Original Article

Predicting the results of a 24-hr human patch test for surfactants: utility of margin-setting in a reconstructed human epidermis model

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ABSTRACT — To predict the results of a 24-hr closed human patch test, we previously recommended the use of *in vitro* test with a reconstructed human epidermis (RhE) model adopted in OECD TG 439, and proposed the margin method, which includes evaluation of twice the concentration to avoid a false positive for surfactants. Therefore, in this study, we used LabCyte EPI-MODEL as a RhE model, and confirmed the reproducibility of this method using five surfactants, including benzalkonium chloride (BC), sodium lauryl sulfate (SLS), and lauryl betaine (LB), for which false negative results have previously been reported, and three different surfactants. For all surfactants, prediction of patch test results using a margin of two revealed that human tests could be performed safely, confirming the utility of the margin method. In addition, we examined the relationship with critical micellar concentration (CMC). The IC₅₀ for cell viability in the RhE model for three types of surfactants (BC, SLS, and LB) was 2.7- to 49.7-times the CMC. Therefore, the range of concentrations in which tests were performed with the present method was within the range of concentrations with high cleansing. Furthermore, we examined the relationship between cell viability and release of the inflammatory mediator interleukin-1 α (IL-1 α). IL-1 α release was associated with cell viability, supporting the results of the human patch test.

Key words: Skin irritation, Reconstructed human epidermis, Cytotoxicity, Surfactant, CMC, IL-1 α

INTRODUCTION

Evaluation of skin irritation is essential when evaluating the safety of cosmetics; however, there is no alternative, effective method to evaluate primary skin irritation associated with quasi-drugs and cosmetics (Japan Cosmetic Industry Association, 2015).

We examined the consistency of results obtained with an *in vitro* test, which uses four types of reconstructed human epidermis (RhE) models described in OECD TG 439, and *in vivo* tests (rabbits, 24 hr exposure) using ingredients for cosmetics as test samples, and observed many false negative results due to differences in exposure time. Conversely, evaluation of correspondence with a 24 hr closed human patch test resulted in improved sensitivity and accuracy compared with a primary skin irri-

tation test (rabbits, 24 hr exposure); an *in vitro* test using a RhE model was assumed to be effective for evaluating irritation for humans. However, false negative results for surfactants were noted, and we proposed a method that sets margin of two as a solution (Sugiyama *et al.*, 2018). As reported in OECD TG 439, a survival rate of 50% or less in an *in vitro* test using the RhE model would be classified as an “irritant” in category 2 on the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS), and judged as Classified (C). If the survival rate exceeds 50%, it would be classified as a “non-irritant”, and judged as Not Classified (NC). A method that uses a margin of two predicts that the result of a patch test is “non-irritant” if an *in vitro* test performed with a concentration twice as high as the test concentration has a survival rate exceeding 50%

and is judged NC. Therefore, if the survival rate is 50% or less at a concentration twice as high (C), the patch test result is predicted to be “irritant” at the test concentration. The concept of the method of setting margin 2 is shown below.

Case	Result of <i>in vitro</i> test		Prediction of irritancy at target dose in 24-hr human patch test
	Target dose A (%)	Two times of the target dose 2A (%)	
1	Classified	Classified	irritant
2	Classified	Not Classified	irritant
3	Not Classified	Classified	irritant
4	Not Classified	Not Classified	non-irritant

In this study, we used LabCyte EPI-MODEL as a RhE model, confirmed the reproducibility of benzalkonium chloride (BC), sodium lauryl sulfate (SLS), and lauryl betaine (LB) for which false negatives were previously reported, and examined the efficacy of the method that increased the surfactant concentration and set a margin of two. Furthermore, we examined the physiochemical state using a two-fold increase in concentration and the release of IL-1 α .

