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Article

In vitro evaluation of the cytotoxicity and eye irritation potential of preservatives widely used in cosmetics

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The consumption of cosmetics has been increasing every year and is expected to reach $675 billion by 2020 at an estimated growth rate of 6.4% per year. Exposure to skin irritants is the major cause of non-immunological inflammation of the skin. Therefore, the safety evaluation of cosmetic preservatives should be increased. Thus, the present work aimed to evaluate the cytotoxicity as the viability endpoint and the eye irritation potential of preservatives used in cosmetics. Cytotoxicity assays were performed using MTT and NRU in human keratinocytes (HaCaT), human dermal fibroblasts, adult (HDFa), and human hepatoma cells (HepG2). The eye irritation potential was evaluated using the Hen's Egg Test-chorioallantoic membrane (HET-CAM). The evaluated preservatives were methylparaben (MP), propylparaben (PP), phenoxyethanol (PE), and a mixture of methylchloroisothiazolinone and methylisothiazolinone (CMI/MI). All preservatives showed cytotoxic potential within the permitted concentrations for use in cosmetic products. In the HET-CAM test, PE and CMI/MI, MP, and PP were classified as severe, moderate, and poor irritants, respectively. Our results indicate that proper safety evaluations are required to ensure the beneficial properties of preservatives on cosmetic products without exceeding exposure levels that would result in adverse health effects for consumers.

Keywords: Preservatives. Cosmetics. Cell culture. Cytotoxicity. HET-CAM test.

INTRODUCTION

The global cosmetic market was evaluated in $460 billion in 2014 and is expected to reach $675 billion by 2020 at an estimated growth rate of 6.4% per year. This rising market requires constant innovation with safe cosmetic products. In this perspective, the safety of ingredients and the absence of microbiological contamination are indispensable. According to the Rapid Alert System (RAPEX) of the European Commission (EC), 62 cosmetic products were recalled between 2008 and 2014 due to contamination by microorganisms. The recalled products were notified by 14 different countries mostly in 2013 and 2014 (Halla *et al.,* 2018).

Preservatives are added to cosmetic products to prevent the growth of bacteria and fungi during shelf life and to ensure that consumers are protected from the harmful effects of microorganisms. Insufficient use of preservatives can lead to deterioration of the product; on the other hand, excessive use of preservatives can increase the risk of adverse reactions (Halla *et al.,* 2018; Herman, 2019).

Exposure to skin irritants is the major cause of non-immunological inflammation of the skin. Dermal exposure to chemical substances can lead to a wide variety of skin reactions such as irritant contact dermatitis, sensitization, altered pigmentation, acne, and cancer. The safety of cosmetic products is generally determined by the known toxicity of the ingredients and skin compatibility (van de Sandt *et al*., 1999).

Parabens are *p*-hydroxybenzoic acid ester compounds that have been used as antimicrobial preservatives in food, pharmaceutical products, and cosmetics for nearly

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10 decades. They are highly valuable substances due to their antimicrobial activity against a wide range of microorganisms, safety (low acute toxicity and low potential for irritation or sensitization), and stability over a wide range of pH values (Aubert *et al*., 2012). Methylparaben (MP) (CAS n. 99-76-3) and propylparaben (PP) (CAS n. 94-13-3) are the most commonly used parabens. However, these and other parabens have been under attack in recent years because of possible adverse effects such as breast cancer, decreased sperm production, and endocrine disruption (Oishi, 2002; Darbre *et al*., 2004; Darbre, Harvey, 2008, 2014; Kodani *et al*., 2016; Tahan *et al*., 2016). Nevertheless, the maximum authorized concentrations for MP (0.4% for one ester or 0.8% when used as a mixture of esters) and PP (0.14%) are considered safe for human health (European Union, 2009; SCCS, 2013).

Phenoxyethanol (PE; CAS n. 122-99-6) is a broad-spectrum preservative that has excellent activity against a wide range of gram-negative and gram-positive bacteria, yeast, and molds, and it is used together with many other preservatives (Shabir *et al.,* 2010). However, the Scientific Committee on Consumer Safety (SCCS) has considered that the maximum PE concentration that is safe to use in cosmetic products is 1% (Lilienblum, 2016).

