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First Phase Inter-Laboratory Validation of the *In Vitro* Eye Irritation Tests for Cosmetic Ingredients: (5) Evaluation of Skin^{2TM} Dermal Model ZK1100

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SUMMARY

A collaborative validation study with 6 laboratories was conducted to evaluate three-dimensional cultured cell method, SKIN^{2TM} Model ZK1100® (SKIN^2), as an alternative to the Draize rabbit eye irritation test (Draize test) using physiological saline and 9 surfactants. MTT (3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyl tetrazolium bromide)-50 values, indicating the concentration of test materials which reduce cell survive to 50% of control, were compared to the results of the Draize test. Physiological saline (S-1) and POE hydrogenated castor oil (S-2) had MTT-50 values greater than 10,000 μg/ml and were classified as non-iritants, which coincided with the classification by the Draize test. Correlation coefficients for comparison of the MTT-50 values for the other eight surfactants with the maximum average Draize total score (MAS), the score at 24-hr, and the area under the Draize score-time curve (AUC) were −0.92, −0.94, and −0.91, respectively. Higher correlations with cornea and conjunctiva scores were noted. Interlaboratory differences in the results were not significant. These results indicate that SKIN^2 test is promising as a useful and reliable alternative method to the Draize test.

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INTRODUCTION

Public concern has increased over the use of animals in safety evaluation of cosmetics[1]. In particular, the Draize rabbit eye irritation test (Draize test) has been widely criticized because of possible pain caused in test animals and the presence of a unnecessary exaggeration factor between test results and symptoms of accidental human exposures[2,3]. Cosmetic manufactures have worked toward development and evaluation of potential replacement methods to the Draize test[4,5,6]. In Japan, a collaborative validation study was com-

menced in 1992 under the guidance of the Ministry of Health and Welfare (MHW). The SKIN^{2TM} Model ZK1100® (SKIN^2) data reported here are part of the results from the first phase of the collaborative study entitled "Studies on the test methods to evaluate the safety of new ingredients of cosmetics"[7].

SKIN^2 is a kit developed by Advanced Tissue Sciences Inc. as an alternative to the Draize test[8,9]. The model has been devised for growing human dermal fibroblasts in a three-dimensional nylon mesh support such that they remain mitotically and metabolically active. Target cells growing in the 3D-matrix are assumed to maintain cell-cell interactions analogous to those in dermis. Furthermore, another potential advantage to this system is that the cells can be exposed from more than just their apical surface. In evaluating this system for eye irritation testing, various measures of toxicity including neutral red uptake, 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) reduction, lactate dehydrogenase release, and PGE_2 release have been used[9]. In this study, the MTT cell viability assay procedure described by T. Mosmann (1983)[10], was employed.

MATERIALS AND METHODS

Test substances

The 10 test substances evaluated are listed in Table I. The test materials included one cationic surfactant, 4 anionic surfactants, 4 nonionic surfactants, and physiological saline. These substances were from the Japanese standards of cosmetic ingredients[11,12,13] or the Japanese pharmacopoeia, and were supplied by the Japan Cosmetic Industry Association (JCIA) to the National Institute of Health Science (NIHS). The substances were coded and supplied by NIHS to each laboratory for testing to obtain objective information about the *in vitro* methods and intra-inter-laboratory variability.

Preparation of test solution

The test substances were prepared in accordance with the protocol recommended by the supplier (Advanced Tissue Sciences Inc.). In the definitive study, the test substances were dissolved at 10, 1, 0.1 and 0.001 mg/ml in assay medium. If it was not possible to obtain a dose-response curve with one dose producing 100–80% cell viability, more than one dose producing 75–25%, and one dose producing 10–0%, then the experiments were repeated with modification of the concentration of test substance.

