Resazurin Metabolism Assay Is a New Sensitive Alternative Test in Isolated Pig Cornea

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Received August 20, 2002; accepted December 10, 2002

The main object of our study was to investigate whether the resazurin metabolism assay is a sensitive surfactant and alcohol toxicity test in isolated pig cornea and to compare this recently developed fluorometric assay with the data collected in the eye irritation reference chemical data bank. Resazurin is a substrate that changes color in response to metabolic activity. Isolated pig corneas were immersed for 10 min in surfactants and alcohol irritant solutions. After incubation, resorufin fluorescence was read and corneal viability was assessed. This corneal viability was compared with the maximal modified average score published in the report of ECETOC. This assay highlighted different concentration-dependent irritation potentials of the three surfactants tested, and the same results were obtained with corneas treated with the alcohols. We observed that the degree of surfactant- and alcohol-induced decrease in corneal viability, using the resazurin reduction test, was correlated with the in vivo irritation measurements as determined by the Draize test and scored with the Modified Maximum Average Score (MMAS). This assay allowed us to classify the ocular irritancy of the tested surfactants and alcohols in the same ranking order as the Draize classification. Corneal viability measurement can be used as a potential alternative for the toxicological assessment of surfactants and alcohols. The nontoxic, nonradioactive resazurin metabolism assay allows rapid assessment of many samples with simple equipment and at reduced cost for continuous monitoring of corneal viability. This assay seems to be suitable as a toxicological screening test for eye irritation determination.

Key Words: isolated corneal model; toxicological assessment; resazurin; surfactants; alcohols.

Commercial products, whether drugs, cosmetics, home and automobile care products, or bulk chemicals, are subject to a variety of tests to determine potential adverse health effects. Historically, toxicological data used in assessing the safety of chemicals to man have been generated by testing the materials on animals. The Draize eye irritation test (Draize et al., 1944) and the low-volume eye irritation test (LVET; Freeberg et al., 1986) are commonly used rabbit eye tests that provide useful information to ensure that products are safe for their intended use and reasonably foreseeable misuse by consumers, are appropriately labeled, and meet regulatory safety testing requirements (Maurer and Parker, 1996).

Regulations now require extensive testing of new chemicals and there are increasing demands for the testing of many chemicals that may have been used for a number of years. The net result of these developments is that the number of animals used in toxicological tests may be expected to increase. Many companies and regulatory authorities share the published concern of the animal welfare organizations. For several years, industry has been modifying the existing in vivo techniques, in order to minimize distress and the number of animals used, until alternative nonsentient methods have been developed that are acceptable to regulatory authorities.

The in vivo rabbit eye irritation test, in particular, has frequently been criticized on animal welfare grounds (Bruner, 1992; Rowan, 1984) and because of its lack of objectivity (Bruner, 1992) and repeatability (Bruner, 1992; Talsma et al., 1988; Weil and Scala, 1971). In recent years, many laboratories have been working to develop in vitro alternatives to this test, and a number of validation exercises have been carried out to assess the reliability and relevance of some of the promising methods (Bruner et al., 1991).

Eye irritation is a local and reversible response of normal living corneal, iris, and conjunctival cells to direct injury caused by contact with an irritant. Approximately 75% of the Draize test scores are derived from corneal effects. Thus, some part of a test battery being developed to refine and/or replace the Draize test should be aimed at this major irritancy factor. We were convinced of the importance of investigating corneal injury. Numerous techniques evaluate it by assays based on the measurement of thickness (Burton, 1972; Kennah et al., 1989b), opacity and permeability (Gautheron et al., 1992; Jester et al., 2001; Muir, 1984, 1985). A three-dimensional in vitro model of cultured human corneal epithelial cells (Krus-
The main object of our study was to investigate whether the resazurin metabolism assay is a sensitive surfactant-toxicity test in isolated pig corneas and to compare this recently developed fluorometric assay with the data collected in the eye irritation reference chemical data bank.

**MATERIALS AND METHODS**

Isolated pig corneas preparation, incubation, rinsing, revealing, and reading procedures were chosen after testing numerous possibilities in preliminary studies.

