—Regular Articles—

Application of the Reconstructed Rabbit Corneal Epithelium Model to Assess the *in Vitro* Eye Irritancy Test of Chemicals

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The rabbit corneal epithelium model (RCE model) was developed as a three-dimensional *in vitro* model to replace animal testing for the assessment of eye irritation. In the model, a stratified culture of rabbit corneal epithelial cells is grown at the air-liquid interface on collagen gel that acts as a parabasal membrane. Histological cross-sections show that the structure of the RCE model closely parallels that of the rabbit corneal epithelium. The eye irritation potency of test samples is estimated from the measurement of viability using the MTT assay in conjunction with the RCE model. A set of 30 chemicals belonging to different families with known *in vivo* Draize score was investigated with the *in vitro* eye irritation test using the RCE model in order to internally validate the protocol. Use of the RCE model at concentrations of 0.05%, 0.50%, and 1.00% and the calculation of the IC_{50} and percentage of viability allowed the irritants to be divided into four classes. The performance of the *in vitro* eye irritation test at a concentration of 0.50% using the RCE model was characterized by good sensitivity (92.3%), good specificity (100%), and good accuracy (93.3%) compared with the irritation classification predicted by *in vivo* Draize score at concentrations of 10% and 100%. These results indicate that the RCE model may provide a useful and sensitive *in vitro* eye irritation test as an alternative method to the Draize test.

Key words——rabbit corneal epithelium model; eye irritation; rabbit corneal epithelial cell; alternative method; MTT assay

INTRODUCTION

The eye constantly comes into contact with diverse substances including cosmetic products and their ingredients. Therefore the evaluation of the eye irritation potential of cosmetic products and ingredients is essential to assure their safety in the case of accidental exposure. The *in vivo* Draize eye test,[1] which has become the international standard assay for acute ocular toxicity (OECD TG 405, 2002) is often criticized for both ethical (painful to rabbits) and scientific reasons (subjective scoring, low inter-laboratory reproducibility, sensitivity differences with humans).[2] Eye irritation is a local, reversible response of normal living corneal and conjunctival cells to direct injury caused by contact with an irritant. Irritation can be associated with the depth of injury in the cornea, which is itself linked to the cytotoxicity of the agent.[3] The kinetics of penetration following topical contact are difficult to evaluate, but clearly chemicals that are able to rapidly injure cells should have a higher irritation potential to the cornea.

The EU stipulated the EU Cosmetics Directive 2003/15/EC (7th amendment to Directive 76/768/EEC) that calls for a ban on animal use for toxicity and allergenic reaction tests for the assessment of cosmetic products in EU countries by 2009 and a further ban on animal use in the safety evaluation of cosmetic products and ingredients by 2013 with the aim of banning the import and sale of cosmetics involving the use of animal-based assessment methods in member states in a stepwise manner.[4] Therefore successful implementation of the 3Rs (reduction in the number of animals used, refinement of techniques and procedures to reduce pain and distress, and replacement of animal techniques with non-animal techniques)[5] is imperative in toxicological safety evaluations of cosmetics. Since eye irritation caused by exogenous chemical irritants usually involves damage to the cornea, conjunctival epithelium, and endothelial cells, it

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can be measured based on endpoint observations of changes in cell activity, membrane integrity, cytosolic enzymes, and the extent of metabolic disorders. A number of new approaches have been submitted to the European Center for the Validation of Alternatives (ECVAM) and Interagency Coordinating Committee on the Validation of Alternative Methods (ICVAM). An extensive list of *in vitro* models that have been proposed as alternatives to the Draize test has been published.[6] Such alternative assays can be categorized as target organ/tissue assays [*e.g.*, the bovine corneal opacity and permeability (BCOP) test, isolated rabbit eye (IRE) test, chicken enucleated eye test (CEET)], organotypic models [*e.g.*, the hen's egg test-chorioallantoic membrane (HET-CAM), chorioallantoic membrane vascular assay (CAMVA), tissue equivalent assay], cytotoxicity assays (*e.g.*, the neutral red assays, red blood cell lysis assay, fluorescein leakage assay), and chemical reaction assays (*e.g.*, the irritation assay system). Although some of the many alternative assays developed have received limited attention, substantial effort has been invested in evaluating a significant number of them. Many validations and evaluation studies were conducted in the European Commission/British Home Office study,[7] a European Cosmetic, Toiletary, and Perfumery Association (COLIPA) study,[8] the Cosmetics, Toiletries and Fragrance Association (CTFA) study,[9] and Interagency Regulatory Alternatives Group (IRAG) study.[10] In Japan, many efforts have been made to find reliable, relevant predictive models such as chorioallantoic membrane (CAM) methods,[11] cell-based cytotoxicity methods,[12,13] and reconstituted tissue models.[14] To date, the Draize eye irritation test has not been fully replaced with *in vitro* methods, in part due to a lack of understanding of the underlying physiologic mechanisms of eye irritation.