MATERIALS AND METHODS

Chemicals

Benzalkonium chloride (BC), cetylpyridinium chloride (CPC), sodium lauryl sulfate (SLS), and lauryl betaine (LB, 35% lauryl dimethylaminoacetic acid solution) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), and laurtrimonium chloride (LC, lauryltrimethylammonium chloride) is a raw material used in cosmetics. Therefore, we used reagents that differed from those used in the previous study (Table 1).

Additional surfactants used were sodium laurate (SL), oleth 10 (OL, Polyoxyethylene [10] oleyl ether), and sodium lauryl glutamate (SLG). SL and OL were purchased from FUJIFILM Wako Pure Chemical Corporation. SLG is a raw material used in cosmetics (Table 2).

Measurement of cell viability using a RhE model

From the four RhE models described in OECD TG 439, we purchased the LabCyte EPI-MODEL24 from Japan Tissue Engineering Limited. The test method followed the LabCyte protocol (LabCyte, 2011).

The purchased RhE model was precultured overnight prior to its use in the test. Three tissues were used to examine 25 μ L of each test sample. Distilled water, which was used to dilute test samples, was used as a negative

control, and 5% SLS aqueous solution was used as a positive control. Following application to the samples for 15 min, test samples were carefully washed with phosphate buffered saline (PBS: Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), and performed post-incubation for 42 hr in a CO₂ incubator. Following post-incubation, culture medium containing 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, FUJIFILM Wako Pure Chemical Corporation) (0.5 mg/mL) was added, samples were placed in a CO₂ incubator for 3 hr, and then extracted with 300 μ L of isopropanol (2-propyl alcohol, FUJIFILM Wako Pure Chemical Corporation). The absorbance of 200 μ L of the extracted liquid was measured at 570 and 650 nm using a microplate reader (BioTek Instruments Inc., Winooski, VT, USA), with the difference in absorbance used as the measurement value. For cell viability, we calculated the viability of each test sample taking the measured value of distilled water as 100%. To evaluate irritation, according to OECD TG 439, a survival rate of 50% or less was classed as a UN GHS category 2 irritant (Classified: C), and a survival rate exceeding 50% was classed as a non-irritant (Not Classified: NC).

For the purpose of examining the reproducibility of cytotoxicity and the utility of the margin 2 method, the test chemicals (BC, CPC, SLS, LB, and LC) included in the previous report (Sugiyama *et al.*, 2018) and additional surfactants (SL, OL, and LG) from which patch test data was available (Kanto *et al.*, 2013) were used. Based on the concentration in the result of the patch test, the concentration was set by the common ratio of 2, mainly in the high concentration region, for the purpose of verifying the margin 2 method and calculating IC₅₀. Table 1 shows the results of the previous study (Run 1; Sugiyama *et al.*, 2018), and those of the reproducibility examination (Run 2, and Run 3).

Measurement of IL-1 α by Enzyme-Linked Immuno Sorbent Assay (ELISA)

To confirm the inflammatory response in the concentration area without a decrease in cell viability predicted to be “irritant” by using the margin 2 method, we measured IL-1 α release levels from chemicals that were false-negative evaluations (BC, SLS, LB), true-positive evaluation (SL), and negative control (OL).

Using LabCyte EPI-MODEL24, we collected the culture medium after washing the test samples (after 15 min application), 6, 24, and 42 hr later. We added new culture medium (0.5 mL) after each collection, and continued culture for up to 42 hr. Collected culture medium was stored at -20°C and thawed for measurement of IL-1 α .

Table 1. Results of skin irritation tests (Cell viability and 24 hr human patch test).