**Reagents.** Eagle’s minimum essential medium (without phenol red) and phosphate buffer were purchased from Biomedia (Boussens, France). Foetal calf serum was obtained from Dominique Dutschter SA (Strasbourg, France); other chemicals were from Sigma (St Louis, MO).

**Isolated pig cornea.** Approximately one hundred fifty enucleated porcine eyes were obtained from a local slaughterhouse within 2 h post mortem. The animals were killed by electroblot, followed by severing of the major neck blood vessels, to permit exsanguination. The eyes were enucleated shortly after. No special instructions were given to the slaughterhouse personnel other than that the eyes should not be washed in hot water and should be placed, as soon as possible, in clear plastic bags, which should then be closed. Because fresh eyes could show significant bacterial infection (Barber, 1984) or could be contaminated with other materials, the globe was immersed in ophthalmic Betadine® solution for 5 min and was rinsed with sterile phosphate buffer solution (PBS, Biomedia, Boussens, France) before use. The cornea was removed by cutting 2–3 mm external to the limbus and the iris was cut from the insertion. The lens was then pulled away with forceps and the cornea was washed in a sterile 24-well plate containing phosphate buffer solution.

**Surfactant treatment.** Solutions of all surfactants were prepared in phosphate buffer solution at the following concentrations: BAC: 10% (100 mg/ml), 5% (50 mg/ml), and 1% (10 mg/ml); SDS: 30% (300 mg/ml), 15% (150 mg/ml), and 3% (30 mg/ml); TX-100: 10% (100 mg/ml), 5% (50 mg/ml), and 1% (10 mg/ml). All solutions were expressed as mass/mass percentage, having a pH of 7.4 and stored at room temperature.

**Alcohol treatment.** Propylene glycol, glycerol, ethanol, isopropanol, n-octanol, and n-butanol were used nondiluted and were stored at room temperature.

**Resazurin metabolism assay.** All assays were conducted using 24-well microtitre plates. Resazurin was prepared as a stock solution of 100 μg/ml in Eagle’s minimum essential medium (without phenol red), containing 5% dextran T40 and used at a final concentration of 10 μg/ml. Stock solution was filtered with a sterile 0.20 μm-pore filter and stored in the dark at 4°C. Isolated pig corneas were immersed for 10 min in 1 ml of surfactant solution or alcohol. During the treatment, the 24-well plate containing treated cornea was agitated. After this time, corneas were removed and rinsed with 2 ml of PBS. After treatment, 2 ml of resazin-reveal solution were added to the corneas in a 24-well plate and incubated at 37°C. Every 2 h each cornea was rinsed in PBS buffer. Six hours after the beginning of incubation, resorufin fluorescence was read using a microplate spectrofluorometer Safire® (TECAN, Trappes, France; λex = 570 nm, λem = 590 nm).

**Eye irritation reference chemicals data bank.** The ECETOC technical report no. 48(2), eye irritation reference chemicals data bank, published in 1998 presents comprehensive rabbit irritation data obtained on 132 chemicals assessed in 149 in vivo tests (ECETOC, 1998). Stringent criteria regarding purity of the chemicals and quality of the in vivo data were applied in selecting the chemicals. The 132 chemicals selected are available at high and consistent purity and are expected stable in storage. They have been tested undiluted in in vivo studies, excepting those chemicals where high concentrations of the substance could be expected to cause severe effects. The in vivo data have been generated since 1981 in studies carried out according to OECD test guideline 405 and following the principles of good laboratory practice. The data presented were obtained from tests normally using at least 3 rabbits evaluated at the same time. Dosing was by instillation of 0.1 ml (or equivalent weight) into the conjunctival sac and observations were made at least 24, 48, and 72 hours after instillation.

The chemicals are ranked for eye irritation potential on the basis of a Modified Maximum Average Score (MMAS). Irritation and chemical data of...
surfactants and alcohols of interest (ECETOC, 1998; Kennah et al., 1989a,b) are summarized in Tables 1 and 2, respectively.

