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두 가지 단계적 접근법을 이용한 30종 화학물질의 안자극성 평가

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Evaluation of Eye Irritation Potential for 30 Chemicals using Two Tiered Approaches

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ABSTRACT. Various alternative test methods are being developed to replace *in vivo* Draize rabbit test that evaluates eye irritation. However, a single alternative method has difficulty to be applied in safety evaluation on substances, because it cannot fully replace the *in vivo* test by simulating only part of the *in vivo* system. For this reason, different studies using several alternative test methods and test results of literatures have been under way. Our previous study suggested effective tiered approaches using three tests among Short Time Exposure (STE), Bovine Corneal Opacity and Permeability (BCOP), Hen's Egg Test-Chorioallantoic Membrane (HET-CAM), re-constructed human cornea epitheliums (RhCE) tests. This study aimed to evaluate eye irritation potential for 30 new test chemicals using two tiered approaches. According to the data generated by direct test performance and literature survey, the accuracy, sensitivity and specificity of the two tiered approaches were 83.3%~86.7%, 93.3%, 73.3%~ 80%, respectively. Furthermore, the accuracy, sensitivity, and specificity of the two tiered approaches by applying combined data against 30 test chemicals in our present study and 47 test chemicals in previous study were 90.9%~92.2%, 95.9%~98.0%, 78.6%~85.7%. Consequently, the two tiered approaches may be used to identify between irritants and non-irritants to replace *in vivo* test.

KEY WORDS: tiered approach, eye irritation, predictive capacity

Introduction

Since the Draize rabbit eye test developed by Draize in 1944 was adopted by the Organization for Economic Cooperation and Development (OECD) Test Guideline (TG) 405 (Draize et al., 1944, OECD TG 405, 1981), it has been used for evaluation of chemicals, cosmetics, medical devices for many years and classifies all ranges of United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) classification on serious eye damage and eye irritation depending on severity of lesions in conjunctiva, cornea, and iris and how long it persists (Settivari et al., 2016, wilson, 2015) after exposure to test chemicals. Starting with ban on animal test on cosmetic products in the European Union (EU) in 2003 (Directive 2003/15/EC, 2003) by implementing the principles of 3Rs (Replacement, Reduction,

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and Refinement), EU prohibited animal testing on all cosmetic ingredients in 2013 (Regulation EC 1223/2009, 2009). Accordingly, other countries have also prohibited animal testing on cosmetic ingredients. The Republic of Korea has also prohibited distributing and selling cosmetic products and cosmetic ingredients for which animal testing was conducted since 2017 under Korean Cosmetics Act. In accordance with these changes, a number of alternative test methods using cell lines, organotypic models, re-constructed human cornea-like epitheliums (RhCE), re-constructed human cornea epitheliums, or macromolecule to replace the Draize test were developed. The test methods using cell lines are Fluorescein Leakage (FL), Short Time Exposure (STE), Cytosensor-Microphysiometer (CM), Neutral Red Release (NRR) test methods, etc. The test methods using organotypic models are Bovine Corneal Opacity and Permeability (BCOP), Isolated Chicken Eye (ICE), Isolated Rabbit Eye (IRE), Hen's Egg Test-Chorioallantoic Membrane (HET-CAM) test methods, etc. The test methods using RhCE are EpiOcular^{TM} Eye Irritation Test (EIT) and the test methods using re-constructed human cornea epitheliums (RhCE) are SkinEthic Human Corneal Epithelium (HCE) EIT, LabCyte-CORNEA-MODEL24 EIT, MCTTHCE^{TM} EIT, or Vitrigel-EIT. Of those, FL test (TG 460, 2017a), STE test (TG 491, 2018a), BCOP test (TG 437, 2017b), ICE test (TG 438, 2018b), EpiOcular^{TM} EIT, SkinEthic HCE EIT, LabCyte-CORNEA-MODEL24 EIT, MCTTHCE^{TM} EIT (TG 492, 2019a), Vitrigel EIT (TG 494, 2019b), and macromolecular test (TG 496, 2019c) were adopted as the officially validated test methods in OECD. These test methods identify Category1 (Cat. 1) and/or No Category (No Cat.).

