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A N I N T E R N A T I O N A L J O U R N A L

A Reassessment of the *In Vitro* Total Protein Content Determination (TPC) with SIRC and 3T3 Cells for the Evaluation of the Ocular Irritation Potential of Shampoos: Comparison with the *In Vivo* Draize Rabbit Test

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ABSTRACT

The aim of this work was to determine the correlation between results obtained from the Draize test and from the Total Protein Content Determination (TPC) to assess the ocular irritancy potential of 20 shampoos. For TPC, two established cell lines (SIRC and 3T3) were used. The concentration that induced 50% inhibition relative to controls (IC_{50}) was calculated for each product. Among shampoos tested with SIRC, only one had a false positive result. However, for the 3T3, three false-negative results were found. Pearson coefficient related to the in vivo *value of maximum average score (MAS) was -0.58 (p=0.007) with SIRC and –0.73 (p=0.007) with 3T3. These results showed that the TPC assay was capable to predict the ocular irritant potential of shampoos, and therefore was a promissory tool to be used as a preliminary assay for the detection of irritant products and to be part of a battery of screening tests to minimize the animal use in the Draize Test.*

Key words: Draize test, ocular irritation, TPC, shampoos, SIRC, 3T3

INTRODUCTION

The political pressure exerted by the part of the scientific community and activists sectors that defend animal rights and contest *in vivo* methods has generated an important impact on the scientific research. In this context, the chemical industry or even government regulatory and quality control agencies are under ever-growing pressure to replace the animal testing by the methods that do not use animals in the toxicological evaluation of health products (Eun and Suh, 2000; Pauwels and Rogiers, 2004).

The evaluation of eye and skin irritation potential is essential to ensure the safety of individuals that are in contact with a wide variety of substances designed for the industrial, pharmaceutical or cosmetic use (Vinardell and Mitjans, 2008). Cosmetics are among the most controversial products which use animal tests to assess the skin and eye irritation. This is a crucial issue, especially in the European Community countries that demand

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new initiatives and commercialization of "cruelty-free cosmetics" (McNamme et al., 2009; Barile, 2010).

The Draize eye irritation test is a test recommended by the international guidelines for the safety assessment of the chemicals (Scott et al., 2010) and it is the only test accepted by some regulatory agencies, e.g., Brazilian National Health Surveillance Agency (ANVISA), to assess the eye irritation potential. However, several aspects of this test have been criticized, including its subjectivity, cruelty and overestimation of its response in comparison to human exposure (Wilhelmus, 2001; Princen, 2006). Consequently, some laboratories have been making efforts to develop and validate *in vitro* assays in order to replace *in vivo* methods. Many methodologies have been devised to evaluate the eye irritation, such as the Chicken Enucleated Eye test (CEE), Isolated Rabbit Eye test (IRE), Bovine Corneal Opacity and Permeability test (BCOP), Cultured Bovine Lens test, Hen´s Eggs Chorioallantoic Membrane test (HET-CAM), Neutral Red Release assay (NRU), the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay (MTT), Red Blood Cell Lysis assay (RBC), etc (Balls et al., 1995; Vian et al., 1995; Pape et al., 1999; Cooper et al., 2001; Burdick et al., 2002; Eskes et al., 2005; Vinardell and Mitjans, 2006; Alves et al., 2008; Tavaszi et al., 2008; Schutte et al., 2009).

Since more than one mechanism can induce eye irritation, only one *in vitro* assay is not sufficient for a complete evaluation of this endpoint. For this reason, a combination of *in vitro* tests will be required to predict the human eye irritancy effectively (Rougier et al., 1992; Earl et al., 1997; Scott et al., 2010). The ideal is to obtain the data related to different outcomes; for example, vascularization (e.g., HET-CAM), opacity/ permeabilization (e.g., BCOP) and cytotoxicity (e.g., RBC) (Debbasch et al., 2005; Barile, 2010). Studies of new *in vitro* ocular toxicity methods are, however, still being compared to the data obtained in the tests that have been performed in the rabbits because adequate human data are not available (Roggeband et al., 2000; Princen, 2006). It is important to note that most of these tests are being used to assess the irritation potential of the isolated ingredients, such as the surfactants used in different formulations of shampoos, soaps and other cosmetics (Sina et al., 1995; Gerner et al., 2005; Martinez et al., 2006; Costa et al., 2009). Such substances are, in most cases, responsible for the induction of toxic effects in the rabbit's eyes (Froebe et al., 1990).