**Statistical analyses.** In corneal viability experiments, background fluorescence was determined on wells containing the dye solution but no corneas, and was deducted from all control and treated wells. Wells containing corneas without any treatment were used as controls. All results obtained were expressed in fluorescence units (FU) and the percentage of viability was calculated using the following equation: (FU treated/FU control) × 100. At each point, values of relative corneal viability were the mean of eight to twelve determinations.

All control wells were compared to each other using a one-way ANOVA test, and no significant difference was found, so control data were pooled to homogenize the results and the graphs. Statistical comparisons were performed using a one-way ANOVA test and a Dunnet test at a 0.05 level of significance. Normality of all groups was evaluated using a Kolmogorov-Smirnov normality test. The results indicate that the data match the pattern expected if they are drawn from a population with normal distribution, so correlation was evaluated with the Pearson correlation test performed with the same software.

**RESULTS**

**Surfactant Treatment**

The corneal viability after surfactant treatment was estimated using the resazurin metabolism assay.

Figure 2 shows that BAC led to a significant decrease in corneal viability with all tested BAC concentrations (1, 5, and 10%). The decrease in resazurin metabolism was 48, 70, and 73%, respectively, compared with control values ($p < 0.01$). A significant decrease in resazurin metabolism was observed with the 3, 15, and 30% SDS concentrations (Fig. 3): 30, 37, or 44%, respectively, compared with control values ($p < 0.01$). The decrease in resazurin metabolism with Triton X-100-treated corneas was 18 and 24%, respectively, compared with the control values ($p < 0.05$), with the two highest concentrations (5 and 10%; Fig. 4). The other concentration (1%) did not induce a significant difference.

**DISCUSSION**

Resazurin is known to act as an intermediate electron acceptor in the electron transport chain between the final reduction of oxygen and cytochrome oxidase by substituting for molecular oxygen as an electron acceptor (Pagé et al., 1993). The deoxygenated product of resazurin, resorufin, exhibits strong emission at wavelengths greater than 550 nm. This transformation of nonfluorescent resazurin to fluorescent resorufin has been utilized as a fluorometric indicator for the determination of cell viability. Although it has been arbitrarily postulated as being reduced by mitochondrial enzymes (De Fries and Mitsubishi, 1995), it is not known whether this occurs intracellularly at the plasma membrane surface or just in the medium as a chemical reaction. Biochemical tests in sperm cells suggest that the mitochondrial diaphorase enzyme is responsive to resazurin reduction (Zalata et al., 1998).

The resazurin metabolism test has been used since the 1950s to assess bacterial or yeast contamination in biological fluids (Erb and Ehlers, 1950) and it is also used to measure the viability of sperm by colorimetry (Carter et al., 1998). It

**Table 1**

Comparative Ocular Irritancy of Surfactants

<table>
<thead>
<tr>
<th>Chemical class</th>
<th>Surfactants</th>
<th>MMAS</th>
<th>Irritation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cationic (extreme)</td>
<td>BAC 10%</td>
<td>108</td>
<td>Extreme</td>
</tr>
<tr>
<td></td>
<td>BAC 5%</td>
<td>83.8</td>
<td>Extreme</td>
</tr>
<tr>
<td></td>
<td>BAC 1%</td>
<td>56.3</td>
<td>Severe</td>
</tr>
<tr>
<td>Anionic (severe)</td>
<td>SDS 30%</td>
<td>60.5</td>
<td>Severe</td>
</tr>
<tr>
<td></td>
<td>SDS 15%</td>
<td>59.2</td>
<td>Severe</td>
</tr>
<tr>
<td></td>
<td>SDS 3%</td>
<td>16</td>
<td>Medium</td>
</tr>
<tr>
<td>Nonionic (moderate)</td>
<td>TX-100 10%</td>
<td>68.7</td>
<td>Severe</td>
</tr>
<tr>
<td></td>
<td>TX-100 5%</td>
<td>33.8</td>
<td>Irritant</td>
</tr>
<tr>
<td></td>
<td>TX-100 1%</td>
<td>1.7</td>
<td>Nonirritant</td>
</tr>
</tbody>
</table>

**Note.** Modified maximum average scores (MMAS) of the Draize rabbit eye irritation test were obtained from the ECETOC Eye Irritation Reference Chemicals Data Bank, 2nd ed., ECETOC, 1998.