The isothiazolinones methylchloroisothiazolinone (CMI; CAS n. 55965-84-9) and methylisothiazolinone (MI; CAS n. 2682-20-4) are used either as a mixture with a 3:1 ratio CMI/MI or as a single substance. They are classified as skin sensitizers according to the European Commission (EC) Regulation No. 1272/2008 (European Union, 2008; Garcia-Hidalgo *et al*., 2018; Towle *et al*., 2018) and are known to cause allergic contact dermatitis (ACD) (Groot *et al*., 1988; Lundov *et al*., 2011; González-Muñoz *et al*., 2014; Yu, Sood, Taylor, 2016; Berthet *et al*., 2017; Garcia-Hidalgo *et al*., 2018). Accordingly, the use of the CMI/MI mixture in leave-on personal care products (PCPs) was prohibited starting the 16th of April 2016 (European Union, 2014), whereas the same ban was applied to MI alone from the 12th of February 2017 (European Union, 2016). For rinse-off products, the maximum allowed concentration is 0.0015% for both the CMI/MI mixture and MI alone (European Union, 2009; Garcia-Hidalgo *et al*., 2018; Towle *et al*., 2018).

Animal models have been replaced in recent years by alternative methods. Because all animal species have evolved to fill specific environmental niches and have divergent genetic backgrounds, physiology, and metabolism, many candidate drugs fail during clinical testing, which is due to not only a lack of efficacy in humans, but also to the identification of unacceptable toxicity issues not detected in pre-clinical animal testing. Furthermore, given that non-human mammals are poor models for predicting toxicity in humans, the cells derived from them cannot be better (Jennings, 2015). For this reason, the 21st century toxicology movement signals the need for an improved toxicological approach to risk assessment that is more precise and relevant to predict the real effect of a substance in humans. To achieve this goal, it is necessary to abandon tests on animals and to perform studies in human cells *in vitro* instead, to understand and assess the real biochemical mechanism of action in our species (Hartung, 2009).

The Hen's Egg Test-chorioallantoic membrane (HET-CAM) is an alternative method to animal experimentation for evaluating corrosives and/or severe ocular irritants that uses the chorioallantoic membrane of an embryonated hen's egg. This test assesses the damage to this membrane to determine the potential of irritation to the conjunctiva. The acute effects of the tested substance on the small vessels and proteins of the soft tissue of the membrane are assumed to be similar to the effects in the eyes of rabbits (Cazedey *et al*. 2009; ICCVAM, 2010).

Some preservatives have been shown to cause allergies, while others are suspected to be endocrine disruptors or to cause resistance to some bacteria (DEPA, 2015). Consumers currently use up to ten products a day, either cosmetics or PCPs. For these reasons, the safety of preservatives should be assessed.

In this study, the preservatives MP, PP, PE, and CMI/MI were evaluated for their eye irritation potential by the HET-CAM test, and cytotoxic effects as the viability endpoint in three cell lines: human keratinocytes (HaCaT) and human dermal fibroblasts, adult (HDFa) for assessing skin irritancy, and human hepatoma cells (HepG2) as a preliminary method to evaluate the hepatotoxicity of cosmetic raw materials.

MATERIAL AND METHODS

Reagents and samples

3-4(4.5-dimethyl-2-thiazolyl)-2.5-diphenyl-2H-tetrazolium bromide (MTT), neutral red solution (0.33%), sodium lauryl sulfate (SLS), and phosphate buffer saline (PBS) were purchased from Sigma–Aldrich; MP (99.8%) and PP (99.7%) (All Chemistry), PE (99.2%) (Clariant), CMI/MI (1.15%/0.35%) (Biovital), penicillin 100 U/mL, streptomycin 0.1 mg/mL, and amphotericin B 25 mg/mL solution, Dulbecco's Modified Eagle's Medium - high glucose (DMEM), and fetal calf serum (FCS) were purchased from Cultilab; trypsin 10x (Gibco), dimethyl sulfoxide (DMSO), isopropanol, glacial acetic acid solution, and absolute ethanol 99.8% were purchased from Synth; fertile white leghorn chicken eggs were purchased from Mário Salviato, Brazil.

Cell lines

HDFa, HaCaT, and HepG2 cells were purchased from Life Technologies, Brazil.

In vitro cytotoxicity assay

Cell culture routine

Each cell line (HaCaT, HDFa, and HepG2) was grown separately in DMEM supplemented with 10% of FCS and penicillin 100 U/mL, streptomycin 0.1 mg/mL, and amphotericin B 25 mg/mL. The cultures were maintained at 37 ± 2 °C and in a 5% CO_2 atmosphere.

When cells reached a confluence of 80%–90%, they were removed from the culture flasks by enzymatic digestion (trypsin/EDTA), and the cell suspension was centrifuged (1200 rpm, 3 min). The cells were then resuspended in culture medium and the cell suspension was adjusted to a density of 5×10^5 cells/mL. Using a multichannel pipette, 100 µL of culture medium (blank) was dispensed into the peripheral wells of a 96-well plate and 100 µL of cell suspension into the remaining wells. The 96-well plates were then incubated for 24 h for complete cell adhesion to the plate.

MTT

The cytotoxic effect of MP, PP, PE, and CMI/MI was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) assay using HaCaT, HDFa, and HepG2 cells. The protocol used was based on ISO 10993-5 (2009).