While many alternative test methods have been developed, a single *in vitro* test has difficulty in evaluating eye irritation of chemicals as the alternative test methods have limitations to replace *in vivo* biological system. Therefore, tiered approach strategies combining different *in vitro* tests were developed. Kolle et al. (2011) presented Top-Down/Bottom-Up approaches combining BCOP test and EpiOcular^{TM} EIT. Hayashi et al. (2012a, 2012b) proposed Bottom-Up approaches which combine STE and BCOP tests or which uses BCOP test after performance of STE test or EpiOcualr^{TM} EIT according to solubility in three solvents (saline, 5% dimethyl sulfoxide (DMSO) in saline, ormineral oil). Settivari et al., (2016) suggested a tiered approach using NRR test and EpiOcular^{TM} EIT conjointly, and Adrianes et al., (2018) published Top-Down/Bottom-Up approaches combining BCOP LLBO and RhCE (SkinEthic HCE or EpiOcular^{TM}) tests or BCOP OP-KIT, Slug Mucosal Irritation (SMI), RhCE tests. Recently, Alépée et al., (2019a) developed Bottom-Up approach combining STE test and BCOP LLBO. The STE test used in the Bottom-Up approach showed improved false negative rate by applying mineral oil as solvent to high volatile substances based on Abo et al., (2018). Also, Bottom-Up approach combining physicochemical properties (water solubility, octanol-water partition coefficient (LogP), vapor pressure, and surface tension), RhCE (SkinEthic, EpiOcular^{TM}) test and BCOP LLBO was proposed by Alépée et al., (2019b). In addition, OECD suggested the Guidance Document No. 263 on an Integrated Approach on Testing and Assessment (IATA) for Serious Eye Damage and Eye Irritation in 2017 (OECD, 2017c). The Guidance Document recommends referring to "Existing information & non-testing methods" (Part 1), and "Weight-of-evidence analysis" (Part 2) in order before performing tests (Part 3) for decision on classification and labelling and contains a detailed description of each module in each part.

Our previous study suggested two tiered approaches combining the STE, BCOP, RhCE tests or the BCOP, HET-CAM, RhCE tests as Top-Down approach, or conducting EpiOcular^{TM} EIT if only certain prediction is not made by two tests after conducting STE and BCOP tests or HET-CAM and BCOP tests simultaneously (Ko et al., 2020). This study aimed to evaluate eye irritation potential for 30 new test chemicals using the two tiered approaches.

Materials and Methods

1. Test chemicals

A total of 30 test chemicals consisting of 7 UN GHS Cat. 1, 8 UN GHS Category 2 (Cat. 2), and 15 UN GHS No Cat. were analyzed in the present study. The 30 test chemicals were selected based on European Centre for Ecotoxicology and Toxicology of Chemicals database. Prior to performing the STE, BCOP, HET-CAM, and EpiOcular™, the test results of the 4 methods were obtained from literatures. If test results on some test chemicals do not exist or show inconsistent results with the *in vivo* UN GHS Category, the 4 tests were conducted for prediction on eye irritation of test chemicals. Also, if test results between literatures were inconsistent or test results are borderline results, the tests were conducted. The list, literature data, and experimental data on the 30 test chemicals are presented in Table 1. Among the test chemicals used to carry out experiment, quinacrine, lauric acid, and 1,2,4-Triazole, sodium salt were purchased from TCI (Tokyo, Japan) and myristyl myristate was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). cis-Cyclo-octene was purchased from Wako Chemicals (Richmond, VA, USA), while the other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2. Test methods

A detailed description of all test protocols using *in vitro* test methods are presented by Ko et al., 2020. Among the four test methods, the STE test was performed according to OECD TG 491 (2015) and Statens Seruminstitut Rabbit Cornea (SIRC) cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). The BCOP test was followed according to OECD TG 437 (2017b) and the eyes were obtained from slaughtered cows (mean age of 31 months old) at an abattoir (Kyungsin Co. Ltd., Kyungsan, Korea). The HET-CAM test was performed as described in DB-ALM Protocol nº 96 (EURL ECVAM, 2010). The EpiOcular™ EIT was followed according to OECD TG 492 (2019a) and EpiOcular™ tissue models were purchased from MatTek Co. (Ashland, OR, USA).

