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First Phase Inter-Laboratory Validation of the *in vitro* Eye Irritation Tests for Cosmetic Ingredients: (7) Evaluation of Cytotoxicity Tests on Primary Rabbit Corneal Epithelial Cells (CornePack)

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SUMMARY

The cytotoxicity test of neutral red (NR) uptake in primary rabbit corneal epithelial cells (CornePack) was evaluated as an alternative method to the Draize rabbit eye irritation test (Draize test). We tested 9 surfactants and isotonic sodium chloride solution (physiological saline) and controlled the test procedures under a common standard operating procedure (SOP) among laboratories. The concentrations of test chemicals that showed 50% reduction in NR uptake relative as compared to control (the median NR uptake concentration: NR_{50}) were determined, and compared with *in vivo* scores.

Six laboratories participated in this study. Each laboratory exist a few quitter and inter-laboratory coefficients of variation (CV) of this assay were above 0.500 on 2 test substances. The correlation and rank correlation coefficients between maximal average Draize total score (MAS) and NR_{50}s of surfactants were −0.619 and 0.846 respectively.

We concluded that this cytotoxicity test on CornePack assay is not reproducible and correlates poorly with Draize scores. However, we improved the protocol by omitting the pre-culture step and changing the NR assay. As a result, the uptake of NR of negative control samples became more stable and higher than the former data.

Because of its higher sensitivity than the other methods validated under the Ministry of Health and Welfare (MHW) project, we consider this assay may be evaluated with a wider range of chemicals used as cosmetic ingredients addition to improvement of methods.

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INTRODUCTION

CornePack is an assay kit developed by

Kurabo Industries Ltd. to evaluate the cytotoxicity of chemicals. Major components of the kit are normal epithelial cells derived from rabbit cornea, serum free culture medium, and reagents for neutral red (NR) uptake assay. This method has been reported to have high sensitivity and good correlationship with the results of the Draize rabbit eye irritation tests (Draize test).

In vitro alternatives to the Draize test described in the scientific literate were evaluated in order to identify appropriate methods for safety evaluation of cosmetic ingredients[1], for inclusion in a validation study of the selected methods by twenty laboratories under the Ministry of Health and Welfare (MHW) project entitled "Studies on the test methods to evaluated the safety of new ingredients of cosmetics". In this paper, we report the results of the first phase inter-laboratory evaluation of CornePack with nine surfactants used as cosmetic ingredients and isotonic sodium chloride solution (physiological saline) as a control.

MATERIALS AND METHODS

Test substances

The name of the 10 test substances used are listed in Table I. They are one cationic surfactant, 4 anionic surfactants, 4 nonionic surfactants and physiological saline[2]. They are from the Japanese standards of cosmetic ingredients[3,4] and supplied from the Japan Cosmetic Industry Association (JCIA) to National Institute of Health Science (NIHS). The coded substances were distributed from NIHS to each laboratory to enable us to get objective information about the methods and intra-/inter-laboratory variation. The test substances, except sodium hydrogenated tallow-L-glutamate (HT-glutamate), were prepared in phosphate-buffered saline (PBS) and filtered before use. HT-glutamate was insoluble in PBS or ethanol used for preparing test samples. The final concentration PBS and ethanol in assay medium did not exceed 10% and 0.1%, respectively.

Procedures of CornePack[5]

Six laboratories participated in this study. CornePack contains normal rabbit corneal epithelial cells (NRCE), serum free medium, neutral red (NR; 5 mg/ml) solution, fixing solution and extraction solution, and was distributed by Kurabo Industries Ltd. to each laboratory. All laboratories used same lot of the cells, culture medium, solutions, reagents and culture plates. One hundred μl of 2.5×10^4 cells/ml suspension were added to each well of 96 well-plates (Nunclon^R: Nunc) and cells were incubated for 3 days at 37°C in

Table I. Test substances

Samle number	Name	Abbreviation	Classification
S-1	Isotonic Sodium Chloride Solution	Physiological saline	-
S-2	Polyoxyethylene Hydrogenated Caster Oil(60 E.O.)	POE hydrogenated caster oil	Nonionic
S-3	Polyoxyethylene Sorbitan Monolaurate (20 E.O.)	Tween 20	Nonionic
S-4	Polyethyleneglycol Monolaurate (10 E.O.)	PEG monolaurate	Nonionic
S-5	Sodium N-Lauroyl Sarcosinate (30% solution)	Lauroyl sarcosinate	Anionic
S-6	Sodium Hydrogenated Tallow - L- glutamate	HT- glutamate	Anionic
S-7	Sodium Lauryl Sulfate	SLS	Anionic
S-8	Sodium Polyoxyethylene Laurylether Sulfate (2 E.O. : 27% solution)	POE laurylether sulfate	Anionic
S-9	Polyoxyethylene Octylphenylether (10 E.O.)	Triton X-100	Nonionic
S-10	Benzalkonium Chloride	Benzalkonium chloride	Cationic

