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Evaluation of Eye Irritation Potential of Solid Substance with New 3D Reconstructed Human Cornea Model, MCTT HCE™

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Abstract

The eye irritation potential of drug candidates or pharmaceutical ingredients should be evaluated if there is a possibility of ocular exposure. Traditionally, the ocular irritation has been evaluated by the rabbit Draize test. However, rabbit eyes are more sensitive to irritants than human eyes, therefore substantial level of false positives are unavoidable. To resolve this species difference, several three-dimensional human corneal epithelial (HCE) models have been developed as alternative eye irritation test methods. Recently, we introduced a new HCE model, MCTT HCE™ which is reconstructed with non-transformed human corneal cells from limbal tissues. Here, we examined if MCTT HCE™ can be employed to evaluate eye irritation potential of solid substances. Through optimization of washing method and exposure time, treatment time was established as 10 min and washing procedure was set up as 4 times of washing with 10 mL of PBS and shaking in 30 mL of PBS in a beaker. With the established eye irritation test protocol, 11 solid substances (5 non-irritants, 6 irritants) were evaluated which demonstrated an excellent predictive capacity (100% accuracy, 100% specificity and 100% sensitivity). We also compared the performance of our test method with rabbit Draize test results and *in vitro* cytotoxicity test with 2D human corneal epithelial cell lines.

Key Words: Eye irritation, Reconstructed cornea model, MCTT HCE™ model, Protocol refinement

INTRODUCTION

The eye irritation potential of new drug candidates or pharmaceutical ingredients should be evaluated during the preclinical stage of development process if there is a possibility of ocular exposure. The representative example is benzalkonium chloride which is the most widely used preservative in the eye drop (Okahara and Kawazu, 2013). In order to evaluate eye irritation potential of ophthalmic agents and other substances with the possibility of direct ocular exposure, the rabbit Draize test (OECD, 2002) has been commonly employed. However, rabbit eyes are more sensitive to eye irritation than human eyes therefore, overestimation of eye irritation potential is unavoidable (Prinsen, 2006; Adriaens *et al*., 2014). In addition, consideration of animal welfare have been increased recently (Jang *et al*., 2014), demanding a new alternative test method to replace rabbit eye Draize test.

Many alternative approaches to rabbit eye Draize test have been developed such as bovine corneal opacity permeability (BCOP) test (Cater and Harbell, 2006) and hen's egg chorioallantonic membrane (HET-CAM) test (Spielmann *et al*., 1993). Validation study for BCOP test (OECD, 2013a), isolated chicken eye test (OECD, 2013b) and fluorescein leakage test (OECD, 2012) had been successfully completed by the European Center for the Validation of Alternative Methods (ECVAM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and these were registered in the 'Organization for Economic Cooperation and Development (OECD) Guidelines for the Testing of Chemicals'. However, these test methods still have many shortcomings such as use of non-human tissue, applicability limited to ocular corrosives and severe irritants, and unsatisfactory predictive capacity for human ocular irritation, reflecting a considerable demand for novel and more human-like alternative tests.

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Table 1. List of solid test substances

Test substance	Abbreviation	CAS No.	MMAS	GHS	Ref.
Phenothiazine	P	92-84-2	0	NI	(1) (2)
Aluminum hydroxide	AH	21645-51-2	12.7		(1) (2)
Potassium tetrafluoroborate	PT	14075-53-7	0		(1)
trans-cinnamic acid	t-CA	140-10-3	-		(2)
Glyceryl stearate (Glycerol triisostearate)	GS	123-94-4	2.0		(2)
Ammonium nitrate	AN	6484-52-2	18.3	Cat 2	(1)
Citric acid	CA	77-92-9	17.4		(2)
Chlorhexidine	CH	55-56-1	82.3	Cat I	(1)
Quinacrine	Q	69-05-6	82		(1) (2)
Promethazine hydrochloride	PH	58-33-3	71.7/84.0		(1)
Zinc gluconate	ZG	4468-02-4	-		(2)

(1) Kaluzhny *et al*., 2011, (2) Alépée *et al*., 2013.

