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Establishment of a new *in vitro* test method for evaluation of eye irritancy using a reconstructed human corneal epithelial model, LabCyte CORNEA-MODEL

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ABSTRACT

Finding *in vitro* eye irritation testing alternatives to animal testing such as the Draize eye test, which uses rabbits, is essential from the standpoint of animal welfare. It has been developed a reconstructed human corneal epithelial model, the LabCyte CORNEA-MODEL, which has a representative corneal epithelium-like structure. Protocol optimization (pre-validation study) was examined in order to establish a new alternative method for eye irritancy evaluation with this model. From the results of the optimization experiments, the application periods for chemicals were set at 1 min for liquid chemicals or 24 h for solid chemicals, and the post-exposure incubation periods were set at 24 h for liquids or zero for solids. If the viability was less than 50%, the chemical was judged to be an eye irritant. Sixty-one chemicals were applied in the optimized protocol using the LabCyte CORNEA-MODEL and these results were evaluated in correlation with *in vivo* results. The predictions of the optimized LabCyte CORNEA-MODEL eye irritation test methods were highly correlated with *in vivo* eye irritation (sensitivity 100%, specificity 80.0%, and accuracy 91.8%). These results suggest that the LabCyte CORNEA-MODEL eye irritation test could be useful as an alternative method to the Draize eye test.

1. Introduction

Chemicals, cosmetics, and pharmaceuticals must be assessed for their irritancy potential and risk to the human eye. For many years, the eye irritation potential of these substances has mainly been evaluated by the Draize eye test, which was developed in the 1940s (Draize et al., 1944). The Draize eye test is the only eye toxicity test officially accepted in the Organization for Economic Co-operation and Development (OECD) test guidelines 405 (OECD, 2002) for regulatory purposes in the classification of eye-irritating chemicals. This test is mainly based on the scoring of observed macroscopic changes in the rabbit cornea, conjunctiva, and iris. However, it is often criticized for both ethical (animal welfare) and scientific reasons (subjective scoring, low inter-laboratory reproducibility, or sensitivity differences with humans) (Christian and Diener, 1996). Therefore, there is a strong need for an *in vitro* evaluation method which can be used to assess eye-irritancy.

To date, a number of *in vitro* assay methods have been developed as alternative methods to the Draize eye test. Above all, the Bovine Corneal Opacity and Permeability (BCOP) test and the Isolated Chicken Eye (ICE) test were found to adequately predict severe irritancy although they were not recommended for the identification of materials not classified for irritation, they were adopted as OECD test guideline 437 and 438 (OECD, 2009a,b). Furthermore, their OECD test guidelines extending the applicability will be revised, which are accepted for the identification of non-irritant chemicals in the field of eye irritation (OECD, 2012a,b). Recently, a rabbit corneal epithelial cell line has been made available as a useful alternative when evaluated by a cytotoxicity assay (Hagino et al., 2008; Takahashi et al., 2008, 2009). Although this assay is easy to perform economically, there are some disadvantages, in that it is often not possible to test insoluble and solid chemicals because they must be dissolved in a culture medium.

To solve these problems, there have been increasing expectations for the development of an eye irritation test (EIT) method using a reconstructed human corneal epithelial (RhCE) model, produced with tissue engineering techniques. In light of this situation, two RhCE models, EpiOcular™ (MatTek, MA, USA) and SkinEthic HCE™ (SkinEthic, France) have already been made commercially available. EpiOcular™ is produced using normal human epidermal keratinocytes which have been cultured to form a stratified and squamous epithelium similar to a cornea (Jones et al., 2001; Stern et al., 1998). When cultivated under certain conditions using a serum free medium, keratinocytes are formed in a multi-layered tissue similar to the structure of a corneal epithelium. On the other hand, the SkinEthic HCE™ model is constituted of immortalized human

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corneal epithelial cell lines and therefore is structurally very similar to the corneal mucosa of the human eye (Van Goethem et al., 2006; Cotovio et al., 2010). It is assumed that the similar structure to the human corneal epithelium for both models has been reproduced, and a validation study on an EIT method using these tissue models is being conducted by European Union Reference Laboratory for alternatives to animal testing (EURL-ECVAM) (Freeman et al., 2010).

Recently, it has been developed a new commercially available RhCE model, the LabCyte CORNEA-MODEL (Japan Tissue Engineering Co., Ltd., Japan), which is produced by using normal human cornea epithelial cells. The LabCyte CORNEA-MODEL displays a high similarity to human corneal epithelium in morphology, histology and biomarker expression (Katoh et al., 2012).

The main objective of this study was to establish a new *in vitro* EIT method using the LabCyte CORNEA-MODEL (the LabCyte CORNEA-MODEL EIT). A test protocol based on a simplified cytotoxic approach was set up in the same manner as an already validated skin irritation testing method (OECD, 2010), so as to promptly and easily predict the eye-irritation potential of a test chemical. Therefore, to develop an optimized EIT protocol using the LabCyte CORNEA-MODEL, two important conditions were examined in order to designate exposure periods and post-exposure incubation periods in the pre-validation study. Furthermore, 61 chemicals were selected from the ECETOC database (ECETOC, 1998) and some previous reports (Van Goethem et al., 2006; Takahashi et al., 2008), and these chemicals were examined to assess the performance of the optimized LabCyte COLNEA-MODEL EIT as a new alternative *in vitro* test method.