Chemicals		In vivo (24 hr human patch test)			In vitro evaluation using LabCyte						
INCI name (CAS RN®)	Abbreviation	Type of surfactants	Conc. (aq)	S.I.I. ¹⁾	M.E.S. ²⁾	Class ³⁾	Run 1 ⁴⁾		Run 3		
							Classification ⁵⁾ Cell viability (%)	Prediction ⁶⁾	Classification ⁵⁾ Cell viability (%)	Prediction ⁶⁾	
Benzalkonium chloride (8001-54-5)	BC	Cationic surfactant	0.01%				NC	96.6 ± 8.2	unpredictable		
			0.05%								
			0.1%	43.4		I	C	43.4 ± 11.9	I	NC	NI
			0.2%			I	C	15.9 ± 1.1	I	108.4 ± 10.1	NC
			0.4%			I				NC	NC
			0.5%			I	C	15.6 ± 3.6	I	95.6 ± 4.7	NC
			0.8%			I				12.0 ± 7.0	NC
Cetylpyridinium chloride (123-03-5)	CPC	Cationic surfactant	1.0%	105.3		I	C	10.7 ± 2.7	I		
			2.0%			I					
			10.0%			I	C	28.1 ± 2.3	I		
Sodium lauryl sulfate (151-21-3)	SLS	Anionic surfactant	0.5%		0.49 ± 0.51	I			NC	I	
			1.0%	85.5		I	NC	54.7 ± 11.2	I	74.0 ± 8.0	I
			2.0%			I	C	10.3 ± 2.6	I	16.2 ± 10.6	I
			5.0%			I	C	7.8 ± 1.4	I	5.2 ± 1.4	I
										0.6 ± 0.6	I
Lauryl betaine (683-10-3)	LB	Ampholytic surfactant	0.50%				NC	79.9 ± 10.0	NC	I	
			1.0%	50.0		I	NC	67.2 ± 13.7	I	79.9 ± 10.0	I
			2.0%			I	C	11.1 ± 0.8	I	6.8 ± 1.4	I
			4.0%			I				4.3 ± 0.3	I
			5.0%			I	C	12.1 ± 2.8	I	3.9 ± 0.4	I
			8.0%			I				3.4 ± 0.1	I

Table 1. (Continued).

Chemicals		In vivo (24 hr human patch test)			In vitro evaluation using LabCyte							
INCI name (CAS RN®)	Abbreviation	Type of surfactants	Conc. (aq)	S.I.I. ¹⁾	M.E.S. ²⁾	Class ³⁾	Run 1 ⁴⁾		Run 2		Run 3	
							Classification ⁵⁾ Cell viability (%)	Prediction ⁶⁾	Classification ⁵⁾ Cell viability (%)	Prediction ⁶⁾	Classification ⁵⁾ Cell viability (%)	Prediction ⁶⁾
Lauritrimonium chloride (112-00-5)	LC	Cationic surfactant	1.0%	34.2		I	C 20.6 ± 0.8	I	C 13.1 ± 0.3	I		
			2.0%			I		C 4.3 ± 0.2	I			
			10.0%			I	C 8.2 ± 1.6	I				

INCI, International Nomenclature for Cosmetic Ingredient; CAS RN®, Chemical Abstracts Service Registry Number®

1) In human patch tests, the application site was judged using Japanese standard (6 points evaluation; -, ±, +, ++, +++, +++++). Sugai's Irritation Index (S.I.I.) was calculated by giving each classification a rating score (-: 0, ±: 0.5, +: 1, ++: 2, +++: 3, +++++: 4), summing the scores of the subjects, dividing the score by the number of subjects, and multiplying 100. (Sugai, 1995)

2) Mean Evaluation Score (M.E.S.) was calculated by giving each classification a rating score (-: 0, ±: 0.5, +: 1, ++: 2, +++: 3, +++++: 4), summing the scores of the subjects, dividing the score by the number of subjects.

3) When S.I.I. was 30 or more, it was classed as an irritant (I). When S.I.I. was less than 30, it was classed as a non-irritant (NI).

When M.E.S. was 0.3 or more, it was classed as an irritant (I). When M.E.S. was less than 0.3, it was classed as a non-irritant (NI). In the absence of a test result, if irritation was revealed by the test at a lower concentration, it was classed as an irritant (I).

4) The data were quoted from (Sugiyama *et al.*, 2018).

5) Classified (C): Viability ≤ 50%. Not Classified (NC): Viability > 50 %

6) When classification of the *in vitro* test was (C), the patch test was predicted as (I).

