# Eye irritation hazard of chemicals and formulations assessed by methods *in vitro*

**Dagmar Jírová [1], Kristina Kejlová [1], Stanislav Janoušek [1], Hana Bendová [1], Marek Malý [1], Hana Kolářová [2], Markéta Dvořáková [1]**

1 National Institute of Public Health, Prague, Czech Republic
2 Faculty of Medicine and Dentistry, Palacký University, Olomouc, Czech Republic

*Correspondence to:* Dagmar Jírová, MD., PhD.
National Reference Center for Cosmetics, National Institute of Public Health,
Šrobárova 48, 100 42 Prague 10, Czech Republic.
tel: +420 267082439; fax: +420 267082386; e-mail: jirova@szu.cz

*Submitted: 2014-09-23* *Accepted: 2014-11-08* *Published online: 2014-11-30*

*Key words:* **eye irritation; skin irritation; chemicals; cosmetics; alternative methods *in vitro***

Neuroendocrinol Lett 2014; **35**(Suppl. 2):133–140 **PMID:** 25638377 NEL351014A15 © 2014 Neuroendocrinology Letters • **www.nel.edu**

**Abstract**

**BACKGROUND:** The aim of this study was to compare human and animal skin irritation data with results of selected in vitro methods, including HET-CAM test, Neutral Red Release Assay, Neutral Red Uptake Assay and EpiOcular™ eye irritation test and with already existing data of eye irritation obtained from animal experiments.
**METHODS:** Chemicals employed in previous skin irritation validation studies and commercially available cosmetic formulations were subjected to further testing using *in vitro* methods Neutral Red Release (NRR) assay, Neutral Red Uptake (NRU) assay, HET-CAM test and EpiOcular™ assay.
**RESULTS:** The study revealed that skin irritants are not necessarily eye irritants; specifically volatile or solid materials may be misclassified. NRR assay provided false negative results in case of substances with fixative effect or not removable under standard washing procedure, emphasizing the role of microscopical evaluation as a crucial additional endpoint. Although overpredictive, HET-CAM test provided the lowest false negative rate. The most aggressive cosmetic formulation was correctly identified by EpiOcular™ assay, in accordance with NRU and NRR assays results, while HET-CAM test correctly identified the mildest formulation.
**CONCLUSIONS:** Each of the *in vitro* methods is related to a specific endpoint of ocular irritation and provides only partial information on the mode of action of the tested material. Despite good reproducibility of individual *in vitro* assays, only the weight-of-evidence approach and results of multiple selected in vitro tests can allow for estimation of eye irritation hazard *in vivo*.

**Abbreviations:**

| | |
|---|---|
| HET-CAM | - Hen´s Egg Test – Chorioallantoic Membrane |
| NRR | - Neutral Red Release Assay |
| NRU | - Neutral Red Uptake Assay |
| SLS | - Sodium lauryl sulphate |
| DMEM | - Dulbecco´s Modified Essential Medium |
| PBS | - Phosphate buffered saline |
| IS | - Irritancy score |
| NC | - non-classified |
| R36 | - irritant ("irritating to eyes") |
| R41 | - severe eye irritant/corrosive ("risk of serious damage to eyes") |
| NI | - non-irritant |
| R38, I | - irritant ("irritating to skin") |
| R34 | - skin corrosive ("causes burns") |



ORIGINAL ARTICLE

## INTRODUCTION

The determination of eye irritation potential is required for the hazard assessment not only for chemicals under the European Union REACH system, but also for cosmetic ingredients. Eye irritation assessment of final formulations also plays a role in consumer product development. Validated or valid alternative methods should be used exclusively for the testing of chemicals produced or imported at between 1 and 10 tonnes per annum (EC 2006). Since the EU ban on animal testing of cosmetic ingredients and formulations came into force, no animal experiments on eye irritation are authorized at all in this field (EC 2009). Various *in vitro* protocols, involving tissue and organotypic models, are currently being assessed, developed or redesigned with the aim to evaluate eye irritation hazard.

Each of the *in vitro* methods available so far provides only partial information related to the individual ocular tissue structures such as cornea, conjunctiva and/or iris (Curren & Harbell 1998). None of the *in vitro* alternatives can reproduce all the aspects of the *in vivo* method and thus are most likely to be used in combination or test batteries (Grindon *et al.* 2008, McNamee *et al.* 2009; Scott *et al.* 2010). To date, no single stand-alone *in vitro* method has been validated to fully replace the conventional Draize eye irritation test. However, a key difficulty in determining the validity of alternative *in vitro* methods is that the *in vivo* animal data are both scarce, highly variable and often of limited utility for hazard prediction for man (Earl *et al.* 1997).

The aim of this study was to compare human and animal skin irritation data with results of selected *in vitro* methods, including HET-CAM test, Neutral Red Release Assay, Neutral Red Uptake Assay and EpiOcular$^{TM}$ eye irritation test, and with animal eye irritation data when existing. Selected chemicals subjected to *in vitro* testing included substances previously tested with regard to their skin irritation potential in the ECVAM validation study on *in vitro* tests for acute skin irritation (Spielmann *et al.* 2007) and in studies focused on skin irritation determined by means of 4-h human patch test (Basketter *et al.* 2004, Jírová *et al.* 2010). In addition, nine commercially available surfactant-based cosmetic formulations were also subjected to *in vitro* testing for eye irritation potential.

