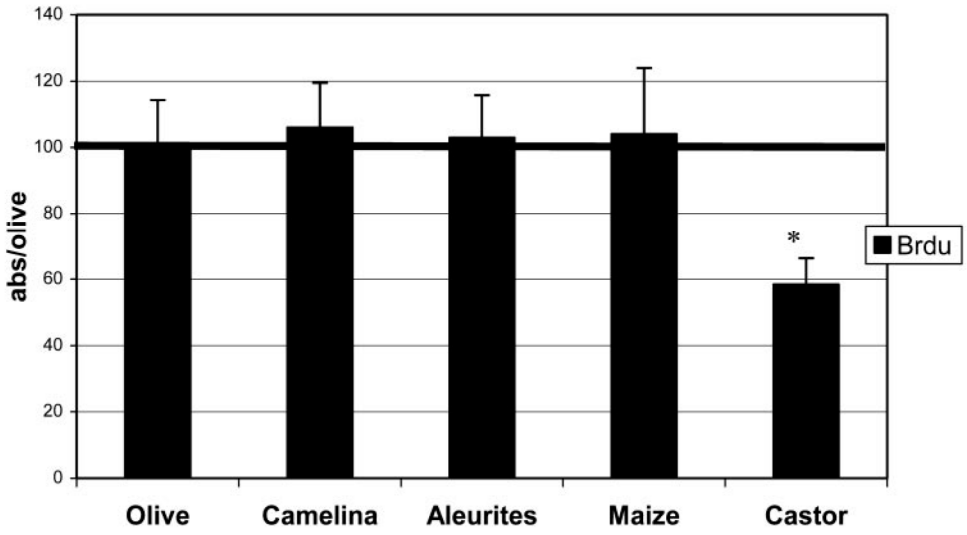

FIGURE 2. Cell proliferation evaluation with BrdU incorporation after incubation with different oils for 15 minutes followed by a 24-hour recovery period. Castor oil inhibited cell proliferation. $^{*}P < 0.001$.

at 4°C. They were then lysed after five freeze–thaw cycles and a cold sonication for 30 to 60 seconds. Cell membranes were isolated by several ultracentrifugations. Total lipids were extracted, and membrane fatty acids were analyzed and quantified by high-pressure liquid chromatography, as previously described.[25,26]

In Vivo Assessment of Ocular Irritation: Draize Test

All procedures in this study were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research. Animal care and experimentation complied with the rules of European Council Guidelines: license for experimental studies on living animals. Eight-week-old male New-Zealand rabbits (Cegav, Saint Mars d'Egrenne, France) were placed in an individual cage and kept in standard laboratory conditions (at 20°C, 12-hour light–dark cycle), fed ad libitum on the standard laboratory diet with free access to water.

Ocular irritation was evaluated by a modified Draize test.[27] Briefly, 0.1 mL of nondiluted (100%) oil was instilled into the conjunctival sac, and the upper and lower lids were gently held together for 10 seconds. The opposite eye of each animal served as the untreated control. Ocular responses (conjunctiva, iris, cornea) were scored at 1, 24, 48, 72, and 96 hours, and at 7 days. A fluorescein stain was used to confirm corneal effects visible by examination at the 24-hour observation and at each subsequent observation period until the cornea failed to exhibit uptake of fluorescein. Irritation was scored according to the method of Draize et al.[27] A score of less than 15 was considered to show that the oil was a nonirritant.

Statistical Analysis

Results were obtained in arbitrary fluorescence units and expressed as a percentage of control values. For cell experiments, oils were tested in six wells, and each experiment was performed in triplicate. Mean values for each concentration were analyzed by one-way ANOVA followed by the Dunnett test.[28,29] All statistical analyses were performed with commercial software (Sigma Stat 2.0; SPSS, Chicago, USA). $P < 0.05$ was considered statistically significant.