The most studied alternative methods for eye irritation are those that assess the cytotoxicity of a substance on a cell monolayer (Guillot, 1992). One of these methods was described by Shopsis and Eng (1985), and is based on a rapid screening procedure of cytotoxicity that evaluates the growth rate reduction reflected by the colorimetric determination of the total protein content stained with Coomassie blue in the cell culture. Over the years, another methodology with the same scientific basis has been proposed using an analogous stain, called Kenacid blue (Clothier et al., 1988; Clothier, 1995; Clothier et al., 2006). In 1995, a similar method called Total Protein Content Determination (TPC) that quantified the cell proliferation by the adsorption of the dye, its elution and measurement of optical density (OD), was evaluated concerning its value to predict the ocular irritancy of the surfactants in mouse embryo fibroblasts (3T3), rabbit cornea1 cells (SIRC) and L929 mouse fibroblasts (Vian et al., 1995). There was an inverse relationship between the cytotoxic effect of the test substance and the content of protein measured.

Despite the well-known advantages of the TPC assay - such as speed, sensibility, low cost and a high degree of automation - studies about its value to effectively predict the human ocular irritancy are scarce and limited to effects induced by the isolated substances. For this reason, the present study evaluated the applicability of this cytotoxicity assay using SIRC and 3T3 cell lines to predict the irritation potential in the finished products. It also aimed to a better understanding of its applicability in combination with other *in vitro* assays in order to minimize the animal use in the Draize eye irritation test.

MATERIAL AND METHODS

Samples

For this study, twenty shampoos – eleven for children and nine for adult use - were acquired from the commercial establishments in Rio de Janeiro (Brazil) and coded 1-20. A description of these products formulations can be found in Table 1. All the samples were tested in blind study in both *in vivo* and *in vitro* tests.

Table 1 - Formulation of the analysed shampoos, and classification of their irritation potential by the Draize test.

Product	Composition	MAS	Final Classification*
Sh01	Disodium laureth sulfosuccinate, sodium lauryl sulfate, PEG-80 glyceryl cocoate, sorbitan laureth, sodium lauryl sarcosinate	1.6	Non-irritant
Sh02	Sodium laureth sulfate, PEG-200 hydrogenated glyceryl palmate, disodium cocoamphodiacetate, PEG-7 glyceryl cocoate, PEG-30 glyceryl cocoate, disodium ricinoleamido MEA-sulfosuccinate, sodium laureth-8 sulfate, magnesium laureth-8 sulfate, magnesium laureth sulfate, magnesium oleth sulfate, sodium oleth sulfate	25.4	Moderate irritant
Sh03	Cocamidopropyl betaine, sodium trideceth sulfate, sorbitan laurate, lauramide/myristamide imidazoline, Polyethylene glycol distearate, sodium laureth carboxylate	11.6	Slight irritant
Sh04	Disodium laureth sulfosuccinate, sodium laureth sulfate, cocamide dea, coco-betaine	36.8	Moderate irritant
Sh05	Decyl glucoside, sodium lauroyl sarcosinate, sodium laureth sulfate/cocamidopropyl betaine	28.6	Moderate irritant
Sh06	Sodium laureth sulfate, cocamidopropyl betaine, cocamide dea/glicerin, sodium laureth sulfosuccinate	41.0	Severe irritant
Sh07	Ammonium laureth sulfate, ammonium lauryl sulfate, stearyl alcohol, ammonium xylenesulfonate	52.2	Moderate irritant
Sh08	Sodium laureth sulfate, alkyl polyglicoside, oleamide dea, ethoxylated mathyl glucoside dioleate, cocamidopropyl betaine	29.0	Moderate irritant
Sh09	Sodium laureth sulfate, Ammonium lauryl sulfate, cocamidopropyl betaine	38.2	Moderate irritant
Sh10	Laureth sulfate, cocamidopropyl betaine, ethylene glycol distearate, sodium laureth sulfate	52.2	Severe irritant
Sh11	Sodium laureth sulfate, cocamidopropyl betaine, ethylene glycol distearate, cocamide dea	32.0	Severe irritant
Sh12	Sodium laureth sulfate, cocamide dea, PEG-150 distearate	7.2	Slight irritant
Sh13	Sodium trideceth sulfate, decyl glucoside, cocamidopropyl betaine, PEG -150 distearate, PEG -120 methyl glucose dioleate	8.8	Slight irritant
Sh14	PEG -120 methyl glucose dioleate, laureth sulfate/sodium sulfosuccinate, cocamidopropyl betaine, decyl glucoside, polysorbate 20	4.8	Slight irritant
Sh15	Sodium myreth sulfate, sodium laureth sulfate, sodium cocoamphocetate, nonoxynol-120	38.4	Moderate irritant
Sh16	Sodium laureth sulfate, laureth sulfosuccinate, cocamidopropyl betaine, PEG -120 distearate, decyl polyglucose	10.2	Moderate irritant
Sh17	Sodium laureth sulfate, cocamidopropyl betaine	12.4	Moderate irritant
Sh18	Sodium laureth sulfate, lauryl polyglucose, cocamidopropyl betaine	34.0	Moderate irritant
Sh19	Sodium laureth, lauryl glucoside, cocamidopropyl betaine	43.4	Severe irritant
Sh20	Sodium laureth sulfate, cocamidopropyl betaine, ethylene glycol distearate, oleamide dea	46.2	Severe irritant