a 5%CO_2 incubator. For each test chemical, solutions of 8 different concentrations including a non-treatment level were prepared. One hundred μl of the test solutions of different concentrations were added into four wells per concentration. After 48 h, 100 μl of NR-medium with 0.15 mg NR/ml (final conc. 50 μg/ml) was added to each well and the cells were incubated for 3 h, during which NR was taken up into viable cells. The cells were washed rapidly after the removal of the dye medium, and fixed with 1% formalin-1% $CaCl_2$. Two hundred μl of a mixture of 1% acetic acid-50% ethanol were added to each well after the removal of formalin. After 20 min., the absorbance was measured with a microplate reader at 540 nm. The ratio of fractional release in wells to the one in the non-treatment wells was calculated for each concentration and the median NR uptake concentration, i.e., NR_{50}, was calculated from average concentration-response curves from two or three experiments.

RESULTS

Two NR_{50} values reported from each laboratory and their averages, SD and coefficient of variations (CV) for the 10 test substances are shown in Table II. These two values were selected by each laboratory, in which definitive experiments were conducted two or three times (Final test from 1 to 3) until satisfactory uptake of NR (more than 0.5 at OD_{540}) in negative control wells was established (Table III). Fig. 1 also shows median and quartile of NR_{50}s on test substances except saline, for which an NR_{50} was not obtained. Among the result obtained, a few outliers were detected with polyoxyethylene hydrogenated caster oil (60 E.O.: POE hydrogenated caster oil), polyoxyethylene sorbitan monolaurate (20 E.O.: Tween 20) and polyoxyethylene octylphenylether (10E.O.: Triton X-100). CVs were above 0.500 for results of Tween 20 and HT-glutamate. Though these outliers were not ommited from comparison of *in vivo* results, NR_{50}s of this test were considered to vary widely as far as we tested substances.

Table IV shows Draize scores for 10% solutions of the 10 test substances[1]. The maximal average Draize total scores (MAS), scores at 24 h after treatment and area under

Table II. The median effective concentration of CornePack assay.

Labo.	A		B		C		D		E		F		(μg/ml)		
Experiment number	1	2	1	2	1	2	1	2	1	2	1	2	Ave.	SD	CV
S-1*	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000	not tested	>400	>400	>10,000	>10,000	>1,000		
S-2	170.0	170.0	152.0	104.0	190.0	170.0	196.5	338.0	123.1	217.3	155.0	160.0	179.0	58.6	0.328
S-3	130.0	120.0	133.0	86.0	142.0	137.0	214.0	395.0	79.5	96.5	170.0	150.0	154.0	84.2	0.545
S-4	67.0	58.0	28.0	42.0	82.0	54.0	73.0	34.3	17.6	34.8	52.0	73.0	51.3	20.2	0.394
S-5	27.0	23.0	31.5	26.0	29.0	31.2	25.0	40.0	27.9	27.7	22.9	23.2	27.9	4.80	0.172
S-6	0.98	0.89	0.96	0.80	0.92	0.77	0.97	3.45	3.8	0.62	0.94	0.56	1.31	1.09	0.832
S-7	1.10	0.68	0.92	0.61	0.84	0.80	1.05	0.70	0.54	1.09	0.79	0.85	0.83	0.18	0.217
S-8	3.60	4.40	4.70	2.80	3.95	4.05	4.48	5.00	4.54	4.94	3.10	3.60	4.10	0.71	0.173
S-9	17.0	15.0	11.6	10.1	18.5	17.3	18.5	0.90	17.6	10.6	11.2	14.2	13.5	5.80	0.430
S-10	1.60	1.20	1.10	0.62	0.68	0.81	1.80	0.77	0.64	0.30	1.45	1.58	1.05	0.48	0.457

* Sample names show in Table I.

Table III. Average of absorbance obtained from negative control of CornePack assay.

Labo.	OD540*					
	Dose finding test	Test 1	Test 2	Test 3	New protcol Test 1	Test 2
A	0.202±0.029	0.321±0.034	0.223±0.026	0.736±0.027	NT	NT
B	0.063±0.010	0.725±0.050	0.684±0.031	NT	0.873±0.075	1.006±0.062
C	0.326±0.222	0.366±0.028	0.745±0.026	0.520±0.036	NT	NT
D	0.947±0.018	0.938±0.034	0.464±0.099	0.784±0.030	NT	NT
E	0.320±0.027	0.534±0.046	0.566±0.057	NT	0.897±0.066	0.625±0.070
F	0.655±0.026	0.589±0.079	0.656±0.062	NT	0.865±0.062	0.938±0.061