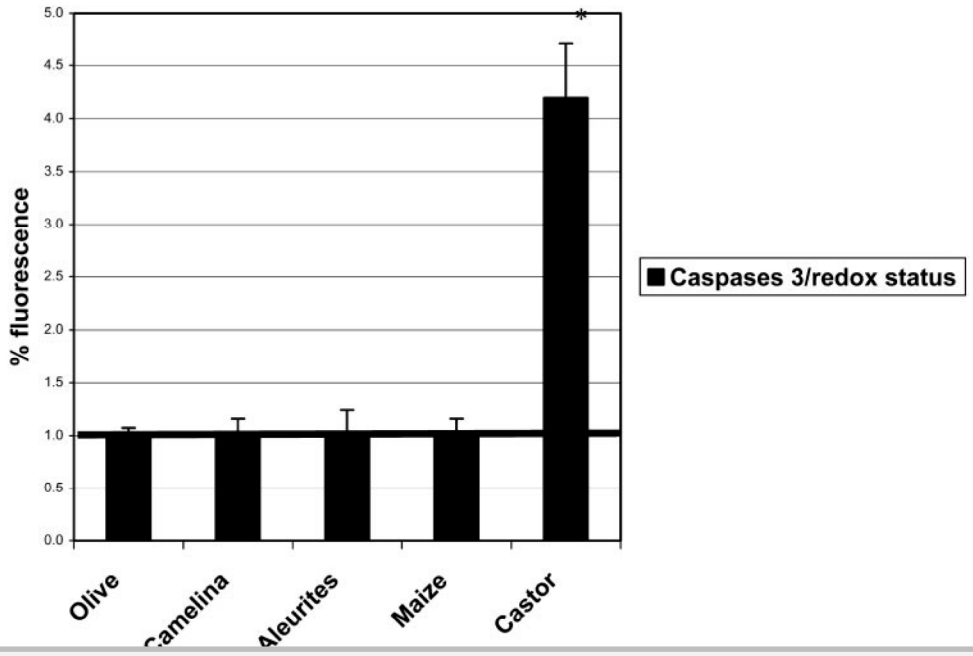

FIGURE 3. Caspase 3 activity evaluated by the rhodamine 110-DEVD fluorogenic substrate after incubation with different oils for 15 minutes followed by a 24-hour recovery period. Results are expressed as a percentage of the control. Castor oil acti-

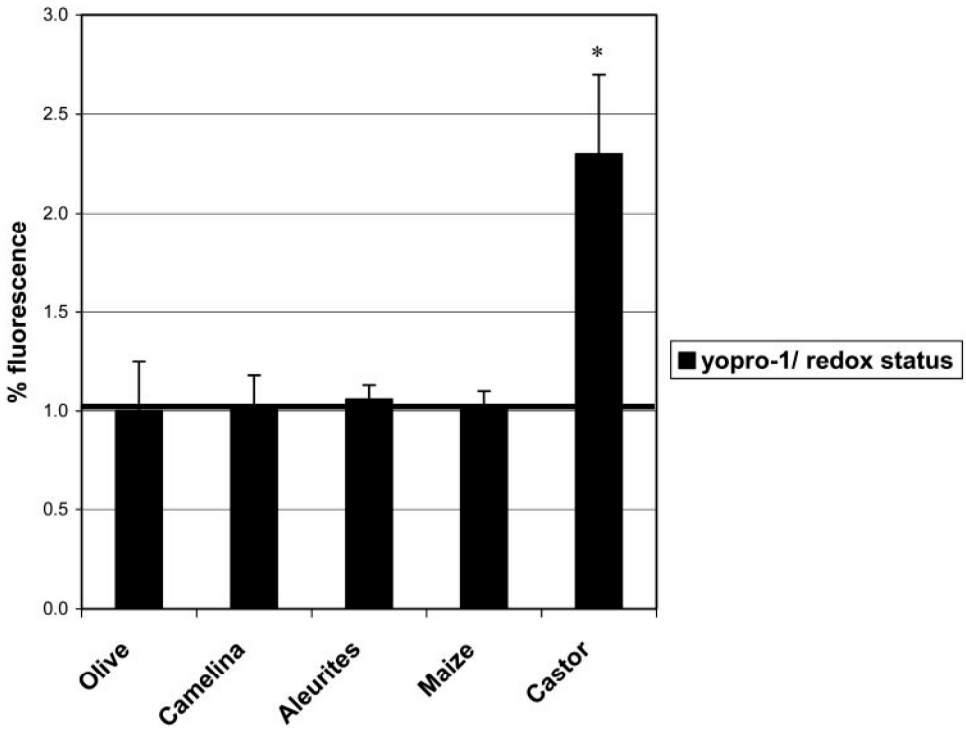

FIGURE 4. P2X7 cell death receptor activation evaluated with the YO-PRO-1 test after incubation with different oils for 15 minutes followed by a 24-hour recovery period. Results are expressed as a percentage of the control. Castor oil activated P2X7 cell death receptor. $^*P < 0.001$.