Sh = shampoo; MAS = maximum average score, according to Draize et al., 1944; *According to Kay and Calandra, 1962. All products contain fragrance, colour, water and preservative in their formulations.

Cell lines

SIRC rabbit cornea1 cells and 3T3 mouse embryo fibroblasts were obtained from American Type Culture Collection (ATCC) and used in this work. SIRC was cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture Ham F-12 (DMEM HAM F12), supplemented with 3 mM L-glutamine, 13.8 mM $NaHCO_3$, 1000 UI penicillin, 2.5 mg/mL amphotericin and 10% of fetal bovine serum (FBS) from Gibco (Eragny, France). 3T3 was cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 3 mM L-glutamine, 20 mM HEPES, 26 mM $NaHCO_3$, 1000 UI penicillin, 2.5 mg/mL amphotericin and 10% FBS. The cells were incubated at 36.5ºC in a CO_2 incubator and subcultured twice a week.

The *in vitro* assay

The process of staining by Coomassie blue was performed as proposed by Margis and Borojevic (1989) and adapted for use in the microplates of 96 wells (Shopsis and Eng, 1985). For this purpose, a modified protocol was established for the TPC assay, as detailed below.

SIRC and 3T3 cells were grown in 96 - wells microplates in a volume of 100µL and concentration of 1.5 x 10^5 cells/ml (SIRC) and 1.0 x 10^5 cells/ml (3T3) in their culture media and maintained for 24 h in a CO_2 incubator at 36.5ºC ± 0.5ºC with 3.5% of CO_2. After that, the media were changed for 100µL dilutions of pre-set of samples in the DMEM HAM F12 with 5% of FBS for SIRC and in the DMEM with 5% of FBS for 3T3. After 24 h of incubation, the microplates were washed with sodium phosphate buffer (SPB, 0.01M) in an automatic washer using a four cycles of washing. Then, the SPB was discarded and 100µl SPB 0.01 M / 4% formaldehyde were added and incubating for 15 minutes. The fixative agent was discarded and the microplate was dry at room temperature. A volume of 50 µl of Coomassie blue R-250 stain (0.2% w/v) solution was added to each well and the microplate was incubated for 30 minutes in the dark. The dye was discarded, the microplate was immediately plunged into a plastic container with distilled water, and soon after, washed by hand three times, swapping the containers for each wash. Then, the microplate was placed in an automatic shaker (500 rpm) for 20 minutes, followed by a final washing with the distilled water. The microplate was dried at room temperature and dye was then eluted by adding 100µL of sodium dodecyl sulphate (SDS) 1% in each cavity and leaving it overnight. The optical density (OD) was measured by spectrophotometer at 595nm.

