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First Phase Inter-Laboratory Validation of the *In Vitro* Eye Irritation Tests for Cosmetic Ingredients: (6) Evaluation of MATREX™

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SUMMARY

The MATREX™ system developed by Organogenesis, INC, has a three-dimensional structure of collagen gel and human fibroblasts. We evaluated this method in three laboratories as an alternative method to the Draize rabbit eye irritation test using coded nine surfactants and isotonic sodium chloride solution. Results were: 1) The mean coefficient of variance among the three laboratories for the ten substances was 0.220, 2) The correlation coefficient between *in vitro* EC_{50} values on inhibit of MTT reduction in MATREX™ and the Draize scores (maximum average Draize total score: MAS) was 0.633. The advantage of the MATREX™ is considered to be the ability to apply water insoluble substances. It was not possible to assess this potential advantage in this study since all test substances done at this phase of validation were water soluble, however this will be checked during the second phase of validation, when insoluble substances will be evaluated.

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INTRODUCTION

MATREX™ was developed as a test kit for eye irritation study by Organogenesis[1–4] and the details were reported by Gay et al[5]. This kit is composed of human dermal fibroblasts in a contracted collagen lattice. The cells maintain their original biochemical activity and retain differentiated physiological functions, morphologics and cytoskeletal organization. The use of MATREX™ as an alternative method for the Draize eye irritation test (Draize test) was also reported and the reliability to distinguish positive or negative irritants was 0.807[6]. Thus, we considered MATREX™ promising as an *in vitro* alternative method to the Draize test.

In the current study, the MATREX™ method was evaluated with nine coded surfactants and isotonic sodium chloride solution by three independent laboratories using the same procedures; test substances were applied either to the surface of the cultures (standard procedure) or in culture medium submerged cultures. The Draize tests were performed by using the same lot of the test substances and

the correlation with *in vitro* results determination.

This is a part of the Ministry of Health & Welfare (MHW) project entitled "Studies on the test methods to evaluate the safety of new ingredients of cosmetics"

MATERIALS AND METHODS

Test substances

Names and abbreviations of the 10 test substances used are listed in Table I. They were one cationic surfactant, 4 anionic surfactants, 4 nonionic surfactants and isotonic sodium chlorides solution (Table I)[7)8)]. They were of Japanese standards of cosmetic ingredients[7)8)]. They were supplied from the Japanese Cosmetics Industry Association (JCIA) to the National Institute of Health Sciences (NIHS). The coded samples were distributed to each laboratory to enable us to get objective information about the methods and inter-laboratory variability.

Preparation of Living Dermal Model (MATREX^{TM})

The MATREX^{TM} was manufactured by Organogenesis Inc. (Canton, MA) and supplied to each laboratory by a one-day delivery system. The fibroblasts used in the MATREX^{TM} fabrication were originally derived from a single human foreskin and propagated in monolayer culture. The dermal component was composed of human dermal fibroblasts in collagen-containing matrix on a 3 micron pore-size polycarbonate membrane (modified Transwell, Costar, Cambridge, MA). While tests were done, the cell matrix in the modified Transwell were placed in a six-well plate. The upper surface of the matrix was exposed to air while the lower portion, resting on the membrane, was in contact with the underlying cell culture medium.

Measurement of cytotoxicity

The cell matrix was placed in the assay plates and 5 ml of the "MATREX^{TM} assay medium" was added to the surface of the matrix for 30 min at room temperature to remove any residual conditioned medium from the matrix. Then, the 5 ml of medium was aspirated and 1.5 ml of fresh assay medium was put underneath each MATREX^{TM} culture. A polyethylene ring was set on the surface of the matrix by silicon sealant to circumscribe the area of exposure (0.8 cm^2). Eighty microliters of test solution was applied to the center of the matrix and the treated cultures were incubated for 24 hrs at 37°C in humidified incubator with 5% CO_2 in air. At the end of the exposure period, the upper surface of the MATREX^{TM} was rinsed with the "MATREX^{TM} assay medium" to remove any traces of test substances. After being washed, the cell matrices were transferred to six-well assay plates containing 1.5 ml of the "MATREX^{TM} assay medium" including 0.333 mg/ml of 3-[4, 5-dimethylthiazol-2-