We developed the rabbit corneal epithelial (RCE) model using cultured rabbit corneal epithelial cells and collagen gel as a scaffold to evaluate the *in vitro* eye irritation potential of chemicals including pharmaceuticals, cosmetics, and their raw ingredients. The collagen gel has good physical strength and elasticity properties and is therefore a useful scaffold for the RCE model. Generally, cytotoxicity tests using cultured cells have the advantage of being simple and quick with a low evaluation cost. However, when using cultured cells alone in media, the examination of water-insoluble materials is sometimes difficult as the test substances may precipitate from the media. In contrast to the conventional monolayer culture system suspended in media, the RCE model employs a dry surface. Therefore it is useful for both soluble and insoluble substances including various forms of cosmetic products. The aim of the development of the RCE model was to evaluate a new three-dimensional epithelial model cultivated from rabbit corneal cells to replace direct animal testing for the assessment of eye tolerance to overcome some of the limitations noted in the existing cytotoxicity methods, such as barrier function and permeation of the corneal layer.

In this study, we evaluated the RCE model, which is cultured using rabbit corneal epithelial cells and collagen gel as a scaffold, to evaluate the *in vitro* eye irritation potential of 30 reference chemicals that have a known degree of eye irritation. The *in vivo* and *in vitro* results were compared and analyzed to study the feasibility of the eye irritation assay using the RCE model to replace the animal-based eye irritation test and provide a scientific basis for the safety evaluation of chemicals using *in vitro* eye irritation methods.

MATERIALS AND METHODS

Test Materials Thirty chemical substances from various categories with available Draize results[15,16] were selected from the Japanese Standard of Cosmetic Ingredients or Japanese Pharmacopoeia for the eye irritation assay using the RCE model. The following chemicals were tested: 2 anionic surfactants [sodium lauryl sulfate (SLS), sodium polyoxyethylene (2) lauryl ether sulfate (SPLE)], 3 cationic surfactants [stearyl trimethylammonium chloride (STAC), benzalkonium chloride (BC), cetyltrimethylammonium bromide (CTAB)], 6 non-ionic surfactants [Triton X-100 (TX-100), polyoxyethylene lauryl ether (10 E.O.) (PLE), polyoxyethylene hydrogenated caster oil (60 E.O.) (PHCO), Tween 80 (TW80), Tween 20 (TW20), polyethylene glycol 400 (PG400)], 1 polyol [glycerin (GLY)], 1 ketone [acetone (AC)], 5 alcohols [benzyl alcohol (BA), isobutyl alcohol (IBA), ethanol (EtOH), isopropyl alcohol (IPA), myristyl alcohol (MA)], 1 ester [ethyl acetate (EA)], 2 amines [monoethanolamine (MEA), triethanolamine (TEA)], 3 salts [potassium laurate (PL), sodium salicylate (SS), saline (SA)], 3 acids [glycolic acid (GA), myristic acid (MA), lactic acid (LA)], 1

alkali [sodium hydrate (SH)], 1 sulfoxide [dimethyl sulfoxide (DMSO)], and 1 oil [mineral oil (MO)] (all purity guaranteed). The evaluation of the eye irritation intensity included all severity classes from non- or mildly irritant to strongly irritant. All of these chemicals were obtained from a supplier of cosmetic ingredients. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was obtained from Wako Pure Chemical Industries (Osaka, Japan).

Cells and Culture Normal rabbit corneal epithelial (NRCE) cells were provided by ArBlast Co. Ltd. (Kobe, Japan) and co-cultured along with inactivated 3T3 fibroblasts, as described previously.[17] The culture medium M-stars A and supplemented hormonal epithelial medium (SHEM) were obtained from ArBlast Co. Ltd. For three-dimensional cell culture, type-I collagen was prepared on ice according to the manufacturer's instructions (Nitta Gelatin Inc., Japan). The type-I collagen mixture was added to polycarbonate membrane culture inserts and allowed to gel at 37℃. After that, the culture inserts were placed in a 12-well plate containing the treated 3T3 fibroblasts, and the cultured rabbit corneal epithelial cells were inoculated on 12-well culture inserts. The culture was submerged into the previously described M-stars A medium for 5 days and then exposed to air by lowering the medium level (airlifting). After airlifting, the suitability of the RCE model was confirmed by examining the multilayer structure formation and the flatness of the most outer layer with light microscopy.

Light Microscopy The samples were fixed with 10% buffered neutral formaldehyde. Then, the samples were routinely processed with a cryostat and stained with hematoxylin and eosin.