When classification with the *in vitro* test was (NC), if classification with the *in vitro* test with double the concentration was (NC), the patch test was predicted as (NI); it would be predicted as (I) with a classification of (C).

Data of the 24 hr human patch test of 1) were quoted from (Sugiyama *et al.*, 2018) and 2) were from (Kanto *et al.*, 2013).

Prediction of surfactant irritation using RhE model

Table 2. Results of skin irritation tests (Cell viability and 24 hr human patch test) for additional surfactants.

Chemicals		Type of surfactant	Conc.(aq)	<i>In vivo</i> (24 hr human patch test)			<i>In vitro</i> evaluation using LabCyte	
INCI name (CAS RN®)	Abbreviation			S.I.I. ¹⁾	M.E.S. ²⁾	Class ³⁾	Classification ⁴⁾ Cell viability (%)	Prediction ⁵⁾
Sodium laurate (193-08082)	SL	Anionic surfactant	1.0%		0.27 ± 0.27	NI	NC 69.8 ± 1.5	I
			2.0%		0.45 ± 0.33	I	C 48.8 ± 18.4	I
			4.0%			I	C 13.1 ± 0.4	I
Oleth-10 (324-33622)	OL	Nonionic surfactat	5.0%		0.18 ± 0.28	NI	NC 83.5 ± 0.4	NI
			10.0%		0.14 ± 0.27	NI	NC 85.1 ± 4.6	unpredictable
Sodium lauryl glutamate (29923-31-7)	SLG	Anionic surfactant	1.0%	9.2		NI	NC 69.5 ± 2.2	NI
			2.0%				NC 80.6 ± 10.0	unpredictable

INCI, International Nomenclature for Cosmetic Ingredient; CAS RN®, Chemical Abstracts Service Registry Number®

1) In human patch tests, the application site was judged using Japanese standard (6 points evaluation; -, ±, +, ++, +++, ++++). Sugai's Irritation Index (S.I.I.) was calculated by giving each classification a rating score (-: 0, ±: 0.5, +: 1, ++: 2, +++: 3, ++++: 4), summing the scores of the subjects, dividing the score by the number of subjects, and multiplying 100. (Sugai, 1995)

2) Mean Evaluation Score (M.E.S.) was calculated by giving each classification a rating score (-: 0, ±: 0.5, +: 1, ++: 2, +++: 3, ++++: 4), summing the scores of the subjects, dividing the score by the number of subjects.

3) When S.I.I. was 30 or more, it was classed as an irritant (I). When S.I.I. was less than 30, it was classed as a non-irritant (NI). When M.E.S. was 0.3 or more, it was classed as an irritant (I). When M.E.S. was less than 0.3, it was classed as a non-irritant (NI). In the absence of a test result, if irritation was revealed by the test at a lower concentration, it was classed as an irritant (I).

4) Classified (C): Viability ≤ 50%, Not Classified (NC): Viability > 50 %

5) When classification of the *in vitro* test was (C), the patch test was predicted as (I). When classification with the *in vitro* test was (NC), if classification with the *in vitro* test with double the concentration was (NC), the patch test was predicted as (NI); it would be predicted as (I) with a classification of (C). The data of the 24 hr human patch test in 2) were from (Kanto *et al.*, 2013) and the data in 1) were from in-house data.

An IL-1α ELISA Kit (Quantikine, R&D Systems, Abingdon, UK) was used to measure IL-1α, we used, and followed the specified protocol. Assay diluent was added to 200 μL of test samples, which were then left for 2 hr at room temperature. After washing three times with washing buffer, we added 200 μL of IL-1α conjugate, and left at room temperature for 1 hr. After washing three times with washing buffer, 200 μL of TMB/H₂O₂ solution was added, samples were shielded from light, and left for 20 min at room temperature. Subsequently, we added reaction stop solution (2N H₂SO₄) and measured the absorbance at 450 and 540 nm within 30 min. Differences in absorbance were taken as the measured values, and the concentration of IL-1α was calculated using a prediction curve (calibration curve) prepared with standard samples.