Since eye irritants affect corneal epithelium directly, the disruption of structural integrity, and decreased viability of the cornea are important indices of eye irritation. Eye irritants can cause lysis of membranes or denaturation of proteins or interaction with macromolecules in the cornea (Kaluzhny *et al*., 2011). In this background, *in vitro* eye irritation tests were developed using human corneal epithelium cell line (Cho *et al*., 2012) or reconstructed human corneal epithelium (HCE). Especially, reconstructed HCE models well recapitulate the stratified and differentiated human cornea epithelium and can be applied to solid substances that are prevalent in ophthalmologic drugs (Liang *et al*., 2011) or medical devices (Postnikoff *et al*., 2014). Three HCE models have been reported up to now; EpiOcular™ (MatTek, Ashland, MA, USA) model prepared with normal human epidermal keratinocyte (Stern *et al*., 1998; Kaluzhny *et al*., 2011), SkinEthic™ Reconstituted Human Corneal Epithelium (SkinEthic, Lyon, France) model with immortalized human corneal epithelial cell line (Van Goethem *et al*., 2006) and LabCyte CORNEA-MODEL (Japan Tissue Engineering Co., Ltd, Gamagori, Aichi, Japan) with normal human corneal epithelial cells (Katoh *et al*., 2013).

Recently, we have reported a new HCE model, MCTT HCE™ (Modern Cell & Tissue Technologies, Seoul, Korea), which is reconstructed from primary-cultured human corneal epithelial cells (Jung *et al*., 2011). MCTT HCE™ model has human cornea-like structure with 3 distinct differentiated layers, i.e., basal cell layer, wing cell layer and superficial squamous cells layer. And the biomarkers for cornea such as CD44v6 and MUC1 are well-expressed. Eye irritation test with MCTT HCE™ model showed the accuracy of 88%, sensitivity of 100% and specificity of 77% for 25 liquid reference compounds but its utility in the evaluation of solid substances has not been demonstrated. In the present study, we established eye irritation test method with MCTT HCE™ model for solid substances and evaluated the predictive capacity of the developed method with 11 known references (Table 1).

MATERIALS AND METHODS

Tissues

MCTT™ HCE, Reconstituted human corneal epithelium tissue, was supplied by MCTT Co. (Seoul, Korea). Human primary limbal epithelial cells obtained from corneal transplantation were cultured on an insert (0.6 cm^2 polycarbonate Millicell® cell culture insert, Millipore, Darmstadt, Germany) for 14 days.

Media and reagents

Maintenance medium was supplied by MCTT Co. Sodium lauryl sulfate for positive control, quinacrine, chlorhexidine, promethazine hydrochloride, potassium tetrafluoroborate, phenothiazine, aluminum hydroxide, t-cinnamic acid, glyceryl stearate, ammonium nitrate, citric acid, zinc gluconate, pyridine and PBS for washing was obtained from Sigma (St. Louis, MO, USA). WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) for measuring cell viability was supplied by Roche (Basel, Switzerland).

Experimental protocol refinement

Overall experimental protocol: After MCTT™ HCE was incubated 22 ± 2 hr at 37°C in 5% CO_2 incubator, 40 mg of solid test article was topically applied on the HCE pre-wetted with 40 μL of PBS (Fig. 1C). Tissues were rinsed with PBS after treatment and post-incubated for 16 ± 1 hr at 37°C in 5% CO_2 incubator. And then HCE was incubated with WST-1 for 3 hr to measure cell viability. Absorbance of formazan was measured at 450 nm using the UV spectrophotometer. The mean of 2 wells for each test substance was calculated.

Determination of the treatment time: Test articles were treated for 10 or 30 min at 37°C in 5% CO_2 incubator. Other procedure was performed as above.

Determination of washing method: Test articles were treated for 10 min and rinsed with several conditions using PBS. For rinsing the tissues, 'Wash 1' method was followed by retention with PBS for 1 min and washing 2 times with 10 mL of PBS and shaking in 4 mL of PBS in the 12 well plate. 'Wash 2' method was followed by washing 4 times with 10 mL of PBS and shaking in 30 mL of PBS in the beaker. 'Wash 3' method was followed by rinsing 4 times with 10 mL of PBS and shaking in 30 mL of PBS in the beaker 2 times, after that soaking in 4 mL of media in the 12 well plate for 10 min at 37°C in 5% CO_2 incubator. Other procedure was performed as above.