**Tab. 1.** Chemicals tested for eye irritation potential by methods *in vitro*.

| Chemical name | CAS number | purity (%) | supplier |
|---|---|---|---|
| Sodium lauryl sulphate (SLS) | 151-21-3 | >99 | Merck |
| Nonanoic acid | 112-05-0 | 96 | Aldrich |
| Heptaldehyde | 111-71-7 | >=95 | Fluka |
| 1-Decanol | 112-30-1 | 99+ | Sigma-Aldrich |
| Decanoic acid (at 37°C liquid) | 334-48-5 | 96 | Aldrich |
| 10-Undecenoic acid (at 37°C liquid) | 112-38-9 | 98 | Sigma |
| Alpha terpineol | 98-55-5 | 95 | Alfa Aesar |
| Butyl methacrylate | 97-88-1 | 99 | Sigma-Aldrich |
| 1-Bromohexane | 111-25-1 | >98 | Fluka |
| Linalyl acetate | 115-95-7 | >95 | Fluka |
| Di-n-propyl disulphide | 629-19-6 | 98 | Aldrich |
| Hexyl salicylate | 6259-76-3 | 98+ | SAFC |
| Terpinyl acetate | 80-26-2 | 95+ | SAFC |
| Di-propylene glycol | 25265-71-8 | 99 | Aldrich |
| Heptyl butyrate | 5870-93-9 | 98+ | SAFC |
| 4-(Methylthio)benzaldehyde | 3446-89-7 | 95 | Aldrich |
| Hydroxycitronellal | 107-75-5 | 95+ | Aldrich |
| 1-Bromo-4-chlorobutane | 6940-78-9 | 99 | Aldrich |
| Naphtalene acetic acid (solid) | 86-87-3 | 97 | Sigma |
| Dodecanoic (lauric) acid (solid) | 143-07-7 | 98+ | SAFC |

## MATERIALS AND METHODS

*Tested chemicals*

Information on the CAS numbers, purity and suppliers of the tested chemicals is given in Table 1. 18 chemicals were applied as liquids and 2 chemicals as solids.

*Tested cosmetic formulations*

The group of tested final formulations comprised nine commercially available surfactant-based cosmetic products, designated as: Baby shampoo (renowned as mild), Anti-dandruff shampoo for greasy hair with zinc pyrithione, Shower gel with panthenol and silk, Regular shampoo, Liquid soap "eco-friendly", Antidandruff shampoo with piroctone olamine, Regular shower gel, Children liquid soap, Shower gel with sea salts.

*Hen´s Egg Test – Chorioallantoic Membrane (HET-CAM)*

The procedure initially described by Luepke (Luepke 1985) was performed with the modifications of Kalweit *et al.* (1990) and Vinardell and Mitjans (2006) according to INVITTOX Protocol No. 47 (http://ecvam-dbalm.jrc.ec.europa.eu). Briefly, fertilised hen eggs (COBB 500, ROSS 308) obtained from a local supplier (Xavergen a.s., Habry, Czech Republic) were incubated until embryonic day 9. After removing the egg shell covering the air cell using a rotating dentist saw blade and cutting through the inner egg membrane, the test substances were applied to the chorioallantoic membrane either directly (solids) or in cellulose rings with inner diameter 5 mm (liquids). Undiluted samples were applied in the amount of 50 μl on at least 6 eggs. Positive controls (0.1 N NaOH, 1% Sodium lauryl sulphate – SLS) and a negative control (0.9% NaCl) were included in each run of the test. Using a stereomicroscope (Nikon SMZ800), reactions of the arteries, veins or blood system of the membrane to the tested substances were continuously


Copyright © 2014 Neuroendocrinology Letters ISSN 0172–780X • www.nel.edu

observed for up to 5 minutes. The time of first appearance of haemorrhage (H), lysis (L)/vasoconstriction (V) or coagulation (C) measured in seconds was recorded. The irritancy score (IS) of the chemical was calculated using the Kalweit´s formula:

$$IS=(301-H)\times 5/300+(301-L/V)\times 7/300+(301-C)\times 9/300$$

The tested substances were classified according to their IS into four categories (Table 2).

*Neutral Red Release Assay (NRR)*

The NRR protocol used was an adaptation of INVITTOX protocol No. 54 (http://ecvam-dbalm.jrc.ec.europa.eu). Briefly, 3T3 Balb/c fibroblasts (L1, ECACC No. 86052701) were cultivated in 96-well plates for 3 days until reaching 100% confluency, preloaded with Neutral Red (50 µg/ml of culture medium DMEM with serum, incubation 3 h), rinsed (200 µl PBS/well) and each well was filled with culture medium (150 µl of DMEM with serum/well). Before application (within 1 h after preloading) the medium was removed, the test samples were applied for 1 min (50 µl/well, 3 wells per each concentration) and immediately rinsed with 200 µl PBS (twice in case of cosmetic formulations). The samples were applied in 7 concentrations (diluted in PBS) and neat with the exception of solids (naphtalene acetic acid and dodecanoic acid, diluted in PBS up to the highest soluble concentration 250 mg/ml and 500 mg/ml, respectively). PBS was used as negative control on each 96-well plate. SLS (concentration range 0.05–10 mg/ml PBS) served as positive control in each run of the assay. Microscopic evaluation (Olympus IX-50-S8F) of the cell culture was performed before the NR destain solution (1 ml acetic acid, 49 ml deionized water and 50 ml ethanol) was added to each well. The plates were shaken for 10 min and the release of NR was measured fluorimetrically using multidetection reader FLx800 (Biotek Instruments, USA). The $NRR_{50}$ value (concentration (mg/ml) causing the release of 50% of the preloaded NR from the cell culture, compared to negative control) was calculated using Phototox Version 2.0 software (obtained from ZEBET, Germany). As no validated prediction model is available yet, in-house classification (Table 3) was based on obtained $NRR_{50}$ values of the tested substances related to the $NRR_{50}$ value of concurrently tested 20% SLS, which is known to be classified as severe irritant/corrosive, EU R41/GHS Cat.1 (ECETOC 1998). Tested materials with $NRR_{50}$ values higher than 1 000 mg/ml were classified as non-irritant, based on the results of a collaborative study on the evaluation of alternative methods to the eye irritation test (Anon 1991).