Measurement of CMC

In order to investigate the physical properties of surfactants at concentrations near the IC₅₀ for which the margin 2 method was valid, we measured the CMCs of BC, SLS and LB which showed false negative evaluations.

We obtained the CMC by measuring the surface tension at 37°C using the Wilhelmy plate method. To measure surface tension, we used a Dynamic Contact Angle

Meter and Tensiometer 21 (Dataphysics Instrument, Filderstadt, Germany). We measured the surface tension of test sample solutions at each concentration, plotted the concentration on a logarithmic plot, and calculated the concentration at which surface tension reached a constant value as the CMC.

RESULTS

Confirmation of reproducibility for cell viability

To confirm the reproducibility of measurements made using LabCyte, we evaluated cell viability with BC, CPC, SLS, LB and LC. The result confirmed reproducibility in a follow-up test using LabCyte excluding 0.1% BC, 1% SLS and 1% LB (Fig. 1).

Utility of the margin two method

Table 1 shows the results of the 24 hr closed human patch test, which were predicted in accordance with a method that set the margin as two, along with *in vitro* test results (survival rate and judgement value). Furthermore, we present the results of a 24 hr human patch test.

For BC, the *in vitro* test was performed twice at 0.1 and 0.2% (Run 2 and Run 3) to predict the 0.1% patch

Fig. 1. Reproducibility of cell viability in *in vitro* test using LabCyte. To confirm the reproducibility, the results of one or two additional tests are shown as Run 1 (◆, Sugiyama *et al.*, 2018), Run 2 (■), and Run 3 (▲). A: Benzalkonium Chloride (BC), B: Sodium Lauryl Sulfate (SLS), C: Lauryl Betaine (LB), D: Laurtrimonium Chloride (LC), E: Cetylpyridinium Chloride (CPC).

test result. The results showed that viability with 0.1% in the three tests (including Run 1) were 43.4, 95.6, and 101.6%, respectively, with UN GHS classifications of C, NC, and NC, which were inconsistent. However, the 0.2% survival rates were 50% or less in Run 2 and Run 3, and were classed as C; thus, the predictions for the 0.1% patch test with the three tests were “irritant”. As such, reproducibility of the method that sets a margin of two was confirmed, and all results were consistent with the patch test results. For CPC, SLS, LB, and LC, reproducibility of the method using a margin of two was con-

firmed, and patch test results were predicted without any false negatives obtained.

Next, to increase the number of surfactant evaluations in the present study, we evaluated SL, OL, and SLG through *in vitro* tests. Table 2 shows the results of the *in vitro* tests and the prediction of patch tests. For SL, we obtained patch test results for 1 and 2%; thus, we performed an *in vitro* test at 1, 2, and 4%. Based on the method that sets a margin of two, the 1% patch test result was predicted as “irritant”. Since patch test results for OL were obtained at 5 and 10%, we performed *in vitro* tests

at 5 and 10%; the survival rate exceeded 50% for all concentrations (NC). However, based on the margin method patch test results were predicted as “non-irritant” at 5%. Solubility was not suitable to evaluate at a concentration of 20%, and the *in vitro* test could not be performed; thus, we were unable to predict patch test results for the 10% concentration. Patch test results for SLG at 1% were obtained and we examined 1 and 2%, where both were determined as NC. Based on the margin method, we predicted that the 1% patch test result would be considered “non-irritant”.

The evaluation of reproducibility, along with the additional test result for surfactants, showed that setting a margin of two predicts the patch test results for all surfactants, without false negatives.