Table I. List of test chemicals

Sample Number	Name	Abbreviation	Classification
S-1	Isotonic Chloride Solution	Physicological saline	-
S-2	Polyoxyethylene hydrogenated Caster Oil (50 E.O.)	POE hydrogenated caster oil	Nonionic
S-3	Polyoxyethylene Sorbitan Monolaurate (20 E.O.)	Tween 20	Nonionic
S-4	Polyethylene glycol Monolaurate (10 E.O.)	PEG monolaurate	Nonionic
S-5	Sodium N-Lauryl Sarcosinate	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	POE laurylether sulfate	Anionic
S-9	Polyoxyethylene Octylphenylether (10 E.O.)	Triton X-100	Nonionic
S-10	Benzalkonium Chloride	Benzalconium chloride	Cationic

yl]-2, 5-diphenyltetrazolium bromide (MTT)[9]. The assay plate was incubated for 3 hrs at 37°C. After the expose of MTT, the center of the cell matrix was excised using an 8 mm diameter skin biopsy punch and MTT-formazan was extracted with 0.3 ml of isopropanol containing 0.04 N HCl. Absorbance at 570 nm was measured with the isopropanol extraction medium as a blank. All data were expressed as the percentage mean±standard deviation (%MTT) for MTT conversion normalized to 100% conversion seen in the appropriate untreated control (no addition control). The median effect concentration (EC_{50}) for each test chemical was estimated from dose-response curve.

In the case of Polyoxyethylene Octylphenylether (10 E.O.) (S-9: Triton X-100), "the dipping method" was also evaluated. Cell matrices were submerged into culture medium containing test substances for 24 hrs at 37°C prior to measurement of MTT metabolism[10-14].

Study design for the validation

Three different laboratories participated in this inter-laboratory evaluation of MATREX™. The test was conducted according to the original SOP of the MATREX™ test. Technical transfer was done by Toyobo Corp. Ltd before the study was begun. A two step approach was taken to estimate the EC_{50} value of the test substances. The first step was a range finding study using 0.01%, 0.1% and 1.0% solutions (w/v in distilled water) of the test substances. According to these results, test substances were classified into eleven categories (Table II). The second step, EC_{50} estimation studies, were performed with several concentration using the scheme which was pre-determined from the categories (Table III), using the MATREX™ application methods as described above.

RESULTS AND DISCUSSION

Results of the MATREX™ study

Results of the MATREX™ study on 9 surfactants and isotonic sodium chloride solution (S-1: Physiological saline) are shown on Table IV. The irritation potential of each chemical was judged using MATREX™ classifications (Table V). EC_{50} values of isotonic sodium chloride solution and polyoxyethylene hydrogenated caster oil (S-2: POE hydrogenated caster oil) were higher than 5%, and these substances were classified into "No effect". Polyoxyethylene sorbitan monolaurate (20E.O.) (S-3: Tween 20), polyethylene glycol monolaurate (10E.O.) (S-4: PEG monolaurate), sodium N-lauroyl sar-

Table II. Classification by the range finding study

Survival ratio			
0.01%	0.1%	1%	Classification
over 60%	over 60%	over 60%	A
over 60%	over 60%	60%~50%	B
over 60%	over 60%	50%~40%	C
over 60%	over 60%	under 40%	D
over 60%	60%~50%	50%~40%	E
over 60%	60%~50%	under 40%	F
over 60%	50%~40%	under 40%	G
60%~50%	50%~40%	under 40%	H
60%~50%	under 40%	under 40%	I
50%~40%	under 40%	under 40%	J
under 40%	under 40%	under 40%	K

Table III. Concentration for the EC_{50} estimation study

Classification*	Concentration(%)				
A	1	5	10	50	100
B	0.25	0.5	1	2.5	5
C	0.25	0.5	1	2.5	5
D	0.10	0.25	0.5	0.75	1
E	0.05	0.1	0.5	1	5
F	0.05	0.75	0.1	0.25	0.5
G	0.025	0.05	0.075	0.1	0.25
H	0.01	0.025	0.05	0.075	0.1
I	0.0075	0.01	0.025	0.05	0.075
J	0.0025	0.005	0.0075	0.01	0.025
K	0.001	0.0025	0.005	0.0075	0.01

* Classification was depended on the result of the range finding study

Table IV. Results of the MATREX methods (EC_{50} value, coefficient variation and classification)