The cells were treated with 100 µL of positive control (10% dimethyl sulfoxide), negative control (DMEM), and five different concentrations obtained by serial dilution of MP, PP, PE, and CMI/MI (Table I). Due to the low solubility of MP and PP in DMEM, ethanol was used to help the solubilization. Therefore, for each ethanol concentration, a negative control containing the same amount of ethanol in DMEM was tested (Chiari *et al*. 2014). The 96-well plates were incubated at 37 ± 2 °C and in a 5% CO_2 atmosphere. DMEM with 5% FCS was used as a treatment medium to ensure good cell health (Repetto *et al*., 2008).

After 24 h of treatment, the plates were gently washed with PBS and 100 µL of MTT solution (1 mg/mL in PBS) was added to each well. The 96-well plates were incubated at 37 ± 2 °C and in a 5% CO_2 atmosphere for 3 h. Then, the MTT solution was decanted and 100 µL of isopropanol was added to each well. The plates were rapidly shaken on a microtiter plate shaker for at least 10 min and the optical density (OD) was measured at 570 nm in a Spectrostar Nano–BMG Labtech spectrophotometer. The cytotoxicity was evaluated by at least three independent assays and, for each one, the treatment was performed in triplicate.

The percentage of dead and live cells was calculated based on the negative control and represents the cytotoxicity of each treatment, as follows (Chiari *et al*., 2014):

$$\%ofcellularviability = \frac{(Abs_{ofnegativecontrol} - Abs_{oftreatment})}{Abs_{ofnegativecontrol}} x100 \quad (1)$$

The concentration that promoted the reduction of cellular viability in 50% (IC_{50}) was calculated by a non-linear regression curve using the concentration (log) versus the percentage of living cells (Table I; Figure 1).

Neutral red uptake (NRU)

The cytotoxic effects of MP, PP, PE, and CMI/MI were evaluated by the NRU assay using HaCaT, HDFa,

and HepG2 cells. The protocol used was based on ISO 10993-5 (2009).

The cells were treated with 100 µL of positive control (SLS), negative control (DMEM), and the same preservative concentrations used for the MTT assay (Table I).

After 24 h of treatment, the plates were gently washed with PBS and 100 µL of NR medium (40 µg/mL, incubated overnight) was added to each well. The 96-well plates were incubated at 37 ± 2 °C and in a 5% CO_2 atmosphere for 3 h. After incubation, the NR medium was removed, and the cells were washed with PBS. PBS was decanted and 150 µL of NR desorb solution (1% glacial acetic acid, 50% ethanol, and 49% H_2O) was added to all wells, including blanks. The plates were rapidly shaken on a microtiter plate shaker for at least 10 min. The OD was measured at 540 nm using a microtiter plate reader. The analysis of the response to different concentrations and calculation of IC_{50} were performed as in the MTT assay (Table I; Figure 2).

SLS was tested in a four-concentration scale: 0.05 mg/ml; 0.1 mg/ml; 0.15 mg/ml; 0.2 mg/ml. The historical mean and IC_{50} of SLS is 0.093 mg/mL. The test met the acceptance criteria if the IC_{50} for SLS was within the 95% CI (Confidence interval; 0.070 mg/ml to 0.116 mg/ml) (ISO, 2009).

Statistical analysis

Statistical analysis was performed using the GraphPad Prism software, and the graphs were plotted in OriginPro. One-way ANOVA and Tukey's multiple comparison test were performed to assess the statistical difference between the means of the preservatives in each cell line. An unpaired two-tailed t-test was performed to assess the statistical difference between the NR and MTT assays (Table I). All values are reported as the mean ± standard deviation (SD). Differences were considered significant when $P < 0.05$. The CI was set at 95%.

Eye irritation potential

The eye irritation potential test was performed using the HET-CAM assay (Luepke, 1985). The protocol was carried out according to ICCVAM (2010).

Freshly fertilized hen eggs were obtained on day zero weighing between 50 and 60 g, and defective eggs (excessively misshapen eggs or eggs with cracked or thin shells) were discarded. The eggs were incubated at 37 ± 0.5 °C and 40% ± 5% humidity for 10 days in the horizontal position to ensure the correct positioning of the embryo (away from the CAM) (McKenzie *et al*., 2015). The exact moment when they were placed in the incubator was marked as T_0. On day 9, the eggs were candled to ensure fertility, and the shell was marked on the line of the airspace. The eggs were incubated for 10 days. Then, each egg was placed in an egg holder with the larger end upwards and the shell was cut just above the marked line of the chorioallantoic membrane using surgical scissors. The inner membrane was then carefully removed using forceps, without causing injury to the blood vessels, to expose the chorioallantoic membrane below. The test solution volume used was 300 µL for PE, CMI/MI, negative control (0.9% (w/v) NaCl in deionized water), and positive control (0.1 N NaOH in deionized water), measured using a pipette. For MP and PP, 100 µL was added directly onto the CAM to ensure that at least 50% of the CAM surface area was covered, and a timer started. Any lysis, hemorrhage, and/or coagulation was documented at different times over a 5 min period after application of the test solution, and any effect was noted and compared with controls (ICCVAM, 2010).