Eye Irritation Tests Using the RCE Model A test sample (100 μl, diluted with PBS or mineral oil) was applied to the surface of the RCE model, and the tissue was cultured in SHEM medium at 37℃ under 5 % CO_2 for 30 min. After incubation, the eye irritation potency of the test samples was estimated by the measurement of cytotoxicity using the MTT assay.[18] Briefly, tissues were quickly blotted on absorbent paper and transferred to MTT 0.5 mg/ml in maintenance medium (1.2 ml/well of a 12-well plate) and incubated for 3 h. RCE models were removed from the MTT solution and again blotted on absorbent paper and transferred to isopropanol (1.5 ml/well and 0.5 ml added onto each tissue) for 2 h at room temperature with protection from evaporation and light (formazan extraction). After 15 min of gentle shaking, 200 μl aliquots were transferred to a flat-bottomed 96-well plate before measuring the optical density at 570 nm with isopropanol as the blank using a microtiter plate reader. The cell viability rate (percentage of control) is expressed as the percentage relative to the PBS or mineral oil non-irritating control.

$$\text{Cell viability rate (\% of control)} = [(\text{OD with irritant} - \text{OD blank}) / (\text{OD without irritant} - \text{OD blank})] \times 100$$

The eye irritation test using the RCE model allowed the calculation of the 50% inhibitory concentration (IC_{50}) value and viability at concentrations of 0.05 %, 0.50%, and 1.00%. From this information, the irritants could be divided into four classes: class A (non-/mildly irritant), class B (weakly irritant), class C (moderately irritant), and class D (strongly irritant) according to the classification shown in Table 1.

Statistical Analysis The inhibitory effect on the cytotoxicity was expressed as the mean±standard error (S.E.) of three independent experiments, and

Table 1. Classification Criteria of Eye Irritation Ratings in the *in Vitro* Eye Irritation Test and Draize Test

Classification		Draize test[a]		RCE test[b]			
				Cell viability (%)			IC_{50}[c]
		100% DS_{100}	10% DS_{10}	1%	0.50%	0.05%	
A	non-/mildly irritant	<15.0	<10.0	CV[e]≧90	CV≧90	CV≧95	IC≧50
B	weakly irritant	≧15.0	<15.0	30≦CV<90	60≦CV<90	85≦CV<95	2.0≦IC<50
C	mderately irritant	EC[d]	≧15.0	5.0≦CV<30	15≦CV<60	60≦CV<85	0.1≦IC<2.0
D	strongly irritant	EC	≧50.0	CV<5.0	CV<15	CV<60	IC<0.1

[a] The Draize score is calculated from the results at concentrations of 10% and 100% in the Draize test. [b] The RCE score is calculated from the results of IC_{50} values and viability at concentrations of 0.05%, 0.50%, and 1.00% in the eye irritation test using the RCE model. [c] 50% inhibition concentration (%). [d] Estimated classification (prediction class based on another concentration). [e] Cell viability (%).

subsequent inspection of the means was evaluated using Student's *t*-test between two groups at a significance level of $p<0.01$.

RESULTS

Preparation of the RCE Model NRCE cells have been used to develop a three-dimensional *in vitro* model of the rabbit corneal epithelium (RCE model). NRCE cells form a stratified culture when grown at the air-liquid interface on collagen gel as a scaffold in a culture insert with serum-free M-stars A medium. The RCE model was confirmed to be a multilayer of well-stratified corneal cells on collagen gel acting as a scaffold by examining a histological vertical section (Fig. 1).

Dose-Effect Relationship of the *in Vitro* Eye Irritation Test in the RCE Model To investigate the *in vitro* eye irritation potential of chemicals, we evaluated the viability of the RCE model with cosmetic integrants. The percentage of viability of the reference chemical SLS obtained at seven concentrations were compared using 6 parallel wells at each concentration. Test data indicated that the percentage of viability calculated from the RCE model decreased in response to an increase in SLS concentration (Fig. 2). As illustrated in Fig. 2, a dose-effect relationship was observed where lower viability was obtained at higher concentrations of SLS ($F=1055.362$ and $p<0.001$ from a single factor analysis of variance of the results).

Eye Irritation Test of Chemicals Using the RCE Model We evaluated the effectiveness of the RCE model to investigate the *in vitro* eye irritation potential of chemicals. Tests were conducted for 30 selected reference chemicals at some concentrations with 6 parallel wells at each concentration. According to the classification shown in Table 1, 30 selected reference chemicals were classified into four classifications based on the results of the Draize test:[15,16] class A, non-/mildly irritant [Draize score at 100% (DS_{100})$<$15.0 or Draize score at 10% (DS_{10})$<$10.0]; class B, weakly irritant ($DS_{100}\geq 15.0$ and $DS_{10}<15.0$); class C, moderately irritant ($15\leq DS_{10}<50.0$); and class D, strongly irritant ($DS_{10}\geq 50.0$) according to the reference[19] (Table 2).

