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Topical Issue

Oily formulations challenge: how to evaluate their beneficial effects in hydrophilic cell-based models?

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Abstract – In the European Union, Israel and India, testing cosmetic products or their ingredients on animals is prohibited. In this context, *in vitro* cell models play a pivotal role in the evaluation of both safety and beneficial effects of cosmetics. Oily formulations, widely used in cosmetics, are complex to study on cell models due to their lipophilic nature that doesn't match with hydrophilic culture medium. Organic solvents are then required to solubilize oily formulations, but they can interfere with the cellular response. To avoid the use of organic solvents, we developed a method based on cells to evaluate potential beneficial effects of oily formulations. Our method, suitable for high throughput screening, consists in: (1) incubating cells with oily formulations for a short time followed by a recovery period in culture medium and (2) studying cell parameters using robust techniques such as cytofluorometry and fluorescence resonance energy transfer (FRET). Depending on the studied cell parameter, various beneficial effects can be revealed like antioxidant, anti-inflammatory and skin regeneration. The field of cell parameters is open and can be extended to new perspectives in the development of oily formulations.

Keywords: oily formulations / solvent / alternative methods / high throughput screening / FRET

Résumé – Le défi des formulations huileuses : comment évaluer leurs effets bénéfiques sur des modèles cellulaires hydrophiles ? Au sein de l'Union Européenne, en Israël et en Inde, il est interdit de tester les produits cosmétiques sur les animaux. Dans ce contexte, les modèles cellulaires *in vitro* jouent donc un rôle primordial dans l'évaluation de la sécurité et des effets bénéfiques des produits cosmétiques. Les formulations huileuses, très largement utilisées dans le domaine des produits cosmétiques, sont complexes à étudier sur des modèles cellulaires du fait de leur nature lipophile alors que le milieu de culture est de nature hydrophile. Par conséquent, des solvants organiques sont nécessaires pour solubiliser les formulations huileuses, mais ces solvants peuvent interférer avec les réponses cellulaires. Afin d'éviter le recours aux solvants organiques, nous avons développé une méthode cellulaire pour évaluer les potentiels effets bénéfiques des formulations huileuses. Notre méthode, utilisable pour le criblage à haut débit, consiste à (1) incuber les cellules avec les formulations huileuses pendant une courte durée suivie d'une période de récupération dans le milieu de culture et (2) étudier les paramètres cellulaires en utilisant des techniques robustes telles que la cytofluorimétrie et la fluorimétrie par transfert d'énergie (FRET). Selon les paramètres cellulaires étudiés, différents effets bénéfiques peuvent être révélés comme les propriétés antioxydantes, anti-inflammatoires ou la régénération cellulaire. Le champ des paramètres cellulaires à étudier est vaste et peut être étendu à de nouvelles perspectives permettant la mise en évidence des effets bénéfiques des formulations huileuses.

Mots clés : formulations huileuses / solvants / méthodes alternatives / criblage à haut débit / FRET

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1 Introduction and context

Since the antiquity, oily formulations have been widely used for cooking, treating diseases, religious ceremonies or beauty care (cosmetics). They are obtained from natural sources: mainly from fruits, nuts or seeds of plants (Poucher, 1992). For example, olive oil, sesame oil and Chia oil were reported by archaeological documents to be used in several civilizations around the world such as Ancient Egyptians, Ancient Greeks, Ancient Romans, Mesopotamian, Chinese, Indian or Aztec civilizations (Bedigian and Harlan, 1986; Salmon and Shipley, 2011; Ullah *et al.*, 2016). Nowadays, the cosmetic industry exploits not only the mechanical properties of oily formulations such as emollience, moisturizing and grooming (Berdick, 1972), but also their biological effects such as antioxidant. Their biological beneficial effects are mainly empirical, and, in most cases, there is a lack of scientific data to support them.

The assessment of cosmetics must be performed using *in vitro* alternative methods to animal testing to respect the ban on animal testing in Europe (Official Journal of the European Union, 2009), Israel and India. Then, demonstrating the potential beneficial effects of cosmetics implies working on cells. The main issue is that cell culture medium is hydrophilic, meaning that lipophilic products like oily formulations cannot be solubilized in cell culture medium. To solubilize oily formulations, dilutions in organic solvents are required but organic solvents can interfere with the cellular response (Tanneberger *et al.*, 2010). These technical issues can explain the lack of scientific data demonstrating the beneficial effects of oily formulations. Besides, to meet consumer demand of "natural products", manufacturers try to limit the use of chemical solvents in their industrial processes to obtain "natural" oily formulations with techniques like supercritical CO_2 extraction, extraction of lipophilic compounds by vector oils... (Chemat *et al.*, 2012; Yara-Varón *et al.*, 2017; Benito-Román *et al.*, 2018). If organic solvents are avoided during the extraction process of oily formulations, it appears logical to avoid them during all the steps of the research process

The cosmetic industry is particularly interested in antioxidant and anti-inflammatory formulations because oxidative stress and inflammation are two major stresses that can alter the skin *via* sun exposure, pollution, injuries... Oxidative stress and inflammation can lead to skin aging, photoaging, atopic dermatitis (Rinnerthaler *et al.*, 2015; Marrot, 2017; Naidoo *et al.*, 2017).