Test kit and procedures

Skin^2 was supplied by Oriental Yeast Co., Ltd. and testing was conducted according to the protocol included in each kit. Briefly, cell cultures were removed from the agarose shipping medium and cultured for 12 hours in fresh culture medium at 37°C in an atmosphere of 5% CO_2 in air. Then, the medium was changed to the test substance solution. Two cell cultures were allocated to each test

Table I. Test substances evaluated

Sample number	Name	Abbreviation	Surfactant Class
S-1	Isotonic sodium Chloride Solution	Physiological Saline	-
S-2	Polyoxyethylene Hydrogenated Castor Oil (60 E.O.)	POE Hydrogenated Castor 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	Acyl Glutamate	Anionic
S-7	Sodium Lauryl Sulfate	SLS	Anionic
S-8	Sodium Polyoxyethylene Laurylether Sulfate (2 E.O.:27% solution)	POE Laurylather Sulfate	Anionic
S-9	Polyoxyethylene Octylphenylether (10 E.O.)	Triton X-100	Nonionic
S-10	Benzalkonium Chloride	Benzalkonium Chloride	Cationic

concentration. After 20 hours of treatment the medium was changed to the MTT regent and the cultures were incubated further at 37°C for 2 hours, while being gently shaken in the CO_2 incubator. Then, the cultures were washed and MTT formazan produced by succinate hydrogenase in mitochondria was extracted from the cells with isopropanol. During the 1-hour extraction, the culture plates were shaken at room temperature. After completion of extraction, aliquots of the alcohol extract were transferred to 96-well plates and the absorbance measured at 540 nm. Control cell cultures were handled in the same manner as treated cultures except that they were treated with culture medium alone. The MTT data (percentage of untreated control values) were plotted as dose-response graphs and the MTT-50 values obtained by interpolation at the 50% response level.

Statistics

After all data were collected from the test facilities, the test substances codes were broken by NIHS and the test substance names identified. The *in vivo* and *in vitro* data sets were compared by regression analysis, with calculation of Pearson's and Spearman's correlation coefficients using Statistical Analysis System (SAS) programming[14].

RESULTS AND DISCUSSION

MTT-50 valued for the 10 test substances from the 6 test facilities are indicated in Table II. The MTT-50 values were greater than 10,000 μg/ml for physiological saline (S-1) and POE hydrogenated castor oil (S-2). These substances were classified as non-irritants. For the substances tested, the *in vitro* results (Table II), spanned more than 4 orders of magnitude, from benzalkonium chloride (S-10, 4μg/ml) to saline (S-1) and POE hydrogenated castor oil (S-2).

No significant inter-laboratory differences were noted in MTT-50 values obtained. Overall, the coefficient of variation (CV) was approx. 20% except for HG-glutamate (S-6) and benzalkonium chloride (S-10). The CVs were 51.7% for HG-glutamate and 32.4% for benzalkonium chloride. These relatively higher variations may have been attributed to large amount of serial dilution required because of their higher cytotoxic potency. The SKIN^2 cell cultures were prepared with normal skin fibroblasts obtained from 6 healthy volunteers, and these cells were mixed in order to get consistent MTT-50 values for a positive control (SLS) during the manufacturing process[15]. This is a possible reason the inter-laboratory differences were minimal. Furthermore, the MTT-50 values for Tween 20 (S-3), SLS (S-7), Triton X-100 (S-9), and benezalkonium chloride were consistent with the results of another study[16]. In summary, the experimental reliability of SKIN^2 was relatively high.

The results of the Draize test conducted as a

Table II. Summary of MTT50 (μg/ml) values from the 6 test laboratories

Sample number	D*	A	K	L	P	U	Mean±S.D. (μg/ml)	C.V. (%)
S-1	>10000	>10000	>10000	>10000	>10000	>10000	-	-
S-2	>10000	>10000	>10000	>10000	>10000	>10000	-	-
S-3	290	510	300	325	402	301	355±86	24.3
S-4	165	250	180	250	248	202	216±39	17.8
S-5	390	405	310	360	538	295	383±87	22.8
S-6	13	22	50	26	20	45	29±15	51.7
S-7	48	54	40	44	44	53	47±6	11.7
S-8	150	210	150	125	170	201	168±35	19.5
S-9	55	62	48	51	54	43	52±7	12.5
S-10	2	7	4	2	5	3	4±1	32.4

* Letters in the upper row indicate the test laboratories.

