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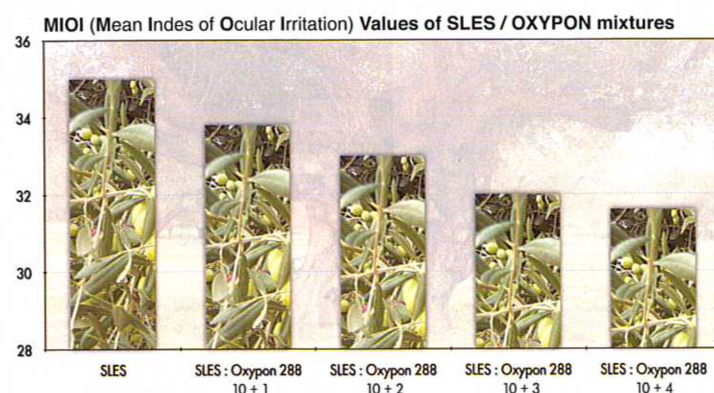
„The perfect combination“

Influence on the irritation potential of SLES in the RBC test shown by use of OXYPON 288



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Skin model dermal irritancy testing of cosmetics

ABSTRACT

The potential for cosmetic formulations to induce skin irritation is an important consideration for cosmetics producers. Irritation potential may be predicted by *in vitro* systems, provided they are sufficiently complex to mimic the skin barrier *in vivo*.
The objective of this work was to evaluate the dermal irritation potential of different cosmetic formulations using the human epidermis model, EpiDerm. Our study showed that *in vitro* tests, as an alternative to animal testing, allow for preliminary assessment of a cosmetic formulation's irritation potential. However, further studies are required to evaluate the usefulness of *in vitro* epidermis models to predict *in vivo* skin irritation.

Skin irritation, accompanied by typical inflammation symptoms like erythema, edema, dryness, desquamation and itching, is the most common adverse effect in humans. The source of the irritant is most often a chemical. The main pathological mechanisms of irritancy include skin barrier disruption, induction of a cytokine cascade and involvement of the oxidative stress network; all of which result in a visible or subclinical inflammatory reaction.[1-3] Interleukin-1α (IL-1α) is believed to be the main switch in the initiation of cutaneous inflammation. It has been hypothesised that IL-1α is derived from the damaged keratinocytes during the interaction between the irritant and epidermal barrier.[1-4]

Until recently, identifying irritants has relied entirely on animal testing, such as the Draize rabbit skin test; a classic example introduced into safety tests for drugs and chemicals 60 years ago, which involves applying the product to a rabbit's skin.[5] In accordance with EU law, a ban on animal testing of cosmetics has been in force since 2004, whereas since 2009 (for repeated dose toxicity test, reproductive toxicity and toxicokinetics until 2013) all animal testing for ingredients used in cosmetics production has been stopped.[6] Therefore, safety tests with the use of *in vitro* method are a field of toxicology which, currently, is undergoing intense development. On the other hand, the Registration, Evaluation and Authorization of Chemicals (REACH) legislation has stimulated the development of alternative tests for the assessment of potential toxicological effects of substances.[7]

The development of molecular investigative techniques led to the introduction of different *in vitro* models for the assessment of irritant properties. Human epidermal equivalents are one of the most extensively studied models to determine the irritant potential of a chemical *in vitro*. Until now, the EpiSKIN, EpiDerm and SkinEthic models have been validated by ECVAM (European Centre for the Validation of Alternative Methods). They consist of fully differentiated reconstructed human epidermis and are able to mimic the human epidermis. The presence of the functional *stratum corneum* in epidermal equivalents means that, in a similar way to human skin, the cultures have a barrier function and therefore test substances (water soluble or insoluble) can be applied topically in a similar manner to patch testing human volunteers.[3,8]

The aim of this study was to evaluate the dermal irritation potential of cosmetics using *in vitro* tests. For that purpose, five cosmetic formulations were tested *in vitro* using the commercially available epidermis equivalent, EpiDerm (now referred to as "the skin model").