In this context, we developed a method based on cells to evaluate potential beneficial effects of oily formulations without the use of any organic solvents. To demonstrate the reliability of our method, we selected oily formulations already known for their beneficial effects. Olive oil majority composed of omega-9 fatty acid and corn oil rich in omega-6 and-9 fatty acids were used as antioxidant oils (Cheung *et al.*, 2007; El-Kholy *et al.*, 2014; Barrouin-Melo *et al.*, 2016; Carnevale *et al.*, 2018); fish oil, rich in omega-3 fatty acids and corn oil were used as anti-inflammatory oils (Tab. 1) (Maroon and Bost, 2006; Odabasoglu *et al.*, 2008; Mullen *et al.*, 2010; Calder, 2011, 2015).

Table 1. Major fatty acid composition of the different tested oils. Data provided by suppliers (Sigma-Aldrich and Polaris).

	Olive oil (Sigma-Aldrich)	Corn oil (Sigma-Aldrich)	Fish oil (Polaris)
C16:0	12%	12%	ND
C18:1 ω9	70%	32%	ND
C18:2 ω6	10%	51%	ND
C20:5 Ω3 EPA	ND	ND	32%
C22:6 Ω3 DHA	ND	ND	24%

ND: non-determined.

2 Materials and methods

2.1 Cell models

HaCaT cells, spontaneously transformed human keratinocytes, were obtained from Cell lines service (Cell lines service-CLS-Germany) and THP-1, a human monocyte-derived cell line, was obtained from the American Type Culture Collection (ATCC® TIB-202™). HaCaT and THP-1 cells were respectively cultured in Dulbecco's modified Eagle's medium (DMEM, ThermoFisher, France) and in RPMI-1640 (ThermoFisher, France) supplemented with 10% fetal calf serum, 2 mM of glutamine, 50 IU/ml of penicillin and 50 IU/ml of streptomycin (ThermoFisher, France). Fetal calf serum was decomplemented for THP-1 cells. Cell cultures were maintained in controlled atmospheric conditions: CO_2 5%, humidity 95% and temperature 37 °C.

When adherent HaCaT cells reached confluency, they were dispersed using trypsin and counted. HaCaT cells were seeded in 96-well microplates at 100 000 cells/mL.

Suspension THP-1 cells were counted and seeded at 400 000 cells/mL in 96-well microplates and were differentiated into macrophages using phorbol myristate acetate (Sigma-Aldrich, France) at 30 ng/ml for 24 h. Cell medium was removed and replaced by fresh medium for 24 h.

HaCaT and macrophages were incubated for 15 minutes with neat oils or cell medium (as negative control), then rinsed with PBS (ThermoFisher, France) and placed in culture medium at 37 °C for a 24 h-recovery period.

2.2 Antioxidant effects

To reveal the antioxidant effects of oily formulations, we induced oxidative stress using a chemical agent, tert-ButylHydroPeroxide tBHP (Sigma-Aldrich, France) at 1.5 mM for 15 minutes.

The antioxidant positive controls, olive oil and corn oil (Sigma-Aldrich, France), were selected according to the literature (Cheung *et al.*, 2007; Barrouin-Melo *et al.*, 2016; Carnevale *et al.*, 2018).

Intracellular ROS were measured using 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA, Life Technologies, France) (LeBel *et al.*, 1992), which is hydrolyzed by cell esterases in 2',7'-dichlorodihydrofluorescein and oxidized by