* Average values of above 8 plates
NT: Not tested

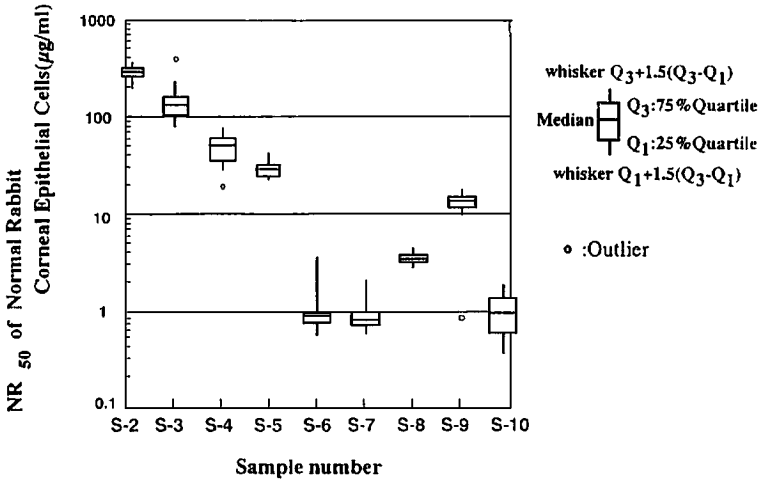


Figure 1. Summary statistics of CornePack assay. Sample names show in Table I.

Table IV. Results of the Draize rabbit eye irritation test on 10% solution of 10 test substances.

Sample number	Maximum average score				24 hrs score				Area under the curve*:%			
	Total	Cornea	Iris	Conjuctiva	Total	Cornea	Iris	Conjuctiva	Total	Cornea	Iris	Conjuctiva
S-1**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
S-2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
S-3	0.7 (1)***	0.0	0.0	0.7 (1)	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.1
S-4	3.3 (1)	0.0	0.0	3.3 (1)	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.2
S-5	10.3 (48)	8.3 (48)	0.0	8.0 (1,4)	8.3	5.0	0.0	3.3	3.4	1.9	0.0	1.5
S-6	26.7 (24)	16.7 (24-72)	1.7 (72-168)	12.0 (4)	26.7	16.7	0.0	10.0	14.9	10.7	0.8	3.5
S-7	15.0 (4)	8.3 (48,72)	0.0	10.0 (4)	14.7	6.7	0.0	8.0	7.1	4.2	0.0	3.0
S-8	10.0 (4)	3.3 (48)	0.0	10.0 (4)	2.7	0.0	0.0	2.7	2.0	0.7	0.0	1.4
S-9	41.3 (72)	30.0 (72)	5.0 (168)	10.0 (4,48)	24.7	15.0	1.7	8.0	26.9	18.4	2.3	6.3
S-10	78.0 (24)	66.7 (24)	5.0 (96-168)	14.7 (96)	78.0	66.7	0.0	11.3	57.3	43.9	2.5	10.9

* The area ratio under the curve means the ratio (%) of the area under the line connecting scores at each observation period to those based on theoretical maximum of Draize total score until 7 days after treatment.

** Sample names show in Table I.

*** Thses values in parenthesis are the time (hour) when the scores became maximum.

the curve (AUC) as shown in this Table were used to compare with the results of CornePack. The correlation coefficients and Spearman rank correlation coefficients between Draize scores and logarithmic transformed NR_{50} values results are given in Table V, and Fig. 2 shows correlation diagrams between MAS and logarithmic transformed NR_{50} values. The correlation coefficients were not high except for conjunctiva. The rank correlation coefficients, except iris, were above 0.649, e.g., for MAS was 0.846. The predictability of CornePack for irritation potential (MAS) was assessed by linear regression for nine test chemicals (Fig. 3). When the cut-off point between negative and positive irritants was set at MAS 15, the corresponding NR_{50} was calculated to be 14.8 μg/ml from the regression line. As shown in Fig. 3, there were no false negative substances but sodium polyoxyethylene laurylether sulfate (2E.O.; 27% solution: POE laurylether sulfate) was indicated to be false positive.

We compared these results with those from other cultured cell test including crystal violet staining in Statens Seruminstitut Rabbit Cornea (SIRC) cells, NR uptake assay in SIRC cells[6], MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide) assay in HeLa cells and crystal violet staining assay in CHL/

Table V. Correlation and Spearman rank correlation matrix between CornePack assay and Draize rabbit eye irritation scores.