Experimental

The skin model was obtained from MatTek Corporation, Ashland, MA, USA. A model of the human epidermis, it was developed at MatTek Corporation and is based on neonatal, foreskin-derived normal human epidermal keratinocytes (NHEK) which have been cultured to form a multilayered, highly differentiated model of the human epidermis. The skin model exhibits *in vivo*-like morphological and growth characteristics which are uniform and highly reproducible. Ultrastructural analysis has revealed the presence of multi-layered *stratum corneum* containing intercellular lamellar lipid layers arranged in patterns characteristic of *in vivo* epidermis.[9]

An *in vitro* skin irritation test was performed according to the Standard Operating Procedure evaluated in the European Centre of the Validation of Alternative Methods (ECVAM) "Skin Irritation Validation Study – *In vitro* Skin Irritation Test: Human Skin Model, EpiDerm-200; version 7:0, Oct 2007". Briefly, tissues are topically exposed to the test substances for 60 minutes. Then, tissues are thoroughly rinsed and blotted to remove the test substances and transferred to a fresh medium. After a 42 hour incubation period, cell viability is assessed with the use of MTT [(3-4,5 dimethyl triazole 2-yl) 2,5-diphenyltetrazoliumbromide] colorimetric test. Relative cell viability is calculated for each tissue as a percentage of the mean of the negative control tissues (%viability=100x[OD (sample)/OD (negative control)).[10-11]

IL-1α secretion in the cell culture medium was measured after 42 hours tissue exposure to test formulation using the quantitative sandwich enzyme immunoassay technique according to SOP "*In vitro* Skin Irritation test: Human Epidermis Model EpiSkin, version:1.2, Sept 2005". IL-1α was measured in culture

Table 1: Prediction Model combining viability and IL-1α release.

In vitro interpretation	Classification
Mean tissue viability is ≤50%	Irritant (I) R38
Mean tissue viability is >50% and amount of IL-1α released ≥50 pg/mL	Irritant (I) R38
mean tissue viability is >50% and amount of IL-1α released <50 pg/mL	Non-irritant (NI) No label

media only for tissues with viability >50% after treatment.[12]

Skin irritation potential of the test material is predicted if the remaining relative cell viability is below 50%. The cut off limit defined for IL-1α is set to 50 pg/mL. The test substance is considered to be an irritant (R38) to skin if the viability is 50% and the amount of IL-1α release is ≥50 pg/mL. The test substance is considered to be a non-irritant (no label) to skin if the viability is >50% and the amount of IL-1α release is <50 pg/mL. Table 1 presents Prediction Model combining viability and IL-1α release.[10-12]

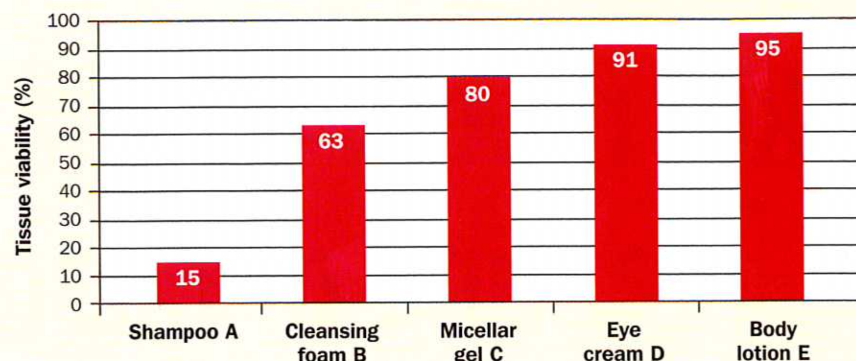

Figure 1: *Tissue viability after exposure to test formulations.*

Test results

In order to evaluate dermal irritation potential of cosmetics, we tested five coded cosmetic formulations in triplicates in blind trials using the reconstructed human epidermis skin model. We studied the effect of test samples on cellular viability (Fig. 1) and measured IL-1α release (Fig. 2).