3. Prediction models of two tiered approaches

The two tiered approaches were carried out using the procedure of Ko et al., (2020). The two tiered approaches consist of the STE, BCOP, HET-CAM, RhCE tests. The tiered approach A begins with the STE test. If some chemicals were classified as No prediction can be made (No pre) or No Cat. in the STE test, the BCOP test was used to classify Cat. 1, No pre and No Cat. as the second stage. If some chemicals were classified as No pre and No Cat. in the BCOP test, the RhCE test was used as the final stage. The use of STE test as the first stage of the tiered approach A is limited to test chemicals that are dissolved or uniformly suspended for at least 5 minutes in three solvents (saline, 5% dimethyl sulfoxide in saline, or mineral oil). Therefore, some test chemicals that are not dissolved or uniformly suspended for at least 5 minutes in the three solvents were began with the BCOP test for predicting Cat. 1. If some chemicals were classified as No pre or No Cat. in the BCOP test, the HET-CAM test should be conducted. If some chemicals were classified as "Slight" or "Mild" in the HET-CAM test, the RhCE test determined the final decision.

In the tiered approach B, the STE and BCOP tests were used simultaneously at the first stage. If the results of both tests are either positive or negative, the eye irritation potency for test chemicals was finally determined from the STE and BCOP tests. However, if the BCOP and STE tests had discordant classification, the RhCE test should be employed at the final step. The final decision of hazard identification is determined by the result of RhCE test. If the test chemicals were insoluble in tiered approach B, the HET-CAM and BCOP tests were used simultaneously at

Table 1. Prediction capacities of eye irritation potency obtained by direct test performance and literature survey against 30 test chemicals

No.	Test chemicals	CAS no.	Physical state	UN GHS Category	Predictions							
					STE		BCOP		HET-CAM		EpiOcular™	
					Exp & Liter.	F D	Exp & Liter.	F D	Exp & Liter.	F D	Exp & Liter.	F D
1	Sodium hydroxide (10%)	1310-73-2	Liquid	Cat. 1	No pre	Δ	Severe[7], Very severe[3], Very severe irritant[12], Cat.1[11]	O	Severe[7,18], Severely irritating[20]	O	Cat. 1/2[11]	Δ
2	Trichloroacetic acid (30%)	76-03-9	Liquid	Cat. 1	No pre	Δ	Cat. 1[11,16], Severe irritant[1], Very severe[7], Very severe irritant[12]	O	Severe[7,18]	O	Cat. 1/2[11]	Δ
3	Quinacrine	69-05-6	Solid	Cat. 1	No pre[15]	Δ	Not labeled[7], Moderate irritant[2],No Cat.[15]	X	Moderate[13,18], Non irritant[7] Severely irritating	Δ	I[8,9,14], No pre[15]	Δ
4	p-Fluoroaniline	371-40-4	Liquid	Cat. 1	–	–	Moderate[3,7], Moderate irritant[1]	Δ	Severely irritating	O	I[8]	Δ
5	Sodium lauryl sulphate (15%)	151-21-3	Liquid	Cat. 1	Severe irritant[5]	O	Mild[3], Severe[7], Severe irritant[12], Not Cat1[11], Moderate irritant[19]	Δ	Severe[7,18]	O	Cat. 1/2[11]	Δ
6	Lauric acid	143-07-7	Solid	Cat. 1	No pre	Δ	No pre[15]	Δ	Severely irritating	O	No pre[15]	Δ
7	1,2,4-Triazole, sodium salt	41253-21-8	Solid	Cat. 1	No pre	Δ	Category 1	O	Moderately irritating	Δ	I	Δ
8	Triton X-100 (5%)	9002-93-1	Liquid	Cat. 2	Moderate irritant[10]	Δ	Severe irritant[17], Moderate/Very severe irritant[12], Very Severe[7], Mild[3], Not Cat1[11,12]	Δ	Severe[7]	O	I[8,9,14], Cat. 1/2[11]	Δ
9	L-Aspartic acid	70-47-3 / 56-84-8	Solid	Cat. 2	–	–	Not labeled[7],NI[1] No Cat	X	Moderately irritating	Δ	NI	X
10	Potassium cyanate	590-28-3	Solid	Cat. 2	No Cat	X	Mild irritant[1]	Δ	Severe[7]	O	I	Δ
11	2,6-Dichlorobenzoyl chloride	4659-45-4	Liquid	Cat. 2	Minimal irritant[6]	X	No accurate/reliable prediction[16], NSI[11], NI or mild irritant[1], Mild irritant[12]	Δ	Severe[7,18]	O	Cat. 1/2[11], I[8,14]	Δ
12	Methyl cyanoacetate	105-34-0	Liquid	Cat. 2	Moderate irritant[5,10] Minimal irritant[10] No Cat.[15] No Cat.	X	Mild[7], Mild irritant[1], No pre[15]	Δ	Moderate[7]; Severe[18] Irritating	Δ	I[8,14], No Cat.[15]	Δ
13	Maneb	12427-38-2	Solid	Cat. 2	–	–	Mild[7] No pre	Δ	Irritating	Δ	I[8,14]	Δ