Prediction model

OD of negative control was converted to 100% of cell vi-

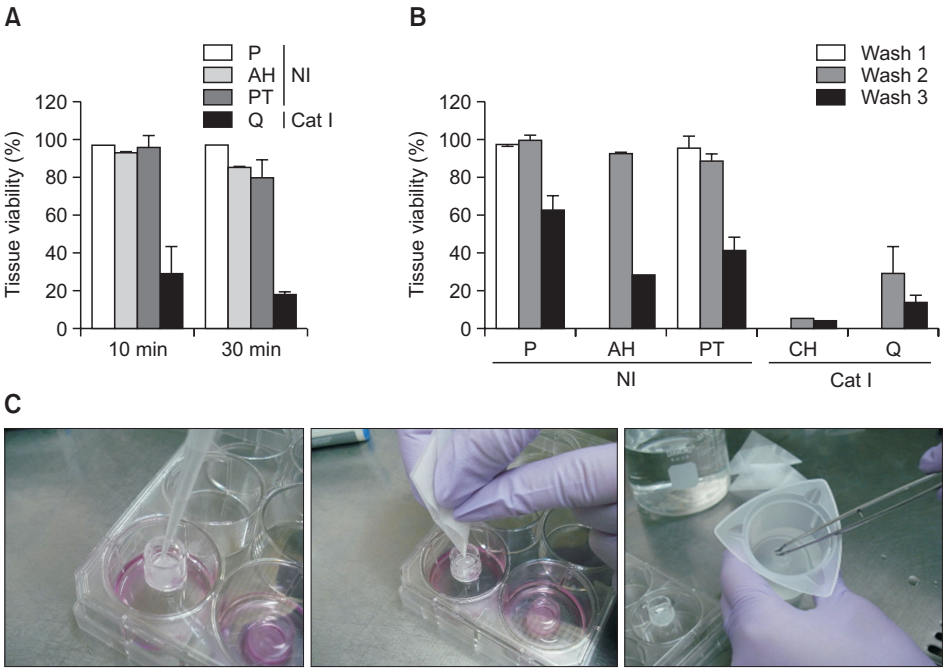

Fig. 1. Optimization of treatment time of test substance and washing method. (A) Effects of application time (B) and washing method on tissue viability (n=2-3). (C) Photographs of treatment of solid substance and washing procedure. Values are mean and error bars indicates absolute value ([Tissue1-Tissue2]/2) (n=2~3).

ability and OD of test material was changed into a percentage. If the cell viability was above than 50%, the test substance was judged to be a non-irritant, otherwise, it was determined an eye-irritant.

Cytotoxicity assessment of human corneal epithelial cells (HCE-T)

Cytotoxicity of some irritants were done according to Cho *et al*. (2012). Human corneal epithelial cells (HCE-T) which were transformed with SV-40 adenovirus vector (Araki-Sasaki *et al*., 1995) established by Dr. Kaoru Araki-Sasaki (Osaka University, Osaka, Japan), were kindly provided by Dr. Choun-Ki Joo (Catholic University of Korea, Seoul, Korea). The cells were cultured in DMEM/F-12 medium (Lonza, NJ, USA) containing 5% FBS (Gibco, California, USA), 5 μg/mL recombinant human insulin (Sigma-Aldrich), 10 ng/mL human EGF (Sigma-Aldrich), 100 ng/ml cholera toxin (Biomol, Allemagne, Germany), and 0.5% DMSO (Sigma-Aldrich) (Seomun and Joo, 2008). HCE-T cells were seeded in 96-well plates(2×10^4 cells/well), and grown for 48 hr in a humidified atmosphere of 5% CO2 at 37°C until the cells attained full confluence. The cells in the 96-well plates were exposed to 200 μL of test material solution for 1 h. After the exposure, the cells were washed 3 times with 200 μL of phosphate-buffered saline (PBS) (Lonza), and then, 100 μL of methylthiazolyldiphenyl tetrazolium bromide (MTT) (Sigma-Aldrich) solution (0.5 mg/mL in cell culture medium) was added. After 3-h incubation, the cells were washed 3 times with PBS. MTT formazan was extracted with 100 μL of 0.04 N HCl-isopropanol (Sigma-Aldrich) for 30 min, and the absorbance of the extract was measured at 570 nm with a spectrophotometer (Molecular Devices, Sunnyvale, USA).