2. Materials and Methods

2.1. Reconstructed human corneal epithelial model

The LabCyte CORNEA-MODEL is a new, commercially available reconstructed cultured human corneal epithelial model produced by Japan Tissue Engineering Co., Ltd. It consists of normal human corneal epithelial cells whose biological origin is normal human corneal tissue supplied by the Rocky Mountain Lions Eye Bank (CO, USA) after exchanging a material transfer agreement. In order for proliferation of the human corneal endothelial cells to occur while maintaining their phenotype, they were cultured with 3T3-J2 cells as the feeder layer (Rheinwald and Green, 1975; Green, 1978). Reconstruction of a cultured human corneal epithelial model is achieved by cultivating proliferating corneal epithelial cells on an inert filter substrate (surface 0.3 cm^2) at the air–liquid interface for 13 days, with an optimized medium containing 5% fetal bovine serum. This results in the construction of a multilayer structure consisting of a fully differentiated epithelium with features of the normal human corneal epithelial tissue (Katoh et al., 2012). The LabCyte CORNEA-MODEL is embedded in an agarose gel containing nutrient solution and shipped in 24-well plates at around 18 °C.

2.2. Quality control for the LabCyte CORNEA-MODEL

All batches of LabCyte CORNEA-MODEL produced are checked for their tissue viability, barrier function and morphology as quality control testing. This paper introduces the results of this check for tissue viability and barrier function.

2.2.1. Tissue viability

The LabCyte CORNEA-MODEL tissues were subjected to (3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay as described in the following. Tissues were put in the wells of 24-well plates containing 0.5 mL of MTT medium in each well (0.5 mg/mL; Dojindo Co., Kumamoto, Japan), and were incubated for 3 h (37 °C, 5% CO_2, humidified atmosphere). The formazan produced in the tissues was extracted with isopropanol (300 μL) and the extract (200 μL) was measured at 570 nm and at 650 nm as a reference absorbance, with isopropanol as a blank.

2.2.2. Barrier function: 50% inhibitory concentration (IC50) assay

To evaluate whether the LabCyte CORNEA-MODEL tissue resists the rapid penetration of the cytotoxic marker chemical sodium lauryl sulphate (SLS), the viability of the epithelial tissue was estimated in terms of the half maximal inhibitory concentration (IC50). Various concentrations (0.1, 0.2, 0.3, and 0.4% (w/v)) of SLS (50 μL) were applied to the LabCyte CORNEA-MODEL for 1 h, and then the cell viability was measured using an MTT assay. All experiments were performed in triplicate.

2.3. Test chemicals

The 61 reference test chemicals (25 No Category, 36 Category 1 or 2) were selected with reference to the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) database (ECETOC 1998) and previous reports (Van Goethem et al., 2006; Takahashi et al., 2008). As a reference for the *in vivo* eye irritancy classification of each chemical, we used the United Nations globally harmonized system (GHS) classification (United Nations, 2003). The "Category 1", "Category 2" and "No Category" described in the GHS classification mean "severe eye irritant (corrosive)", "irritant" and "materials not classified for irritation", respectively. The details of the 61 chemicals tested are shown in Table 1.

2.4. Optimization of the test protocol for the LabCyte CORNEA-MODEL EIT

The LabCyte CORNEA-MODEL tissues were shipped from the supplier on Mondays and delivered to recipients on Tuesdays. Upon receipt, the tissues were aseptically removed from the transport agarose medium, transferred into wells on 24-well plates (BD Biosciences, CA, USA) with the assay medium (0.5 mL) and incubated overnight (37 °C, 5%, CO_2, humidified atmosphere).

2.4.1. Optimization of suitable time periods for liquid chemicals combining sample exposures and post-exposure incubation

On the next day after receipt, the tissues were topically exposed to the liquid test chemicals. Liquids (50 μL) were applied with a micropipette. Viscous liquids were applied using a positive displacement-type tip with a micropipette, such as the MICROMAN® (Gilson Inc., France). Each test chemical was applied to three tissues. In addition, three tissues serving as negative controls were treated with 50 μL distilled water. In order to determine optimal exposure periods for the liquid chemicals, tissues were incubated for varying time periods (1, 3, 5, 10 and 20 min). After exposure, each tissue was carefully rinsed with PBS (Invitrogen, CA, USA) ten times or more, using a washing bottle to remove any remaining test chemical from the tissue surface. The blotted tissues were then transferred to new wells on 24-well plates containing 0.5 mL of fresh assay medium. In order to determine optimal post-exposure incubation periods, the tissues were post-exposure incubated for various time periods (0, 2 and 24 h) under the culture conditions of 37 °C, 5% CO_2, and with a humidified atmosphere. After the post-exposure incubation periods, blotted tissues were transferred to new wells on 24-well plates containing 0.3 mL of freshly prepared 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) in PBS solution (1:10 dilution of Cell Counting Kit-8; Dojindo Co., Japan) for a WST-8 assay (Ishiyama et al., 1997). WST-8 produces a highly water-soluble formazan dye upon reduction in the presence of an electron mediator. Tissues were incubated for 5 h at 37 °C under