RESULTS

Cell Viability, Apoptosis, and Oxidative Stress

Cell viability results, as evaluated with the neutral red assay (Fig. 1), indicated that maize, *Aleurites moluccana*, and camelina oils did not induce any cellular necrosis when compared with the control olive oil. Although an increase in neutral red incorporation was observed for the three oils when compared with olive oil, no proliferative effect was detected by the specific proliferation BrdU assay (Fig. 2). On the other hand, the neutral red and BrdU assays indicate that castor oil significantly decreased cell viability ($P < 0.001$). In the same way, assays for apoptosis revealed that maize, *Aleurites moluccana*, and camelina oils did not activate the P2X7 cell death receptor and caspase 3 (Fig. 3), whereas castor oil significantly induced apoptosis through both P2X7 cell death receptor activation (+200%, $P < 0.001$; Fig. 4) and caspase 3 activation (+301%, $P < 0.001$; Fig. 3).

Finally, when compared with control olive oil, maize, *Aleurites moluccana*, and camelina oils did not alter intracellular redox status, whereas castor oil significantly decreased intracellular redox potential (−42.3%; Fig. 5). Assays with the H_2O_2-sensitive DCF fluorogenic probe confirmed that castor oil, but not other oils, significantly increased intracellular ROS production (+1400%; Fig. 6) that was surely responsible for the alteration of global redox status measured by the Alamar blue assay.

Fatty Acid Incorporation in Membranes

When compared with olive oil, cells incubated with camelina or *Aleurites* oils significantly increased the incorporation rate of α-linolenic acid in their membranes, and concomitantly, a decrease in the membrane arachidonic acid content was observed (Table 1). Because of the strong necrotic effect of castor oil, the modification in membrane fatty acid composition of

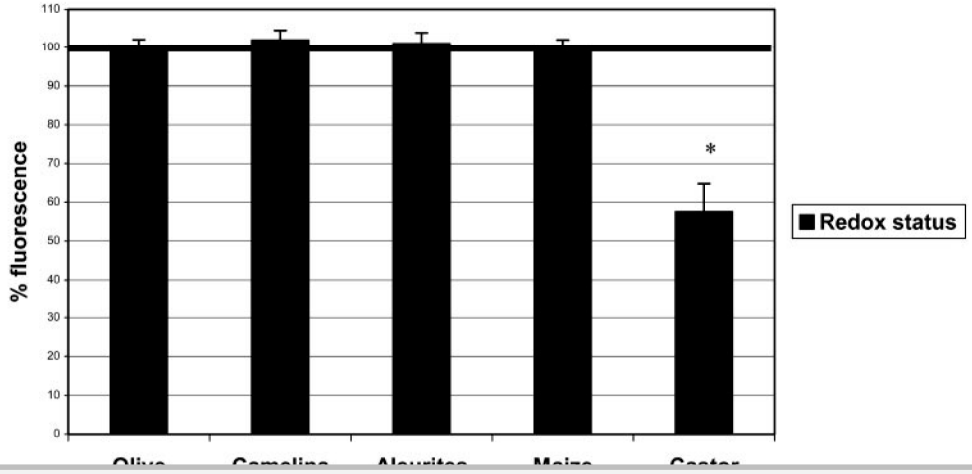

FIGURE 5. Redox status evaluation using the Alamar blue test after incubation with different oils for 15 minutes followed by a 24-hour recovery period. Results are expressed as a percentage of the control. Castor oil altered intracellular redox status.