All reference chemicals decreased the viability when tested in the RCE model in a dose-dependent manner (Figs. 3–6). The inhibitory effects of the viability decreased in the following order with the results of Draize test: class D (SH, STAC, BC, CTAB)$>$class C (TX-100, PL, PLE, GA, MEA, MA, BA, SLS, SPLS)$>$class B (LA, SS, AC, IBA, EtOH, IPA, MA, EA)$>$class A (PHCO, DMSO, TEA, TW-80, TW-20, PE400, MO, GLY, SA) in the RCE model. From the eye irritation test using the RCE model, the IC_{50} values and percentage of viability were calculated using information from three concentrations (0.05%, 0.50%, 1.00%). From this information, four classes of irritants were identified: class A (non-/mildly irritant), class B (weakly irritant), class C (moderately irritant), and class D (strongly irritant) (Table 2). The classes based on the viability at a concentration of 0.05% in the *in vitro* eye irritation test using the RCE model correctly identified 8 of the 9 substances in class A, 3 of the 8 substances in class B, 9 of the 9 substances in class C, and 4 of the 4 substances in class D from the Draize test. The classes based on the viability at a concentration of 0.5% from the *in vitro*

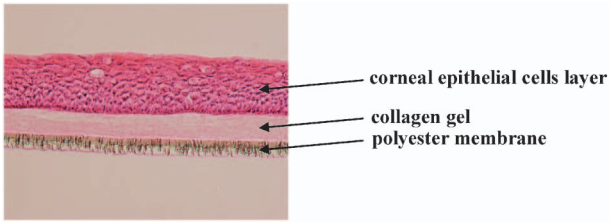

Fig. 1. Microscopic View of a Histological Vertical Section of the Rabbit Corneal Epithelium (RCE) Model (×200)
The vertical section was stained with hematoxylin/eosin.

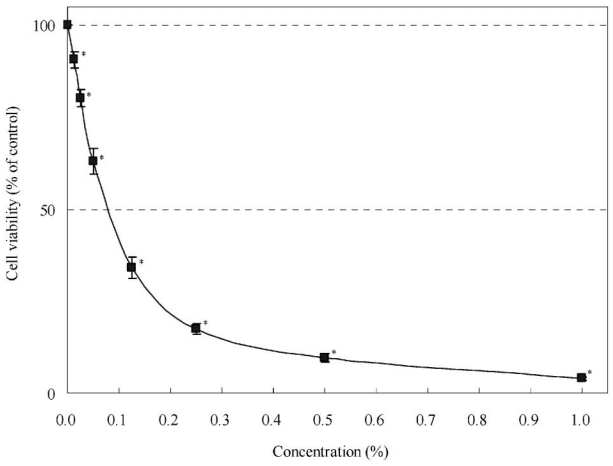

Fig. 2. Cell Cytotoxicity of Sodium Lauryl Sulfate (SLS) in the *in Vitro* Eye Irritation Test Using the RCE Model
Cell cytotoxicity was measured using the MTT assay. The cell viability represents the mean±S.D. of six experiments compared with the control group. *Significantly different from the control group at $p<0.001$.

Table 2. Results from the *in Vitro* Eye Irritation Test and Draize Test[a] for Reference Cosmetic Ingredients

