Eye Irritation

The Neutral Red Release (NRR) assay is a quantitative colorimetric cytotoxicity test using cell lines to measure the immediate toxic effect of a test substance on the cell membrane.

Objective & Application

**TYPE OF TESTING**: Replacement (partial)

**LEVEL OF ASSESSMENT**: Toxic potential

The NRR assay was developed as an alternative to the Draize eye irritation test (OECD TG 405, 2012; Method B.5 of Annex to the Test Methods Commission Regulation 440/2008/EC, EU 2008; Draize et al., 1944). In 1999, the French authorities have accepted the NRR assay using the SIRC for the evaluation of cosmetic products (Balls et al., 2002), but in 2009 the ESAC stated that the evidence available so far is insufficient to support a recommendation that NRR is ready for consideration for regulatory use (ESAC, 2009).

Basis of the Method

In principle the vital dye release method for assessing short time exposures was established by Reader et al. (1989, 1990) at the FRAME Alternatives Laboratory at the University of Nottingham. The test has been further developed from the NRU assay as developed by Borenfreund et al. (1984).

The NRR is based on the observation that some substances that are damaging the eye appear to be cytotoxic to a number of cell types (e.g., corneal epithelium, corneal endothelium). This assay experimentally foresees the evaluation of the loss of lysosomal integrity in cultivated cells by measuring spectrophotometrically the amount of preloaded Neutral Red (3-amino-7-dimethylamino-2-methylphenzaine hydrochloride) released from damaged cells (Harbell et al., 1997), this dye, in fact, is used as a marker for cell viability and accumulates in the lysosomal compartment of an intact cell due to existing pH gradients.

Cultured cells are pre-loaded with the dye and exposed to a test substance. The amount of dye released from the cells indicates the membrane damage induced by the test substance. The toxicological endpoint of the method is the concentration of test substance inducing 50% release of pre-loaded dye (NRR50). This concentration is also named IC50 in context with the Predisafe™ test kit.

Experimental Description

**Biological and Endpoint Measurement:**

CELL VIABILITY: NRR measured spectrophotometrically

**Endpoint Value:**

IC50: the concentration of test substance inducing 50% release of pre-loaded dye in the Predisafe™ test kit

NRR50: the concentration of test substance inducing 50% release of pre-loaded dye in the NRR assay

**Experimental System:**

3T3-L1 FIBROBLASTS (MOUSE): commercially available murine cell line

NHEK: commercially available cell line

SIRC CELL LINE: commercially available cell line derived from rabbit cornea

The NRR assay has been established using primary cell cultures as well as established cell lines from different species such as murine 3T3-L1 cells, SIRC from rabbit origin, or a cell line derived from human NHEK (Eskes et al., 2005). The standard protocol (DB-ALM Protocol N. 54) proposes the 3T3 mouse fibroblast cell line in low passage numbers (<50). Cells were plated in 96 well flat bottomed plates and incubated for 3-4 days until cultures reach confluency, since rapid cell proliferation is not a feature of undamaged eye tissue. At the confluent state, medium is removed from the cells and NR solution (final concentration 50 µg/ml) is added for 3 hours. Cells are washed to remove excessive dye and the test
substance is added for 1 to 5 min. in serial dilutions (1:10) to the cells. Reader et al. (1989) recommended diluting water-soluble solids and water-miscible solutions in PBS and poorly soluble material in mineral oil.

After the exposure time, cells are again washed and destain solution (1% acetic acid, 50% Ethanol, 49% distilled water) is added to the treated cells. Plates are incubated on a shaker for 15-20 min to disperse the released dye. The optical density of each well is read at 546 nm with a reference measurement at 405 nm. It is recommended to assess the substances in triplicates, and to have several controls (cell-free blank, solvent control, and PBS control) (Reader et al., 1989; DB-ALM Protocol N. 54).

A SOP is available in DB-ALM as Protocol No. 54: "Frame Neutral Red Release Assay"

**Data Analysis/Prediction Model**

There is no formally recognised prediction model available for the NRR assay (Zuang, 2001). However, Zhuang (2001) proposed to use the available data of the CTFA study Phase III as a "rough guide".