**Table 2**

Comparative Ocular Irritancy of Alcohols

<table>
<thead>
<tr>
<th>Alcohols</th>
<th>MMAS</th>
<th>Irritation</th>
<th>Chemical formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propylene glycol</td>
<td>1.3</td>
<td>Nonirritant</td>
<td>C,H,(OH)</td>
</tr>
<tr>
<td>Glycerol</td>
<td>1.7</td>
<td>Nonirritant</td>
<td>C,H,(OH)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>24</td>
<td>Medium</td>
<td>C,H,OH</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>30.5</td>
<td>Irritant</td>
<td>C,H,OH</td>
</tr>
<tr>
<td>n-Octanol</td>
<td>41</td>
<td>Irritant</td>
<td>C,H,OH</td>
</tr>
<tr>
<td>n-Butanol</td>
<td>60.8</td>
<td>Severe</td>
<td>C,H,OH</td>
</tr>
</tbody>
</table>

**Note.** Modified maximum average scores (MMAS) of the Draize rabbit eye irritation test were obtained from the ECETOC Eye Irritation Reference Chemicals Data Bank, 2nd ed., ECETOC, 1998.
has been commercialized since 1993 as Alamar blue® dye (O’Brien et al., 2000). It has been assessed several times on different types of cells for its cytotoxicity reliability, i.e. fibroblasts (Voytik-Harbin et al., 1998), immortalized and cancer-cell lines (Nakayama et al., 1997; Pagé et al., 1993), tumor necrosis factor (TNF)-hypersensitive cells (Shahan et al., 1994), and for its cell proliferation reliability on mouse and human lymphocytes (Ahmed et al., 1994; De Fries and Mitsuhashi, 1995), and primary neuronal cell culture (White et al., 1996). Resazurin reduction has even been used to monitor the viability of corneal endothelial cells (Larson et al., 1997).

Resazurin fluorescence detection offers several advantages over other methodologies. It is reproducible, simple (since the dye is directly added to the cornea), and the fluorescence is measured after a few hours without any other operation or calculation (Pagé et al., 1993). It is inexpensive, because the dye is the only reagent that needs to be purchased. As resazurin is sensitive to metabolic functions (De Fries and Mitsuhashi, 1995), it is important to minimize any bacterial contamination in the incubation media. To avoid this potential problem, all solutions should be sterile filtered, and pig corneas should be decontaminated prior to use.

The pig cornea consists of four layers: a multi-layered surface epithelium, a connective tissue stroma containing sparse flattened keratocytes, a posterior limiting Descemet’s membrane, and a single-layered posterior endothelium of flattened cells. The avascular stroma accounts for 90% of the thickness of the cornea in most mammals. It consists of parallel bands of collagen

FIG. 2. BAC corneal viability measurement. Isolated pig corneas were treated with BAC solutions at concentrations ranging from 1 to 10%. After incubation, resorufin fluorescence was read at 590 nm emission wavelengths with 570 nm excitation wavelengths. Results were expressed in fluorescence units (FU) and percentage of viability, calculated using the following equation: (FU treated/FU control) × 100. **p < 0.01; n = 12.

FIG. 3. SDS corneal viability measurement. Isolated pig corneas were treated with SDS solutions at concentrations ranging from 3 to 30%. After incubation, resorufin fluorescence was read at 590 nm emission wavelengths with 570 nm excitation wavelengths. Results were expressed in fluorescence units (FU) and percentage of viability, calculated using the following equation: (FU treated/FU control) × 100. **p < 0.01; n = 12.
fibers, which are about 78% hydrated. Consequently, the major part of resazurin metabolism occurs in the surface epithelium.

The use of slaughter pig as eye donor satisfied animal protection and Creutzfeldt-Jakob safety preoccupations, because the pigs would have been killed anyway and thus far no prion contamination has been described in the pig. Moreover, the viability of cells in the corneal preparations was uniformly high across pig specimens; no statistical difference was found between the different cornea control groups. Use of slaughter pigs does not induce intraspecies variations.