Category	Sample		Draize score[a]			RCE score[b]							
						Cell Viability (%)			IC_{50} value (%)	Classification[d]			
			100%	10%	Classification[d]	1%	0.50%	0.05%		1%	0.50%	0.05%	IC_{50}
Anionic surfactant	Sodium lauryl sulfate	SLS	EC[b]	15.0	C	4.0±0.4	9.7±1.5	63.0±3.6	0.085	C	D	C	D
	Sodium polyoxyethylene laurylether sulfate (2 E.O.) (27%)	SPLS	ND[c]	10.0	C	10.0±2.3	20.3±1.3	67.6±2.0	0.135	C	C	C	C
Cationic surfactant	Stearyl trimethylammonium chloride	STAC	EC	91.3	D	2.6±2.7	5.2±3.1	53.3±1.5	0.075	D	D	D	D
	Benzalkonium chloride	BC	EC	78	D	4.0±2.0	7.3±2.5	56.6±1.6	0.078	D	D	D	D
	Cetyl trimethylammonium bromide	CTAB	EC	76.7	D	4.6±2.4	10.2±1.8	58.2±2.6	0.081	D	D	D	D
Non-ionic surfactant	Triton X-100	TX-100	EC	41.3	C	11.0±2.3	9.7±3.2	71.3±2.1	0.145	C	D	C	C
	Polyoxyethylene lauryl ether (10 E.O.)	PLE	EC	28.0	C	15.2±3.0	29.2±3.0	75.3±3.6	0.195	C	C	C	C
	Polyoxyethylene hydrogenated caster oil (60 E.O.)	PHCO	13.3	0.0	A	98.6±2.1	99.0±2.1	101.3±1.9	—	A	A	A	A
	Tween 80	TW80	4.7	0.0	A	94.1±1.9	94.5±1.9	95.3±2.2	—	A	A	A	A
	Tween 20	TW20	4.0	0.7	A	92.2±2.5	93.0±1.9	94.7±2.0	—	A	A	B	A
	Polyethylene glycol 400	PG400	4.0	0.0	A	98.0±3.2	98.6±2.7	96.3±2.0	—	A	A	A	A
Polyol	Glycerin	GLY	1.7	0.0	A	97.6±1.8	98.3±2.2	99.6±1.7	—	A	A	A	A
Ketone	Acetone	AC	65.8	3.0	B	71.3±3.1	80.3±2.2	94.3±2.0	3.277	B	B	B	B
Alcohol	Benzyl alcohol	BA	31.0	23.0	C	20.2±3.0	43.6±2.2	80.3±2.3	0.295	C	C	C	C
	Isobutylalcohol	IBA	60.0	1.0	B	73.2±1.9	82.6±2.4	95.7±1.3	3.669	B	B	A	B
	Ethanol	EtOH	32.7	0.0	B	76.2±2.2	85.2±2.9	97.0±1.6	4.560	B	B	A	B
	Isopropylalcohol	IPA	30.0	1.0	B	75.2±2.2	88.9±3.1	95.6±1.2	4.391	B	B	A	B
	Myristyl alcohol	MA	27.3	8.7	B	79.6±2.3	87.6±2.0	98.3±1.8	4.883	B	B	A	B
Ester	Ethyl acetate	EA	15.0	3.0	B	82.6±1.5	88.9±1.9	98.9±1.6	5.752	B	B	A	B
Amine	Monoethanol amine	MEA	EC	23.3	C	9.0±1.5	18.2±3.1	65.3±1.6	0.110	C	C	C	C
	Triethanol amine	TEA	8.0	0.0	A	99.1±1.9	101.7±1.8	99.6±2.6	—	A	A	A	A
Salt	Potassium laurate	PL	EC	38.0	C	7.4±2.0	15.6±2.6	62.3±2.1	0.100	C	C	C	C
	Sodium salicylate	SS	83.7	0.0	B	68.2±3.7	78.3±2.0	93.9±1.7	2.524	B	B	B	B
	Saline	SA	0.0	EC	A	103.2±2.2	98.3±2.0	100.6±2.0	—	A	A	A	A
Acid	Glycolic acid	GA	EC	25.0	C	12.2±2.3	26.6±2.0	72.6±2.2	0.155	C	C	C	C
	Myristic acid	MA	EC	23.0	C	9.4±1.9	19.3±2.5	68.2±2.3	0.122	C	C	C	C
	Lactic acid	LA	102.7	9.7	B	57.1±1.9	67.2±2.3	87.2±2.1	1.882	B	B	B	C
Alkali	Sodium hydrate	SH	EC	108	D	1.0±1.7	3.3±3.3	32.6±1.6	0.035	D	D	D	D
Sulfoxide	Dimethyl sulfoxide	DMSO	9.7	EC	A	98.8±2.2	99.6±2.6	100.7±1.8	—	A	A	A	A
Oil	Mineral oil	MO	3.3	EC	A	99.9±3.1	102.7±1.1	98.7±2.0	—	A	A	A	A

[a] Sources: Ohno Y., Kaneko T., Inoue T. *et al.*[15]; Van Goethem F., Adriaens E., Alépée N. *et al.*[16] [b] Estimated classification (prediction class based on another concentration). [c] 50% inhibition concentration (%). [d] No data available. [e] The present study classified the reference cosmetic ingredients into four severity ratings for eye irritation, *i.e.*, A, B, C, and D, which indicate non-/mildly irritant, weakly irritant, moderately irritant, and strongly irritant, respectively.

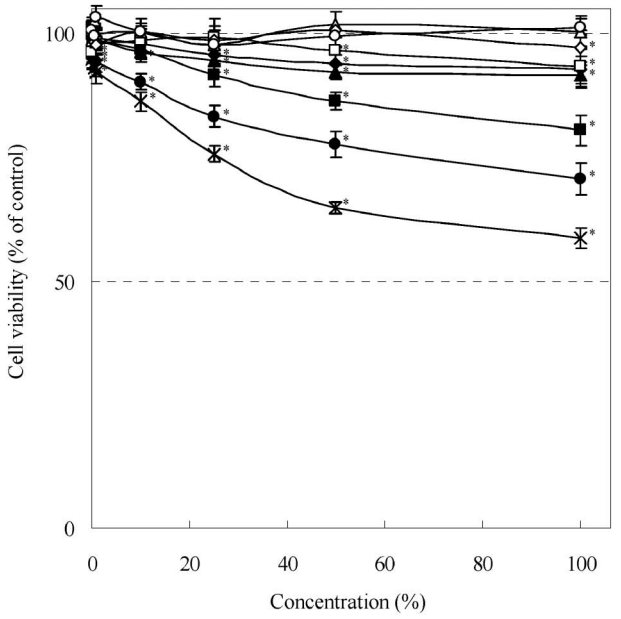

Fig. 3. Cell Cytotoxicity of Reference Cosmetic Ingredients in Class A (Non-/Mildly Irritant) from the *in Vitro* Eye Irritation Test Using the RCE Model

■, PHCO; ▲, DMSO; ◆, TEA; ●, TW80; ×, TW20; □, PG400; △, MO; ◇, GLY; ○, SA. Cell cytotoxicity was measured using the MTT assay. The cell viability represents the mean±S.D. of six experiments compared with the control group. *Significantly different from the control group at $p<0.01$.