The prediction model of the Predisafe™ assay is based on a mathematical algorithm of 47 historical eye irritation data of the Draize test. Courtellement et al. (1999) could show that the best mathematical algorithm was obtained when the \( \text{in vivo} \) values (MMAS) were plotted *versus* the natural logarithm of \( \text{IC}_{50} \) values. The following prediction model for the Predisafe™ assay has been established:

\[
\text{MMAS} = 63.75 - 13.5 \ln (\text{IC}_{50})
\]

<table>
<thead>
<tr>
<th>Draize MMAS</th>
<th>classification</th>
<th>Predisafe IC(_{50}) in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 15</td>
<td>Slightly irritant</td>
<td>&gt; 50%</td>
</tr>
<tr>
<td>15 – 30</td>
<td>Moderately irritant</td>
<td>25% - 50%</td>
</tr>
<tr>
<td>30 – 110</td>
<td>Irritant</td>
<td>&lt; 25%</td>
</tr>
</tbody>
</table>

Classification according to Courtellement et al. (1999).

**Test Compounds**

Cosmetic raw material and formulations, surfactant-based formulations have been used.
Modifications

Several different protocols have been published concerning the use of different cell lines such as murine 3T3, SIRC, MDCK cells or NHEK (Reader et al., 1989; Guyomard et al., 1994; Jones et al., 2001; Cheng et al., 1995), serum-free or serum-containing (1%-10%) media (Harbell et al., 1997; Reader et al., 1989; Getting et al., 1994), exposure times ranging from 30 seconds to 30 minutes (Reader et al., 1989; Guyomard et al., 1994; Harbell et al., 1997; Courtellement et al., 1999; Jones et al., 2001), and additional endpoint values (NRR20, NRR80) (Reader et al., 1989; Balis et al., 1990; Clothier et al., 1995). The commercially available Predisafe™ test kit has been developed by Guyomard et al. (1994) (at Biopredict, Rennes, France) based on the NRR method of Reader et al. (1989). Predisafe™ is a ready-to-use kit containing the confluent cell layer of SIRC. Three reference compounds (SDS at 0.01%, 0.05%, 0.2% or formic acid) as quality controls and all diluents and reagents for the experiment. The QC substance has to lie within predefined acceptance ranges that are initially defined by historical data. The quality control substance ensures the proper setting of the experiment.

Discussion

The NRR assay is rapid to perform, and easy to establish in a laboratory without special technical training (Guyomard et al., 1994; Eskes et al., 2005; DB-ALM Protocol N. 54). This cell-based method is designed to identify cytotoxic substances causing a response after a brief contact in a high concentration. The short exposure times of the assay limit it to identify only rapid occurring effects (Eskes et al., 2005; DB-ALM Protocol N. 54). For this reason the NRR is proposed by Clothier et al. (1995) to be performed in conjunction with other tests such as the KB assay (DB-ALM Protocol N. 15) or the FL assay (DB-ALM Protocol N. 71). Grabarz et al. (1999) proposed the use of a test battery of the NRR in combination with the EpiOcular model (MatTek, Ashland, USA) to identify surfactant-based formulations, which are mild ocular irritants.

Status

The NRR assay is used as part of an in-house battery for a selected category of formulations in a number of industrial laboratories (Zuang et al., 2001). Jones et al. (2001) claimed that the NRR assay is less predictive than other alternative methods (IRE, BCOP, FL, EpiOcular) concerning the evaluation of hair care formulations. According to the authors (Jones et al., 2001), the results of the NRR assay correlated poorly with the in vivo ocular irritancy of surfactants.

In the European pre-validation study (CEC, 1991) only one laboratory performed the NRR, which impedes a between-laboratory comparison. Ranking of tested substances according to the irritating potency was compared to the NRU assay, which is a long-term test. For several compounds relative potency was different in the NRR and NRU (CEC, 1991).

In the COLIPA validation study, Predisafe™ showed a better correlation with cosmetic formulations than with ingredients (Courtellement et al., 1999). The authors (Courtellement et al., 1999) explained that some false positives results of surfactants or surfactant-based formulations are due to the high sensitivity of the NRR assay. They proposed to reduce the exposure time to 30 sec. to avoid overpredictions (Courtellement et al., 1999). Furthermore, the authors could show a high relevance and reliability of Predisafe™ in the screening of ocular irritation potential of cosmetic formulations. Brantom et al. (1997) could not confirm the between-laboratory reproducibility since only three laboratories conducted the Predisafe™ assay in the COLIPA study.

The NRR assay has correctly predicted all hydroalcoholic-based formulations in the CTFA study Phase I (hydroalcoholic-based formulations) (Gettings et al., 1991), but was not well performing with oil/water emulsions in the CTFA Evaluation Phase II (Gettings et al., 1996). In the CTFA study on surfactant-based formulations (Phase III) the Predisafe™ belonged to the tests with the least disagreement with results of the Draize test when testing surfactant-based formulations (Gettings et al., 1996).