*Neutral Red Uptake Assay (NRU)*

The NRU Assay, based on INVITTOX Protocol No. 46 (http://ecvam-dbalm.jrc. ec.europa.eu), was employed for formulation testing only. 3T3 Balb/c fibroblasts (L1, ECACC No. 86052701) were cultivated in 96-well plates for 24 h until reaching cca 60% confluency. Before application, the culture medium (DMEM with serum) was removed and replaced by 200 µl of the tested materials, diluted in medium without serum (4 wells for each concentration). After 24 h, the cells were rinsed (200 µl PBS/well) and incubated with Neutral Red solution (50 µg/ml of culture medium DMEM without serum, incubation 3 h). DMEM without serum was used as negative control on each 96-well plate. Sodium lauryl sulphate (concentration range 1-20 µg/ml DMEM without serum) served as positive control in each run of the assay. Microscopic evaluation of the cell culture was performed before the NR desorb solution (1 ml acetic acid, 49 ml deionized water and 50 ml ethanol) was added to each well. The plates were shaken for 10 min and the uptake of NR was measured fluorimetrically using multidetection reader FLx800 (Biotek Instruments, USA). The $NRU_{50}$ value (concentration (µg/ml) resulting in 50% inhibition of NR uptake compared to negative control) was calculated using Phototox Version 2.0 software (obtained from ZEBET, Germany). As no validated prediction model is available yet, in-house classification (Table 3) was based on obtained $NRU_{50}$ values of the tested substances related to the $NRU_{50}$ value of concurrently tested 20% SLS, which is known to be classified as severe irritant/corrosive, EU R41/GHS Cat.1 (ECETOC 1998). Tested materials with $NRU_{50}$ values higher than 10 000 µg/ml were classified as non-irritant and without negative effects on the eye (Jones *et al.* 1999, Spielmann *et al.* 1996).

*Ocular Irritation Assay – EpiOcular™ Tissue Model*

The EpiOcular™ Assay was conducted according to protocols issued by the tissue model (EpiOcular™ OCL-200) supplier MatTek Corporation, Ashland, MA, USA (www.mattek.com). Chemical substances were tested according to protocol for EU labeling with regard to

**Tab. 2.** Prediction model for HET-CAM test.

| Classification | | IS |
|---|---|---|
| NC / GHS no category | non-irritant | 0.0–0.9 |
| R36-II / GHS Cat.2B | slight irritant | 1.0–4.9 |
| R36-I / GHS Cat.2A | moderate irritant | 5.0–8.9 |
| R41 / GHS Cat.1 | severe irritant | 9.0–21.0 |

**Tab. 3.** In-house prediction models for NRR Assay and NRU Assay.

| Classification | $NRR_{50}$ (mg/ml) | $NRU_{50}$ (µg/ml) |
|---|---|---|
| NC / GHS no category | ≥1 000 | ≥ 10 000 |
| R36 / GHS Cat.2A, 2B | <1 000 and >$NRR_{50}$(SLS 20%) | <10 000 and >$NRU_{50}$ (SLS 20%) |
| R41 / GHS Cat.1 | ≤$NRR_{50}$(SLS 20%) | ≤$NRU_{50}$ (SLS 20%) |

REACH legislation (MatTek Corporation 2007), while the cosmetic formulations were tested using the ocular irritation protocol for water soluble materials (MatTek Corporation 2005).

Upon receipt, the EpiOcular™ tissues were stored at 2–8 °C. Before use the tissues were equilibrated overnight at 37 °C, 5% $CO_2$ in assay medium.

Chemical substances were applied undiluted to the surface of duplicate tissues, pretreated with 20 µl PBS for 30 min, in the amount of 50 µl for 30 min (liquids) or 50 mg (one leveled spoonful) for 90 min (solids). Sterile deionized water served as negative control, methyl acetate (CAS 79-20-9) was used as positive control. After exposure the tissues were extensively rinsed in PBS and allowed to post-soak submerged in assay medium for 12 min. Next, the tissues were transferred into 1 ml of fresh medium for post exposure incubation (2 h for liquids, overnight for solids). Following post-incubation, the MTT assay for cell viability was performed (Mosman 1983). The tissues were transferred to 24-well plates containing MTT medium (1 mg/ml). After a 3 h incubation, the blue formazan salt formed by cellular mitochondria was extracted with 2 ml of isopropanol per well and the optical density was determined by spectrophotometer Varian Cary UV-VIS 1E (Varian Inc., USA) at 570 nm. The prediction model developed by MatTek Corporation, based on the test article-treated tissues viability relative to negative control-treated tissue viability, is given in Table 4.