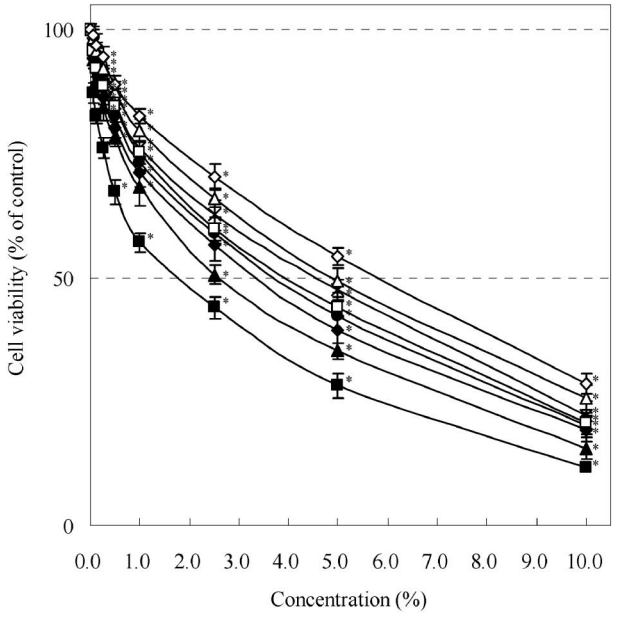

Fig. 4. Cell Cytotoxicity of Reference Cosmetic Ingredients in Class B (Weakly Irritant) from the *in Vitro* Eye Irritation Test Using the RCE Model

■, LA; ▲, SS; ◆, AC; ●, IBA; ×, EtOH; □, IPA; △, MA; ◇, EA. Cell cytotoxicity was measured using the MTT assay. The cell viability represents the mean±S.D. of six experiments compared with the control group. *Significantly different from the control group at $p<0.01$.

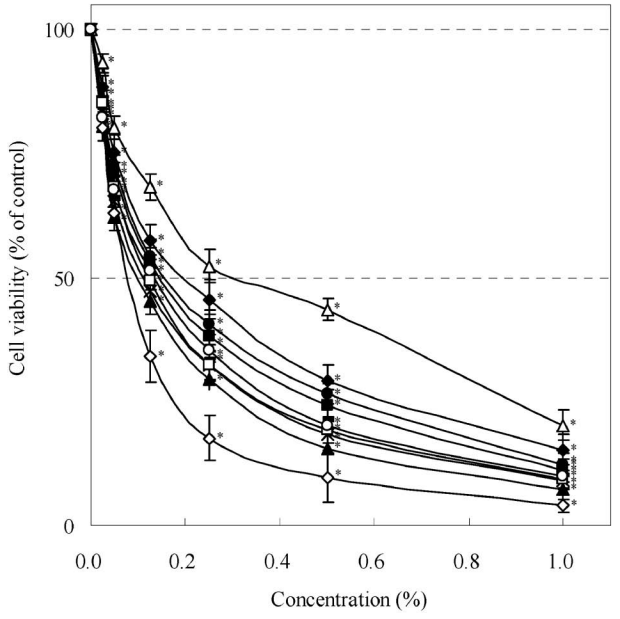

Fig. 5. Cell Cytotoxicity of Reference Cosmetic Ingredients in Class C (Moderately Irritant) from the *in Vitro* Eye Irritation Test Using the RCE Model

■, TX-100; ▲, PL; ◆, PLE; ●, GA; ×, MEA; □, MA; △, BA; ◇, SLS; ○, SPLS. Cell cytotoxicity was measured using the MTT assay. The cell viability represents the mean±S.D. of six experiments compared with the control group. *Significantly different from the control group at $p<0.01$.

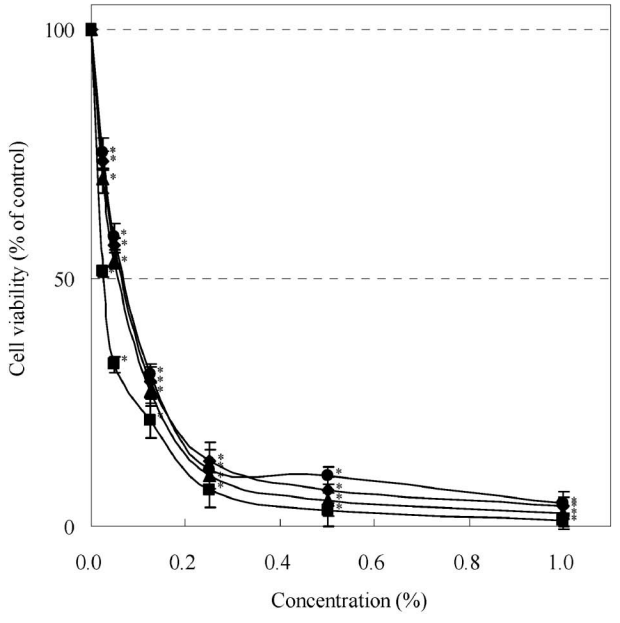

Fig. 6. Cell Cytotoxicity of Reference Cosmetic Ingredients in Class D (Strongly Irritant) from the *in Vitro* Eye Irritation Test Using the RCE Model