An initial curve was obtained with eight concentrations of each sample diluted in the culture medium (100, 10, 1, 0.1, 0.01; 0.001; 0.0001 and 0.00001 mg/ml). Thereafter, a second curve with a closer range of eight concentrations was conducted from the outcome of the first curve. In this second curve, the concentration in which there was a reduction of 50% in the absorbance compared to the untreated cells (control) was considered the concentration of cytotoxic effect (i.e. IC_{50} or the 50% inhibitory concentration). The value of IC_{50} for each product tested was calculated using the average of three independent tests.

The *in vivo* test

Five male or female New Zealand albino rabbits, weighing 2-3 kg, were used in the *in vivo* test for each product. The animals were maintained in individual cages, with water and food *ad libitum*, at 20 ± 2ºC and humidity of 70%. The protocol of the ocular irritation test was approved by the Animal Use Ethics Commission (CEUA/FIOCRUZ). One hundred microlitres of each product were instilled into one of the eyes, followed by massaging for 30 seconds, while the other eye was used as control. The readings were performed at 24, 48, 72 h and 7 days after the application, and the corneal, iris and conjunctival average of alterations were graded according the Draize scale (Draize et al., 1944): non-irritant (MAS ≤ 14.9), slight irritant (MAS ≥ 15 to ≤ 24.9), moderate irritant (MAS ≥ 25 to ≤ 49.9), severe irritant (MAS ≥ 50 to ≤ 79.9) and maximum irritant (MAS ≥ 80 to ≤ 110). To finally classify the eye irritation potentials of the products, the Kay and Calandra methodology was used, which took into account the persistence and consistence of the irritation response (Kay and Calandra, 1962). For this, the following guideline was used: "non-irritant" when the MAS in 24 hours ≤ 2.4, "slight irritant" when the MAS in 48 and 72 hours ≤ 2.4; "moderate irritant" when reading the 7th day ≤ 10 in at least three animals; "severe irritant" when the reading on day 7 > 30 in at least one animal or when the reading on the 7th day ≤ 30 in at least three animals; "maximum irritant" if the reading on day 7 > 60 in at least one animal. After the last reading, the animals were sacrificed (thiopental 100mg/kg, *i.v.*).

Statistical Analysis

The results obtained in this study were analysed by using the performance comparisons between the *in vitro* (TPC) and *in vivo* (Draize eye irritation) tests, derived from the contingency table. The following values were calculated: sensitivity (the ratio of *in vivo* irritants classified *in vitro* as irritants); specificity (the ratio of *in vivo* non-irritants classified *in vitro* as non-irritants); accuracy (the ratio of product classes [irritants and non-irritants] correctly classified *in vitro*); false positives and false negatives. Pearson correlation coefficients were used to compare the results obtained *in vitro* and *in vivo*.

RESULTS

The number of surfactants in the products analyzed in this study ranged from two to eleven (Table 1). In the case of shampoos classified *in vivo* as non-irritant and slight irritant, the number of surfactants in their compositions varied from three to six, while in the category of moderate and severe irritant, they were composed by two to eleven surfactants. Table 2 shows the distribution of the studied shampoos, as well as their potential for eye irritation in the Draize test. It could be seen that the unique shampoo classified as non-irritant *in vivo* was for children's use. Among the four slight irritant shampoos, just one was for adult's use. In the case of shampoos classified *in vivo* as moderate (50%) and severe (25%) irritants, there was certain homogeneity between the results obtained both in the adults and children shampoos. Table 3 shows the averages of the IC_{50} values obtained from three *in vitro* independent experiments using SIRC and 3T3 cells. When using the cellular lineage SIRC (N = 20 shampoos), the averages of the IC_{50} values ranged from 0.205 to 2.676mg/ml. In the case of the use of 3T3 cells (N = 12 shampoos), the *in vitro* results ranged from 0.161 to 2.475mg/mL. In both the cellular lineages, the highest IC_{50} mean value was obtained for the same shampoo (Sh01), classified *in vivo* as non-irritant.