Statistics

Values are mean and error bars indicates absolute difference between duplicate tissue ([Tissue1-Tissue2]/2) (n=2)

RESULTS

Optimization of treatment time of test substance

Firstly, experiments were performed according to the method developed for liquid test articles, i.e., exposure periods of 10 min and 4 times of washing and post-incubation time of 16 hr (Jung *et al*., 2011). In order to optimize treatment time for solid substance, test articles were treated for 10 or 30 min and post-incubated for 16 hr after washing. As a result, the exposure time appeared not to affect the assay results (Fig. 1A). The tissue viabilities following the treatment of aluminum hydroxide were determined to be 93.0 ± 1.0% and 85.3 ± 1.2% for the treatment times of 10 and 30 min, respectively. And the viabilities of potassium tetrafluoroborate were measured to be 90.4 ± 1.4% and 79.6 ± 10.0% for the treatment times of 10 and 30 min, respectively. Modified Maximum Average Score (MMAS) represents the average scoring of severity observed on cornea (0-80), iris (0-10) and conjunctiva (0-20) at 24, 48 and 72 hr after chemical treatment. MMAS of phenothiazine and potassium tetrafluoroborate is 0.0, and MMAS of aluminum hydroxide is 12.7 (Kaluzhny *et al*., 2011). c.f. No/mild irritation MMAS <25; moderate irritation 25≤MMAS<59; and strong irritant MMAS ≥ 59. Moreover, tissue viability of these 3 test articles was about 100% in the EpiOcular™ and SkinEthic HCE™ model. Our results fell within the range of non-irritant, however, we fixed treatment time to 10 min to be identical to the method used in liquid substances. Fig. 1C shows the treatment of solid substances (with PBS pre-loading) and washing procedure.

Modification of washing method

Some of solid chemicals, aluminum hydroxide, quinacrine and chlorhexidine, were difficult to wash off remaining powders from the treated tissue with the original washing method (wash 1). In order to wash powder cleaner for the correct prediction of eye irritant, we tried to modify washing protocol as shown as 'materials and method'. As quinacrine and chlorhexidine are severe eye irritants (Cat 1), these were not affected by washing methods (Fig. 1B). However, cell viability of phenothiazine-treated tissues diverged substantially to 99.8 ± 3.0% and 62.7 ± 8.1% following 'wash 2' and 'wash 3' protocol, respectively. Likewise, cell viability of aluminum hydroxide was 93.0 ± 1.0% and 28.4 ± 0.2% with 'wash 2' and 'wash 3', respectively. Although aluminum hydroxide was not removed completely, 'wash 3' method was thought to be too harsh for non-irritants, so 'wash 2' protocol was selected for washing solid substances.

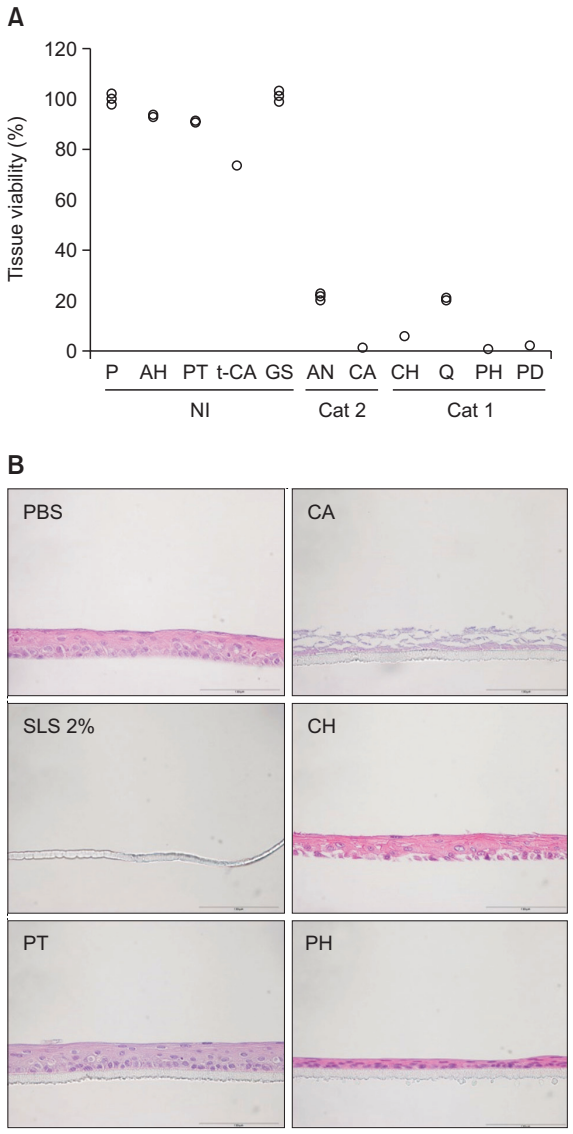

Fig. 2. Tissue viability obtained with the optimized eye irritation protocol. (A) Tissue viability results for 11 reference substances (n=3). (B) Histology of treated tissues (representative photograph). 2% SLS: 2% Sodium Lauryl Sulfate for positive control.