14	2-Methyl-1-pentanol	105-30-6	Liquid	Cat. 2	Moderate irritant[4,5,6], No pre[15]	Δ	Severe irritant[17],No pre[15]	Δ	Irritating	Δ	I[8,9,14], No pre[15]	Δ
15	n-Hexanol	111-27-3	Liquid	Cat. 2	Moderate irritant[4,5,6]	Δ	No pre Severe irritant[17], Severe[7], Very severe[7] No pre	O	Severe[7]	O	I[8,9,14]	Δ
16	2-Ethoxyethyl methacrylate	2370-63-0	Liquid	No Cat.	No Cat	X	No Cat	X	Slightly irritating	X	I	Δ
17	1,6-Dibromohexane	629-03-8	Liquid	No Cat.	No Cat	X	No Cat	X	Slightly irritating	X	NI	X
18	1-Bromo-4-chlorobutane	6940-78-9	Liquid	No Cat.	No Cat	X	No pre	Δ	Slightly irritating	X	I	Δ
19	Myristyl myristate	3234-85-3	Solid	No Cat.	No Cat	X	No Cat	X	Slightly irritating	X	NI	X
20	n-Hexyl bromide	111-25-1	Liquid	No Cat.	No Cat	X	No pre	Δ	Slightly irritating	X	NI	X
21	Ethyleneglycol diethyl ether	629-14-1	Liquid	No Cat.	No Cat	X	No pre	Δ	Slightly irritating	X	I	Δ
22	4,4-Methylene bis-(2,6-di-tert-butylphenol)	118-82-1	Solid	No Cat.	–	–	No Cat	X	Slightly irritating	X	NI	X
23	p-Methylthiobenzaldehyde	3446-89-7	Liquid	No Cat.	–	–	No pre	Δ	Slightly irritating	X	I	Δ
24	2,4-Difluoronitrobenzene	446-35-5	Liquid	No Cat.	–	–	No Cat	X	Slightly irritating	X	NI (borderline)[8] NI	X
25	cis-Cyclo-octene	931-87-3	Liquid	No Cat.	No Cat	X	No pre	Δ	Slightly irritating	X	NI (borderline)[8] NI	X
26	2,4-Pentanediol	625-69-4	Liquid	No Cat.	Minimal irritant[5]	X	No pre	Δ	Moderately irritating	Δ	NI[8]	X
27	Di-iso-butyl ketone	108-83-8	Liquid	No Cat.	Minimal irritant[5]	X	No pre	Δ	Slightly irritating	X	NI[8,14]	X
28	3-Methylhexane	589-34-4	Liquid	No Cat.	Minimal irritant[5]	X	No Cat	X	Slightly irritating	X	NI[8,14]	X
29	Iso-Octyl acrylate	29590-42-9	Liquid	No Cat.	Minimal irritant[5]	X	No Cat	X	Slightly irritating	X	NI[8,14]	X
30	n-Octyl bromide	111-83-1	Liquid	No Cat.	Minimal irritant[5]	X	No Cat	X	Slightly irritating	X	NI[8]	X