Cosmetic formulations were tested at 20% dilution (w/v) in sterile deionized water according to the ocular irritation protocol for water soluble materials. An initial time range finding experiment was performed on duplicate tissues with exposure time of 16 min. From the results, exposure times for a definitive test were selected (1 min, 4 min, 64 min, or 256 min). Each sample was applied to the surface of duplicate tissues in the amount of 100 µl per tissue. Deionized water served as negative control, 0.3% Triton X-100 (MatTek Corp., USA) was used as positive control. After exposure the tissues were extensively rinsed in PBS and allowed to post-soak submerged in assay medium for 10 min. Next, the MTT assay for cell viability was performed (Mosman 1983). The tissues were transferred to 24-well plates containing MTT medium (1 mg/ml). After a 3 h incubation, the blue formazan salt formed by cellular mitochondria was extracted with 2 ml of isopropanol per well and the optical density was determined by spectrophotometer Varian Cary UV-VIS 1E (Varian Inc, USA) at 570 nm. The prediction model developed by MatTek Corporation (Table 5) is based on determination of the time of exposure needed for the test water-soluble formulation (20% dilution) to reduce the tissue viability to 50% of control tissues (ET50).

**Tab. 4.** Prediction model for EpiOcular™ Assay (chemicals).

| Classification | viability (% of negative control) |
|---|---|
| non-irritant / GHS no category | > 60% |
| irritant / GHS Cat.1, 2A, 2B | ≤ 60% |

**Tab. 5.** Prediction model for EpiOcular™ Assay (water-soluble materials).

| Classification | | ET50 (min.) |
|---|---|---|
| NC / GHS no category | non-irritant | >256–26.5 |
| R36-II / GHS Cat.2B | slight irritant | <26.5–11.7 |
| R36-I / GHS Cat.2A | moderate irritant | <11.7–3.45 |
| R41 / GHS Cat.1 | severe irritant | <3.45 |

*Statistical analysis*

Statistical analysis of agreement between classification using individual methods included determination of percentage of agreement and kappa coefficient for chemicals and determination of Spearman´s rank-order correlation for formulations (Siegel & Castelan 1988).

## RESULTS

*Chemicals*

Table 6 summarizes available *in vivo* data and results obtained by methods *in vitro* for 20 tested chemicals.

The $NRR_{50}$ value could be determined only for 5 chemicals causing desintegration of cell membranes and NR release. Such clear concentration dependent effect was elicited by SLS, alpha terpineol, dipropylene glycol, nonanoic acid and 4-(Methylthio)benzaldehyde. For a number of chemicals a toxic effect without the release of NR was observed, leading to false negative result of the NRR assay. Light microscopy revealed that the false negative results are caused either by absorption of the released NR by the tested chemical, unremovable under the standard washing procedure (e.g. hydroxycitronellal and heptaldehyde), or by fixative effect on cell membranes preventing the NR release (decanoic acid, 1-decanol, 10-undecenoic acid and linalyl acetate). Two crystallic substances (naphtalene acetic acid and dodecanoic acid), although applied up to the highest soluble concentrations, caused no toxic effect confirmed microscopically. The EpiOcular™ assay results classified 11 of the 20 tested chemicals as irritants for cornea. The HET-CAM test exhibited the highest number of positive results, suggesting the classification R36/R41(GHS Cat.1, 2A, 2B) for conjunctiva in case of 16 of the 20 tested chemicals.

Statistical analysis of agreement between classification revealed significant correlation between results of HET-CAM and NRR assays (75%), less significant agreement was observed between results of NRR and EpiOcular™ assays (70%). The percentage of agreement and kappa coefficients are given in Table 7.