■, SH; ▲, STAC; ◆, BC; ●, CTAB. Cell cytotoxicity was measured using the MTT assay. The cell viability represents the mean±S.D. of six experiments compared with the control group. *Significantly different from the control group at $p<0.01$.

eye irritation test using the RCE model correctly identified 9 of the 9 substances in class A, 8 of the 8 substances in class B, 7 of the 9 substances in class C, and 4 of the 4 substances in class D from the Draize test. The class based on the viability at a concentration of 1.0% from the *in vitro* eye irritation test using the RCE model correctly identified 9 of the 9 substances in class A, 8 of the 8 substances in class B, 9 of the 9 substances in class C, and 4 of the 4 substances in class D from the Draize test. Thus the four results are similar to the classifications based on the Draize scores. The inconsistencies were mainly found with alcohols and acids. The performance of the *in vitro* eye irritation test using the RCE model according to the IC_{50} (50% inhibitory concentration) values and viability at concentrations of 0.05%, 0.50%, and 1.00% was characterized by good sensitivity (92.3%, 76.2%, 92.3%, 100%), good specificity (100%, 88.9%, 100%, 100%), and good accuracy (93.3%, 80.0%, 93.3%, 100%) compared with the irritation classification predicted by *in vivo* Draize score at concentrations of 10% and 100% (Table 3). These results indicate that the RCE model may prove to be a useful and sensitive *in vitro* eye irritation test as an alternative to the Draize test.

DISCUSSION

We developed the RCE model as a three-dimensional *in vitro* model using a stratified culture of rabbit corneal epithelial cells grown at the air-liquid interface on collagen gel as a parabasal membrane. This allows an artificial corneal epithelium (reconstituted rabbit corneal epithelium) to be prepared which exhibits barrier characteristics and paracellular permeability similar to those of a native rabbit cornea (Fig. 1). Eye irritation caused by exogenous chemical irritants usually involves damage to the cornea, conjunctival epithelium, and endothelial cells. As the decrease in the percentage of cell viability with increasing concentrations of SLS (a known irritant) indicates, the degree of eye irritation caused by chemicals can be simulated using the RCE model as an *in vitro* eye irritation test.

The present study tested and divided 30 chemicals into four classes of varying irritant severity, *i.e.*, non-/mildly irritant, weakly irritant, moderately irritant, and strongly irritant, and the results were compared with those from the Draize test. The *in vitro* eye irritation test results from the RCE model correctly identified 28, 24, 28, and 30, respectively, of the 30 chemicals using IC_{50} values and percentage of viability at concentrations of 0.05%, 0.50%, and 1.00%. Inconsistencies with the *in vivo* results were found for acids and alcohols, which could have been caused by a pH dilution of the acids during buffer preparation. Another reason might be changes in the potential toxicity of test substances through a reaction with chemicals in the buffer as a result of direct contact between the RCE model and the test substances.[20] In addition, as a result of the volatile nature of the alcohols, a cytotoxicity reading that is lower than expected may be obtained *in vitro*, while the anesthetic effect of alcohols was more likely to cause eye damage in whole-animal tests.[21]

The performance of the *in vitro* eye irritation test with the RCE model using results of the percentage of viability at a concentration of 1.00% were characterized by better sensitivity, specificity, and accuracy compared with the irritation classification predicted by the *in vivo* Draize score at concentrations of 10% and 100%. However, IC_{50} values and viability results from the *in vitro* test obtained at concentrations of 0.05% and 0.50% did not compare as well with the results from the Draize score. Taken together, these results indicate that the classification based on results from the percentage of viability at a concentration of 1.00% from the *in vitro* eye irritation test using the RCE model could be the most useful as part of an evaluation system for eye irritation.

In conclusion, the RCE model was prepared by cul-

Table 3. Comparison of the Classification Based on Results from the *in Vitro* Eye Irritation Test Using the RCE Model Compared with the Classification Based on the Draize Test and the Performance of the *in Vitro* Eye Irritation Test

			In vitro classfication			
			1%	0.50%	0.05%	IC_{50}
In vivo classfication	No Irritant	A	9/9	9/9	8/9	9/9
	Irritant	B	8/8	8/8	3/8	7/8
		C	9/9	7/9	9/9	8/9
		D	4/4	4/4	4/4	4/4
	Total		30/30	28/30	24/30	28/30
Sensitivity (%)			100	92.3	76.2	92.3
Specificity (%)			100	100	88.9	100
Accuracy (%)			100	93.3	80.0	93.3

Results were compared using the IC_{50} values and viability at concentrations of 0.05%, 0.50%, and 1.00% in the RCE model with the results from the Draize test at concentrations of 10% and 100%.

turing rabbit corneal epithelial cells on collagen gel in a cell culture insert. The eye irritancy effects of chemicals were determined by measurement of the cell viability using the RCE model as a guideline. The results of this test were comparable with those of the Draize test, and thus this modification of the RCE model may provide a useful, sensitive *in vitro* eye irritation test to replace the animal-based eye irritation test.