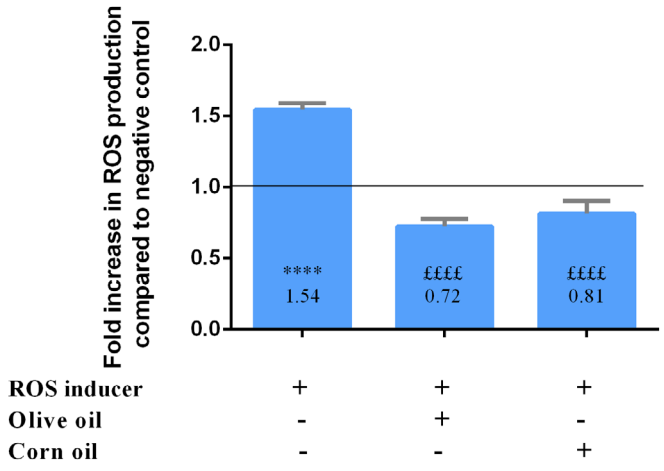

Fig. 1. Antioxidant effects of oily formulations. Cells are incubated with olive or corn oil for 15 minutes, followed by a 24-hour recovery period in culture medium. At the end of the recovery period, oxidative stress is induced by a ROS inducer (tBHP 1.5 mM for 15 minutes). ROS production was quantified by cytofluorometry (H2DCF-DA assay). Negative control was fixed at 1. ****$p < 0.0001$ compared to negative control, ££££ $p < 0.0001$ compared to ROS inducer (ANOVA followed by Tukey test, $n = 3$).

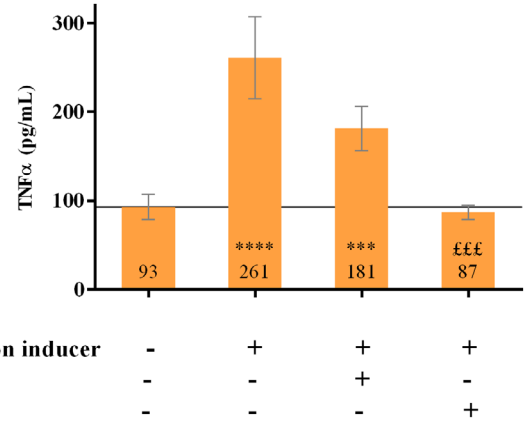

Fig. 2. Anti-inflammatory effects of oily formulations. Cells are incubated with corn or fish oil for 15 minutes. After oils removal, the cells were incubated with either culture medium or an inflammation inducer (poly(I:C) 1 µg/mL) for 24 hours. Cell supernatants were collected and TNFα was quantified using FRET technology. ****$p < 0.0001$ and $p < 0.001$ compared to negative control, £££ $p < 0.001$ compared to ROS inducer (ANOVA followed by Tukey test, $n = 3$).

ROS in fluorescent 2',7'-dichlorofluorescein. A 10 µM solution of H2DCF-DA was distributed into wells (200 µL/well). After a 20-minute incubation period at 37 °C, the fluorescence signal was read (λex = 485 nm, λem = 535 nm) using a cytofluorometer (Spark, Tecan, Switzerland).

2.3 Inflammation measurement

To reveal the anti-inflammatory effects of oily formulations, we induced inflammation using a chemical agent, poly(I:C) (Sigma-Aldrich, France) at 1 µg/mL for 24 hours.

The anti-inflammatory positive controls, fish oil (Polaris, France) and corn oil (Sigma-Aldrich, France), were selected according to the literature (Odabasoglu *et al.*, 2008; Calder, 2015; Mullen *et al.*, 2010).

TNFα cytokine secretion was quantified in macrophage supernatants by fluorescence resonance energy transfer (FRET) technology (HTRF® Cisbio Biosassays, France). FRET technology depends on the transfer of energy between two fluorophores, a donor and an acceptor, which are coupled with biomolecules. The energy transfer occurs only when the donor and the acceptor are close enough together to interact. Briefly, when the donor and the acceptor come close enough to each other, excitation of the donor by a fluorometer laser triggers an energy transfer towards the acceptor, which in turn emits specific fluorescence which is measured. Manufacturer's instructions were followed to perform the quantification of secreted TNFα.

2.4 Statistics

Statistical analysis was performed on at least three independent experiments with GraphPad Prism 6. A one-way ANOVA followed by Tukey assays with a risk α at 5% was used. Thresholds of significance were ****$p < 0.0001$, ***$p < 0.001$ compared to culture medium (negative control) and ££££$p < 0.0001$, £££$p < 0.001$ compared to the positive control (ROS inducer: tBHP 1.5 mM or inflammation inducer poly(I:C) 1 µg/mL).

3 Results

3.1 Antioxidant effects of oily formulations

Human keratinocytes were incubated according to the method described above with olive oil and corn oil, and then stressed with tBHP, a ROS inducer (Fig. 1).

As expected, tBHP led to a 1.54-fold increase in ROS production by keratinocytes compared to negative control. This ROS overproduction was significantly decreased by both olive oil and corn oil (−53% and −47%, respectively).

3.2 Anti-inflammatory effects of oily formulations

Human macrophages were incubated according to the method described above with corn oil and fish oil, and then stressed with poly(I:C), an inflammation inducer (Fig. 2).

As expected, poly(I:C) increased the secretion of TNFα (261 pg/mL *versus* 93 pg/mL in negative control). This TNFα oversecretion by macrophages is partially inhibited by corn oil (181 pg/mL *versus* 261 pg/mL) and fully inhibited by fish oil (87 pg/mL *versus* 261 pg/mL).