Table III. Summary of Draize rabbit eye test responses to 10% solutions of the 10 test substances

Sample number**	Maximum Average Score				24 hr Score				Area Ratio Under Curve (AUC):%*			
	Total	Cornea	Iris	Conjuctiva	Total	Cornea	Iris	Conjuctiva	Total	Cornea	Iris	Conjucliva
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	0.0	0.0	0.0	0.0
S-4	3.3 (1)	0.0	0.0	3.3 (1)	0.0	0.0	0.0	0.0	3.3	0.0	0.0	0.3
S-5	10.3 (48)	8.3 (48)	0.0	8.0 (1,4)	8.3	5.0	0.0	3.3	4.5	1.5	0.0	3.1
S-6	26.7 (24)	16.7 (24-72)	1.7 (72-168)	12.0 (4)	26.7	15.7	0.0	10.0	15.0	2.4	0.6	8.9
S-7	15.0 (4)	8.3 (48,72)	0.0	10.0 (4)	14.7	6.7	0.0	10.0	9.1	3.3	0.0	3.9
S-8	10.0 (4)	3.3 (48)	0.0	10.0 (4)	2.7	0.0	0.0	2.7	3.2	0.5	0.0	2.7
S-9	41.3 (72)	30.0 (72)	35.0 (168)	10.0 (4,48)	24.7	15.0	1.7	8.0	25.6	14.4	1.8	12.4
S-10	78.0 (24)	66.7 (24)	5.0 (96-168)	14.7 (96)	78.0	66.7	0.0	11.3	57.9	34.5	2.0	21.5

* This 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 shows in Table I.

*** Values in parentheses indicate the time (hours) when the scores became maximal.

part of this validation study are indicated in Table III[7]. The *in vivo* irritancy of the surfactants covered a full range, from saline (S-1) and POE hydrogenated castor oil (S-2) which were non-irritating to the strongly irritating benzalkonium chloride (S-10). For examination of *in vivo*: *in vitro* responses, the MTT-values were plotted against maximum scores (Figure 1), 24-hr scores (Figure 2) and AUC scores (Figure 3). With respect to the maximum score (Figure 1), the correlation Pearson's coefficient were −0.92 for the maximum average Draize total score (MAS), −0.89 for the cornea score, −0.76 for the iris score, −0.81 for the conjunctiva score. Across the comparisons, the correlation was relatively higher for cornea and conjunctiva scores than for iris scores. It is clear from the *in vivo* data that full ranges of responses were produced in cornea and conjunctiva by the test surfactants with only minimal iris effects. Thus, the *in vitro* results correlate best with responses of the ocular target tissues (corner, conjunctiva).

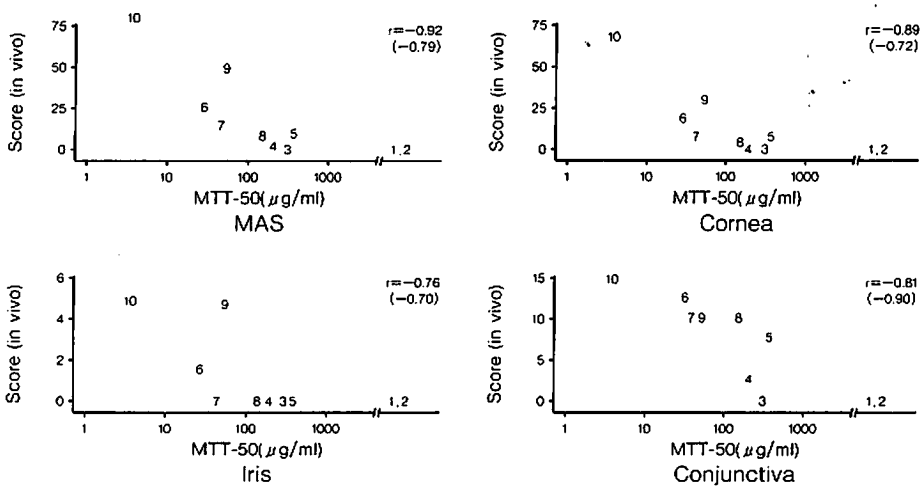

Figure 1. Correlation between MTT-50 values and MAS scores. Average MTT-50 values (X-axis; Table II) are compared to maximum average total (MAS) or tissue (cornea, conjunctiva, iris) scores (Y-axix; Table III). Each test material is indicated by a unique number (Table I).

