**ORIGINAL ARTICLE**

**An in Vitro Evaluation Method to Test Ocular Irritation using a Human Corneal Epithelium Model**

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**Abstract**

Recently, safety evaluation tests that do not involve animal experiments have been prosperously developing. However, the optimal evaluation materials and methods for assessing ocular irritancy have not been well investigated. In this study, we determined the optimal evaluation method for testing ocular irritation using a human cultured corneal epithelium model (corneal model). In order to assess adequate treatment conditions for the corneal model, we used cetylpyridinium chloride (CPC), which has been recognized as an irritant chemical by the Draize eye test. The irritancy elicited by multiple concentrations of CPC was evaluated by a cytotoxicity assay under nine treatment conditions and compared to the Draize score. The treatment conditions that included a 5-second exposure period followed by a 24-hour post-incubation period (hereafter called protocol “5-sec+24-h”) showed a significant correlation between cytotoxicity and the Draize score. Furthermore, the dose-dependent cytotoxicity of six test chemicals was assessed by protocol “5-sec+24-h” and found to correlate with the Draize eye test results.

**Key words:** ocular irritation, corneal model, Draize eye test, cytotoxicity assay, dose-dependency

**Introduction**

A classical and reliable method to evaluate ocular irritation is an eye mucosal irritation assay (Draize eye test) devised by J. H. Draize (Draize et al., 1944). According to guideline 405 of the OECD (Organization for Economic Cooperation and Development), the Draize eye test should be performed by observing the cornea, conjunctiva, and iris for three days after applying 100 μL of liquid test chemicals with an eye dropper or 100 mg of powder test chemicals into one eye of a rabbit (OECD, 2002). The damage level of each eye section is used to assign a Draize score (110 total points). Since the development of the Draize eye test, five irritancy groups (none, mild, moderate, severe or very severe) were classified in accordance with the Draize score and used to assess the irritancy levels of various chemicals (Kay et al., 1962). However, these animal experiments often involve the suffering of laboratory animals and are against with 3R’s concept (Reduction, Refinement and Replacement). Therefore, an in vitro evaluation method for assessing ocular irritancy is greatly needed.

To date, a number of in vitro assays have been developed as alternative methods to the Draize eye test. Above all, it was shown that the bovine corneal opacity and permeability (BCOP) test and isolated chicken eye (ICE) test lead the correlative results with the Draize score when severe irritants or corrosive test chemicals were adopted by the NICEATM (The NTP Interagency Center for the
Evaluation of Alternative Toxicological Methods) and the ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM, 2006a, 2006b, Merrill et al., 2006)). Furthermore, the international validation studies were performed successfully and these two tests were accepted by the ESAC, scientific advisory committee of ECVAM (European Centre for the Validation of Alternative Methods) (ECVAM, 2007). However, these tests are not considered complete in vitro assays because animal-derived tissues are still used. Recently, a rabbit corneal epithelial cell line was reported as a useful alternative when evaluated by a cytotoxicity assay (Ohno et al., 1999). Although this assay is easy to perform and moderate in price, there are some disadvantages; insoluble and powder chemicals and stock solutions are often not testable because they must be dissolved in culture medium.

To correct these problems, we focused on a human cultured corneal epithelium model. In this study, we determined the optimal treatment conditions to assess cytotoxicity using a human cultured corneal epithelium model (corneal model) and demonstrated that dose-dependent ocular irritancy could be detected by our evaluation method.

Materials and Methods

1. Human cultured corneal epithelium model (the corneal model)

The corneal model was supplied as a kit by Japan Tissue Engineering Co., Ltd. Corneal epithelial cells were prepared from the normal human corneal epidermis which was provided by the Human & Animal Bridging Research Organization (Tokyo, Japan). The corneal epithelial cells were proliferated by co-culturing them with mouse 3T3-J2 cells as a feeder. The corneal model consisting of the proliferated corneal epithelial cells was cultivated on a cell culture inert (surface 0.3cm², BD Biosciences, CA, USA) in an optimized medium containing 5% fetal bovine serum, and kept at the air-liquid interface for 4 days. The corneal model was embedded in an agar gel containing nutrient solution and shipped at around 18°C. In order to eliminate the stress that follows shipment, the corneal models were incubated in sterile 24-well culture plates containing pre-warmed assay medium for 2 hours before starting the assay. During the experiments, the corneal models were maintained under the standard incubation conditions at 37°C with 5% CO₂ in humidified atmosphere. It is recommended that the corneal models be preserved at 4°C and used within 24 hours of arrival.