Table 2 - Classification of the potential for eye irritation of 20 shampoos by *in vivo* test.

	NI	**SI**	**MI**	**SeI**	**HI**
Children shampoos	1 (5 %)	3 (15 %)	5 (25 %)	2 (10 %)	0
Adults shampoos	0	1 (5 %)	5 (25 %)	3 (15 %)	0
Total	1 (5 %)	4 (20 %)	10 (50 %)	5 (25 %)	0

NI: Not Irritant; SI: Slight Irritant; MI: Moderate Irritant; SeI: Severe Irritant and HI: High Irritant.

Table 3 - The values of IC_{50} obtained in the *in vitro* test with SIRC and 3T3 cells.

Product	Category	SIRC (mg/ml) *	3T3 (mg/ml) *
Sh01	Child shampoo	2.676 ± 0.322	2.475 ± 1.200
Sh02	Child shampoo	0.357 ± 0.035	0.884 ± 0.199
Sh03	Child shampoo	0.536 ± 0.005	1.278 ± 0.158
Sh04	Child shampoo	0.562 ± 0.084	0.894 ± 0.211
Sh05	Child shampoo	0.410 ± 0.008	0.397 ± 0.138
Sh06	Child shampoo	0.470 ± 0.058	0.664 ± 0.163
Sh07	Adult shampoo	0.205 ± 0.026	0.424 ± 0.181
Sh08	Adult shampoo	0.412 ± 0.077	0.845 ± 0.101
Sh09	Adult shampoo	0.484 ± 0.074	0.161 ± 0.065
Sh10	Adult shampoo	0.338 ± 0.016	0.686 ± 0.320
Sh11	Adult shampoo	0.375 ± 0.019	0.663 ± 0.035
Sh12	Adult shampoo	0.740 ± 0.048	0.964 ± 0.464
Sh13	Child shampoo	1.019 ± 0.133	ND
Sh14	Child shampoo	0.759 ± 0.065	ND
Sh15	Child shampoo	0.643 ± 0.080	ND
Sh16	Child shampoo	0.525 ± 0.046	ND
Sh17	Adult shampoo	0.553 ± 0.048	ND
Sh18	Adult shampoo	0.431 ± 0.020	ND
Sh19	Child shampoo	0.463 ± 0.037	ND
Sh20	Adult shampoo	0.255 ± 0.074	ND

* Three independent trials (mean ± standard deviation), ND = not determined.

Predictive ability

Some alternative methods, such as the RBC assay, have cut-off values, previously established during the development /standardization of the method. Such cut-off values are useful in identifying the positive (irritants) or negative (non-irritants) responses, and to classify the irritant potential of a positive substance, according to the severity of the

observed effects (Alves et al., 2008). For the cytotoxicity tests, however, there was no consensus in the scientific literature concerning an IC_{50} value to be considered as a cut-off to discriminate between the irritant and non-irritant substances. One of the few articles already published in this issue is from Vian and collaborators (Vian et al., 1995). These authors evaluated the effects of surfactants on the SIRC and 3T3 cells and proposed a value of 0.700mg/ml as the best cut-off that distinguished *in vitro* an irritant (IC_{50} < 0.700 mg/ml) of a non-irritant (IC_{50} > 0.700 mg/ml) substance.

Many authors consider that the degree of injury induced in the eye of the rabbit after the application of substances classified as slightly irritant is negligible and represents a low risk situation when extrapolated to the circumstances of human exposure. This is because the experimental conditions of the Draize test is maximized, i.e., the substance-test is applied without dilution, is instilled directly into the conjunctival sac of the animal and the exposure is longer when compared to the exposure situation in humans (Freeberg et al., 1986; Roggeband et al., 2000). Therefore, to establish a cut-off value to carry out a comparison between the *in vitro* and the *in vivo* assays, *in vivo* shampoos were classified as non-irritant and slightly irritant in a single category (called "non-irritants") and the moderate and severe irritant shampoos in another category (called "irritants"). The *in vitro* cut-off established in the present study was the same of Vian and collaborators (Vian et al., 1995), i.e., 0.700mg/ml. Table 4 shows the classifications of shampoos in the *in vitro* assay (with SIRC and 3T3 cells) and *in vivo* after the establishment of the cut-off value. The predictability of the TPC *in vitro* assay by using SIRC and 3T3 cells is summarized in Table 5.