The evaluation is based on the development of each of the three HET-CAM endpoints at fixed time intervals (Luepke, 1985) of 30, 120, or 300 s. The numerical scores for lysis, hemorrhage, and coagulation (Table II) obtained according to the appearance time were summed to give a single numerical value (Table III), indicating the Irritation Score (IS) of the test substance on a scale with a maximum value of 21, according to Oliveira *et al*. (2012).

The score for each egg is the sum of lysis, hemorrhage, and coagulation scores. The rating of a product tested is the arithmetic mean rounded to one decimal of the scores obtained on four eggs, with a maximum rating of 21, which corresponds to an irritation category based on the irritating potential of the test product on the chorioallantoic membrane, as shown in Table IV. Once this 300 s period was over, the remaining eggs were placed in a sealed bag for subsequent

incineration. Each egg was treated in this way until all eggs had been tested and destroyed (McKenzie *et al*., 2015; Derouiche, Abdennour, 2017).

A test was considered acceptable if the negative and positive controls induced a response that fell within the classification of nonirritating and severely irritating, respectively. Historical control studies indicate that the IS value is 0.0 when using 0.9% NaCl as a negative control, and 19 when using 0.1 N NaOH as a positive control. When using the IS analysis method, the severe irritancy classification for a test substance is assigned when the value is greater than nine (ICCVAM, 2010).

RESULTS AND DISCUSSION

In vitro cytotoxicity assays

Generally, published data for the selected preservatives are limited. Many of the available data are old (back from the 1980s–1990s) and with animal testing, and literature studies show that very few new data have been published for these substances (DEPA, 2015). These arguments reinforce the need to reevaluate the safety of commercially available preservatives through alternative methods.

The neutral red uptake assay is based on the ability of viable cells to incorporate and bind the supravital dye neutral red. This weakly cationic dye penetrates cell membranes by nonionic passive diffusion and concentrates in the lysosomes, where it binds by electrostatic hydrophobic bonds to anionic and/or phosphate groups of the lysosomal matrix. (Repetto *et al*. 2008). The MTT assay makes use of the fact that succinate dehydrogenase present in the mitochondria of viable cells metabolizes yellow water-soluble MTT to a blue, insoluble formazan derivative (Mosmann, 1983; Triglia *et al*., 1991). The amount of formazan produced is proportional to the number of viable cells remaining in the culture. According to the t-test (unpaired, two-tailed), the means of the IC_{50} values obtained in both assays were different, except for PP in the HepG2 cell line, for which the means were considered equal ($P < 0.05$).

TABLE I - IC_{50} % (w/v) values obtained in cytotoxicity assays in different cell lines

Preservatives	IC_{50} % MTT/HaCat	IC_{50} % MTT/HDFa	IC_{50} % MTT/HepG2	IC_{50} % NRU/HaCat	IC_{50}% NRU/HDFa	IC_{50} % NRU/HepG2	Concentrations used % (w/v)	Maximum concentration[1] % (w/v)
PE	0.1636[a] ±0.0102	0.1516[a] ±0.0115	0.2494[a] ±0.0126	0.2174[a] ±0.0077	0.2029[a] ±0.0075	0.2076[a] ±0.0091	0.12-2.0	1.0
CMI/MI	0.0005[a] ±1E-05	0.0011[ab] ±1.8E-04	0.0008[ac] ±7.53E-05	0.0004[a] ±8.8E-06	0.0003[a] ±6.80E-05	0.0005[ae] ±1.18E-05	0.00018-0.0030	0.0015
MP	0.0369[a] ±0.0026	0.0730[a] ±0.0002	0.0315[ad] ±0.0093	0.0413[a] ±0.0004	0.0735[a] ±0.0055	0.0809[a] ±0.0081	0.025-0.40	0.80– 0.40
PP	NC[2]	0.0194[ab] ±4.9E-05	0.0145[acd] ±0.0012	NC[2]	0.0158[a] ±0.0006	0.0142[ae] ±0.0004	0.012-0.20	0.14

Mean ± standard deviation (SD) considering three independent experiments. [a]Significantly different values (Tukey's test – 95% confidence interval). [b,c,d,e]Significantly equal values (Tukey's test – 95% confidence interval). PE: Phenoxyethanol; CMI/MI: Mixture of methylchloroisothiazolinone and methylisothiazolinone; MP: Methylparaben; PP: Propylparaben. [1]Maximum concentration according to Annex V of Regulation European Commission (EC) No. 1223/2009 (European Union, 2009); [2]Not calculable (NC): cell viability less than 50% at the lowest concentration tested.