Predictive capacity of the eye irritation model

Additional 7 solid test materials were evaluated with this modified eye irritation test. When 50% of cell viability was set as the criteria for eye irritation potential evaluation according to that for liquid substances, all of the solid chemicals were predicted correctly (Fig. 2A, Table 2). Histological examination also supported the clear manifestation of irritation on the tissue (Fig. 2B). Moreover, repeated runs showed an excellent consistency between runs (Fig. 2A). This approach resulted in excellent sensitivity (100%) and specificity (100%). Consequently, an overall accuracy of 100% was obtained (Table 2). Pearson correlation analysis of tissue viability versus MMAS exhibited a high and significant correlation (Fig. 3A, correlation coefficient=-0.771, p=0.009) but the test appears to overestimate the toxicity of Cat 2 irritants compared to MMAS, which may reflect potential risk of high false negative rates (high sensitivity but low specificity). In addition, we also evaluated the cytotoxicity of several irritants that could be solubilized in culture medium (Fig. 3B). The results showed that cytotoxicity and tissue viability obtained in MCTT HCE™ for the solid substances were comparable. However, since test articles are applied as it is (i.e., at 100% concentration) in MCTT HCE™, it was difficult to discriminate Cat 2 from Cat 1. Overall protocol for eye irritation test is described in Fig. 4.

DISCUSSION

Many *in vitro* alternative test methods have been developed to replace conventional animal tests. However, for the most of *in vitro* cell-based assays, solid substances cannot be tested as it is but should be dissolved and diluted in appropriate vehicles for treatment. Poor solubility can pose a critical obstacle to the test conduct and the results can vary substantially depending on vehicles. In HCE models, solid test substances can be directly applied, circumventing these problems. However, to apply tissue engineered models to real *in vitro* test, the

Table 2. Predictive capacity of eye irritation test with MCTT HCE™ for 11 solid substances

Test substance	GHS	MCTT HCE
Phenothiazine	NI	NI
Aluminum hydroxide	NI	NI
Potassium tetrafluoroborate	NI	NI
t-cinnamic acid	NI	NI
Glyceryl stearate	NI	NI
Ammonium nitrate	Cat 2	I
Citric acid	Cat 2	I
Chlorhexidine	Cat I	I
Quinacrine	Cat I	I
Promethazine hydrochloride	Cat I	I
Zinc gluconate	Cat I	I
Sensitivity (%)		100 (6/6)
Specificity (%)		100 (5/5)
Accuracy (%)		100 (11/11)

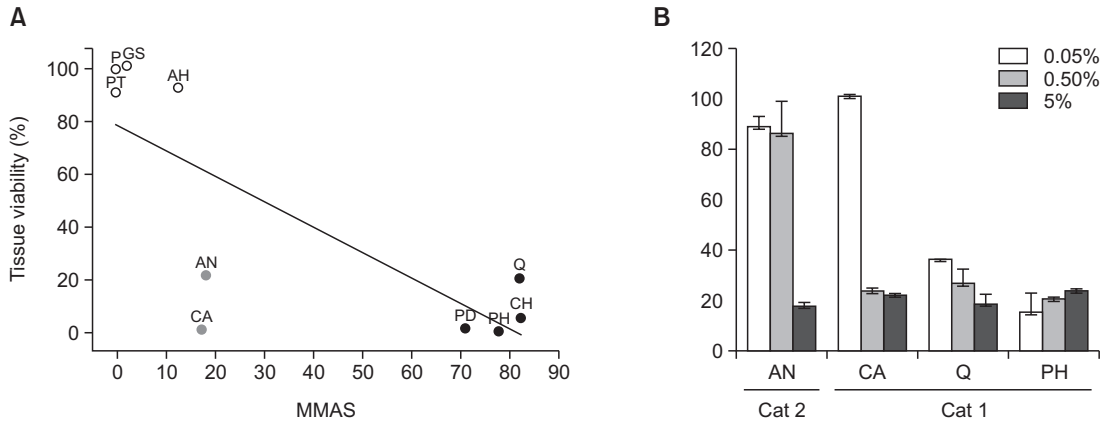

Fig. 3. Comparison with *in vivo* MMAS and cytotoxicity on 2D HCE-T cells. (A) Tissue viability versus MMAS (substances with MMAS data available are presented in Table 1). (B) Cytotoxicity of eye irritants on human corneal cell-line, HCE-T. Mean ± SD (n=3).

