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First Phase Inter-Laboratory Validation of the *In Vitro* Eye Irritation Tests for Cosmetic Ingredients: (8) Evaluation of Cytotoxicity Tests on SIRC cells

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SUMMARY

Cytotoxicity tests using SIRC cells and crystal violet (CV) staining or neutral red (NR) uptake as their endpoints were primarily validated using nine surfactants and physiological saline by six and seven laboratories, respectively, under the Ministry of Health and Welfare (MHW) project on alternatives to the Draize rabbit eye irritation test (Draize test) for cosmetic ingredients. The inter-laboratory coefficient of variation of EC_{50}s of the test substances by CV staining and the NR uptake method were 0.262 and 0.304, respectively. These two endpoints gave similar EC_{50}s and the correlation coefficient between them was high (r=0.996). Correlation coefficients between maximal average Draize total scores (MAS) of test substances in Draize tests and their EC_{50}s in CV staining and NR uptake were −0.894 and −0.913, respectively.

From these results, we concluded these cytotoxicity tests using SIRC cells are promising as alternative methods to the Draize test for cosmetic ingredients, and the CV staining and NR uptake methods gave almost identical results. However, further validation of these methods is needed using a wider range of cosmetic ingredients.

INTRODUCTION

The Draize rabbit eye irritation test (Draize test)[1] has been criticized from the viewpoint of animal welfare and many investigators have been searching for alternatives to the test. Since testing using established cell lines is easy to conduct and to evaluate, various cytotoxicity tests have already been proposed and applied as alternatives to the Draize test.

SIRC cells are a well-known established cell line derived from the cornea of rabbit

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eyes[2-7]. The crystal violet (CV) staining method was developed by Saotome et al.[8] for evaluating the cytotoxicity of chemicals such as drugs for injection. Itagaki et al.[7] reported that the CV staining method was applicable to predict the eye irritancy of surfactants. The major advantages of the CV staining method over other cytotoxicity tests are that the morphological changes in cultured cells can be observed by phase microscopy and the tested microplates can be stored for reference, if necessary. The neutral red (NR) method developed by Borenfreund et al.[9,10] is based on the uptake of NR and its accumulation in lysosomes of viable uninjured cells. This method is the basis of a commercial kit for evaluating the cytotoxicity of chemicals[11].

We have conducted a first-phase inter-laboratory validation of the CV staining method and the NR uptake method with SIRC cells using blinded nine surfactants and physiological saline solution as a negative control in six or seven independent laboratories under the same standard operating procedure (SOP). The results are presented and discussed in this report. This forms a part of the Ministry of Health and Welfare (MHW) project entitled "Studies on the test methods to evaluate the safety of new ingredients of cosmetics"[12].

MATERIALS AND METHODS

Six or seven laboratories participated in the validation of these cytotoxicity tests using SIRC cells. The test procedures in each participating laboratory were strictly controlled under the common SOP. Cooperating researchers received SOP training and conducted a preliminary experiment to get used to the procedures before starting the main experiments. They were required to repeat the same experiments twice to get information about intra-laboratory reproducibility.

Materials

The 10 test substances used in this study are listed in Table I. They comprised one cationic surfactant, four anionic surfactants, four nonionic surfactants and isotonic sodium chloride solution (physiological saline)[13]. They complied with the Japanese standards of cosmetic ingredients[14,15] and were supplied by the Japan Cosmetic Industry Association (JCIA) to the National Institute of Health Sciences (NIHS). The substances were coded by the Test Substance Control Committee and supplied to all participating each laboratories, which were blinded as to the nature of the test materials.

Every member of the validation project used the same lot of Eagle's MEM and calf serum from GIBCO Laboratories, NY, USA (lot 64N5521 and 45K4613, respectively) and 96-well microplates from Becton, Dickinson & Co., NJ, USA (lot 12871112). Other chemicals were of the highest grade available.