Tissue viability data are presented on Figure 1. Sample A (shampoo) led to very strong repression in cell viability (85%), so it is considered to be an irritant to skin. This formulation contains a relatively high concentration of detergents and surfactants sodium lauryth sulfate and cocoamidopropyl betaine, thus demonstrating its potential to irritate the skin. Sample B (cleansing foam), containing cocoamidopropyl betaine, triggered weaker reaction (37% reduction in tissue viability). Solution C (micellar gel), containing surfactant polysorbate 20- polyoxyethylene sorbitan monolaurate and fragrance composition with potential allergens, led to 20% reduction in cell viability. The adverse effects following treatment with sample D (eye cream) and E (body lotion) were weak – no significant changes in the viability were observed.

In the next step for sample B-E, which caused >50% reduction in cell viability, IL-1α release was measured (Fig. 2). The IL-1α secretion for rinse-off products (cleansing foam and micellar gel) was slightly above cut-off limit (50 ng/mL). Samples D and E triggered moderate IL-1α release. According to Prediction Model (Table 1), formulations A-C were considered irritant to the skin. Table 2 summarises tissue viability and IL-1α release results for tested formulations and its *in vivo* classification.

Discussion

According to the recent legislation, the manufacturer of a cosmetic product is obliged to conduct a safety assessment of the product being introduced to the market. Evaluation of the skin irritancy potential of a cosmetic formulation and ingredient is a necessity in the safety assessment of cosmetic products.

In vitro tests for skin irritating properties are performed on models of the epidermis. The use of epidermal models for skin irritation testing involves topical application of test materials onto the surface of the skin equivalent and the subsequent assessment of their effects on cell viability by using the MTT assay. The MTT assay is based on the reduction of yellow tertazolium salt 3-(4,5-dimethylthialzol-2-yl) 2,5-diphenyl tetrazolium bromide to the purple formazan dye by mitochondrial succinate dehydrogenase in viable, metabolically active cells. If an irritant substance results in cytotoxicity, it will result in a corresponding decrease in mitochondrial activity. Measurements of mitochondrial succinate dehydrogenase has become a standard assay for measuring the degree of cytotoxicity of a given test substance. However, the use of other, more mechanistic endpoints, such as interleukin 1α (IL-1α) or lactate dehydrogenase (LDH) production, has also been evaluated.[3-4]

At the time of this publication, *in vitro* models validated for predicting irritant properties by ECVAM are EpiDerm, Episkin and SkinEthic. In April 2007, ESAC endorsed two alternative methods (Episkin and EpiDerm Skin Irritation Tests) as replacements of the *in vivo* rabbit skin irritation test. The Episkin model was considered reliable and relevant for being used as a replacement of the Draize skin irritation test. It can be used as stand-alone tests to distinguish between skin irritating (R38, similar to GHS Category 2) and non-irritating (no-label) chemicals. EpiDerm reliably identifies skin irritants, but negative results required further testing.[13-14] In December 2008, ESAC endorsed the scientific validity of the Modified EpiDerm Skin Irritation Test (SIT) and concluded that it has sufficient accuracy and reliability for the prediction of skin irritating and non-irritating test substances. The major modification is an extended chemical exposure time from 15 to 60 minutes that reflects the robust barrier function of the EpiDerm model. The new exposure time provides an improved sensitivity of the *in vitro* EpiDerm SIT and better correlation with *in vivo* Draize rabbit skin irritation results.[15-16]

In order to determine dermal irritation potential of tested cosmetic formulations, Modified EpiDerm Skin Irritation Test (SIT) was performed. Formulations A-C, which are rinse-off products, were considered irritating, whereas formulations D-E (leave-on products) were non-irritating. In EpiDerm SIT method the exposure time, i.e. the period that the epidermal surface is treated with tested substance, is extended to 60 minutes. In our opinion, rinse-off products study protocol should reflect their cosmetic application; the exposure time should be less than 60 minutes and/or samples should be tested diluted. We are in line with recent MatTek's recommendation. MatTek suggests that the ECVAM-validated EpiDerm SIT method is useful for hazard identification, whereas testing substances over multiple time points: 2, 5, and 18 hours provides irritation potential assessment ideal for use in formulation development application. Therefore, in order to confirm dermal irritation potential of formulations A-C, further tests over multiple time points should be preformed.