The corneal viability study was carried out on a small number of surfactants, representative of the various classes of such substances and the different degrees of irritation described in the Draize eye test, whose mechanism of toxicity is well known (Furrer et al., 2000; Kennah et al., 1989a; Xu et al., 2000). The resazurin metabolism assay allowed ranking the different irritation potentials of the three surfactants tested, in the following order: BAC > SDS > TX-100. These results were correlated with in vivo irritancy measurements as determined by the Draize test. Furthermore, this assay provides results similar to those of other test systems with a variety of irritation endpoints which have characterized the cytotoxic order of surfactants as cationic > anionic > nonionic (Kemp et al., 1983; North-Root et al., 1982).

The same results were obtained with alcohol-treated corneas, for which the resazurin metabolism assay allowed rank-
ing the different irritation potentials of the six alcohols tested, in the following order: n-butanol > n-octanol > isopropanol > ethanol > glycerol, and propylene glycol. These data were highly correlated with the MMAS values and are in agreement with other studies (Gautheron et al., 1992; Pasternak and Miller, 1995).

Figure 6 shows the relation between MMAS and percentage of corneal viability of all tested irritants. The correlation between these two parameters was $r^2 = 0.701$, and the mathematical modeling was represented by the linear equation: $y = -0.6174x + 90.804$.

Resazurin metabolism only reflects the in vitro corneal injury while MMAS is a composite score obtained using in vivo corneal, conjunctival, and iris individual scores. Comparing results obtained with the resazurin metabolism assay with the corneal individual score calculated using the grading scale of Draize might be more relevant than comparison with total MMAS score.

However, as maximal corneal effects contribute 73% to the total MMAS score, in our study $r^2$ value calculated between the MMAS score and the corneal individual score was 0.982. Consequently, correlation between resazurin metabolism assay and corneal individual score ($r^2 = 0.709$) was comparable with correlation between resazurin metabolism assay and total MMAS score ($r^2 = 0.701$).

Unlike corneal individual score, the MMAS score is an OECD recognized measurement, and, as such, has been chosen as the in vivo reference for comparison in numerous in vitro studies. This is why we decided to compare corneal viability percentage with total MMAS score.

Our results are comparable with the correlation obtained by Gautheron et al. (1992) with the bovine cornea opacity and permeability test (BCOP), which, until the problems of possible prion contamination, was often part of the battery of tests used to assess eye irritation of chemicals. Nevertheless the resazurin metabolism methodology is simpler to use than the BCOP assay.

This suggests that the resazurin metabolism corneal viability test is a suitable model for evaluating the ocular irritation potential of mild, moderate, severe, and extreme irritant solutions, and establishing the feasibility of using this system as an alternative to the in vivo Draize test. Application of the test solutions to isolated corneas seems to be sufficient to predict the degree of ocular irritation elicited by the substances. The resazurin metabolism test can be used to evaluate corneal viability. This corneal viability measurement can be used as a potential alternative for the toxicological assessment of surfactants and alcohols. The nontoxic, nonradioactive resazurin metabolism assay allows rapid assessment of large numbers of samples, with simple equipment and at reduced cost for continuous monitoring of corneal viability. This assay seems to be suitable as a toxicologic screening test for eye irritation determination.

However, as with numerous other alternative tests, resazurin metabolism assay doesn’t reproduce physiological conditions, because epithelium, but also endothelium, are exposed to the test substance. Published cornea organoculture models (Collin et al., 1995; Foreman et al., 1996) can be adapted to the resazurin metabolism assay to approach physiological conditions. Using this kind of models brings numerous other advantages, such as testing solid or insoluble substances or evaluating chronic effects. Moreover, the resazurin metabolism assay may be easily completed with the measure of cornea thickness using a portable pachymeter.

However, a precise fine-tuning of the scoring system is needed in order to quantitatively assess the potency of the
ocular-irritant response elicited by the test products. This will only be possible after more extensive intralaboratory and interlaboratory evaluation of the experimental model, aimed to optimize its performance.

ACKNOWLEDGMENTS

This work was financed by grants from Aedebiopharm (Paris). Technical support by TECAN France was of great assistance. We thank Dr. Marcel Debray, Laboratoire de Mathématiques, Faculté de Pharmacie, Université Paris V for helpful statistical analysis.

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