	1	2	3	Average± S.D.	CV	Classification
S-1	>100	>100	>100	100± 0	0	no effect
S-2	21.5	26.5	45	31± 12.4	39.9	no effect
S-3	0.061	0.057	0.072	0.0633± 0.00777	12.3	moderate
S-4	0.058	0.06	0.063	0.0603± 0.00252	4.2	moderate
S-5	0.32	0.25	0.217	0.262± 0.0526	20.0	moderate
S-6	0.0041	0.00385	0.0018	0.00325± 0.00126	38.8	severe
S-7	0.0018	0.0015	0.0017	0.00167± 0.00153	9.2	severe
S-8	0.06	0.047	0.06	0.0557± 0.00751	13.5	moderate
S-9	0.06	0.0285	0.034	0.0408± 0.0168	41.2	severe
S-10	0.0016	0.0023	0.00178	0.00189± 0.00036	19.2	severe

*Unit of EC_{50} value was %
Unit of coefficient variance was %

Table V. Classification of the surfactant by EC_{50} value on the MATREX study

Range of EC_{50} value	Classification
$5\% \leqq EC_{50}$	No Effect
$0.5\% \leqq EC_{50} < 5\%$	Mild
$0.05\% \leqq EC_{50} < 0.5\%$	Moderate
$EC_{50} < 0.05\%$	Severe

cosinate (S-5: Lauroyl sarcosinate) and sodium polyoxyethylene laurylether sulfate (S-8: POE laurylether sulfate) were classified into "Moderate". The remaining substances, sodium hydrogenated tallow l-glutamate (S-6: HT-glutamate), sodium lauryl sulfate (S-7: SLS), Triton X-100 and benzalkonium chloride (S-10: Benzalkonium chloride) were classified into "Severe". The average coefficient of variation (CV) value for the test chemicals was 0.22 among the three participating laboratories.

Correlation between the results of the MATREX™ and the Draize test

The data of the Draize test for test substances are shown in Table VI, which is derived from Ohno et al[15]. The correlation coefficient between EC_{50} values in MATREX™ and the results of the Draize test are shown in Table VII. The correlation coefficients between EC_{50} value and Draize MAS, cornea, iris, and conjunctiva total scores were 0.633, 0.580, 0.486 and 0.767, respectively (also in Figure 1). These four criteria calculated on the basis of the scores at 24 hrs after exposure were 0.587, 0.578, 0.496 and 0.627 respectively. Those on the basis of area under the curve (AUC; explanation as shown in Table VI) were 0.634, 0.585, 0.045 and 0.735, respec-

Table VI. The Draize test scores

Sample number	Maximum Average Score (MAS)				Scores at 24hrs after Exposure				Area Under the Curve(AUC)****			
	Total*	Cornea	Iris	Conj.**	Total	Cornea	Iris	Conj.	Total	Cornea	Iris	Conj.
S-1	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
S-3	0.7(1)	0	0	0.7(1)	0	0	0	0	0.1	0	0	0.1
S-4	3.3(1)	0	0	3.3(1)	0	0	0	0	0.2	0	0	0.2
S-5	10.3(48)	8.3(48)	0	8(1,4)	8.3	5	0	3.3	3.4	1.9	0	1.5
S-6	26.7(24)	16.7(24,48,72)	1.7(72)	12(4)	26.7	16.7	0	10	14.9	10.7	0.8	3.5
S-7	15(4)	8.3(48,72)	0	10(4)	14.7	6.7	0	10	7.1	4.2	0	3.0
S-8	10(4)	3.3(48)	0	10(4)	2.7	0	0	2	2.0	0.7	0	1.4
S-9	41.3(72)	30(72)	5(168)	10(48)	24.7	15	1.7	8	26.9	18.4	2.3	6.3
S-10	78(24)	66.7(24)	5(96-168)	14.7(96)	78	66.7	0	11.3	57.3	43.9	2.5	10.9

* "Total" means sum of cornea, iris and conjunctiva score
**Conjunctiva ***Observation time of MAS for total
****The area under the curve (AUC) stands for the area under the line connecting scores plotted at each observation period. The parameter used in this study was the ratio of AUC of test chemicals to those based on theoretical maximum of the Draize total score, cornea, iris score and conjunctivae score, respectively.