Comparison of CMC and IC₅₀

We obtained the CMC for BC, SLS, and LB (Table 3). To determine the IC₅₀ of the *in vitro* test using LabCyte, we joined the two points before and after the 50% viability with a straight line, and calculated concentration which made 50% viability. Using these values, we divided IC₅₀ by the CMC (IC₅₀/CMC) (Table 3). IC₅₀ values for the *in vitro* test were 0.09-0.16% for BC, 0.71-1.11% for SLS, and 0.70-1.31% for LB, while the IC₅₀/CMC values were 2.7-4.9, 4.7-7.4, and 26.8-49.7 for BC, SLS, and LB, respectively. All IC₅₀ values were higher than the CMC.

Release of IL-1 α

We measured IL-1 α release over time following the application of BC, SLS, LB, SL, and OL to the RhE model, and present the cumulative release in Fig. 2. The results showed that IL-1 α release increased with time with all materials. However, OL resulted in low release at all concentrations, with no dependence on concentration (Fig. 2 [E]).

BC, SLS, LB, and SL presented similar characteristics on graphs. With concentrations of 0.1% or less, BC resulted in low IL-1 α release, with high release at a concentration of 0.2% (Fig. 2 [A]). SLS resulted in low IL-1 α release at 0.5%, and high release at 1% or higher, with no dependence on concentration (Fig. 2 [B]). LB resulted in low IL-1 α release at 0.5%, but high release at 1% or higher, and there was no dependence on concentration (Fig. 2 [C]). SL resulted in low IL-1 α release at 2% or less, with no dependence on concentration, but high release at 4% (Fig. 2 [D]). In all graphs, excluding OL, IL-1 α release was affected by some concentrations of surfactants.

Fig. 3 shows the cumulative IL-1 α release and cell viability at the end of culture. The pattern of cumulative IL-1 α release and cell viability with test samples, other than OL, was similar, where IL-1 α release increased with a decrease in survival rate.

When distilled water was used as the medium, very low levels of IL-1 α were released (2.67-14.98 pg/tissue).

DISCUSSION

Classification of irritation in the 24 hr closed patch test

In the 24 hr closed patch test, there is no set cut-off value to determine an “irritant” from a “non-irritant” (Japan Cosmetic Industry Association, 2015). Therefore, in a previous study, we defined an irritation index (Sugai’s Irritation Index: S.I.I.) of 30 or more as an “irritant”, and of less than 30 as a “non-irritant” (Sugiyama *et al.*, 2018) according to the classification made by Sugai (Sugai, 1995). For the present study, published values for the 24 hr closed patch test were identified (Kanto *et al.*, 2013) for the surfactants added and the concentration used; however, these results were presented using a mean evaluation score (M.E.S.). Patch test results can be pre-

Table 3. The CMC and IC₅₀ for 3 surfactants.

Chemicals		Run	IC ₅₀ (%)	CMC (%)	IC ₅₀ /CMC
INCI name (CAS RN®)	Abbreviation				
Benzalkonium chloride (8001-54-5)	BC	1	0.09	0.033	2.7
		2	0.15		4.7
		3	0.16		4.9
Sodium lauryl sulfate (151-21-3)	SLS	1	1.11	0.149	7.4
		2	0.71		4.7
Lauryl betaine (683-10-3)	LB	1	1.31	0.026	49.7
		2	0.70		26.8
		3	0.91		34.6

CMC, Critical Micellar Concentration; IC₅₀, 50% Inhibitory Concentration
INCI, International Nomenclature for Cosmetic Ingredient; CAS RN®, Chemical Abstracts Service Registry Number®

sented in various ways, and M.E.S. were calculated by summing the scores based on the judgment value of each subject (-: 0, ±: 0.5, +: 1, +++: 2, +++: 3, ++++: 4) and dividing by the number of subjects. This was calculated for each hour of observation. In this study, we used a cut-off value of 0.3 with reference to S.I.I., and used this to examine consistency with the *in vivo* test. Classification

of patch test irritation is shown in Tables 1 and 2.