Copyright © 2014 Neuroendocrinology Letters ISSN 0172–780X • www.nel.edu

**Tab. 6.** Summary table of eye/skin irritation data – chemicals.

| Chemical | Eye irritation | | | | Skin irritation (3) | | |
|---|---|---|---|---|---|---|---|
| | ECETOC (1) *in vivo* | EpiOcular™ viability (%) | NRR$_{50}$ (mg/ml) | HET-CAM (IS) | ECETOC (2) *in vivo* | EpiDerm™ 60 min. | 4 h Human patch test |
| 3% SLS | 3% – NC | – | 177.5±16.1 | 15.1±1.8 | R38 (20%) | R38 (20%) | I (20%) |
| 20% SLS | 15% – R41 | 2.7±0.2 | 21.3±0.8 | 13.2±2.1 | | | |
| 99% SLS | 30% – R36 | – | 2.7±1.1 | 12.3±3.2 | | | |
| Nonanoic acid | | 43.8±3.8 | 46.1±2.7 | 14.8±2.5 | R34/R38 | R38 | I |
| Heptaldehyde | | 14.0±4.1 | 780.4* | 6.9±1.8 | R38 | R38 | I |
| 1-Decanol | | 71.6±5.0 | irritant* | 7,5±3,3 | R38 | R38 | NI |
| Decanoic acid (37 °C liquid) | | 13.2±8.4 | irritant* | 7.1±1.4 | R38 | R38 | I |
| 10-Undecenoic acid (37 °C liquid) | | 19.9±0.5 | irritant* | 8.6±2.4 | R38 | R38 | NI |
| Alpha terpineol | | 10.7±4.3 | 22.5±11.4 | 7.5±1.9 | R38 | R38 | NI |
| Butyl methacrylate | | 70.4±7.2 | >1000 | 8.6±2.5 | R38 | R38 | NI |
| 1-Bromohexane | | 76.8±5.7 | >1000 | 3.2±1.8 | R38 | R38 | I |
| Linalyl acetate | | 78.2±7.9 | irritant* | 5.4±2.1 | R38 | NI | NI |
| Di-n-propyl disulphide | NC | 93.4±3.4 | >1000 | 2.5±2.1 | R38 | NI | NI |
| Hexyl salicylate | | 99.4±3.2 | >1000 | 0.7±0.2 | R38 | NI | NI |
| Terpinyl acetate | | 73.1±3.4 | >1000 | 5.6±2.3 | R38 | NI | NI |
| Dipropylene glycol | | 69.9±0.7 | 697.3±74.9 | 15.8±3.2 | NI | NI | NI |
| Heptyl butyrate | | 107.0±6.2 | >1000 | 0.4±0.2 | NI | NI | NI |
| 4-(Methylthio)benzaldehyde | NC | 41.6±4.2 | 437.7±112.3 | 3.9±1.8 | NI | R38 | NI |
| Hydroxycitronellal | | 16.9±0.7 | irritant* | 8.3±2.7 | NI | NI | NI |
| 1-Bromo-4-chlorobutane | NC | 47.9±1.2 | >1000 | 8.8±3.3 | NI | R38 | NI |
| Naphtalene acetic acid (solid) | R41 | 15.4±4.1 | >250 non-irritant$^{\Delta}$ | 0.3±0.1 | NI | NI | NI |
| Dodecanoic (lauric) acid (solid) | R41 | 7.2±2.9 | >500 non-irritant$^{\Delta}$ | 0.5±0.2 | NI | NI | NI |

Eye irritation: NC – non-classified, GHS no category, R36 – irritant, GHS Cat.2, R41 – severe irritant/corrosive, GHS Cat.1.
Skin irritation: NI – non-irritant, GHS no category, R38, I – irritant, GHS Cat.2, R34 – corrosive, GHS Cat.1
* false negative results produced by fixative effect of chemicals preventing NR release
$^{\Delta}$ negative result at the highest soluble concentration, negative effect of pure chemical confirmed microscopically
(1) – ECETOC, 1998, (2) – ECETOC, 1995, (3) – Jírová et al., 2010.

*Cosmetics*

Summary eye irritation results of 9 surfactant-based formulations are documented in Table 8.

The EpiOcular™ assay identified "Anti-dandruff shampoo for greasy hair with zinc pyrithione" as the only product with the potential of eye irritation. The mildest formulations identified by EpiOcular™ assay were renowned mild "Baby shampoo" and "Liquid soap eco-friendly". Similar classification was obtained using both cytotoxicity tests, NRR and NRU. HET-CAM assay classified all products as irritant, but the best compatibility was proved also for the renowned mild "Baby shampoo". The classification of other tested formulations turned out to be equivocal using individual *in vitro* tests. The numerical data obtained in case of cosmetic formulations were statistically analysed using Spearman's rank correlation coefficient. Statistically significant correlation was proved between results of NRR/EpiOcular™ and NRU/EpiOcular™ assays. Only a medium correlation between NRR and NRU was recognized (Table 9).

**Tab. 7.** Statistical evaluation of agreement in classification of eye irritation (chemicals).

| Methods | agreement (%) | kappa | *p*-value |
|---|---|---|---|
| HET-CAM vs. NRR | 75.0 | 0.4681 | 0.0067* |
| HET-CAM vs. EpiOcular™ | 55.0 | 0.0426 | 0.4111 |
| NRR vs. EpiOcular™ | 70.0 | 0.3939 | 0.0391* |

* statistically significant

**Tab. 8.** Summary table of eye irritation results - cosmetic formulations.

| Formulation | NRR $NRR_{50}$ (mg/ml) | EpiOcular™ ET50 (min.) | NRU $NRU_{50}$ (mg/ml) | HET-CAM (IS) |
|---|---|---|---|---|
| Liquid soap "eco-friendly" | 544.4±174.5 | 84.4 | 193.6±78.0 | 7.6±1.9 |
| Children liquid soap | 343.1±63.3 | 41.9 | 61.2±3.7 | 15.4±1.5 |
| Baby shampoo (renowned as mild) | 332.4±105.8 | 44.9 | 382.8±33.9 | 4.8±2.5 |
| Regular shampoo | 172.0±44.4 | 38.3 | 15.3±1.6 | 10.9±2.0 |
| Shower gel with sea salts | 131.9±52.8 | 38.5 | 99.3±18.9 | 8.8±0.7 |
| Antidandruff shampoo with piroctone olamine | 85.4±30.4 | 41.8 | 59.6±4.0 | 10.0±2.0 |
| Shower gel with panthenol and silk | 83.1±26.1 | 32.4 | 56.4±9.6 | 16.5±1.6 |
| Anti-dandruff shampoo for greasy hair with zinc pyrithione | 60.1±6.1 | 16.1 | 9.8±3.7 | 8.8±3.1 |
| Regular shower gel | 51.7±18.0 | 33.5 | 51.2±3.7 | 7.8±0.6 |
| SLS 3% | 177.5±16.1 | N/A | 463.0±89.1 | 15.1±1.8 |
| SLS 20% | 21.3±0.8 | N/A | 61.7±9.2 | 13.2±2.1 |
| SLS | 2.7±1.1 | N/A | 7.6±4.6 | 12.3±3.2 |