Cells and culture condition

SIRC cells derived from rabbit cornea, obtained from the American Type Culture Collection (ATCC), were cultivated and dis-

Table I. List of the test substances

No.	Test substances	Abbreviation	Classification
S-1	Isotonic Sodium Chloride Solution	Physiological saline	-
S-2	Polyoxyethylene Hydrogenated Castor Oil (60E.O.)	POE hydrogenated castor oil	Nonionic
S-3	Polyoxyethylene Sorbitan Monolaurate (20E.O.)	Tween 20	Nonionic
S-4	Polyethyleneglycol Monolaurate (10E.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 (2E.O.) (27% solution)	POE laurylether sulfate	Anionic
S-9	Polyoxyethylene Octylphenylether (10E.O.)	Triton X-100	Nonionic
S-10	Benzalkonium Chloride	Benzalkonium chloride	Cationic

tributed from the Japanese Cancer Research Resources Bank (JCRB) to each laboratory. The passage numbers of cells used in each laboratory were from 417 to 424. The absence of contamination with mycoplasma was checked prior to the experiment by JCRB and after the experiment by several laboratories in this project.

SIRC cells were cultured in Eagle's MEM supplemented with calf serum (10%) in a CO_2-air (5: 95, v/v) incubator at 37°C. The doubling time of the cells, determined in every laboratory that participated, varied from 12.0 to 24.0 hours (mean±S.D.: 18.95±3.59). The colony formation rates determined in five laboratories were from 29.0 to 72.0% (mean±S.D.: 48.38±19.56), the mode peak of chromosome obtained in four laboratories was 66, and two laboratories confirmed the isozyme pattern of SIRC cells.

Cytotoxicity test by the CV staining method

The culture medium mentioned above (100 μl) was poured into each well except for the first well of a 96-well microplate and 100 μl of a test chemical dissolved in culture medium was added into the first and the second wells. Serial two-fold dilution was performed directly on the plate by transferring 100 μl of the mixed solution from the second well to the third, from the third to the fourth, and so on. SIRC cells were harvested from preculture bottles by trypsinization, washed once, and resuspended (2×10^5 cells/ml) in culture medium. A 100 μl aliquot of the cell suspension was gently introduced into each well. The plates were kept undisturbed for 20 min to allow the cells to settle onto the bottom of the well. They were then cultured in a CO_2 incubator (5% in air) for 3 days. Dead cells were washed off phosphate-buffered saline (PBS (-)), and the cells attached to the bottom of the plate were fixed and stained with 0.4% crystal violet solution in methanol for 30 min. The plate was washed with water, and absorbance at about 590 nm was measured by an automatic microplate reader. The mean absorbance of 10 wells containing no test substance was regarded as the control value, and the percent ratios of absorbance of the other wells were calculated. Five wells were used for each concentration of test substances. The concentrations of the test substances that decreased the absorbance by 50% (EC_{50}) were obtained from the dose-response curves.

Cytotoxicity test by the NR uptake method

Procedures from preparation of test chemicals to incubation with SIRC cells were the same as in the CV method. After incubation for 3 days, the medium was replaced with 250 μl of culture medium containing NR (50 μg/ml final concentration) and incubated for another 3 hr. Then, the medium was removed and the cells were rapidly washed with an aqueous solution containing both 1% formaldehyde and 1% $CaCl_2$. Neutral red incorporated into viable cells was extracted with 200 μl of 1% acetic acid-50% ethanol mixture. After 15 min at room temperature, the microplates were gently agitated by a microplate shaker and the absorbance at 540 nm was measured by an automatic microplate reader. The other procedures were the same as in the CV staining method.

In vivo test

In vivo testing was performed by the conventional Draize eye irritation test method and the results have been separately reported by Ohno et al[16].

RESULTS AND DISCUSSION

Intra-laboratory reproducibility

Absorbance, which is correlated to the number of viable cells, decreased with increasing concentration of the test chmicals except for physiological saline. Tables II and III show the EC_{50}s, i.e., the concentrations by which the CV staining and NR uptake were inhibited by 50%, respectively. Two EC_{50} values, obtained by repetition of the same experiments, are given for each chemical. The