Table VII. Correlation coefficient and rank correlation between EC_{50} value and the Draize test result

	MAS*	Score at 24 hrs	AUC**
Total	0.633(0.850)	0.587(0.848)	0.634(0.850)
Cornea	0.580(0.741)	0.578(0.833)	0.585(0.814)
Iris	0.486(0.767)	0.496(0.137)	0.045(0.744)
Conjunctiva	0.767(0.932)	0.627(0.860)	0.735(0.850)

* MAS means Maximum Average Score
**Explanation of AUC was shown at Table 6.

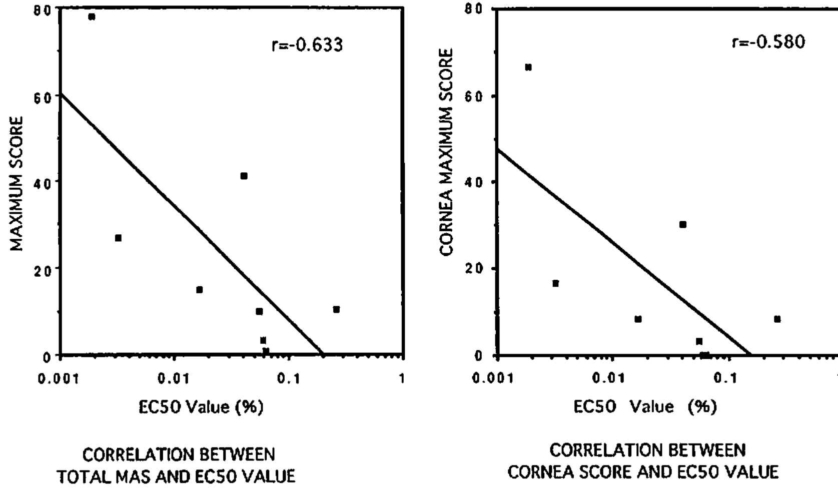

Figure 1-1. Correlation between the draize maximum average score or part of eye score and EC_{50} value

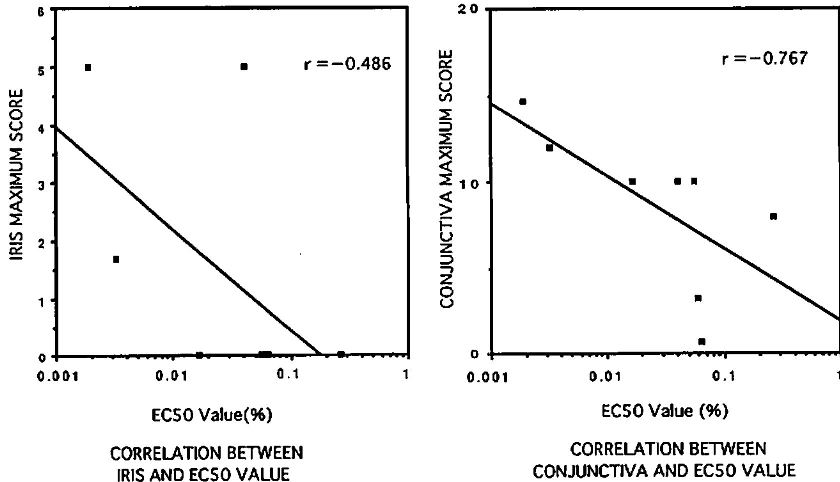

Figure 1-2. Correlation between part of eye score and EC_{50} Value

tively. These data suggest that the results of MATREX™ may correlate better with the changes in conjunctiva than the other parameters.

The rank correlation between the EC_{50} values and Draize test are also shown in Table VII. The rank correlation coefficients between EC_{50} values and the Draize cornea, iris and conjunctiva total scores were 0.850, 0.844, 0.487 and 0.932, respectively. These