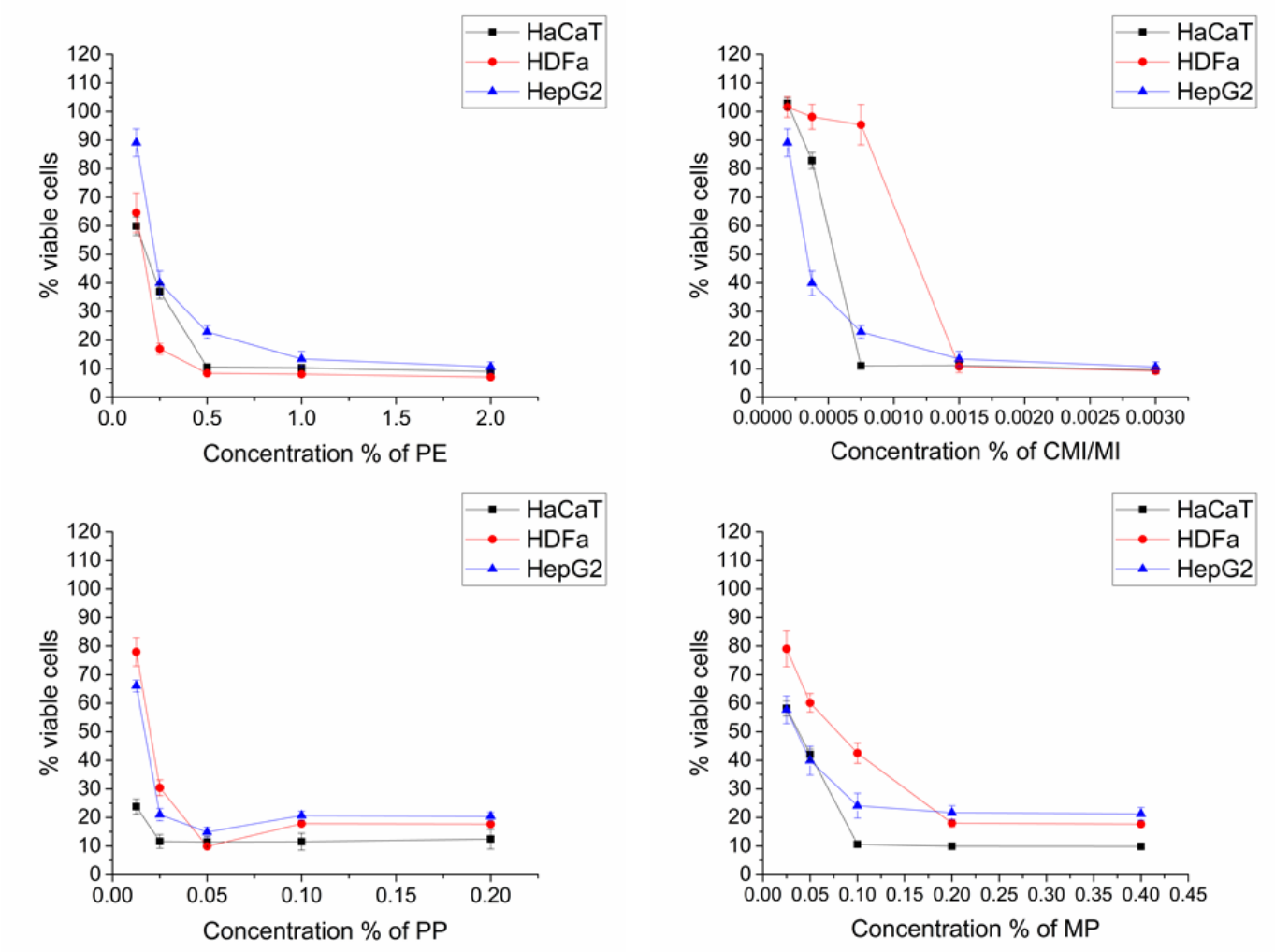

FIGURE 1 - MTT assay: viability of HaCaT, HDFa, and HepG2 cells treated with PE, CMI/MI, PP, and MP.

Results are expressed as mean ± standard deviation (SD), considering three independent experiments. PE, phenoxyethanol; CMI/MI, mixture of methylchloroisothiazolinone and methylisothiazolinone; PP: propylparaben; MP: methylparaben.