**Tab. 9.** Statistical evaluation of correlation between *in vitro* methods for eye irritation (cosmetics).

| Methods | correlation coefficient | *p*-value |
|---|---|---|
| NRR vs. EpiOcular™ | 0.8667 | 0.0025* |
| NRR vs. NRU | 0.4909 | 0.1497 |
| NRR vs. HET-CAM | –0.2432 | 0.4984 |
| NRU vs. EpiOcular™ | 0.8516 | 0.0036* |
| NRU vs. HET-CAM | –0.3769 | 0.2830 |
| EpiOcular™ vs. HET-CAM | –0.3874 | 0.3029 |

* statistically significant

## DISCUSSION

Eye irritation potential has been traditionally scored using the Draize rabbit eye test (Draize *et al.* 1944). However, over the past decades ethical concerns have led to development of numerous *in vitro* methods and to their validation assessing the reliability and reproducibilty to predict eye irritation without animal experiments. A large variety of *in vitro* test methods have been examined and underwent validation process (Balls *et al.* 1999; ICCVAM 2006), however, the predictive performance of each individual assay was not sufficient to fully replace the rabbit Draize eye test. Despite the lack of a single formally validated eye irritation test, distinct valid methods are accepted by regulatory agencies for specific purposes (Anon 1996) and employed in tier testing strategies. The Bovine Corneal Opacity and Permeability (BCOP) and the Isolated Chicken Eye Test (ICE) methods (OECD Test Guidelines 437 and 438) are regulatory accepted as screening tests for identification of ocular corrosives and severe irritants (OECD 2009a,b). Based on a retrospective data analysis, two cell-based *in vitro* assays (Cytosensor Microphysiometer and Fluorescein Leakage) have been scientifically validated and may be used within a tiered testing strategy as described in the ESAC statement (ECVAM 2009). In order to extend the available information on chemicals that were recently examined for skin irritancy (Jírová *et al.* 2010), we assessed their eye irritation potential using selected *in vitro* methods suggested for tier-testing strategies (Scott *et al.* 2010). Unfortunately, for the 20 selected chemicals the available rabbit data on eye irritation are very limited (ECETOC 1998). Moreover, for reference positive control (SLS) a certain discrepancy in classification *in vivo* occurs (15% SLS classified as R41/GHS Cat.1, 30% SLS classified as R36/GHS Cat.2).

Our study confirmed previous findings that skin irritants with reference *in vivo* skin irritation data are not necessarily eye irritants (Williams 1985). The widely accepted rule that skin irritants are also eye irritants is not valid in all cases. Di-n-propyl disulphide classified on rabbit as skin irritant was not classified in the Draize eye irritation test as eye irritant. The absence of eye irritation potential of this chemical was confirmed in our *in vitro* study using NRR and EpiOcular™ assays. Other rabbit skin irritants, e.g. terpinyl acetate, hexyl salicylate, butyl methacrylate or 1-bromohexane, did not exhibit toxic effects in NRR and EpiOcular™ assays and elicited only slight to moderate irritation in HET-CAM assay. These results suggest that distinct volatile or solid materials may be misclassified in the rabbit test due to their physicochemical characteristics. This conclusion is also supported by findings in case of crystallic substances (naphthalene acetic acid and dodecanoic acid) classified in the rabbit eye test as severe irritants. The severely irritant effect on rabbit

 Copyright © 2014 Neuroendocrinology Letters 

eye might have been caused by scratching of cornea *in vivo* as possible mechanical damage was observed also on the reconstructed tissue cells of EpiOcular™ model *in vitro*. These solid substances were not able to elicit real chemically induced eye irritation, as documented by HET-CAM and NRR assay results. In this case, the rabbit classification may not reflect relevant exposure conditions in man, where thorough rinsing after accidental exposure is expected. On the contrary, in the Draize eye irritation test the lids of the experimental animal should be held together after instillation of the test material and the eye may be rinsed only after one hour of exposure (EC 2008).

Commercially available reconstructed human corneal models (e.g. EpiOcular™ or SkinEthic™ HCE) are nonkeratinized epithelial-like tissues made of human cells. The structure of these three-dimensional models is intended to simulate the epithelial covering of the cornea and became an important part of tiered approach for evaluation of eye irritancy *in vitro* (McNamee *et al.* 2009). The EpiOcular™ model proved to be reasonably predictive of ocular irritation in recent studies (Stern *et al.* 1998, Pfannenbecker *et al.* 2013). In our study, EpiOcular™ model results exhibited significant agreement with results of NRR assay (70% agreement). It is not surprising as both test methods are based on the assessment of the potential of a test material to disrupt cellular membranes, causing cell death and subsequent structural damage to eye tissue.