Table 4 - Comparison of classifications between the *in vitro* assay and the *in vivo* Draize test after the establishment of the cut-off value.

Product	SIRC	3T3	*In vivo*
Sh01	Non-irritant	Non-irritant	Non-irritant
Sh02	Irritant	Non-irritant	Irritant
Sh03	Irritant	Non-irritant	Non-irritant
Sh04	Irritant	Non-irritant	Irritant
Sh05	Irritant	Irritant	Irritant
Sh06	Irritant	Irritant	Irritant
Sh07	Irritant	Irritant	Irritant
Sh08	Irritant	Non-irritant	Irritant
Sh09	Irritant	Irritant	Irritant
Sh10	Irritant	Irritant	Irritant
Sh11	Irritant	Irritant	Irritant
Sh12	Non-irritant	Non-irritant	Non-irritant
Sh13	Non-irritant	ND	Non-irritant
Sh14	Non-irritant	ND	Non-irritant
Sh15	Irritant	ND	Irritant
Sh16	Irritant	ND	Irritant
Sh17	Irritant	ND	Irritant
Sh18	Irritant	ND	Irritant
Sh19	Irritant	ND	Irritant
Sh20	Irritant	ND	Irritant

Table 5 - Predictability of the *in vitro* TPC assay for 20 shampoos in SIRC cells and for 12 shampoos in 3T3 cells.

Parameter	SIRC (%)	3T3 (%)
Sensitivity	100.0	66.7
Specificity	80.0	100.0
Accuracy	95.0	75.0
False negatives	0	25.0
False positives	5.0	0

When comparing the *in vitro* assay performed with SIRC cells with the *in vivo* Draize test, a sensitivity of 100% was observed, whereas among the 20 shampoos tested, only one (Sh03) showed a false positive response, producing a specificity of 80% and an accuracy of 95%. When comparing the *in vitro* assay performed with 3T3 cells with the *in vivo* Draize test, three false negative responses (Sh2, Sh4 and Sh8) were observed among the twelve tested shampoos, leading to the values of 67% of sensitivity, 100% of specificity and 75% of accuracy (Tables 4 and 5). The concordance and discordance frequency between *in vivo* and *in vitro* assays after using the SIRC and 3T3 cells is shown in Tables 6 and 7, respectively.

Table 6 - Contingency table using data from *in vitro* test using SIRC cells.

		In vivo classification	
		Irritant	Non-irritant
In vitro classification	Irritant	15	1
	Non-irritant	0	4

Table 7 - Contingency table using data from *in vitro* test using 3T3 cells.

		In vivo classification	
		Irritant	Non-irritant
In vitro classification	Irritant	6	0
	Non-irritant	3	3

Correlation between the values of IC_{50} obtained *in vitro* and the results of the Draize test

Pearson correlation coefficients between the IC_{50} values obtained *in vitro* using 3T3 and SIRC cells and the scores of the three ocular structures (cornea, iris and conjunctiva) and the maximum average score (MAS) of the *in vivo* test are illustrated in Table 8. The Pearson correlation coefficient related to the value of MAS was -0.58 ($p = 0.007$) when using the SIRC cells and -0.73 ($p = 0.007$) when 3T3 cell line was used. With regard to the ocular structures, both for SIRC and 3T3 cells the best correlation coefficient value was found on the conjunctiva [-0.71 ($p = 0.000$) for the SIRC and -0.86 ($p = 0.000$) for 3T3], as shown in Table 8.

Table 8 - Correlation coefficient (Pearson) linking the *in vitro* values of IC_{50} with the scores of the eye structures of the *in vivo* test.