Exp & Liter : Experimental data & Literature data; FD : Final decision
O: Severe irritant, severely irritating, Cat 1; △: Moderate irritant, mild irritant, no prediction can be made, irritating, moderately irritating; X: No Category, minimal irritant, slightly irritating -: Insoluble chemicals in saline, 5% DMSO in saline, or mineral oil; I: Irritant; NI: Non-irritant; No pre: No prediction can be made;
[1]Verstraelen *et al*., 2013; [2]Gautheron*et al*., 1994; [3]Southee*et al*., (1998); [4]Takahashi*et al*., 2009; [5]Takahashi*et al*., 2011; [6]Sakaguchi*et al*., 2011; [7]Balls*et al*., 1995; [8]Kaluzhny*et al*., 2011; [9]Pfannenbecker*et al*., 2013; [10]Kojima*et al*., 2013; [11]Kolle*et al*., 2011; [12]Schrage*et al*., 2011; [13]Gilleron*et al*., 1996; [14]Kaluzhny*et al*., 2015; [15]Adrianes*et al*., 2018; [16]OECDTG437(2013a);[17]VanGoethem*et al*. (2006); [18]Gilleron*et al*., 1997; [19]Lee*et al*., 2007; [20]Steiling*et al*., 1999

the first stage. If the results of both tests are either positive or negative, the decision for eye irritation potency is based on the results of the BCOP test. However, if the BCOP and HET-CAM tests had discordant classification, the RhCE test should be employed in the final step.

4. Predictive capacities of in vitro eye irritation test methods and the two tiered approaches

This study evaluated the predictive capacities of *in vitro* test methods and two tiered approaches by comparing them with UN GHS Category of 30 test chemicals. Predictive capacities were calculated with sensitivity (the percentage of positives that are correctly identified), specificity (the percentage of negatives that are correctly identified) and accuracy (the percentage of all that correctly identified).

Results

1. Performance of in vitro test methods for classification of eye irritation

In this study, chemicals not having eye irritation data, having inconsistent results with the *in vivo* UN GHS Category, having inconsistent results between literatures, or having borderline results were tested by the *in vitro* test methods respectively. We performed experiment directly with 13 test chemicals in the STE test, 20 test chemicals in the BCOP test, 24 test chemicals in the HET-CAM test, 13 test chemicals in the EpiOcular™ EIT and the result of each test through direct test performance is presented in Table 1~4 of supplementary material.

Prior to conducting the STE test, solubility assay for 19 test chemicals was carried out using Saline, 5% DMSO in saline, or mineral oil according to OECD TG 491 (2015). As a result, 6 test chemicals were not dissolved in all three solvents. Thus, these test chemicals were excluded from the STE test and finally 13 test chemicals were tested. As a result, 7 out of the 13 test chemicals were consistent with the *in vivo* UN GHS classification. However, 6 test chemicals (No. 1, 2, 6, 7, 10 and 12) were under-predicted as No pre or No Cat. (Table 1).

The BCOP test was performed with 20 test chemicals. As a result, 12 out of the 20 test chemicals tested were consistent with the *in vivo* UN GHS classification. However, 1 chemical (No. 9) was under-predicted as No Cat. And 7 chemicals (No. 18, 20, 21, 23, 25, 26 and 27) were over-predicted as No pre (Table 1).