The agreement between classification using EpiOcular™ and HET-CAM was not significant as the HET-CAM assay is based on the measurement of different endpoints such as protein coagulation and vascular changes. Although overpredictive, HET-CAM assay provides the lowest false negative rate and offers valuable results related to conjunctiva. This method is used for number of years to prove absence of eye irritation potential in case of cosmetic products intended for the eye area (Anon 1996). It has the greatest potential to distinguish non-classified substances (products) from irritants. Nevertheless, using the standard scoring system (Kalweit´s formula) based on evaluation of three endpoints, one dominant effect may mask other parameters, resulting in paradoxical irritation score higher in case of lower concentrations of a chemical (e.g. SLS 3% versus SLS 20%, see Table 6). This may happen not only due to one endpoit obscuring the others, but may also be caused by an extremely rapid response of the chorioallantoic membrane preventing correct detection of all three endpoints. In this case, the maximum score of the dominant effect may describe the irritative effect better than the summary score including all parameters.

NRR assay results were found to be in agreement with results of both HET-CAM (75%) and EpiOcular™ assays. This sensitive cell-based assay detects integrity of cellular mebranes after a short term application of the test chemical, simulating the duration of accidental exposures in humans. However, this frequently used screening method exhibits distinct methodological imperfections and protocol improvements are required (Zuang 2001). This method may provide false negative results in case of substances with fixative effect preventing the NR release before destaining solution is applied (e.g. 1-decanol, decanoic acid, linalyl acetate, 10-undecenoic acid), or in case of substances absorbing NR and not removable under standard test protocol washing procedure (e.g. hydroxycitronellal, heptaldehyde). Microscopical evaluation is recognized as a crucial additional endpoint for correct result assessment.

For evaluation of surfactant-based cosmetic formulations (Table 8), the NRU assay was additionally employed. This test may provide more valuable information compared to NRR as it detects not only membrane damage, but may identify possible impairment of metabolic function on various cellular levels. The NRR assay protocol, when testing formulations, again exhibited the need of repeated washing steps as the residues of viscous materials, trapping the released NR, may lead to false negative results.

Considering the product composition including characteristics of specific active ingredients, the EpiOcular™ assay correctly identified the most aggressive formulation ("Anti-dandruff shampoo for greasy hair with zinc pyrithione"), supported by NRU and NRR results, while HET-CAM assay correctly identified the mildest formulation ("Baby shampoo renowned as mild"). "Baby shampoo renowned as mild" and "Liquid soap eco-friendly" with compositions regardful to human tissues or the environment exhibited in all the *in vitro* tests results suggesting the best foreseen in-use compatibility.

The results of the study on cosmetic formulations document that all the employed *in vitro* methods provide useful estimation of corneal/conjunctival effects. Statistical evaluation using Spearman´s rank-order correlation revealed strong correlation between EpiOcular™ and NRR/NRU assays. A correlation between NRR and NRU results was recognized, however only medium, probably due to the low number of tested products. The low agreement between EpiOcular™ and HET-CAM assays may be attributed to the testing conditions. While formulations in the EpiOcular™ assay are tested in 20% aqueous dilutions (simulating the use concentration), the HET-CAM protocol employs the application of tested materials undiluted.

Each of the *in vitro* methods is related to a separate endpoint of ocular irritation and can provide only a partial information about the mode of action of the tested material. Despite good reproducibility of individual assays, only the weight-of-evidence approach considering physicochemical and chemical characteristics of the tested material and multiple results of several *in vitro* tests can be employed for estimation of final *in vivo* ocular effects.

## ACKNOWLEDGMENTS

The study was funded by institutional support of the Ministry of Health of the Czech Republic (1RVO-SZÚ-2014).