	Pearson correlation	
	SIRC *	**3T3 ****
Cornea	-0.53($P = 0.015$)	-0.67($P = 0.017$)
Iris	-0.44 ($P = 0.053$)	-0.58 ($P = 0.049$)
Conjunctiva	-0.71($P = 0.000$)	-0.86 ($P = 0.000$)
MAS	-0.58 ($P = 0.007$)	-0.73 ($P = 0.007$)

MAS = maximum average score; * N =20 shampoos; **N =12 shampoos.

DISCUSSION

All the shampoos were tested undiluted in the *in vivo* assay, as this reflects the situation of consumer's use. The types of surfactants used in the formulation of a cosmetic product will certainly influence the ocular irritation properties of the final product. However, the surfactants may be used at various concentrations within different products and, depending on the concentration used, some surfactants can contribute to other useful functions to the cosmetic formulation (such as stabilizers of the emulsion or regulators of viscosity). Thus, it may be observed that the varying concentrations, and not only the types and the number of the surfactant used in the cosmetic formulations, can influence the potential of ocular irritation of a final product. This can be seen by comparing the results of Sh01 with those obtained with Sh09. Even though Sh09 contained only three

surfactants in its formulation, it showed very higher irritation potential (both *in vivo* and *in vitro*) than Sh01, which contained five surfactants.

It is well known that the maximized conditions of the Draize test represent an important limitation of this test. In spite of this, the present results showed that a significant part of the shampoos studied did not demonstrate to be safe, at least from the point of view of ocular toxicity. Moreover, the present study confirmed the importance of testing the products that were often used by the human populations. Among the 20 shampoos evaluated in the *in vivo* test, only one was classified as non-irritant; 15 (75%) showed moderate to severe irritant potential, being seven of them addressed to children's use.

The 405 OECD Test Guideline recommends a sequential testing strategy, in which the *in vivo* test is only needed when the prior assessments have produced negative results, to assess slightly to moderately irritating compounds (OECD, 2002). In this context, the main goal of the present study was to compare the results obtained by using the *in vivo* Draize test with those obtained with TPC assay, not only helping to validate the latter as a preliminary test capable of selecting the most irritating products, thus avoiding their evaluation by the Draize test, but also to evaluate the *in vitro* assay as a possible test to be included in a battery of assays to replace the Draize test.

For this reason, the *in vitro* test was initially standardized, by adapting the process of staining with Coomassie blue proposed by Margis and Borojevic for the use of 96 - wells microplates as Shopsis and Eng, by setting up a protocol with the changes in volumes of each reagent used, in the use of automatic microplate washer and in the scheme of wash of microplates after the staining process. Furthermore, SIRC and 3T3 cell lines were chosen and then their use was standardized, in the concentrations of the cell suspensions used, in the period of time of their growth after subculture and before the treatment with the test substances and also in the period of time by which the cell was exposed to the different dilutions of the shampoos. It should be noted that the choice of SIRC cells was mainly due to the fact that it has been one of cell lineage widely used by many researchers in the studies of physiology of the cornea, as well as in the immunological and toxicological studies. Moreover, it is extensively used to predict the eye irritation, showing an excellent correlation between the effects *in vivo* and *in vitro* caused by the surfactants and cosmetics (North-Rooth et al., 1985; Tani et al., 1999; Hutak et al., 2003; Takahashi et al., 2009). In addition to its ophthalmic origin, the advantages of this cell line include their commercial availability and ease of cultivation. With respect to 3T3 cell line, it was chosen because of its ease of obtaining, handling and due its ability to remain stable even after many passages. Furthermore, this cell line is widely used in other tests for evaluating the cytotoxicity induced by the products and cosmetic ingredients, which allows the comparison of results with those described in the literature (Dickson et al., 1993; Spielmann et al., 1996; Geurtsen et al., 1998; Clothier et al., 2006).