Table II. The EC_{50} values of the CV method obtained from each laboratory

Laboratory / Sample No.		A	B	C	D	E	F	Mean	SD	CV
S-1	1	>10000	>10000	>100000	>500000	>10000	>500000			
	2	>10000	>10000	>50000	>500000	>10000	>500000			
	Mean	>10000	>10000	>50000	>500000	>10000	>500000	—	—	—
S-2	1	2665.2	(>10000)	3080.1	3400	2100.0	3561.00			
	2	3224.2	2792.0	3893.8	3300	2650.0	3811.60			
	Mean	2944.7	2792.0	3487.0	3350.0	2375.0	3686.3	3105.8	490.0	0.158
S-3	1	556.8	739.0	806.3	630	700.0	1220.30			
	2	523.6	849.0	667.0	620	650.0	1235.65			
	Mean	540.2	794.0	736.7	625.0	675.0	1228.0	766.5	242.6	0.316
S-4	1	321.2	444.1	232.3	230	280.0	569.42			
	2	338.5	367.3	257.4	225	330.0	578.73			
	Mean	329.9	405.7	244.9	227.5	305.0	574.1	347.8	127.9	0.368
S-5	1	420.1	596.7	332.6	430	370.0	633.45			
	2	488.0	383.1	342.0	430	480.0	355.92			
	Mean	454.1	489.9	337.3	430.0	425.0	494.7	438.5	57.5	0.131
S-6	1	142.1	115.1	83.1	(90)	77.0	250.15			
	2	142.5	120.1	143.1	(94)	104.5	218.89			
	Mean	142.3	117.6	113.1	(92.0)	90.8	234.5	139.7	56.1	0.402
S-7	1	174.2	168.3	116.6	140	190.0	200.72			
	2	188.5	175.6	117.3	157	190.0	194.08			
	Mean	181.4	172.0	117.0	148.5	190.0	197.4	167.7	30.1	0.179
S-8	1	696.8	545.6	802.9	750	710.0	782.54			
	2	674.2	577.2	927.0	720	820.0	761.99			
	Mean	685.5	561.4	865.0	735.0	765.0	772.3	747.4	72.3	0.097
S-9	1	25.1	42.7	32.2	54	17.5	54.89			
	2	28.3	33.2	14.3	65	47.0	46.98			
	Mean	26.7	38.0	23.3	59.5	32.3	50.9	38.4	14.2	0.369
S-10	1	15.7	18.29	13.6	16.5	14.5	28.32			
	2	16.6	32.05	12.8	13.5	16.5	29.70			
	Mean	16.2	25.2	13.2	15.0	15.5	29.0	19.0	6.5	0.340
SLS	1	172.2	171.9	116.9	126.0	187.0	197.86			
	2	180.3	171.9	117.7	120.0	187.0	—			
	Mean	176.3	171.9	117.3	123.0	187.0	197.9	162.2	33.9	0.209

*The results are presented in μg/ml.

**Results obtained after the key code had been opened are given in the parentheses.

***Since we specified neither the supplier of SLS nor its lot, SLS was used as available in each laboratory.

Table III. The EC_{50} values of the NR method obtained from each laboratory

Laboratory / Sample No.		G	H	I	J	K	L	M	Mean	SD	CV
S-1	1	>10000	>10000	>10000	>10000	>10000	>10000	>500000			
	2	>10000	>10000	>10000	>10000	>10000	>10000	>500000			
	Mean	>10000	>10000	>10000	>10000	>10000	>10000	>500000	—	—	—
S-2	1	1337.3	>10000	6440.6	3600	2295.1	1550.0	4184.85			
	2	1198.2	1992	5935.0	3400	1845.5	1550.0	3444.10			
	Mean	1267.8	1992.0	6187.8	3500.0	2070.3	1550.0	3814.5	2911.8	1603.8	0.551
S-3	1	835.5	839	925.9	800	933.51	820.0	1358.30			
	2	756.5	814	1230.8	820	932.73	720.0	1456.40			
	Mean	796.0	826.5	1078.4	810.0	933.1	770.0	1407.4	945.9	229.8	0.243
S-4	1	336.7	367.3	675.4	370	556.78	300.0	709.05			
	2	344.5	344.8	317.4	380	530.59	320.0	434.96			
	Mean	340.6	356.1	496.4	375.0	543.7	310.0	572.0	427.7	106.7	0.250
S-5	1	491.9	635.6	475.1	560	130.46	350.0	634.03			
	2	467.1	444.1	416.0	550	127.64	390.0	543.20			
	Mean	479.5	539.9	445.6	555.0	129.1	370.0	588.6	444.0	157.2	0.354
S-6	1	159.1	127.9	147.3	175	98.97	95.0	232.08			
	2	157.2	130.7	148.9	168	89.61	163.0	167.88			
	Mean	158.2	129.3	148.1	171.5	94.3	124.0	200.0	146.5	34.5	0.236
S-7	1	186.6	175.6	115.0	180	188.70	160.0	183.50			
	2	178.9	171.9	119.0	174	199.92	175.0	183.63			
	Mean	182.8	173.8	117.0	177.0	194.3	167.5	183.6	170.9	25.2	0.148
S-8	1	699.7	559.2	895.8	700	733.39	450.0	738.02			
	2	652.1	535.6	893.2	700	744.90	450.0	499.00			
	Mean	675.9	547.4	894.5	700.0	739.1	450.0	618.5	675.1	133.9	0.198
S-9	1	26.8	52.8	43.0	63	25.77	22.0	63.31			
	2	27.8	39.3	55.6	63	26.78	21.0	55.11			
	Mean	27.3	46.1	49.3	63.0	26.3	21.5	59.2	41.8	16.8	0.402
S-10	1	14.1	13.82	12.5	14.2	16.46	11.7	28.78			
	2	14.0	34.86	12.6	16.2	18.22	14.5	29.61			
	Mean	14.1	24.3	12.6	15.2	17.3	13.1	29.2	18.0	6.4	0.354
SLS	1	175.9	183.1	114.8	170	199.99	145.0	—			
	2	175.0	179.3	122.0	175	190.90	175.0	186.20			
	Mean	175.5	181.2	118.4	172.5	195.4	160.0	186.2	169.9	25.3	0.149