REFERENCES

1 Anon (1991). Collaborative Study on the Evaluation of Alternative Methods to the Eye Irritation Test, DOC. XI/632/91 - V/E/1/131/91, CEC, Brussels. Commission of the European Communities (CEC), 1991.
2 Anon (1996). Méthode officielle d'évaluation du potentiel irritant par application sur la membrane chorio-allantoïdienne de l'oeuf de poule. J.O. de la République Francais du 26/12/1996, 19137–19138.
3 Balls M, Berg N, Bruner LH, Curren RD, de Silva O, Earl LK *et al.* (1999). Eye irritation testing: the way forward. The report and recommendations of ECVAM workshop 34. ATLA **27**: 53–77.
4 Basketter DA, York, M, McFadden JP, Robinson MK (2004). Determination of skin irritation potential in the human 4-h patch test. Contact Dermatitis **51**: 1–4.
5 Curren RD, Harbell JW (1998). *In vitro* alternatives for ocular irritation. Environ Health Persp **106**(suppl. 2): 485–492.
6 Draize JH, Woodward G, Clavery HO (1944). Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. J Pharmacol Exp Ther **82**: 377–390.
7 Earl LK, Dickens AD, Rowson MJ (1997). A critical analysis of the rabbit eye irritation test variability and its impact on the validation of alternative methods. Toxicol in Vitro **11**: 295–304.
8 EC (2006). Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. Official Journal **L 396**: 1–849.
9 EC (2008). Commision Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), 2008. Official Journal **L 142**: 400–413.
10 EC (2009). Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products. Official Journal **L 342**: 59–209.
11 ECETOC (1995). Skin Irritation and Corrosion: Reference Chemicals Data Bank. ECETOC Technical Report No. 66, 1995. European Centre for Ecotoxicology and Toxicology of Chemicals, Brussels, Belgium.
12 ECETOC (1998). Technical Report No 48 (2). Eye Irritation: Reference Chemicals Data Bank. Brussels, June 1998, European Centre for Ecotoxicology and Toxicology of Chemicals, Brussels, Belgium.
13 ECVAM (2009). Statement on the scientific validity of cytotoxicity/cell function based *in vitro* assays for eye irritation testing. European Centre for the Validation of Alternative Methods, 10th July 2009, Ispra, Italy. Available at: http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/validation-regulatory-acceptance/docs-eye-irritation/ESAC31_CBA_eye-irritation_20091005.pdf (9.9.2014)
14 Grindon C, Combes R, Cronin MTD, Robert DW, Garrod JF (2008) An integrated decision-tree testing strategy for eye irritation with respect to the requirements of the EU REACH legislation. ATLA **36**: 81–92.
15 ICCVAM (2006). ICCVAM Test Method Evaluation Report – In Vitro Ocular Toxicity Test Methods for Identifying Severe Irritants and Corrosives. NIH Publication No: 07-4517. Available at: http://ntp.niehs.nih.gov/iccvam/docs/ocutox_docs/OTeval/OTevalrpt.pdf (9.9.2014)
16 INVITTOX Protocol No. 47 – available from: http://ecvam-dbalm.jrc.ec.europa.eu
17 INVITTOX protocol No. 54 – available from: http://ecvam-dbalm.jrc.ec.europa.eu
18 Jírová D, Basketter D, Liebsch M, Bendová H, Kejlová K, Marriott M *et al.* (2010). Comparison of human skin irritation patch test data with *in vitro* skin irritation assays and animal data. Contact Dermatitis **62**: 109–116.
19 Jones PA, Bracher M, Marenus K, Kojima H (1999). Performance of the Neutral Red Uptake Assay in the COLIPA International Validation Study on Alternatives to the Rabbit Eye Irritation Test. Toxicol in Vitro **13**: 325–333.
20 Kalweit S, Besoke R, Gerener I, Spielmann H (1990). A national validation project of alternative methods to Draize Rabbit Eye Test. Toxicol in Vitro **4**: 702–706.
21 Luepke NP (1985). Hen´s Egg Chorioallantoic Membrane Test for irritation potential. Food Chem Toxicol **23**: 287–291.
22 MatTek Corporation (2005). Ocular Irritation Protocol: Dilution Method for Use with EpiOcular$^{TM}$ Tissue Model (OCL-200). MK-24-007-0002. 09/06/05: 1–6. Available at: www.mattek.com
23 MatTek Corporation (2007). Topical Application Ocular Irritation Assay for EU Labeling Using the EpiOcular$^{TM}$ Human Cell Construct. 11/12/07: 1–10. Available at: www.mattek.com
24 McNamee P, Hibatallah J, Costabel-Farkas M, Goebel C, Araki D, Dufour E *et al.* (2009). A tiered approach to the use of alternatives to animal testing for the safety assessment of cosmetics: Eye irritation. Regul Toxicol Pharm **54**: 197–209.
25 Mosman T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods **65**: 55–63.
26 OECD (2009a). Test No. 437: Bovine Corneal Opacity and Permeability Test Method for Identifying Ocular Corrosives and Severe Irritants, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing. doi: 10.1787/9789264076303-en
27 OECD (2009b). Test No. 438: Isolated Chicken Eye Test Method for Identifying Ocular Corrosives and Severe Irritants, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing. doi: 10.1787/9789264076310-en
28 Pfannenbecker U, Bessou-Touya S, Faller C, Harbell J, Jacob T, Raabe H *et al.* (2013). Cosmetics Europe multi-laboratory pre-validation of the EpiOcular™ reconstituted human tissue test method for the prediction of eye irritation. Toxicol in Vitro **27**: 619–626.
29 Scott L, Eskes C, Hoffmann S, Adriaens E, Alepée N, Bufo M *et al.* (2010). A proposed eye irritation testing strategy to reduce and replace *in vivo* studies using Bottom–Up and Top–Down approaches. Toxicol in Vitro **24**: 1–9.
30 Siegel S, Castellan NJ (1988). Nonparametric Statistics for the Behavioral Sciences. McGraw-Hill, New York, USA.
31 Spielmann H, Hoffmann S, Liebsch M, Botham P, Fentem J, Eskes C *et al.* (2007). The ECVAM International Validation Study on *In Vitro* Tests for Acute Skin Irritation: Report on the Validity of the EPISKIN and EpiDerm Assays and on the Skin Integrity Function Test. ATLA **35**: 559–601.
32 Spielmann H, Liebsch M, Kalweit S, Moldenhauer S, Wirnsberger T, Holzhütter H-G *et al.* (1996). Results of a validation study in Germany on two *in vitro* alternatives to the Draize eye irritation test, the HET-CAM test and the 3T3-NRU cytotoxicity test. ATLA **24**: 741–858.
33 Stern M, Klausner M, Alvarado R, Renskers K, Dickens M (1998). Evaluation of the EpiOcular$^{TM}$ tissue model as an alternative to the Draize eye irritation test. Toxicol in Vitro **12**: 455–461.
34 Vinardell MP, Mitjans M (2006). The chorioallantoic membrane test as a model to predict the potential human eye irritation induced by commonly used laboratory solvents. Toxicol in Vitro **20**: 1066–1070.
35 Williams (1985). Changing concepts of ocular irritation evaluation: pitfalls and progress. Food Chem Toxicol **23**: 189–193.
36 Zuang V (2001). The neutral red release assay: a review. ATLA **29**: 575–599.


Copyright © 2014 Neuroendocrinology Letters ISSN 0172–780X • **www.nel.edu**