Efficacy of the margin two method

Overall good reproducibility of the cell viability assessment was confirmed (Fig. 1). For BC, SLS, and LB, the difference in IC₅₀ was at least two-fold (Table 3). When the margin was set as two, the reproducibility of predict-

Fig. 2. Cumulative amount of IL-1α release over time after treatment with surfactants in *in vitro* test using LabCyte. IL-1α release over time following the application of surfactants, just after washing of test samples (after 15 min application), 6, 24, and 42 hr later after washing, were shown. A: Benzalkonium Chloride (BC), B: Sodium Lauryl Sulfate (SLS), C: Lauryl Betaine (LB), D: Sodium Laurate (SL), E: Oleth-10 (OL).

Prediction of surfactant irritation using RhE model

Fig. 3. Cumulative amount of IL-1 α release and cell viability at the end of culture (42 hr) in *in vitro* test using LabCyte. A: Benzalkonium Chloride (BC), B: Sodium Lauryl Sulfate (SLS), C: Lauryl Betaine (LB), D: Sodium Laurate (SL), E: Oleth-10 (OL). The bar chart shows cell viability (%) and the line indicates released IL-1 α .

ed results of BC, CPC, SLS, LB, and LC with available patch test was confirmed.

Furthermore, for all test materials, the patch test predictions and results were consistent, with no false negatives.

For SLS, the viability for the 1% *in vitro* test (Run 1) was 54.7% (NC); however, the viability for 2%, which is two-fold increase in concentration, was only 10.3% (C). Therefore, the patch test result was predicted to be “irritant”. OECD TG 439 states that Run 2 should be performed for a survival rate close to 50% ($50 \pm 5\%$). Pre-

diction results for the 1% patch test in Run 1 and Run 2 were consistent; however, if the result is not clear with the *in vitro* test, the test should be repeated according to the protocol.

Since CPC and LC presented a survival rate of less than 50% (C) at 1%, the patch test result is predicted to be “irritant” up to 0.5%, which is half of 1%. S.I.I. for 1% CPC is high at 105.3; however, based on the method that sets the margin as two, when the 0.5% *in vitro* test result exceeds 50%, the patch test results for half that value (0.25%), can be predicted as “non-irritant”. Therefore,

the highest concentration in this test where the patch test can be safely performed would be 0.25%, which is one-quarter of 1%.

OL has been reported to present very low irritability to skin based on data obtained with 5 and 10% by Kanto *et al.* (2013); however, evaluation by the Cosmetic Ingredient Review reported potential for mild irritation in a 100% primary skin irritation test (rabbits, 24 hr exposure) (Andersen, 1999). In an *in vitro* test of 5 and 10% OL, survival rates were 83.5 and 85.1%, respectively, with no clear decline observed. However, Shibata *et al.* (1997) evaluated Oleth-5, a monolayer culture system that uses keratinocyte with a similar structure to OL, and reported severe irritation ($EC_{50} = 0.58 \mu\text{g/mL}$ [0.000058%]) (Shibata *et al.*, 1997). The difference between those findings and the present study may be due to the different culture system (monolayer culture and RhE model) and exposure time (24 hr direct exposure and 15 min application to the RhE model followed by washing). It is difficult to determine the irritability of a test substance with $\text{LogKow} > 3.5$ using the *in vitro* test with the RhE model (Sugiyama *et al.*, 2018). Since OL has a LogKow of 6.13, its irritability is difficult to determine using the RhE model.

Comparison of CMC and IC_{50}

A previous review (Effendy and Maibach, 1995) reported that phospholipids in the cell membrane become soluble, damaging cells, which in turn leads to cytotoxicity. Similarly, events such as the elution of protein and the release of inflammatory mediators, cause inflammation. At low concentrations, monomolecularly dispersed monomers impact the fluidity of the cell membrane. Thus, to detect inflammation using liposomes or monolayer culture, concentrations near the CMC indicate cytotoxicity (Itagaki and Yamaguchi, 1989) (Aránzazu Partearroyo *et al.*, 1990). Conversely, lipid removal and adsorption to keratin protein may be performed effectively at concentrations higher than the CMC (Kawai and Okamoto, 1978; Ananthapadmanabhan *et al.*, 1996).