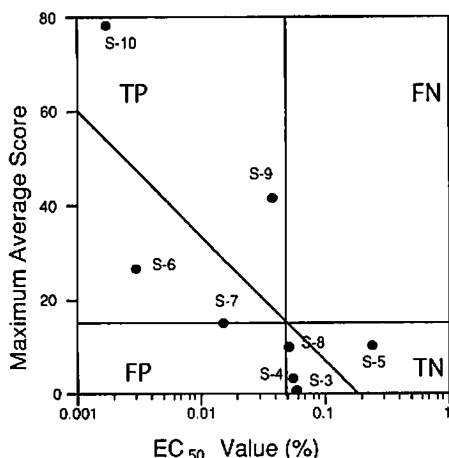

Figure 2. Reproducibility of EC50 value to maximal average score

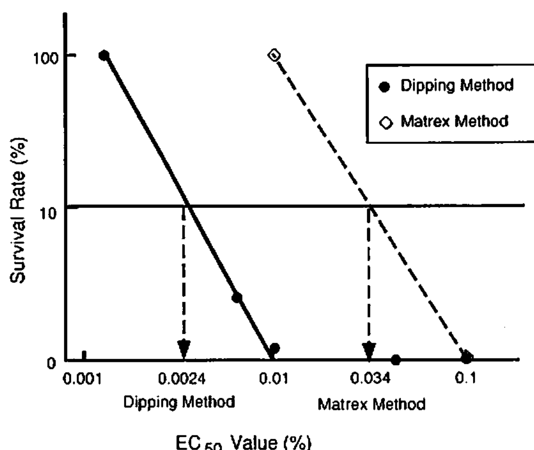

Figure 3. Effect of different exposure method on EC_{50} value at Triton X-100

four criteria calculated on the basis of scores at 24 hrs after exposure were 0.848, 0.833, 0.137 and 0.860 for the rank correlations and against the results of AUC were 0.850, 0.814, 0.744 and 0.850, respectively. Thus, the correlation of total scores, particularly conjunctiva, from Draize test results against EC_{50} values was considered to be high.

For the purpose of comparing these results with those of the other *in vitro* methods validated in the MHW project, predictability of MATREX™ of irritation potential was also assessed by a linear regression formula and the maximum total scores for 8 test chemicals (Figure 2). The results of physiological saline and polyoxyethylene POE hydrogenated caster oil were excluded because of large deviations of EC_{50} values of these substances from those of the other test samples. The cut-off point was set at MAS fifteen, at which score damage to the cornea was not observed. Neither false positive nor false negative results could be shown by this calculation (Figure 2). However, the results for Tween20, PEG monolaurate Lauroyl sarcocinate PEG monolaurate and Triton X-100 seemed to deviate from the regression line. Deviation with Triton X-100 may be related with its lower protein denaturation activity. The reason for the other deviations are uncertain.

Effect of application method on EC_{50} value

The distinctive feature of the MATREX™ method was the ability to apply a test chemical on the surface of the living dermal model. To determine what kind of differences in results may occur, we compared the results of the standard MATREX™ method with those obtained by dipping the whole cell matrix in assay medium containing Triton X-100. The results are shown on Figure 3. The EC_{50} value obtained by the standard MATREX™ method was 0.034%. On the other hand, the EC_{50} value by the dipping method was 0.0024%. Although, both results were classified as "severe" by the MATREX™ classification sheme, the differences in sensitivity was rather large. It seemed to be caused by the differences in the penetration of fthe test substance into the cell matrix. A couple of explanations were considered for the differences in toxicity between the two methods. One possibility is that Triton X-100, which is known to have low protein denaturation activity, was quickly passing through the cell matrix and less damaging to the fibroblasts compared to the dipping application method. The second explanation was opposite to the former explanation: The penetration rate of the chemical compound considerably was slow in the standard method, so that, the chemical could not expose to the cells in the MAT-

REX™ collagen gel by the MATREX™ method than the dipping method. Both explanations were based on penetration rate. Either quick penetration or slow penetration could make the amount of exposure of Triton X-100 less than the dipping method. If we could control penetration rate in MATREX™ to be adjusted to be similar to *in vivo* eye, the detection reliability for eye irritation potential for the MATREX™ method would be increased.

In the case of skin irritation, Jackson suggested that for a chemical having some degree of cytotoxicity potential but a low percutanous absorption rate, the cytotoxicity of the chemical observed *in vitro* studies can be ignored[16]. The same thing may apply to eye irritation; more data are needed to address this point.

In spite of its rather lower correlation coefficient with Draize test, the MATREX™ method may have an advantage of being able to obtain information related to the penetration of chemicals. We propose to evaluate this method for these by using wider range of chemicals during second phase of interlaboratory validation.

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