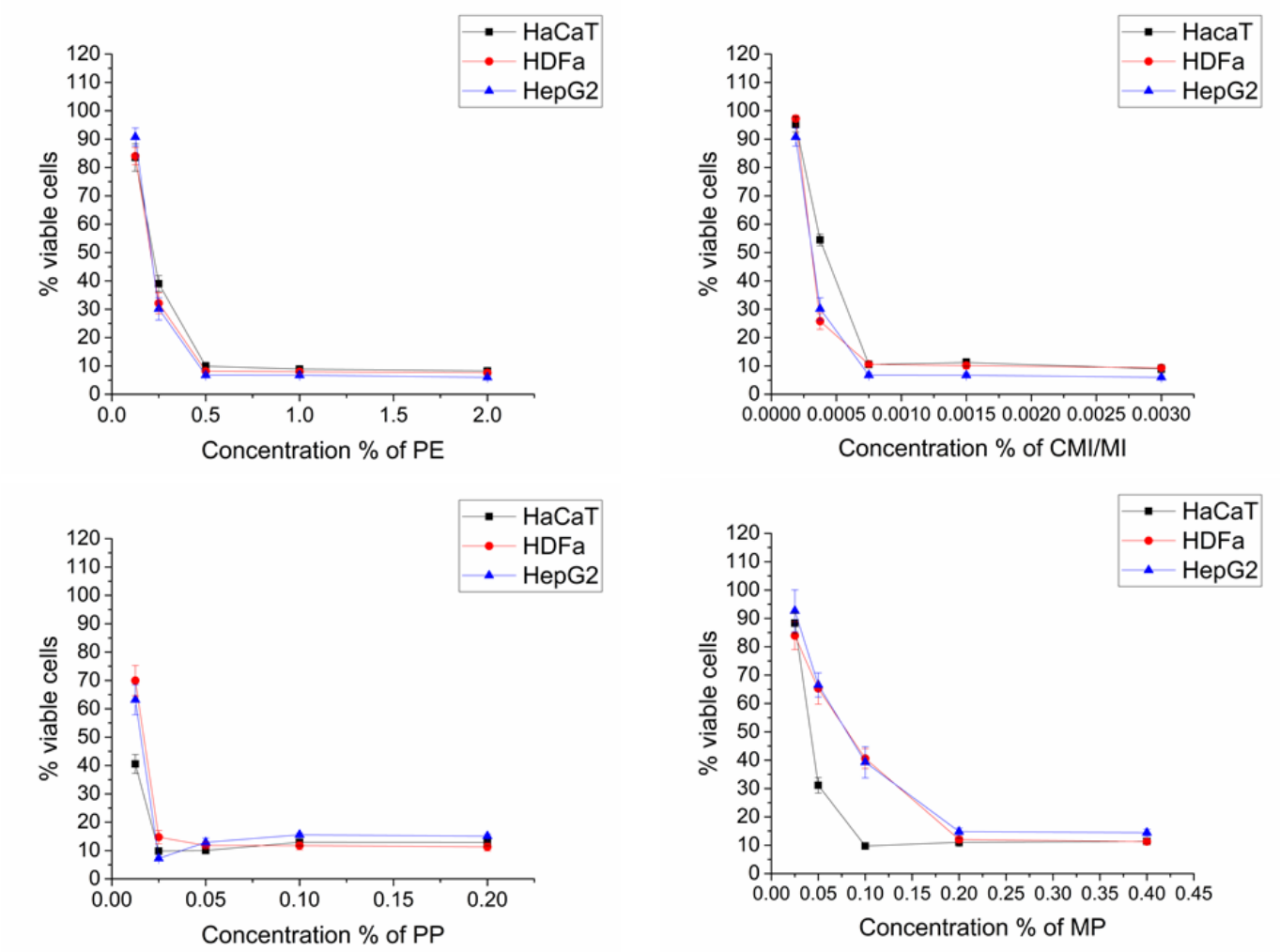

FIGURE 2 - NRU assay: viability of HaCaT, HDFa, and HepG2 cells treated with PE, CMI/MI, PP, and MP.

Results are expressed as mean ± standard deviation (SD), considering three independent experiments. PE, phenoxyethanol; CMI/MI, mixture of methylchloroisothiazolinone and methylisothiazolinone; PP: propylparaben; MP: methylparaben.

Multiple mechanisms and cell types are involved in the induction of skin toxicological responses (van de Sandt *et al*., 1999). The complex phenomenon of skin irritation involves resident epidermal cells, dermal fibroblasts, and endothelial cells as well as invading leukocytes that interact with each other under the control of a network of cytokines and lipid mediators (Bos, Kapsenberg, 1993).