*The results are presented in μg/ml.

**Since we specified neither the supplier of SLS nor its lot, SLS was used as available in each laboratory.

Table IV. The ratio of the two EC_{50} values of the CV method obtained from each laboratory

Laboratory	A	B	C	D	E	F	Mean
Sample No.							
S－1	－	－	－	－	－	－	－
S－2	1.210	－	1.264	1.030	1.262	1.070	1.167
S－3	1.063	1.149	1.209	1.016	1.077	1.013	1.088
S－4	1.054	1.209	1.108	1.022	1.179	1.016	1.098
S－5	1.162	1.558	1.028	1.000	1.297	1.780	1.304
S－6	1.003	1.043	1.722	1.165	1.357	1.143	1.239
S－7	1.082	1.043	1.006	1.121	1.000	1.031	1.047
S－8	1.034	1.049	1.155	1.042	1.155	1.027	1.077
S－9	1.127	1.286	2.252	1.204	2.685	1.168	1.621
S－10	1.057	1.752	1.063	1.222	1.138	1.049	1.214
S L S	1.047	1.000	1.007	1.050	1.000	－	1.021
Mean	1.084	1.232	1.281	1.087	1.315	1.144	1.188

*Since we specified neither the supplier of SLS nor its lot. SLS was used as available in each laboratory.

Table V. The ratio of the two EC_{50} values of the NR method obtained from each laboratory

Laboratory	G	H	I	J	K	L	M	Mean
Sample No.								
S－1	－	－	－	－	－	－	－	－
S－2	1.116	－	1.085	1.059	1.244	1.000	1.215	1.120
S－3	1.104	1.031	1.329	1.025	1.301	1.139	1.072	1.100
S－4	1.023	1.065	2.128	1.027	1.049	1.067	1.650	1.284
S－5	1.053	1.431	1.420	1.018	1.022	1.114	1.167	1.175
S－6	1.012	1.022	1.011	1.042	1.104	1.511	1.382	1.169
S－7	1.043	1.022	1.035	1.034	1.059	1.094	1.001	1.041
S－8	1.073	1.037	1.003	1.000	1.016	1.000	1.479	1.087
S－9	1.037	1.344	1.293	1.000	1.039	1.048	1.149	1.130
S－10	1.007	2.522	1.008	1.141	1.107	1.239	1.029	1.293
S L S	1.005	1.021	1.063	1.029	1.048	1.207	－	1.062
Mean	1.047	1.277	1.238	1.038	1.069	1.152	1.236	1.146

*Since we specified neither the supplier of SLS nor its lot. SLS was used as available in each laboratory.

ratios of the two EC_{50}s, indicating intra-laboratory variance, are also shown in Tables IV and V. Mean values of the ratios in the CV staining and NR uptake methods were 1.188 and 1.146, respectively. These results indicated that the intra-laboratory variance was small by both methods.

Inter-laboratory reproducibility

In order to assess inter-laboratory reproducibility, the average of the two EC_{50} results obtained in each laboratory, and the mean and standard deviation (S.D.) of these averages were calculated, and the results are shown in Tables II & III. Mean values of the inter-laboratory coefficient of variation of the CV method and the NR method for the nine test substances (S2-S10) were 0.262 and 0.304, respectively. These results indicated that the inter-laboratory reproducibility was also good in both test methods.