In this study, we measured the CMC and found that SLS was slightly lower than previous reports of 0.2% (Lémery *et al.*, 2015) and 0.23% (Khan and Shah, 2008), and the result obtained with BC was similar to that obtained previously, at 0.02% (Deutschle *et al.*, 2006).

When we compared the IC_{50} of BC, SLS, and LB with the CMC, we found that the concentration range was higher than the CMC (Table 3). The surfactant concentration used in the present study had high cleansing action, and effectively removed lipid; thus, it effectively weakened barrier function. There was a difference of several-fold between the CMC of SL and LB; however, the IC_{50}

was similar. This is likely due to the very high cleansing action of SLS and its protein solubility.

Yanochko *et al.* (2010) used monolayer culture and a three-dimensional model (stratified culture model) to examine the IC_{50} (applied concentration-cytotoxicity curve) following application of BC for the same exposure time, and reported that with monolayer culture, cells were impacted at a concentration 1/100 that of the three-dimensional model. The IC_{50} of SLS was reported to be $44.67 \mu\text{g/mL}$ by cytotoxicity evaluation in a 24 hr exposed monolayer culture that used the MTT incorporation method with keratinocyte (Sanchez *et al.*, 2004); this was 1/156-1/248 that observed in the present examination. It was thought that the dose of surfactant monomers hardly increased by making the dose twice as high as the target dose because the concentration around IC_{50} , where the margin 2 method is useful, is much higher than the CMC. Therefore, when test samples with concentrations above the CMC were used, adsorption to stratum corneum protein and stratum corneum barrier function diluted the exposure concentration of surfactant to keratinocyte, and the concentration that impacted keratinocyte was estimated to be substantially lower than the CMC for the monomer.

Release of IL-1 α

To evaluate irritation caused by surfactant using a biochemical approach, the utility of inflammatory mediators, IL-1 α and IL-8, has been reported (Shibata *et al.*, 1997; Coquette *et al.*, 2003). IL-1 α , an inflammatory mediator, is released when skin is irritated, and acts as the first 'switch' to initiate inflammation (Coquette *et al.*, 2000).

In the present study, there was an associated between cumulative IL-1 α release and cell viability at the end of culture; cumulative IL-1 α release increased with decreasing cell viability. However, even when there was no clear decrease in cell viability, IL-1 α release may still occur. With 0.5% SLS, the amount of IL-1 α released up to 24 hr post-culture was low, but increased subsequently, and at 42 hr post culture, cumulative IL-1 α release increased to the same level, or higher, than that observed with 1% SLS. The same trend was observed for 0.5% LB and $\leq 2\%$ SL. The pattern of IL-1 α release when there was no effect on cell viability was biphasic. IL-1 α is stored in cytoplasm or bound in membrane in keratinocytes and released when cells are damaged. Furthermore, endogenous IL-1 α interacts with IL-1 receptor I in the cell membrane following its release, producing more IL-1 α , with secondary production of IL-6 and IL-8 (Dinarello, 1998; Shivji *et al.*, 1994; Sims *et al.*, 1993). Therefore, 0.5% SLS, 0.5% LB, and $\leq 2\%$ SL resulted in the release

of endogenous IL-1 α during cell death, and subsequently, the secondary production of keratinocyte led to IL-1 α release of. Thus, even when irritability was predicted to be weak based on cell viability, IL-1 α may be released, causing an inflammatory reaction. Even if the cell viability does not decrease at the target dose, an inflammatory reaction may occur when IL-1 α is released. This suggested the validity of judging the target dose as “irritant” by using the margin 2 method.

In conclusion, when performing an *in vitro* test that uses the RhE model to predict the result of a 24 hr closed human patch test, the margin method that doubles the concentration of surfactant is effective for avoiding false negatives.

Conflict of interest---- The authors declare that there is no conflict of interest.

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