The HET-CAM test was performed with 24 test chemicals. As a result, 23 out of the 24 test chemicals tested were consistent with *in vivo* UN GHS classification. However, 1 chemical (No. 26) was over-predicted as moderately irritating (Table 1).

The EpiOcular™ EIT was performed with13 test chemicals. As a result, 8 out of 13 test chemicals tested were consistent with *in vivo* UN GHS classification. However, L-aspartic acid (No. 9) was shown to be false negative and 4 chemicals (No. 16, 18, 21 and 23) were shown to be false positive (Table 1).

2. Predictive capacities of each in vitro test method against 30 test chemicals

This study collected eye irritation data through literature for test chemicals, and some data were obtained by performing experiments directly. In case different prediction results were generated on a chemical, final decision was made by the majority of the prediction results. The result of the final decision for each test method was shown in Table 1, and the predictive capacities were shown in Table 2A~B.

The predictive capacities of the STE test were accuracy of 87.5% (21/24), sensitivity of 75% (9/12), and specificity of 100% (12/12). In the BCOP test, the accuracy, sensitivity and specificity were 70.0% (21/30), 86.7% (13/15), 53.3% (8.15), respectively; and the HET-CAM test were 96.7% (29/30), 100% (15/15), and 93.3% (14/15), respectively. In the EpiOcular™ EIT, the accuracy, sensitivity

Table 2A. Results of the STE test through direct test performance

No.	Test chemicals	CAS No.	UN GHS Category	Solvent	5% CV	0.05% CV	Prediction
1	Sodium hydroxide (10%)	1310-73-2	Cat. 1	Saline	12.1 ± 2.9	111.1 ± 13.4	No pre
2	Trichloroacetic acid (30%)	76-03-9	Cat. 1	Saline	9.6 ± 0.3	104.9 ± 2.2	No pre
3	Lauric acid	143-07-7	Cat. 1	MO	19.8 ± 5.6	95.4 ± 12.8	No pre
4	1,2,4-Triazole, sodium salt	41253-21-8	Cat. 1	Saline	10.0 ± 2.4	89.0 ± 4.3	No pre
5	Potassium cyanate	590-28-3	Cat. 2	Saline	101.2 ± 12.1	92.74 ± 3.16	No Cat.
6	Methyl cyanoacetate	105-34-0	Cat. 2	Saline	104.9 ± 13.7	107.7 ±4 .01	No Cat.
7	2-Ethoxyethyl methacrylate	2370-63-0	No Cat.	MO	90.1 ± 9.4	97.2 ± 10.5	No Cat.
8	1,6-Dibromohexane	629-03-8	No Cat.	MO	95.5 ± 10.8	112.8 ± 10.8	No Cat.
9	1-Bromo-4-chlorobutane	6940-78-9	No Cat.	MO	93.7 ± 4.2	97.5 ± 6.9	No Cat.
10	Myristyl myristate	3234-85-3	No Cat.	MO	97.0 ± 7.4	106.9 ± 3.4	No Cat.
11	n-Hexyl bromide	111-25-1	No Cat.	MO	91.8 ± 5.0	97.1 ± 9.7	No Cat.
12	Ethyleneglycol diethyl ether	629-14-1	No Cat.	Saline	89.6 ± 3.9	111.4 ± 7.7	No Cat.
13	cis-Cyclo-octene	931-87-3	No Cat.	MO	99.1 ± 7.8	105.7 ± 3.4	No Cat.