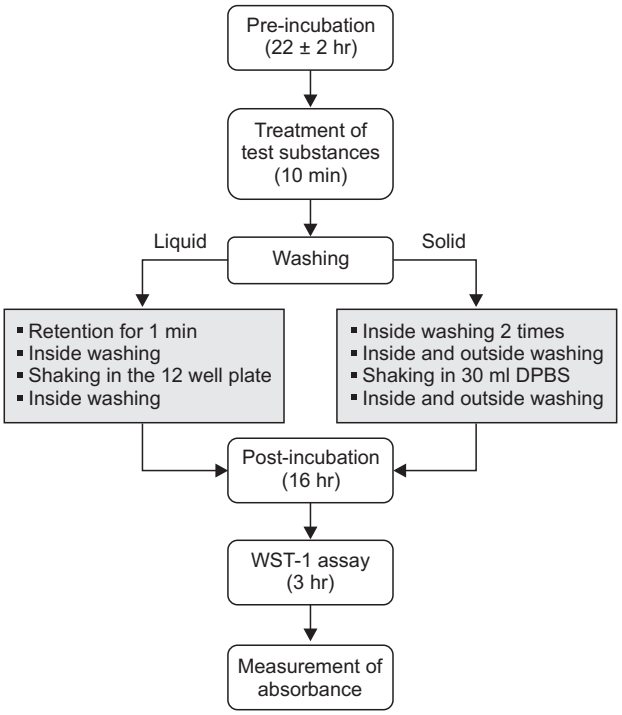

Fig. 4. Overview of the optimized eye irritation test method for MCTT HCE™ model.

development of optimized test method and refinement of test protocol is indispensable to attain appropriate performance and accuracy of the test results (Jung *et al*., 2014). Here we developed a new test method for the determination of eye irritation of solid substances with MCTT HCE™ model and with the developed method, we demonstrated that MCTT HCE™ model could predict the eye irritation of 11 solid chemicals quickly and correctly in comparison with other HCE models.

Solid test materials are supposed to be applied for 90 min and then post-incubated for 18 hr in the EpiOcular™ model (Kaluzhny *et al*., 2011; Pfannenbecker *et al*., 2013). And in the LabCyte CORNEA-MODEL, another recently developed HCE model, solid substances are treated for 24 hr without post-incubation (Katoh *et al*., 2013), reflecting that considerable time can be saved with MCTT HCE™ model. In addition, in MCTT HCE™ model, we could employ identical treatment time and post-incubation time for solid as used in liquid test substances through deploying separate washing schemes, which would be convenient and practical for the conduct of test. Most HCE models need separate treatment procedures for solid substances except for SkinEthic HCE™ where different treatment time is applied according to eye peptide reactivity assay (EPRA) results (Alepee *et al*., 2013). Generally, HCE models are provided in a 24 well format and 12 test substances can be tested in one kit of HCE model (duplicate). However, time setting for solid different from that for liquid substances can be problematic in terms of sample treatment layout and resultantly, models can be unnecessarily wasted. In this regard, eye irritation test using MCTT HCE™ model can be more economic than other HCE models.

BCOP and ICE tests can only identify Category 1 (severe) eye irritants which is a critical limitation for the application to the evaluation of drug substances and cosmetic ingredients. The eye irritation test with MCTT HCE™ could distinguish severe to moderate eye irritants (Category 1 & Category 2) from non-irritants, yet the substances of Category 2, moderate to weak eye irritants, showed somewhat low cell viability, suggesting a potential risk of high false positive rate. This was further corroborated by additional test with 2D monolayer cell system, where substance of Category 2 could be differentiated. More substances of Category 2 and NI should be evaluated to clearly assess the predictive capacity of this model for the discrimination of Category 2 irritants. In addition, incorporation of secondary markers like IL-8 (Debbasch *et al*., 2005), cornifelin expression (Choi *et al*., 2014) or occludin expression (Meloni *et al*., 2010) along with cell viability measurement may be helpful to enhance predictive capacity of this model for the determination of Category 2 eye irritants although further studies are necessary to confirm it.