4 Discussion

In research, the use of laboratory animals tends to decrease for ethical and economical reasons leading to a boom in

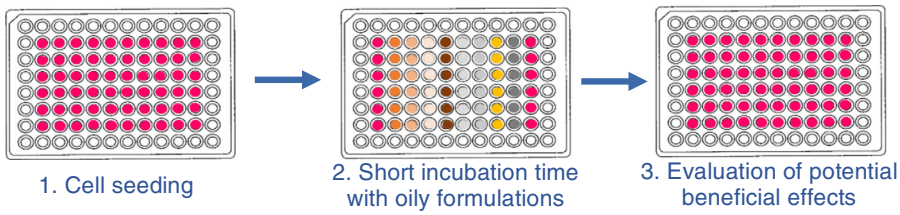

Fig. 3. Schematic representation of the incubation protocol. First, cells are seeded in microplates; second, cells are incubated with oily formulations for a short time followed by a 24-hour recovery period; third, the potential beneficial effects of oily formulations are evaluated.

cellular alternative methods. Besides, animal testing is completely prohibited for the evaluation of safety and beneficial effects of cosmetics in the European Union, Israel and India. Oily formulations are more and more used in cosmetics, but there is a lack of scientific data to support their claims. Evaluating cellular effects induced by oily formulations can be very tricky because of some major technical limitations. Indeed, oily formulations are not compatible with hydrophilic cell culture medium unless they are solubilized in organic solvents. The problem is that organic solvents can modify and alter the cellular response (Blachley *et al.*, 1985; Luo *et al.*, 1999; Elisia *et al.*, 2016). In light of this, we developed a method that doesn't require any organic solvent. It consists in incubating living cells with oily formulations during a short incubation time to avoid cell suffocation, followed by a 24-hour recovery period in cell medium to give cells time to end all triggered mechanisms (Fig. 3).

Depending on the study, the choice of the best-adapted cell model is essential. For example, skin cells are selected to study wound healing whereas immune cells are preferred to evaluate the inflammatory response. Human cellular models are chosen over animal cellular models to be closer to the final use of the oily formulations. Indeed, some metabolization enzymes differ from one specie to another (Martignoni *et al.*, 2006).

Our method is suitable for high throughput screening (HTS) in 96- and 384-well microplates, allowing the evaluation of multiple concentrations and/or multiple formulations at once. In addition, small amounts of oily formulations are required to perform the assays, which is a significant advantage when working with expensive and/or small samples. We used robust techniques such as cytofluorometry and FRET. Nondestructive cytometric assays are preferred, meaning that fluorescence signal is quantified directly on living adherent cells. Here we presented results from FRET experiments, but ELISA is also suitable as these two techniques are equivalent in terms of sensitivity and specificity. We selected FRET because:

- it is more convenient for minimal sample volume;
- it is able to measure 384 samples simultaneously, and;
- process time is quicker than ELISA (Einhorn and Krapfenbauer, 2015).

We focused on two important claims for the cosmetic industry: antioxidant and anti-inflammatory effects. Oxidative stress is a major actor in skin aging process (Rinnerthaler *et al.*, 2015), therefore, the identification of antioxidant properties is of high interest for the development of antiaging cosmetics. To demonstrate the ability of our method to reveal antioxidant effects, we chose two known antioxidant oils: olive and corn oil (Barrouin-Melo *et al.*, 2016; Carnevale *et al.*, 2018). Both were able to decrease the ROS overproduction induced by an oxidant agent. Therefore, this method could be applied to oily formulations suspected to be antioxidant.

We also focused on inflammation. As we previously said, inflammation, often linked with oxidative stress, plays a key role in many skin disorders like photoaging (Pillai *et al.*, 2005) or atopic dermatitis. Therefore, inflammation is a key cell endpoint for the development of cosmetic formulations such as antiaging. To demonstrate the ability of our method to reveal anti-inflammatory effects, we chose two known anti-inflammatory oils: fish and corn oil (Odabasoglu *et al.*, 2008; Mullen *et al.*, 2010; Calder, 2015). Both were able to decrease the oversecretion of TNFα induced by an inflammatory agent, fish oil being more efficient than corn oil. Therefore, this method could be applied to oily formulations suspected to be anti-inflammatory.

5 Conclusion

We developed an innovative method based on alternative methods to animal testing to evaluate different beneficial effects of oily formulations, without the use of any organic solvent. Our method bypasses the technical issues encountered with the experimental use of oily formulations and cell culture requirements such as hydrophilic medium and oxygen during *in vitro* studies. Oily formulations being used in nutrition, medical devices and drugs, our method can also be applied to these other fields and then represents a promising opportunity for diverse research departments.

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