2. Test chemicals

The chemicals used in this study were previously evaluated by the Draize eye test (Ohno et al., 1999). The irritancy level of each test chemical was identified using at least two or more concentrations. The test chemicals were purchased from the following suppliers: cetylpyridinium chloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan: Wako), benzyl alcohol (Acros Organics, Morris Plains, NJ, USA), calcium thioglycolate (Wako), m-phenylenediamine (Wako), sodium salicylate (Wako), lactic acid (Wako), and ethanol (Wako). Distilled water was used as a solvent or negative control.

3. Reagents and antibodies

MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was purchased from Sigma-Aldrich, Milwaukee, USA, Dulbecco’s phosphate-buffered saline (D-PBS) from Gibco Laboratories, Grand Island, NY, USA and isopropanol from Wako. Antibodies used for immunohistochemical staining were purchased from the following suppliers: anti-human cytokeratin-3 mouse monoclonal antibody (BIOMOL Research Laboratories Inc., Plymouth Meeting, PA, USA) and anti-human mucin mouse monoclonal antibody (MUC-1, Novus Biologicals Inc., Littleton, Co., USA). These antibodies were diluted with D-PBS containing 1% bovine serum albumin (Takara Shuzo Co., Ltd., Kyoto, Japan) at 1:100 or 1:50, respectively.

4. Immunohistochemical staining

The corneal models were fixed with two different fixative solutions overnight at room temperature. The bottom and upper layers were treated simultaneously with 10% formalin neutral buffer solution (Mildform, Wako) and an original fixative solution of SUPER FIX (KURABO Industries, LTD., Osaka, Japan), respectively. After fixation, the corneal models were paraffin-embedded as previously described (Nishi et al., 2007; Yamamoto et al., 2008). The human corneal tissue was provided by an unbiased volunteer, and 3-micron paraffin sections of corneal tissue were prepared using a sliding microtome (REM-700, Yamato Kohki Industrial Co., Ltd., Saitama, Japan) and mounted on silanized glass slides (DAKO Japan Co., Ltd., Kyoto, Japan). After the dewaxing process, the sections were blocked with a non-specific staining
Fig. 1 Comparison between the corneal model and human corneal tissue
a) H&E staining. b, c) Immunohistochemical staining by human cytokeratin-3 or MUC-1 antibodies.
The scale bars represent 100 μm.
regent (DAKO Japan Co., Ltd.) and then probed with primary antibody for one hour at 37°C. After washing with D-PBS, immunofluorescence staining was performed using Alexa Fluor 488 conjugated anti-mouse IgG antibody (Invitrogen Corporation, Carlsbad, CA, USA). Each sample was counterstained with DAPI (VECTASHIELD, Vector Laboratories Inc., Burlingame, CA, USA), and images were acquired on an Olympus BX41 inverted microscope with a fluorescent attachment (Olympus Japan Co., Ltd., Tokyo, Japan) and Photonic Science CCD camera (DP50, Olympus Japan) controlled by DP controller software (Olympus Japan).

5. Treatment with test chemicals
Thirty μL of liquid or 10 mg of powder test chemicals were applied directly onto the corneal models. Liquid test chemicals were dropped by a micropipette and powder test chemicals were sprinkled with a microspatula onto the corneal models. At the end of each exposure period, the corneal models were washed gently with D-PBS and incubated in fresh assay medium during the post-incubation period.

6. Cytotoxicity assay
Cytotoxicity was quantified using an MTT assay. The corneal models were placed in a 24-well plate containing 500 μL of MTT working solution (0.5 mg/mL in assay medium) and incubated for 3 hours at 37°C protected from light. At the end of the incubation period, a 6-mm diameter punch out was obtained by biopsy trepan (Kai Industries Co., Ltd., Gifu, Japan) together with membrane filters. To extract the intracellular formazan crystals, samples were incubated with 300 μL of isopropanol overnight at 4°C.

Two hundred μL of each extraction solution was dispensed into 96-well microplates (MICROTEST Flat Bottom, BD Falcon, San Jose, CA, USA) for colorimetric analysis. The optical density was read at 570 nm using a spectrophotometer (SoftMax Pro, Molecular Devices, Sunnyvale, CA, USA), and cell viability was calculated.