		Correlation* coefficients	Rank correlation coefficients
Maximal average Draize total scores (MAS)	Total	− 0.619	0.846
	Cornea	− 0.554	0.730
	Iris	− 0.393	0.215
	Conjuctiva	− 0.919	0.894
Scores of 24 h after	Total	− 0.635	0.788
	Cornea	− 0.559	0.649
	Iris	0.035	0.333
	Conjuctiva	− 0.892	0.846
Area under the curve(AUC):%**	Total	− 0.576	0.818
	Cornea	− 0.538	0.764
	Iris	− 0.404	0.236
	Conjuctiva	− 0.644	0.818

* Logarithmic transformed NR_{50} values used.
**AUC indicates the area under the line connecting scores plotted at each observation period. The parameter used in this study was ratio of AUC of test substances to those based on theoretical maximum of the Draize score, cornea, iris and conjuctiva score, respectively.

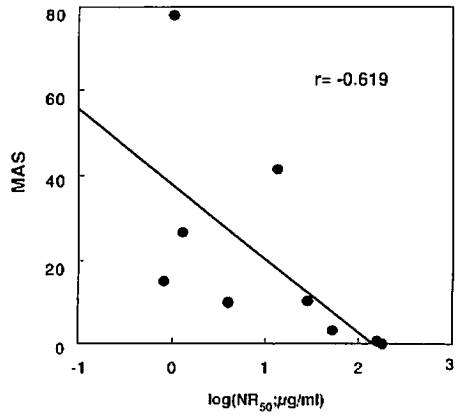
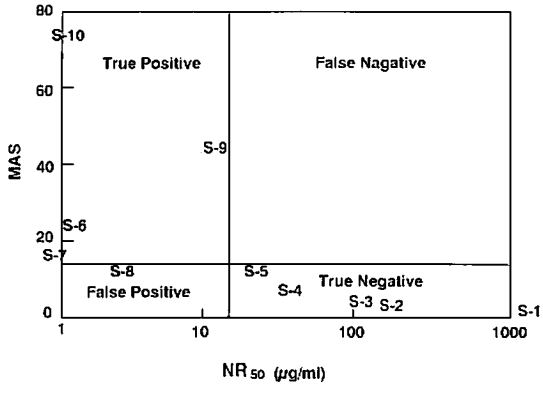


Figure 2. Correlation between maximal average Draize total scores (MAS) and CornePack assay.

IU cells[7]. Table VI shows that the correlation of CornePack with these other methods was low (0.557–0.700) as compared those among themselves (0.932–0.998).

DISCUSSION

Many papers have reported the availability of NRCE as an alternative method to the Draize test. The all have been evaluated using various endpoints of cytotoxicity, including the highest tolerated dose[8], plasminogen activator[9–12], increase in light absorbance at 360 nm[12], wound closure of cultured corneal epithelial cells[14], colony formation[14,15,16] and ^{51}Cr release[17], and results compared with *in vivo* Draize scores for surfactants, alcohols,



Simple regression: $\log(NR_{50}) = -0.220MAS + 1.51$

Figure 3. Reproducibility of CornePack assay to maximal average Draize total scores (MAS). For the discrimination points of MAS 15, the NR_{50} of CornePack is 14.8 μg/ml. Sample names show in Table I.

Table VI. Correlation coefficient among the cytotoxicity tests.

	HeLa -MTT	CHL/IU -CVS	SIRC -CVS	SIRC -NR	CornePack
HeLa-MTT	1.000				
CHL/IU-CVS*	0.961	1.000			
SIRC-CVS	0.990	0.949	1.000		
SIRC-NR**	0.986	0.932	0.998	1.000	
CornePack	0.680	0.557	0.669	0.700	1.000

CVS* :Crystal violet staining assay
NR** :Neutral red uptake assay

aldehydes and other chemicals. Although there were a few chemicals which did not correspond with *in vivo*, many data sets showed high correlation coefficients with Draize scores[10,14,16,17].

Although our results with CornePack did not show a high correlation with most of the individual scores of the Draize test, correlation with conjunctiva scores were relatively good. Reproducibility of the method evaluated by intra-/inter-laboratory variations was also not satisfactory. These variations seemed to be caused by the differences in techniques of cell harvest after the pre-culture, which caused diversity of cell growth rates in the cultures (data not shown). From these results, we improved the protocol to omit the pre-culture step to supply enough frozen cells to inoculate each well for evaluation. We also changed the procedures for starting the NR assay, such that, uptake of NR in negative controls became more stable and higher than seen before (Table III). These improved methods will be adopted for the second validation study.

Concerning the cytotoxicity of surfactants in different cell types including SIRC[6], HeLa and CHL/IU cells[7], NRCE in CornePack showed results that were relatively independent from the others (Table VI). These differences may be caused by the serum free culture medium used in CornePack. Because the sensitivity of CornePack was about 50–60 times higher than the other cell culture method adding serum into the culture medium. Although the results from CornePack did not correlate well to those of the Draize test in this primary validation, there is the possibility that the method may show better results for compounds with lower irritation potency. Thus, we conclude that this test should be evaluated with the improved protocol for a wider range of chemicals used as cosmetic ingredients.

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