Harbell et al. (1997) stated in the IRAG working group 4 (cell cytotoxicity assays) that the NRR assay looks promising as a screen for testing surfactant-based material in the range of toxicities normally found in personal care products. According to Harbell et al. (1997) and Eskes et al. (2005) the assay may be useful in screening surfactants in the mild to very mild range. The authors however point out, that the NRR assay has to be applied with caution for non-surfactant formulations (Harbell et al., 1997).

The short-term NRR assay has been recommended by several scientists to be combined with a longer-term assay (e. g. KB, FL, MTT) for a precise substance classification (Clothier et al., 1995; Zebet Protocol N. 256). Rosenkranz and Cunningham (2000) proposed a battery of cell toxicity assays to predict
ocular irritancy in vitro.

In 1999, the French authorities have accepted the NRR assay using the SIRC for the evaluation of cosmetic products (Zebet Protocol N. 265).

In 2001, the ESAC recommended the NRR for further validation studies for surfactant-based formulations, if the protocol and the prediction model is standardised (Zuang, 2001; Eskes et al. 2005).

In 2005, the ECVAM initiated a retrospective evaluation for the following four in vitro methods: the NRR assay, the RBC test, the FLT, and the SM. The final evaluation of DB-ALM Protocol No. 54 and Predisafe™ (both concerning the NRR Method) done by the Validation Management Team indicates that "... the available evidences are not sufficient to support a recommendation for a regulatory use consideration (ESAC, 2009)."

Abbreviations & Definitions

3T3: A standard fibroblast cell line
BCOP: Bovine Corneal Opacity and Permeability assay
COLIPA: European Cosmetics, Toiletries and Perfumery Association
CTFA: Cosmetic, Toiletry and Fragrance Associations
EC: European Commission
ECVAM: European Centre for the Validation of Alternative Methods
ESAC: ECVAM Scientific Advisory Committee
EU: European Union
FL: Fluorescein Leakage
FLT: Fluorescein Leakage Test
FRAME: Fund for the Replacement of Animals in Medical Experiments
IRAG: Interagency Regulatory Alternatives Group
IRE: Isolated Rabbit Eye
KB: Kenacid Blue
MDCK: Madin-Darby Canine Kidney
MTT: Methyl Thiazol-Tetrazolium
NHEK: Normal Human Epidermal Keratinocytes
NR: Neutral Red
NRR: Neutral Red Release
NRU: Neutral Red Uptake
OECD: Organisation for Economic Co-operation and Development
PBS: Phosphate Buffered Saline
QC: Quality Control
RBC: Red Blood Cell
SDS: Sodium Dodecyl Sulphate
SIRC: Rabbit Corneal Cell line
SM: Silicon Microphysiometer
SO: Standard Operating Procedure
TG: Test Guideline

Contact Details

Dr. Richard Clothier
Reader in Cell toxicology and Trustee of FRAME
Fund for the Replacement of Animals in Medical Experiments
FRAME
Clifton Boulevard
Nottingham NG7 2UH
United Kingdom
e-mail: richard.clothier@btinternet.com

FRAME FRAME Alternatives Laboratory (FAL)
Fund for the Replacement of Animals in Medical Experiments
FRAME
Russell & Burch House
96-98 North Sherwood Street
Nottingham NG1 4EE
United Kingdom
Bibliography

- Babich H. and Borenfreund E. (1992)
  Neutral Red assay for toxicology in vitro.
  *In Vitro Methods of Toxicology*, 237-251

  *Alternatives to Laboratory Animals (ATLA)* 27, 53-77

  Non animal alternative toxicity tests for detergents: genuine replacements or mere prescreens?
  *Journal of Chemical Technology and Biotechnology* 50, 423-433

  Evaluation of a group of petrochemicals using Clonetics neutral red bioassay to predict irritancy.
  *In Vitro Toxicology: Tenth Anniversary Symposium of CAAT; Serialtitle: Alternative Methods in Toxicology* 9, 225

  Use of Clonetics neutral red bioassay to optimize components of serum-free medium for normal human anchorage-dependent cells.
  *In Vitro Cellular and Developmental Biology* 27, 160

  A simple quantitative procedure using monolayer cultures for cytotoxicity assays (HTD/NR90).
  *Journal of Tissue Culture Methods* 9, 7-9

  Comparisons of two in vitro cytotoxicity assays - the neutral red (NR) and tetrazolium MTT tests.
  *Toxicology In Vitro* 2, 1-6