Results
1. The corneal model expresses human corneal epithelium-specific molecular markers
It is well established that human corneal epithelium is constructed of four to six cell layers and expresses several specific molecular markers (Inatomi et al., 1995; Chen et al., 2004; Vascotto et al., 2006). Our histopathological findings suggested that the corneal model has a layered structure similar to human corneal epithelium (Fig. 1-a). The corneal model expresses cytokeratin-3, a specific molecular marker of human corneal epithelium (Fig. 1-b) and MUC-1, a mucosal glycoprotein secreted by human corneal epithelium cells that helps keep the cornea surface humid (Fig. 1-c). Intriguingly, the histological localization of cytokeratin-3 and MUC-1 in the corneal model was consistent with human corneal epithelium.

2. The optimal treatment condition is protocol “5-sec+24-h”
To determine the optimal treatment conditions for the corneal model, cetylpyridinium chloride (CPC) was used as a test chemical in the cytotoxicity assay. As shown in Table 1, the corneal damage elicited by multiple concentrations of CPC was previously scored using the Draize eye test (Ohno et al., 1999). A high score indicates severe irritancy, and the maximum score was defined as 80. Based on the Draize eye data, CPC is considered to induce dose-dependent corneal damage. In order to identify adequate treatment conditions for the corneal model, the dose-dependent cytotoxicity elicited by CPC was examined under nine different

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Chemical Number</th>
<th>Concentration</th>
<th>Draize Score (cornea)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cetylpyridinium chloride</td>
<td>1- 0.1</td>
<td>0.1%</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1- 1</td>
<td>1%</td>
<td>21.7</td>
</tr>
<tr>
<td></td>
<td>1- 10</td>
<td>10%</td>
<td>80</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>2- 1-10</td>
<td>1%</td>
<td>0</td>
</tr>
<tr>
<td>Calcium thioglycolate</td>
<td>3- 10</td>
<td>10%</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>3- 100</td>
<td>as is</td>
<td>31</td>
</tr>
<tr>
<td>Sodium salicylate</td>
<td>4- 10</td>
<td>10%</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4- 100</td>
<td>as is</td>
<td>66.7</td>
</tr>
<tr>
<td>m-Phenylene diamine</td>
<td>5- 10</td>
<td>10%</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5- 100</td>
<td>as is</td>
<td>1.7</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>6- 10</td>
<td>10%</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>6- 100</td>
<td>as is</td>
<td>66.7</td>
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<tr>
<td>Ethanol</td>
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<td>0</td>
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</table>
treatment conditions (Fig. 2). The exposure period was fixed at 5, 30 or 120 seconds, followed by a post-incubation period that was fixed at 1, 12 or 24 hours. The dose-dependent cytotoxicity was detectable under conditions where the post-incubation period was fixed at 12 or 24 hours. In particular, protocol “5-sec+12-h”, protocol “30-sec+12-h” and protocol “5-sec+24-h” gave a significant correlation between cytotoxicity and the Draize score. After accounting for the general-purpose of experimental procedures, it was concluded that protocol “5-sec+24-h” provided optimal treatment conditions for the corneal model.

3. Dose-dependent cytotoxicity is evaluable for six test chemicals by protocol “5-sec+24-h”

To clarify the utility of the above-mentioned test protocol, six additional test chemicals (Table 1) were examined for dose-dependent

![Figure 2](image1.png)

**Fig. 2** Cytotoxicity assay results with nine treatment conditions
a) Post-incubation for an hour. b) Post-incubation for 12 hours. c) Post-incubation for 24 hours.
The exposure period was fixed as follows: ♦=5-sec, ■=30-sec, ●=120-sec.
The results are presented as the means ± SD for three samples from two independent experiments per chemical. The bar graph indicates the Draize score.