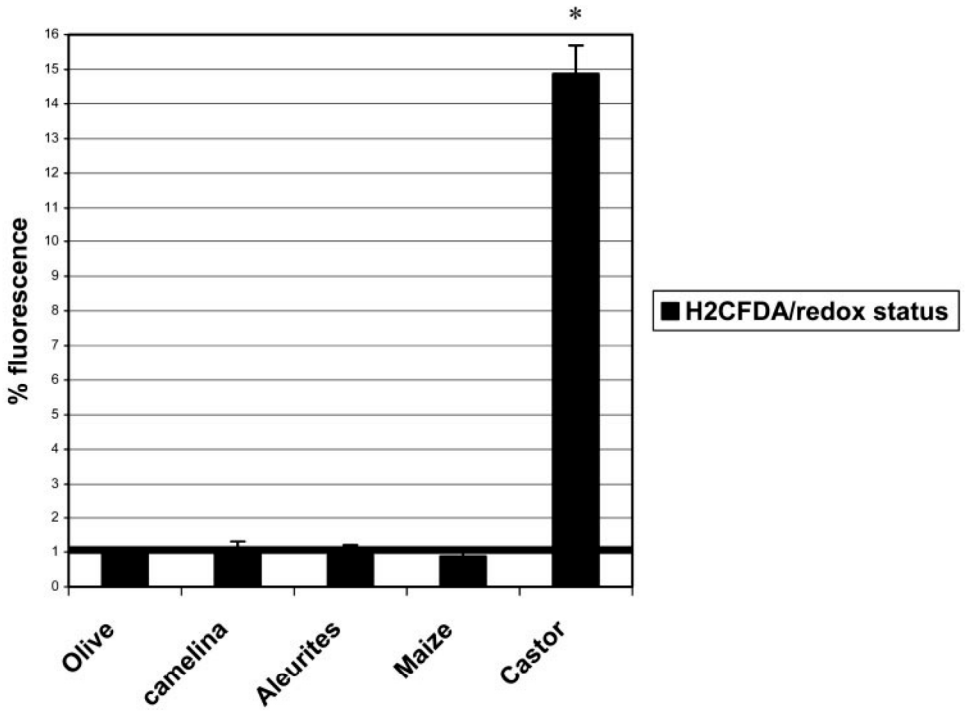

FIGURE 6. ROS production evaluated by DCF-DA test after incubation with different oils for 15 minutes followed by a 24-hour recovery period. Results are expressed in DCF/NR fluorescence ratio. Castor oil increased ROS production. $^*P < 0.001$.

cells maintained for 30 minutes in the presence of this oil was impossible to determine in our experimental conditions.

In Vivo Assessment of Ocular Irritation: Draize Test

As indicated in Table 2, none of the five tested oils induced any noticeable in vivo ocular irritation. For each oil, the score remained ≤2, whereas positivity for this test corresponds to a score of ≧15 or higher.

DISCUSSION

The results of our study demonstrate that castor oil exhibited significant in vitro cell toxicity in comparison with olive oil. Other tested oils (i.e., camelina, *Aleurites moluccana,* and maize oils) did not exhibit such deleterious effects, since there was no difference in the necrotic and apoptotic processes or the alteration of ROS production between these oils and olive oil.

When it occurs, eye drop intolerance can result from either the active component or the vehicle. As an example, benzalkonium chloride, a well-known preservative, is known to induce apoptosis in certain ocular cells; recent published studies from our laboratory demonstrated that benzalkonium chloride activates P2X7 cell death receptor, leading to fluoroquinolone eye drop intolerance.[30] Available cyclosporine eye drops contain maize oil or castor oil as the vehicle.[31] Reports indicate that ophthalmic formulations containing castor oil are more cytotoxic than those containing maize oil.[32] Our present results showed that castor oil induced necrosis, apoptosis (P2X7 receptor and caspase 3 activations), and intracellular H_2O_2 overproduction in an in vitro model of conjunctival cells. As a hypothesis, the cytotoxic effects of castor oil could be due to its high ricinoleic acid content.

TABLE 1. Fatty Acid Incorporation in Conjunctival Cell Membranes after Incubation with Different Oils

Fatty Acid	Control	Olive	Maize	Camelina	Aleurites
Arachidonic acid 20:4 (n-6)	6.75	4.62	4.45	4.09	1.65
α-Linolenic acid 18:3 (n-3)	0.60	0.60	2.82	7.20	13.52

Incubation lasted 30 minutes followed by a 24-hour recovery period. The different oils modified cell membrane fatty acid composi-

Indeed, high concentrations of ricinoleic acid (≥0.1 mM) were shown to be cytotoxic in isolated hamster intestinal epithelial cells.[4] The lack of in vivo toxicity (Draize test) may be due to the single application of castor oil, which is not sufficient to induce a toxic effect and ocular irritation. As a further study, in vivo assays testing repeated applications of castor oil (as well as ricinoleic acid) might show toxicity and explain clinical observations. Indeed, some patients who use eye drops containing castor oil exhibit discomfort and ocular irritation after long-term treatment. Use of nontoxic lipophilic eye drop vehicles, instead of castor oil, could suppress such adverse effects. The other tested oils presented better in vitro tolerance than castor oil and could be used in ophthalmic formulations instead of castor oil. Maize oil is not cytotoxic and is already used in cyclosporine eye drop preparations at the French Ophthalmologic National Center (Hôpital des Quinze-Vingts, Paris, France). Our in vitro results confirm the tolerance of this oil and suggest that long-term treatment of patients would be safe.

Omega-3 fatty acids are critical for membrane functions and were shown to exhibit anti-inflammatory effects.[33] In response to proinflammatory stimuli, enhanced catalytic activity of phospholipase A2 degrades membrane phospholipids to form free arachidonic acid that is converted into prostaglandins and other eicosanoids through activation of the oxidative enzymes cyclooxygenases and lipoxygenases. All these enzymes are found in ocular conjunctiva.[34–37] Replacement of arachidonic acid by DHA and EPA in cell membranes reduces the inflammatory process related to proinflammatory prostaglandin synthesis, since products of the catalytic action of phospholipase

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