  A Summary Report of the COLIPA International Validation Study on Alternatives to the Draize Rabbit Eye Irritation Test.
  *Toxicology In Vitro* 11, 141-179

  Effect of tear proteins on preservative toxicity to epithelial cells.
  *Journal of Toxicology - Cutaneous and Ocular Toxicology* 14, 287-297

  Soaps and Detergents - alternatives to animal eye irritation tests.
  *Journal of the American College of Toxicology* 15, 1-44

  The evaluation of pesticide ingredients and formulation in vitro and correlations with in vivo data.
  *Alternatives to Laboratory Animals (ATLA)* 23, 667-675

- Commission of the European Communities (1991)
  Collaborative study on the evaluation of alternative methods to the eye irritation test.
  *EC Document XI/632/91, V/E/131/91*

  Relevance and reliability of the PREDISAFE assay in the COLIPA eye irritation validation program (Phase 1).
  *Toxicology In Vitro* 13, 305-312

  The use of the neutral red bioassay using normal human keratinocytes to detect material requiring metabolic activation.
  *The Toxicologist* 11(1)
Draize J.H., Woodard G.K. and Calvery H.O. (1944)
Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes.
*Journal of Pharmacology and Experimental Therapeutics* 82, 377-390

ESAC (2009)
*ECVAM Scientific Advisory Committee.*

EU (2008)
*Official Journal of the European Communities* L 142, 141-443

Eye irritation.
*Alternatives to Laboratory Animals (ATLA)* 33, Suppl. 1, 47-81

The CFTA evaluation of alternatives program: An evaluation of in vitro alternatives to the Draize primary eye irritation test (phase I) hydroalcoholic formulations (Part 2); Data analysis and biological significance.
*In Vitro Toxicology* 4, 247-288

The CFTA evaluation of alternatives program: An evaluation of in vitro alternatives to the Draize primary eye irritation test. (Phase II). Oil/water emulsions.
*Food and Chemical Toxicology* 32, 943-976

*Food and Chemical Toxicology* 34, 79-117

Successful use of a battery of in vitro tests to screen for ocular safety before human trials.
*Alternatives to Animal Testing II*, 192

Evaluation of PREDISAFE, a cell kit for predicting eye irritancy of cosmetic raw materials and formulations.
*Cell Biology and Toxicology* 10, 375-379

*Food and Chemical Toxicology* 35, 79-126

Comparative evaluation of five in vitro tests for assessing the eye irritation potential of hair-care products.
*Alternatives to Laboratory Animals (ATLA)* 29, 669-692

MTT-assay and neutral red release (NRR)-assay: Relative role in the prediction of the irritancy potential
of surfactants.
Life Sciences 55, 533-540

- OECD (2012)
  adopted on 2nd October 2012. Link to document (last access 29.10.2012)
  OECD Guidelines for the Testing of Chemicals, Section 4, Health Effects

- Reader S.J. (1988)
  Ph.D. thesis, University of Nottingham

- Reader S.J., Blackwell V., O'Hara R., Clothier R.H., Griffin G. and Balls M. (1990)
  Neutral Red Release from pre-loaded cells as an in vitro approach to testing for eye irritancy potential.
  Toxicology In Vitro 4, 264-266

  A Vital Dye Release Method for Assaying the Short-term Cytotoxic Effects of Chemicals and
  Formulations.
  Alternatives to Laboratory Animals (ATLA) 17, 28-33

- Silva, O. de, Cottin, M., Dami, N., Rouget, R., Catroux, P., Toufic, A., Sicard, C., Dossou, K. G., Gerner, I.,
  Evaluation of eye irritation potential: statistical analysis and tier testing strategies.
  Food and Chemical Toxicology 35, 159-164

  Evaluation of the human epidermal keratinocyte neutral red release and neutral red uptake assay using
  the first 10 MEIC test materials.
  Toxicology In Vitro 6, 367-371

- Zentralstelle zur Erfassung und Bewertung von Ersatz- und Ergänzungsmethoden zum Tierversuch
  (ZEBET); BfR (2001)
  The neutral red release assay for the in vitro assessment of the eye irritation potential of chemical
  substances. Method No. 265, (10.06.01).
  ZEBET Website: http://www.bfr.bund.de/cd/1591 (as of 11 May 2006)

- Zhuang, V. (2001)
  The neutral red release assay: a review.
  Alternatives to Laboratory Animals (ATLA) 29, 575-599

Last update: January, 2010