Keratinocytes are a biologically relevant target for skin irritants, as they are the first living cells to come into contact with substances applied topically (Taofiq *et al*., 2019). HaCaT cells are an immortalized cell line that, in conventional monolayer culture assays with a panel of skin irritants, exhibited a toxicity ranking remarkably similar to normal keratinocytes, measuring MTT reduction as the viability endpoint. However, they showed higher sensitivity, ranging between that of 3T3 cells and normal keratinocytes (van de Sandt *et al*., 1999). This indicates that they are a good cellular model for assessing skin irritation and toxicity under conventional culture conditions.

HDFa cells belong to a dermis cell type with mesenchymal origin and are found in all connective tissues. These cells play a critical role in healing as well as in the synthesis and secretion of extracellular matrix proteins and collagen under cell culture conditions (Abdian *et al*., 2015). Skin fibroblasts contribute to the

fibroblast–keratinocyte–endothelium complex that not only repairs wounds but also maintains the integrity and youth of the skin (Kim *et al*., 2007).

In vitro tests with conventional keratinocyte or fibroblast cultures aimed at evaluating the cutaneous irritancy of various surfactants have shown a good correlation with the irritating effect observed *in vivo* (Gueniche, Ponec, 1993).

HepG2 is a metabolically competent human hepatocellular carcinoma cell line widely applied to simulate the liver function of the human organism *in vitro*. It shows phase I and phase II enzyme activity, including cytochrome P450 enzymes (Sassa *et al*., 1987; Natarajan, Darroudi, 1991; Dauer *et al*., 2003; Hewitt, Hewitt, 2004). The use of this cell line to analyze cosmetic raw materials may seem unnecessary, as active cosmetic substances are expected to not permeate the skin. However, when *in vitro* tests are used to evaluate skin permeation of cosmetic products, the use of this strain is indispensable (Chiari *et al*., 2012).

Parabens are aromatic carboxylic acids containing a carboxyl group bonded directly to a benzene ring; a hydroxy group is present on position 4 with varying alkyl radicals as side chains. Increasing chain length decreases water solubility and thus the desired preservation activity (Fransway *et al*., 2019). PP showed more cytotoxicity than MP, which is consistent with SCCS concerns about the safety of PP. HaCaT cells treated with PP showed cell viability lower than 50% at the lowest concentration tested (0,012%, Table I; Figures 1 and 2). However, we must consider that keratinocyte monolayers are sensitive because of the lack of a stratum corneum (van de Sandt *et al*., 1999). Furthermore, PP has a longer hydrocarbon alkyl chain than MP, and paraben biocidal activity increases with the length of the hydrocarbon alkyl chain, probably contributing to the high cytotoxicity of PP (Fransway *et al*., 2019).

Isothiazolinones (CMI/MI) have low molecular weights (CMI: 150.56 g/mol; MI: 115.15 g/mol), allowing for easy penetration in the epidermis, which, followed by a reaction with the skin macromolecules, may trigger an ACD (Garcia-Hidalgo *et al*., 2018). Isothiazolinone activity is related to the thiol and amine groups of their structures (Halla *et al*., 2018). According to the Cosmetic Ingredient Review (CIR), clinical and nonclinical studies supported that CMI/MI could be safely used in rinse-off products at a concentration up to 0.0015% (Towle *et al*., 2018). Nevertheless, the results obtained in our cytotoxicity assays showed that at this same concentration, cell viability was lower than 50% (Table I; Figures 1 and 2). Undeniably, CMI/MI in a ratio of 3:1 was classified as corrosive or irritating at high concentrations, and no adequate data are provided to support safe use at the maximum authorized concentration of 0.0015% in rinse-off cosmetic products (SCCS, 2009).

Anselmi *et al*. (2002) evaluated the *in vitro* induction of apoptosis vs. necrosis by widely used preservatives, including PE and CMI/MI in a human promyelocytic cell line (HL60). Their results showed that cells treated with low concentrations of CMI/MI (0.001–0.01%) and PE (0.01–0.5%) displayed all the characteristic alterations of apoptosis. In particular, PE showed a drastic decrease in cell viability at time zero, suggesting the sudden death of the cells. These results were observed at a 1% concentration, which is the concentration considered safe by SCCS (Lilienblum, 2016). Furthermore, studies on rabbit skin have shown that PE is potentially a mild irritant (SCCS, 2016). In our study, PE showed high cytotoxicity in all cell lines. and its IC_{50} value, considering both cell lines and assays, was ~0.200 (Table I; Figures 1 and 2).

In our experiments, all preservatives have shown cytotoxic potential within the permitted concentrations for use in cosmetic products. However, the assay simulates the application of several concentrations of the same preservative in a single cell layer for 24 h, without considering the normal structure of human skin and the aggregate exposure. A consumer may have aggregate exposure to the same preservative using multiple personal care and cosmetic products applied to the same body site (Towle *et al*., 2018). The concentrations used in our tests simulate actual use concentrations; however, it is necessary to also consider the amount of product applied and the type of product (leave-on or rinse-off).