Along with the accuracy of 88% for liquid chemicals with MCT HCE™ model (Jung *et al*., 2011), this test method showed high accuracy of 100% for solid substances, reflecting that this model may be versatile for the test of diverse substances. We are continuing to expand the reference data employing

various test substances to prove the predictive capacity and wide applicability of this model. Furthermore, intra- and inter-laboratory validation with 3 participating laboratories and the evaluation of accuracy are currently in progress.

In conclusion, we optimized *in vitro* eye irritation test with MCTT HCE™ model for solid substances through modifying the time of application and the methods of washing. Ten min was fixed for the application time of solid chemicals and suitable washing method (wash 2) was established for rinsing off solid substances on condition that test article was well-removed and cell viability was not decreased rapidly. Through this effort, we could demonstrate that this model could predict the eye irritation potential of 11 solid test substances with high accuracy (~100% accuracy), which suggests that MCTT HCE™ can be useful for the test of diverse drug candidates and cosmetic ingredients.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

REFERENCES

Adriaens, E., Barroso, J., Eskes, C., Hoffmann, S., McNamee, P., Alepee, N., Bessou-Touya, S., De Smedt, A., De Wever, B., Pfannenbecker, U., Tailhardat, M. and Zuang, V. (2014) Retrospective analysis of the Draize test for serious eye damage/eye irritation: importance of understanding the in vivo endpoints under UN GHS/EU CLP for the development and evaluation of in vitro test methods. *Arch. Toxicol.* **88**, 701-723.

Alepee, N., Bessou-Touya, S., Cotovio, J., de Smedt, A., de Wever, B., Faller, C., Jones, P., Le Varlet, B., Marrec-Fairley, M., Pfannenbecker, U., Tailhardat, M., van Goethem, F. and McNamee, P. (2013) Cosmetics Europe multi-laboratory pre-validation of the SkinEthic reconstituted human corneal epithelium test method for the prediction of eye irritation. *Toxicol. In Vitro* **27**, 1476-1488.

Araki-Sasaki, K., Ohashi, Y., Sasabe, T., Hayashi, K., Watanabe, H., Tano, Y. and Handa, H. (1995) An SV40-immortalized human corneal epithelial cell line and its characterization. *Invest. Ophthalmol. Vis. Sci.* **36**, 614-621.

Cater, K. C. and Harbell, J. W. (2006) Prediction of eye irritation potential of surfactant-based rinse-off personal care formulations by the bovine corneal opacity and permeability (BCOP) assay. *Cutan. Ocul. Toxicol.* **25**, 217-233.

Cho, S. A., An, S., Lee, E., Shin, K., Cho, J. C. and Lee, T. R. (2012) A new cell-based method for assessing the eye irritation potential of chemicals: an alternative to the Draize test. *Toxicol. Lett.* **212**, 198-204.

Choi, S., Lee, M., Lee, S. H., Jung, H. S., Kim, S. Y., Chung, T. Y., Choe, T. B., Chun, Y. J. and Lim, K. M. (2014) Identification of cornifelin and early growth response-1 gene as novel biomarkers for in vitro eye irritation using a 3D reconstructed human cornea model MCTT HCE. *Arch Toxicol.* [Epub ahead of print]

Debbasch, C., Ebenhahn, C., Dami, N., Pericoi, M., Van den Berghe, C., Cottin, M. and Nohynek, G. J. (2005) Eye irritation of low-irritant cosmetic formulations: correlation of in vitro results with clinical data and product composition. *Food Chem. Toxicol.* **43**, 155-165.

Jang, Y., Kim, J. E., Jeong, S. H. and Cho, M. H. (2014) Towards a strategic approaches in alternative tests for pesticide safety. *Toxicol. Res.* **30**, 159-168.

Jung, K. M., Lee, S. H., Jang, W. H., Jung, H. S., Heo, Y., Park, Y. H., Bae, S., Lim, K. M. and Seok, S. H. (2014) KeraSkin-VM: a novel reconstructed human epidermis model for skin irritation tests. *Toxicol. In Vitro* **28**, 742-750.