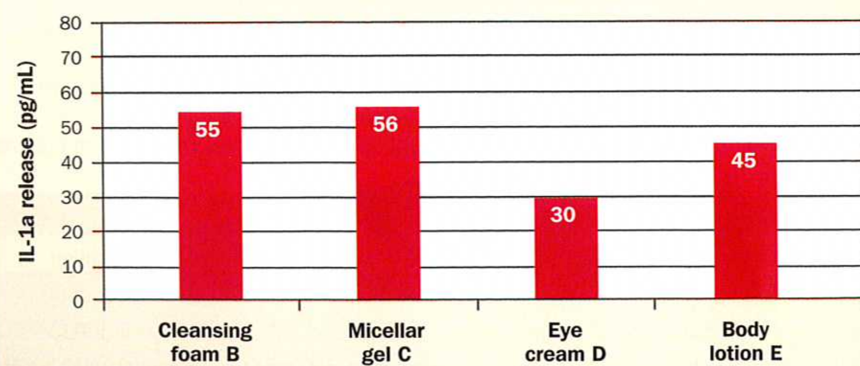

Figure 2: *Tissue IL-1α release after exposure to test formulations.*

Conclusion

The results of our study revealed that EpiDerm *in vitro* tests could be used as a preliminary assessment of dermal irritation after the application of skin care products prior to clinical tests. Nevertheless, further studies are required to adapt the human epidermis models to predict *in vivo* skin irritation caused by cosmetics. PC

Table 2: Summary of tissue viability, IL-1α release results and *in vivo* classification.

Test formulation	Criteria for *in vitro* interpretation		Classification
	Tissue viability (% of control)	IL-1α release (pg/mL)	
Shampoo A	15		Irritant (I) R38
Cleansing foam B	63	55	Irritant (I) R38
Micellar gel C	80	56	Irritant (I) R38
Eye cream D	91	30	Non Irritant (NI) No label
Body lotion E	95	45	Non Irritant (NI) No label

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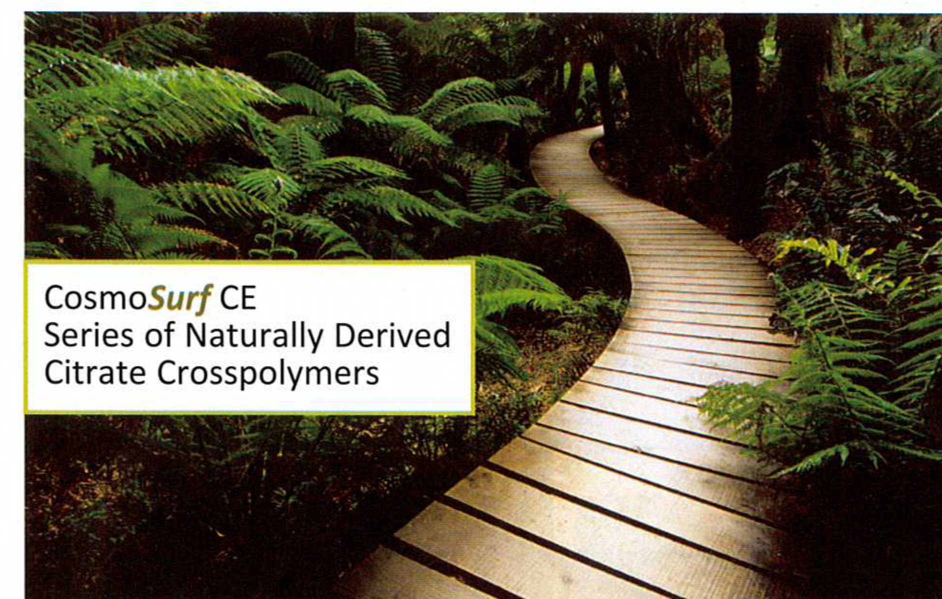

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