![Figure 3](image2.png)

**Fig. 3** Evaluation of dose-dependent ocular cytotoxicity for three liquid test chemicals
Test chemicals were applied directly to the corneal models. The results are presented as the means ± SD for three samples from two independent experiments per chemical. The bar graph indicates the Draize score.
cytotoxicity. Dose-dependent cytotoxicity was detected for benzyl alcohol, lactic acid, and ethanol (Fig. 3-a, b and c). However, calcium thioglycolate, sodium salicylate, and m-phenylenediamine failed to show cytotoxicity when tested in bulk (Fig. 4-a, b and c). The difference between these successful and unsuccessful test chemicals was whether their bulk physical state was a liquid or powder. Therefore, a treatment procedure for powder test chemicals was reconsidered. In the last analysis, one of our trials revealed that a suitable treatment procedure for powder test chemicals was to cover the corneal models with filter paper after applying the powder test chemicals. During this analysis, it was recommended that a 6-mm diameter filter paper (2.5-mm thick) be incubated at 37°C in D-PBS for 3 hours before use. Just after applying the powder test chemicals, the filter paper was mounted on the surface of the corneal models for 5 seconds, a time equivalent to the exposure period for liquid test chemicals. At the end of the mounting period, the filter paper was removed with forceps after the addition of D-PBS. Using this process, the filter paper floated to the liquid level and the corneal models were protected from extrinsic damages such as friction or pressure induced by direct removal with forceps. As a result, no cytotoxicity was detected after filter paper application alone (data not shown). Following this revised procedure, the cytotoxicity of powder test chemicals could also be detected (Fig. 4-d, e and f).

Fig. 4 Evaluation of dose-dependent ocular cytotoxicity for three powder test chemicals
a, b, c) Test chemicals were applied directly to the corneal models. d, e, f) Filter paper was mounted on the corneal models after applying the powder test chemicals. The results are presented as the means ± SD for three samples from two independent experiments per chemical. The bar graph indicates the Draize score.
4. The cytotoxicity level is predictable using the corneal model
The relationship between cytotoxicity data, obtained in Figure 3 and 4, and the Draize score is shown in Figure 5. Each test chemical is displayed in order of the irritancy level as determined by the Draize eye test and the cytotoxicity data has been plotted individually. As a result, the cytotoxicity data and Draize score could be approximately correlated for all test chemicals. Therefore, our ocular irritation testing method using the corneal model predicts not only the existence or nonexistence of cytotoxicity potential but also the irritancy level.

Discussion
The current commercially available human corneal epithelium models include tests such as EpiOcular (MatTek Corporation, USA) and HCE (SkinEthic Laboratories, France). Both models have steric structures similar to human corneal epithelium using immortal human corneal cells. Previous reports have shown that the HCE model has an intermediate filament, desmosomal junction or hemidesmosomal junction and expresses cytokeratin-3, as confirmed by immunoblot analysis (Nguyen et al., 2003). In this study, we demonstrated that MUC-1 and cytokeratin-3 were expressed in the corneal model prepared from the normal human corneal epidermis and localized to sites that were consistent with human corneal epithelium. This finding suggests that the corneal model undergoes a similar differentiation as human corneal epithelium and is a potential alternative material for evaluating human ocular irritancy.

According to OECD guideline 405, it is recommended that the rabbit eyelid be closed for one second after applying the test chemicals and that damage to each eye section be observed for three days. In order to conduct our test under conditions similar to the Draize eye test, we established a particular test protocol that includes a short exposure period and a post-incubation period. To date, 20 test chemicals have been examined by ocular irritation testing using HCE and 80% of these tests were in concordance with the cytotoxicity assay results (Van Goethem et al., 2006). Thus, we adopted the cytotoxicity assay as a preliminary evaluating indicator.

As a result, it was demonstrated that the optimal treatment condition for the corneal model was protocol “5-sec+24-h” and that dose-dependent cytotoxicity could be detected for liquid test chemicals. However, cytotoxicity could not be detected for powder test chemicals. We attributed this failure to the histological differences between the corneal model and a rabbit eye. Because corneal models have no eyelid, we inferred that cytotoxicity was not observed with powder test chemicals because of insufficient humidity or external eyelid-like pressure. For this reason, filter paper was applied and the cytotoxicity of powder test chemicals became detectable.
In this study, we illustrated that the corneal model can assess a range of ocular irritancy levels by a cytotoxicity assay alone using the same test chemicals concentrations as the Draize eye test. To further confirm the utility of our test protocol, more test chemicals should be evaluated in order to obtain a range of in vitro data with the corneal model. In addition, evaluation indicators other than cytotoxicity should be identified in case a novel chemical with unique properties other than cytotoxicity is discovered.

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ICCVAM Background Review Document (2006a)

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