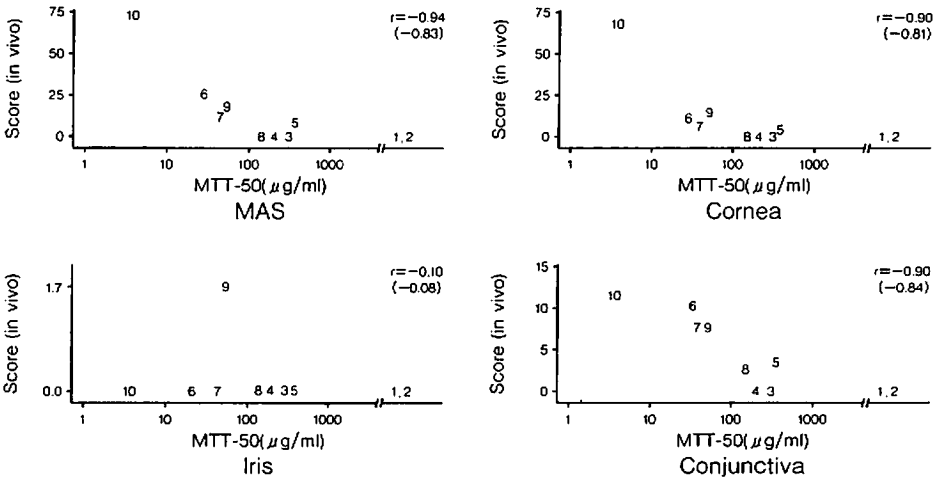

Figure 2. Correlation between MTT-50 values and 24 hour scores. Pearson's and Spearman's (in parentheses) correlation coefficients are indicated.

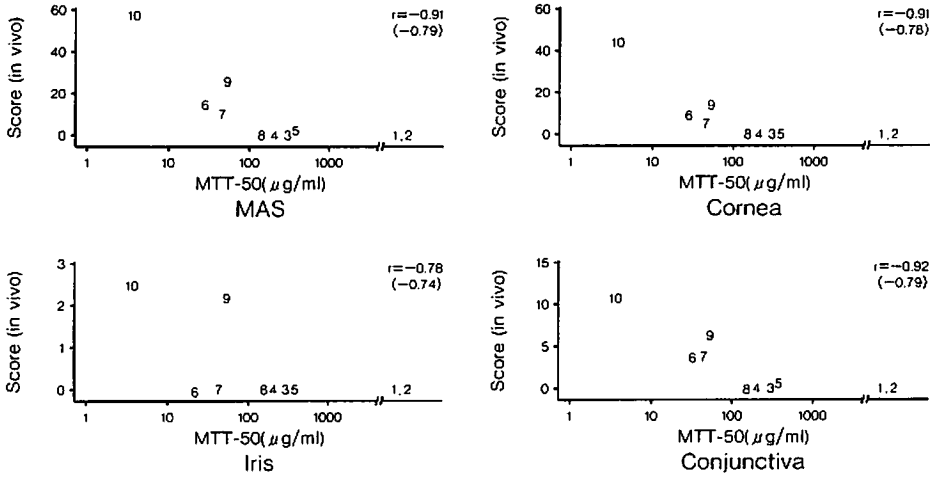

Figure 3. Correlation between MTT-50 values and AUC scores. Pearson's and Spearman's (in parentheses) correlation coefficiants are indicated.

The capacity of *in vitro* tests to discriminate positive and negative eye irritants is one important consideration in the evaluation of alternative methods. To address this point, the comparison between MTT-50 values from SKIN[2] and MAS from the Draize test (Figure 1, upper left) was examined. It has been suggested that adverse reactions are noted in cornea if the MAS exceeds 15 points[17]. Thus we selected 15 as cut-off value between positive and negative irritants. The MTT-50, equivalent to 15 points of the MAS on the regression line (Y=3.98X+249 (p=0.0001)) for 8 substances for which MTT-50 values were obtained, was 58.8 μg/ml. According to this classification, false positive results were obtained in Tween 20 (S-3), PEG monolaurate (S-4), lauroyl sarcosinate (S-5), and POE lauryl ether sulfate (S-8), but no test substance showed false negative results.

In 24-hr and AUC scores, similar results were noted, with high correlation coefficients for MAS, cornea and conjunctiva scores.

As mentioned above, SKIN[2] results showed a high degree of correlation with *in vivo* irritancy from the Draize test, and no remark-

able interlaboratory differences were noted. These results suggest that this dermal model might be a rapid and useful quantitative method to evaluate the eye irritation potential of cosmetic ingredients such as surfactant. Recently, the results of another collaborative study[18] also indicated the usefulness of SKIN^2. However, the number of the test substances was 10 in the present validation study and 4 in the study reported by Y. Taniguchi et al. (1994)[18]. Thus, further extensive studies are planned to evaluate this dermal model.

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