REFERENCES

1) Draize J. H., ''Appraisal of the Safety of Chemicals in Food, Drugs and Cosmetics, Vol. 46,'' ed. by the Association of Food and Drug Officials of the United States, Austin, TX, 1959.
2) Christain M. S., Diener R. M., *J. Am. Coll. Toxicol.*, **15**, 1–44 (1996).
3) Maurer J. K., Parker R. D., Jester J. V., *Regul. Toxicol. Pharm.*, **36**, 106–117 (2002).
4) Eskes C., Zuang V., *ATLA*, **33**, 19–20 (2005).
5) Michal B., Alan M., Goldberg J. H., *ATLA*, **23**, 838–866 (1995).
6) Bruner H. L., Shadduck J., Essex-Sorlie D., In: Dermal and Ocular Toxicology: Fundamentals and Methods (ed. by Hobson D. W,). Boca Raton, CRC Press, FL, 1991, 585–606.
7) Balls M., Botham P. A., Bruner L. H., Spielmann H., *Toxicol. in Vitro*, **9**, 871–929 (1995).
8) Brantom P. G., Bruner L. H., Chamberlain M., De Silva O., Dupuis J., Earl L. K., *Toxicol. in Vitro*, **11**, 141–179 (1999).
9) Gettings S. D., DiPasquale L. C., Bagley D. M., Casterton P. L., Chudkowski M., Curren R. D., *Food Chem. Toxicol.*, **32**, 943–976 (1994).
10) Bradlaw J., Gupta K., Green S., Hill R., Wilcox N., *Food Chem. Toxicol.*, **35**, 175–178 (1997).
11) Hagino S., Kinoshita S., Tani N., Nakamura T., Ono N., Konishi K., Iimura H., Kojima H., Ohno Y., *Toxicol. in Vitro*, **13**, 99–11 (1999).
12) Uchiyama T., Akiyama J., Miyai E., Sakamoto K., Takino Y., Ohnuma M., Ohkosi K., Okamoto Y., Morito Y., Kojima H., Okumura H., Sawamura J., Ikeda N., Sumida Y., Chiba K., Makino I., Kawakami K., Yamamoto R., Torishima H., Yanase H., Miyajima A., Sunouchi M., Hayashi M., Ohno Y., *Toxicol. in Vitro*, **13**, 163–173 (1999).
13) Tani N., Kinoshita S., Okamoto Y., Kotani M., Itagaki H., Murakami N., Sugiura S., Usami M., Kato K., Kojima H., Ohno T., Saijo K., Kato M., Hayashi M., Ohno Y., *Toxicol. in Vitro*, **13**, 175–187 (1999).
14) Ohuchi J., Kasai Y., Sakamoto K., Ohnuma M., Kitamura M., Kawasaki Y., Kakishima H., Suzuki K., Kuwahara H., Imanishi Y., Tatsumi H., Kotani M., Kinoshita S., Tani N., Kojima H., Nakamura T., Suzuki K., Ishibashi T., Hori H., Takahashi H., Nishikawa T., Kitano Y., Ohno Y., *Toxicol. in Vitro*, **13**, 153–162 (1999).
15) Ohno Y., Kaneko T., Inoue T., Morikawa Y., Yoshida T., Fujii A., Masuda M., Ohno T., Hayashi M., Momma J., Uchiyama T., Chiba K., Ikeda N., Imanishi Y., Itagaki H., Kakishima H., Kasai Y., Kojima H., Matsukawa K., Nakamura T., Ohkoshi K., Okumura H., Saijo K., Sakamoto K., Suzuki T., Takano K., Tatsumi H., Tani N., Usami M., Watanabe R., *Toxicol. in Vitro*, **13**, 73–98 (1999).
16) Van Goethem F., Adriaens E., Alépée N., Straube F., De Wever B., Cappadoro M., Catoire S., Hansen E., Wolf A., Vanparys P., *Toxicol. in Vitro*, **20**, 1–17 (2006).
17) Rheinwald J. G., Green H., *Cell*, **16**, 331–343 (1975).
18) Mossman T., *J. Immunol. Methods*, **65**, 55–63 (1983).
19) Kay J. H., Calandra J. C., *J. Soc. Cosmetic Chem.*, **13**, 281–289 (1962).
20) Okumura H., Arashima M., Ohuchi J., Kasai Y., Tsukumo K., Kakishima H., Kotani M., Kojima H., Kurishita A., Hayashi M., Miyajima A., Sunouchi M., Ohno Y., *Toxicol. in Vitro*, **13**, 199–208 (1999).
21) Chiba K., Makino I., Ohuchi J, Kasai Y., Kakishima H., Tsukumo K., Uchiyama T., Miyai E., Akiyama J., Okamoto Y., Kojima H., Okumura H., Tsurumi Y., Usami M., Katoh K., Sugiura S., Kurishita A., Sunouchi M., Miyajima A., Hayashi M., Ohno Y., *Toxicol. in Vitro*, **13**, 189–198 (1999).