The analysis of the 20 shampoos in the *in vitro* test using SIRC cells showed that, under the conditions of the present work, the results presented were consistent with the irritant potential of shampoos established *in vivo*, with one exception, the children shampoo Sh03 that had a false positive result. With regard to the 12 shampoos tested on 3T3 cells, three results (25%) proved to be false-negative, demonstrating that although the *in vitro* test with 3T3 cells had a specificity of 100%, this assay demonstrated no suitability so far. However, because this test with 3T3 cells was performed after the Draize test and the TPC assay with SIRC cells, it was not possible to test all the 20 shampoos due to loss of validity and the fact that there was no other of the same lot to buy. Hence, the number of shampoos tested with 3T3 cells was limited (only 12). Therefore, it would be necessary to test a greater number of shampoos to confirm their ability to predict the results *in vivo*. Vian and collaborators (Vian et al., 1995) also found three false negative results, not only with 3T3 cells, but also with SIRC and L929, and in three different cytotoxicity tests (MTT, NRU and TPC).

The correlations between the MAS for the different structures of the eye and the parameter estimated *in vitro* (Table 8) showed that: (i) the *in vitro* IC_{50} with SIRC cells was moderate correlated with the MAS; (ii) the *in vitro* IC_{50} with 3T3 cells was clearly correlated with the MAS; and (iii) of the three ocular structures, the conjunctiva gave the best correlations both with SIRC and with 3T3 cells. Moreover, although the test with 3T3 cells did not present a good accuracy (75%) when compared with the SIRC cells (95%), the Pearson test showed that TPC assay with 3T3 cells was better correlated with the test results *in vivo* than when using the cell line SIRC. This raised the

question that the value of cut-off chosen, as not was a consensus in the international literature, might not reflect adequately the situation of use in different cell lines. Possibly for each different cell line it would be necessary to establish a specific cut-off.

Another issue of concern was that the *in vivo* MAS value was not considered to be the most suitable endpoint for the final evaluation of *in vitro* test models**,** since recovery and/or irreversibility were not taken into account (Prinsen, 1999). Hence, in this work the Kay and Calandra methodology was used (Kay and Calandra, 1962) to classify the eye irritation potentials of the products, which took into account the irritation response persistence and consistence. In other words, not always the absolute values of the MAS assessed by the classical methodology, originally proposed by Draize and collaborators (Draize et al., 1944), fully indicated the degree of eye irritation, since these results were part of the first stage of evaluation that must be confirmed by the second stage proposed by Kay and Calandra. For example, the shampoo Sh16 with a MAS of 10.2 was classified as moderate irritant, not as non-irritant when the Kay and Calandra methodology was used (Table 1).

Probably, the difference between the Pearson correlations among the two cell lines and differences in comparisons of performance, among other factors might be due to the use of MAS scores in the Pearson correlations, and in the case of analysis of performance, the use of the final classification by Kay and Calandra. However, it would be necessary to analyze a larger number of products and perform inter-laboratory studies to confirm our results.

CONCLUSIONS

The results of this study showed that the cytotoxicity assay by TPC was a promissory tool to be used routinely as a preliminary assay for the detection of irritant products. The principal advantages of this assay were: (*i*.) its ability to predict the ocular irritation potential of shampoos with formulations containing surfactants with high precision; (*ii*.) its simplicity of execution and reproducibility; and (*iii*.) the good correlation between the parameters evaluated *in vitro* and *in vivo*.

Bearing in mind the inter-assay accuracy, the TPC assay in SIRC cells showed the best agreement with the results *in vivo* (95%), differentiated better shampoos in terms of irritation severity and showed greater difference between the irritant and non-irritant shampoos in terms of IC_{50}. However, the TPC assay was better correlated with the *in vivo* test when it was performed with 3T3 cells rather than when SIRC cells were used. With regard to the ocular structures, both for SIRC and 3T3 cells the best correlation coefficient values were found on the conjunctiva. It was also concluded that the varying concentrations of the surfactants in the formulation, rather than just the types, could influence the potential ocular irritation of these products.

The results confirmed the important characteristics of the TPC assay such as the speed, sensibility, low cost and a high degree of automation. However, one must take into account the necessity to analyze a larger number of shampoos and other types of products, to perform the inter-laboratory studies, and to review the parameters of comparison and correlation between *in vivo* and *in vitro* assays.

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