Correlation between in vivo and in vitro tests

The relationships between the Draize scores taken from Ohno et al.[16] (Table VI) and the mean EC_{50}s in the CV and NR methods were examined by linear regression analysis (Table VII). Based on the average correlation coefficient for three parameters (maximum scores, 24 hr scores and the area under the curve (AUC)), the EC_{50} shows good correlations with conjunctivae scores (CV: −0.847, NR: −0.865), total scores (CV: −0.806, NR: −0.824) and cornea scores (CV: −0.768, NR −0.785). The EC_{50} values of both methods also showed good correlations with the iris scores except for 24 hr scores. There was no significant difference in related-

Table VI. Results of the Draize eye irritation test on the ten samples

Sample No.	Maximum score				24 hr score				Area ratio under the curve*			
	Total	Cornea	Iris	Conjunctiva	Total	Cornea	Iris	Conjunctiva	Total	Cornea	Iris	Conjunctiva
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 (24.48.72)	1.7 (72)	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 (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 the theoretical maximum Draize total score until 7 days after treatment.
**: The values in parenthesis are the time (hour) at which the scores became maximum.

Table VII. Correlation between *in vivo* and *in vitro* results

Draize scores		CV method		NR method	
		regression line	correlation coefficient	regression line	correlation coefficient
total scores	(maximum)	y=100.18 -33.060*Logx	-0.894	y=102.41 -33.746*Logx	-0.913
	(24 hr)	y=90.996 -30.640*Logx	-0.837	y=93.295 -31.369*Logx	-0.858
	(AUC)	y=70.450 -24.100*Logx	-0.865	y=72.007 -24.570*Logx	-0.883
cornea scores	(maximum)	y=80.569 -27.315*Logx	-0.858	y=82.478 -27.994*Logx	-0.877
	(24 hr)	y=71.389 -24.560*Logx	-0.784	y=73.253 -25.165*Logx	-0.804
	(AUC)	y=52.176 -17.999*Logx	-0.846	y=53.351 -18.355*Logx	-0.864
iris scores	(maximum)	y= 7.854 -2.722*Logx	-0.858	y= 7.950 -2.745*Logx	-0.866
	(24 hr)	y= 1.088 -0.373*Logx	-0.451	y= 1.070 -0.364*Logx	-0.439
	(AUC)	y= 3.742 -1.300*Logx	-0.863	y= 3.793 -1.312*Logx	-0.871
conjuctivae scores	(maximum)	y=22.073 -5.998*Logx	-0.792	y=22.710 -6.218*Logx	-0.829
	(24 hr)	y=18.549 -5.707*Logx	-0.858	y=15.972 -5.840*Logx	-0.880
	(AUC)	y=14.532 -4.801*Logx	-0.907	y=14.863 -4.903*Logx	-0.927

*The relationship between *in vivo* and *in vitro* results, except for SI, was analyzed by linear regression analysis.

Table VIII. The rank order of test chemicals in both *in vitro* test, and *in vivo*.

Sample No.	Draize total scores		SIRC-CV method		SIRC-NR method	
	maximum	rank	EC_{50}	rank	EC_{50}	rank
S-1	0.0	I-II	>10000	I	>10000	I
S-2	0.0	I-II	3105.8	II	2911.8	II
S-3	0.7	III	766.5	III	945.9	III
S-4	3.3	IV	347.8	VI	427.7	VI
S-5	10.3	VI	438.5	V	443.9	V
S-6	26.7	VIII	139.7	VIII	146.5	VIII
S-7	15.0	VII	167.7	VII	170.8	VII
S-8	10.0	V	747.4	IV	675.1	IV
S-9	41.3	IX	38.4	IX	41.8	IX
S-10	78.0	X	19.0	X	18.0	X
Spearman's rank correlation coefficient with Draize scores			0.961		0.961	

*Test chemicals were ranked from I (least toxic) to X (most toxic).