The results of this analysis suggest that in the case preservatives are able to penetrate the stratum corneum and interact with skin macromolecules, they can trigger adverse reactions (e.g., mild irritation of the skin by estrogenic activity), as shown by the cytotoxicity in the HaCaT and HDFa cell lines, but it is unclear whether

aggregate exposure and the use of other cosmetic ingredients can facilitate the penetration of some substances or intensify adverse reactions.

Eye irritation potential

The HET-CAM test is used to identify potential nonirritating or mildly irritating materials (formulations and raw materials). The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM, 2010) recommends the HET-CAM test for non-regulatory validation or optimization studies, and in countries that authorize or require a Draize test for cosmetic products, HET-CAM can be used as a pretest before deciding on an animal study. However, the 2013 ban on animal testing of cosmetic products in Europe, according to Regulation (EC) No.1223/2009 (European Union, 2009), imposed the need to use *in vitro* methodologies that can mimic the results obtained *in vivo* as much as possible.

Although the HET-CAM test is an established and reliable test for screening purposes, it cannot completely replace the Draize test. The current Draize eye irritation test evaluates the changes observed on the anterior segment of the eye, including the density and area of corneal opacification and the severity of iritis, conjunctival redness, edema, and discharge. HET-CAM evaluates only one segment of the eye (conjunctiva) and must be complemented with corneal models (Derouiche, Abdennour 2017).

The IS obtained with the negative control (0.9% NaCl) and the positive one (0.1 N NaOH), 0.0 and 19, respectively, satisfied the criteria for an acceptable test (ICCVAM, 2010).

TABLE II - Scores for lysis, hemorrhage, and coagulation for HET-CAM test

Phenomenon	Score		
	30 s	120 s	300 s
Lysis	5	3	1
Hemorrhage	7	5	3
Coagulation	9	7	5

Score defined as a function of time (seconds) of occurrence of each phenomenon.
The score is defined as a function of the time (seconds) of occurrence of each phenomenon.

TABLE III - Irritation category according to HET-CAM score range

Score range	Irritation category
0.0 to 0.9	Nonirritant
1.0 to 4.9	Mild irritant
5.0 to 8.9	Moderate irritant
9.0 to 21.0	Severe irritant

TABLE IV - Classification of preservatives submitted to the HET-CAM test

Preservatives	Time[1] (s)	IS	Irritation category
PE	21*	16	Severe irritant
CMI/MI	24*	11	Severe irritant
MP	121*	6	Moderate irritant
PP	300**	0	Nonirritant

PE: Phenoxyethanol; CMI/MI: Mixture of methylchloroisothiazolinone and methylisothiazolinone; MP: Methylparaben; PP: Propylparaben. IS: Irritative score. [1]Time, in seconds, when * or not ** the first change in the chorioallantoic membrane occurred.

The preservatives considered irritants were PE (IS: 16) and CMI/MI (IS: 11). Lysis, hemorrhage, and coagulation phenomena were observed in the chorioallantoic membrane, thus demonstrating the irritation potential of these preservatives. Accordingly, CMI/MI at a ratio of 3:1 is considered corrosive or irritating at high concentrations (SCCS, 2009) and the results obtained are comparable to those of animal tests: in the Draize test, PE produced clear signs of eye irritation, and corneal opacity was observed in all animals (SCCS, 2016).

MP was considered moderately irritating (IS: 6), as only the lysis and hemorrhage phenomena were observed. Likewise, MP at 100% concentration was slightly irritating when instilled into the eyes of rabbits, and a primary eye irritation study in humans showed MP to be nonirritating at concentrations up to 0.3% (CIR, 2008; SCCS, 2013). On the other hand, PP was practically nonirritating (IS: 0), since the blood vessels of the chorioallantoic membrane showed no signs of irritation, similarly to the negative control. Several rabbit eye irritation studies have been conducted on products containing methylparaben, ethylparaben, propylparaben, and/or butylparaben at concentrations of 0.1% to 0.8%. Most products produced no signs of eye irritation, while others produced slight or minimal eye irritation (CIR, 2008). Moreover, parabens are used at low concentrations, which could decrease irritative effects when solubilized in a cosmetic formulation.

PE was cytotoxic in the three cell lines analyzed and classified as a severe eye irritant. This preservative is being used to replace parabens, which are less cytotoxic and have a lower ocular irritation potential. Therefore, proper safety evaluations are required to ensure the beneficial properties of preservatives in cosmetic products without exceeding safe exposure levels, which would result in adverse health effects for consumers.

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