Jung, K. M., Lee, S. H., Ryu, Y. H., Jang, W. H., Jung, H. S., Han, J. H., Seok, S. H., Park, J. H., Son, Y., Park, Y. H. and Lim, K. M. (2011) A new 3D reconstituted human corneal epithelium model as an alternative method for the eye irritation test. *Toxicol. In Vitro* **25**, 403-410.

Kaluzhny, Y., Kandarova, H., Hayden, P., Kubilus, J., d'Argembeau-Thornton, L. and Klausner, M. (2011) Development of the EpiOcular(TM) eye irritation test for hazard identification and labelling of eye irritating chemicals in response to the requirements of the EU cosmetics directive and REACH legislation. *Altern. Lab. Anim.* **39**, 339-364.

Katoh, M., Hamajima, F., Ogasawara, T. and Hata, K. (2013) Establishment of a new in vitro test method for evaluation of eye irritancy using a reconstructed human corneal epithelial model, LabCyte CORNEA-MODEL. *Toxicol. In Vitro* **27**, 2184-2192.

Liang, H., Pauly, A., Riancho, L., Baudouin, C. and Brignole-Baudouin, F. (2011) Toxicological evaluation of preservative-containing and preservative-free topical prostaglandin analogues on a three-dimensional-reconstituted corneal epithelium system. *Br. J. Ophthalmol.* **95**, 869-875.

Meloni, M., Pauly, A., Servi, B. D., Varlet, B. L. and Baudouin, C. (2010) Occludin gene expression as an early in vitro sign for mild eye irritation assessment. *Toxicol. In Vitro* **24**, 276-285.

OECD (2002), Test No. 405: Acute Eye Irritation/Corrosion, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris. DOI: http://dx.doi.org/10.1787/9789264070646-en.

OECD (2012), Test No. 460: Fluorescein Leakage Test Method for Identifying Ocular Corrosives and Severe Irritants, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris. DOI: http://dx.doi.org/10.1787/9789264185401-en.

OECD (2013a), Test No. 437: Bovine Corneal Opacity and Permeability Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris. DOI: http://dx.doi.org/10.1787/9789264203846-en.

OECD (2013b), Test No. 438: Isolated Chicken Eye Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris. DOI: http://dx.doi.org/10.1787/9789264203860-en.

Okahara, A. and Kawazu, K. (2013) Local toxicity of benzalkonium chloride in ophthalmic solutions following repeated applications. *J. Toxicol. Sci.* **38**, 531-537.

Pfannenbecker, U., Bessou-Touya, S., Faller, C., Harbell, J., Jacob, T., Raabe, H., Tailhardat, M., Alepee, N., De Smedt, A., De Wever, B., Jones, P., Kaluzhny, Y., Le Varlet, B., McNamee, P., Marrec-Fairley, M. and Van Goethem, F. (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.

Postnikoff, C. K., Pintwala, R., Williams, S., Wright, A. M., Hileeto, D. and Gorbet, M. B. (2014) Development of a curved, stratified, in vitro model to assess ocular biocompatibility. *PLoS One* **9**, e96448.

Prinsen, M. K. (2006) The Draize Eye Test and in vitro alternatives; a left-handed marriage? *Toxicol. In Vitro* **20**, 78-81.

Seomun Y. and Joo C. K. (2008) Lumican induces human corneal epithelial cell migration and integrin expression via ERK 1/2 signaling. *Biochem Biophys Res Commun* **372**, 221-225.

Spielmann, H., Kalweit, S., Liebsch, M., Wirnsberger, T., Gerner, I., Bertram-Neis, E., Krauser, K., Kreiling, R., Miltenburger, H. G., Pape, W. and Steiling, W. (1993) Validation study of alternatives to the Draize eye irritation test in Germany: Cytotoxicity testing and HET-CAM test with 136 industrial chemicals. *Toxicol. In Vitro* **7**, 505-510.

Stern, M., Klausner, M., Alvarado, R., Renskers, K. and 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.

Van Goethem, F., Adriaens, E., Alepee, N., Straube, F., De Wever, B., Cappadoro, M., Catoire, S., Hansen, E., Wolf, A. and Vanparys, P. (2006) Prevalidation of a new in vitro reconstituted human cornea model to assess the eye irritating potential of chemicals. *Toxicol. In Vitro* **20**, 1-17.