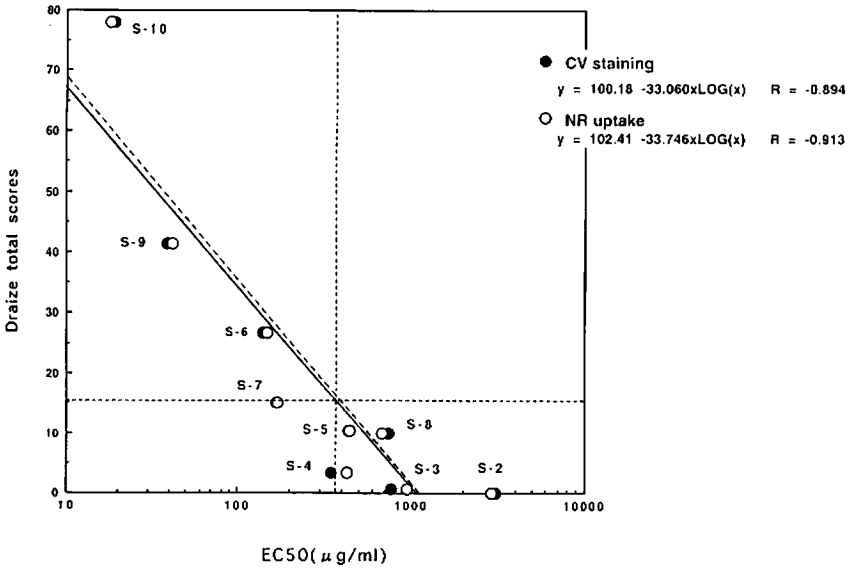

Figure. 1. The relationships between maximum total scores of test substances in Draize tests and their EC_{50}s in the CV staining method (● data, ——regression line) and the NR uptake method (○ data, ------regression line).

ness among the parameters other than iris score. Comparison with maximum scores, 24 hr scores and AUC also gave similar relatedness except for the case of iris, scores.

The values of Spearman's rank correlation coefficient with EC_{50} and maximal average Draize total scores (MAS) were very high (CV; −0.961, NR; −0.961) in both test methods (Table VIII), indicating that comparison with proper standard substances may allow an appropriate classification of test substances according to their eye irritancy.

Comparisons of two endpoints in the cytotoxicity test

These two *in vitro* methods gave similar EC_{50}s and the correlation coefficient between them was high (r=0.996). The rank orders of the potency of test chemicals agreed fairly well with each other (Table VIII). These results suggest that although the two methods adopt physiologically/biochemically different endpoints, they give similar EC_{50} values, at least when surfactants are used as test compounds.

Compatibility between in vivo and in vitro test results

The classification of the irritation potential of ten test chemicals, predicted by using linear regression formulae, was compared with that based on the maximum total scores of the Draize test (Fig. 1). The regression lines were y=100.18−33.060 log x for the CV method and y=102.41−33.746 logx for the NR method, where y is the maximum total score and x is the *in vitro* EC_{50} for a given agent. When a value of fifteen was taken as the cut-off point of MAS, the EC_{50} calculated from the regression lines was 377.16 for the CV method and 389.25 for the NR method. Good compatibility between *in vivo* and *in vitro* test results was revealed for the test chemicals examined except for PEG monolaurate (S4) in the CV method (false positive). On the above basis, PEG monolaurate (S4) fell into a different class in terms of *in vitro* reaction in the CV method. However, the values for S4 in the CV and NR methods are very similar, and that in the CV method is only just outside the cut-off point, so we consider these two methods are essentially identical when surfactants are used as test compounds.

Towards further validation

It is necessary that alternative test methods should be based on scientifically proven principles. Cytotoxicity tests may be a suitable alternative to the eye-irritation test in this regards. They can afford information about the overall effects of test substances on many basic biochemical and physiological mechanisms of cells, which may correlate with the direct effects on the constituent cells of the eye. Since testing using established cell lines is easy to conduct and the evaluate, various cytotoxicity tests have already been proposed and applied as alternatives to the Draize test.

We selected SIRC cells for the tests, because they are derived from rabbit cornea, one of the target cell types in the eye-irritation test. However, Kojima et al.[7] indicated that differences in cell type do not seem to cause any significant differences in the results of the test as an alternative to the Draize test, at least when the test substances are limited to surfactants.

Good intra-/inter-laboratory variances were found in the CV staining method and NR uptake method using SIRC cells. The EC_{50}s obtained from both methods also showed good correlations with the Draize eye irritation test results. In addition, it was proved that these different endpoint assays showed similar EC50 values, when surfactants were used as test compounds.

We conclude that these cytotoxicity tests on SIRC cells are useful as an alternative method to predict eye irritation of surfactants. However, further validation is needed using a wider range of cosmetic ingredients.

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