CV: Cell viability; No pre: No prediction can be made; MO: mineral oil

Table 2B. Results of the BCOP test through direct test performance

No.	Test chemicals	CAS No.	UN GHS Category	Opacity	Permeability	IVIS	Prediction
1	1,2,4-Triazole, sodium salt	41253-21-8	Cat. 1	82.5 ± 3.3	2.1 ± 0.2	113.3 ± 5.5	Cat. 1
2	L-Aspartic acid	70-47-3 / 56-84-8	Cat. 2	0.3 ± 0.4	0.0 ± 0.0	0.6 ± 0.7	No Cat.
3	Maneb	12427-38-2	Cat. 2	23.3 ± 0.4	0.1 ± 0.0	24.5 ± 0.2	No pre
4	2-Methyl-1-pentanol	105-30-6	Cat. 2	13.1 ± 0.2	1.7 ± 0.1	38.1 ± 1.8	No pre
5	n-Hexanol	111-27-3	Cat. 2	17.2 ± 4.7	1.9 ± 0.1	37.2 ± 9.3	No pre
6	2-Ethoxyethyl methacrylate	2370-63-0	No Cat.	1.6 ± 0.5	0.0 ± 0.0	1.7 ± 0.1	No Cat.
7	1,6-Dibromohexane	629-03-8	No Cat.	0.5 ± 0.6	0.0 ± 0.0	0.6 ± 0.9	No Cat.
8	1-Bromo-4-chlorobutane	6940-78-9	No Cat.	4.1 ± 0.2	0.0 ± 0.1	4.6 ± 1.1	No pre
9	Myristyl myristate	3234-85-3	No Cat.	1.8 ± 1.4	0.0 ± 0.0	1.9 ± 1.4	No Cat.
10	n-Hexyl bromide	111-25-1	No Cat.	4.9 ± 1.1	0.2 ± 0.0	8.2 ± 0.7	No pre
11	Ethyleneglycol diethyl ether	629-14-1	No Cat.	7.0 ± 2.5	1.9 ± 0.1	35.9 ± 1.6	No pre
12	4,4-Methylene bis-(2,6-di-tert-butylphenol)	118-82-1	No Cat.	1.4 ± 2.3	0.1 ± 0.0	2.2 ± 2.5	No Cat.
13	p-Methylthiobenzaldehyde	3446-89-7	No Cat.	4.8 ± 0.9	0.0 ± 0.0	5.1 ± 0.9	No pre
14	2,4-Difluoronitrobenzene	446-35-5	No Cat.	1.6 ± 1.3	0.0 ± 0.0	1.6 ± 1.4	No Cat.
15	cis-Cyclo-octene	931-87-3	No Cat.	4.2 ± 1.0	0.3 ± 0.0	7.2 ± 3.6	No pre
16	2,4-Pentanediol	625-69-4	No Cat.	3.3 ± 0.6	0.1 ± 0.0	4.5 ± 1.3	No pre
17	Di-iso-butyl ketone	108-83-8	No Cat.	3.6 ± 0.8	0.1 ± 0.1	4.8 ± 1.4	No pre
18	3-Methylhexane	589-34-4	No Cat.	0.7±0.8	0.0 ± 0.0	1.2 ± 0.9	No Cat.
19	Iso-Octyl acrylate	29590-42-9	No Cat.	-0.2±0.7	0.0 ± 0.0	-0.8 ± 0.7	No Cat.
20	n-Octyl bromide	111-83-1	No Cat.	0.0±1.0	0.0 ± 0.0	-0.3 ± 1.2	No Cat.

No pre: No prediction can be made

Table 2C. Results of the HET-CAM test through direct test performance

No.	Test chemicals	CAS No.	UN GHS Category	Q, S score	Irritation score	Prediction
1	Quinacrine	69-05-6	Cat. 1	S	16	Severely irritating
2	p-Fluoroaniline	371-40-4	Cat. 1	S	18	Severely irritating
3	Lauric acid	143-07-7	Cat. 1	S	16	Severely irritating
4	1,2,4-Triazole, sodium salt	41253-21-8	Cat. 1	S	8.0	Moderately irritating
5	L-Aspartic acid	70-47-3 / 56-84-8	Cat. 2	S	11	Moderately irritating
6	Methyl cyanoacetate	105-34-0	Cat. 2	Q	1.2	Irritating
7	Maneb	12427-38-2	Cat. 2	S	15	Irritating
8	2-Methyl-1-pentanol	105-30-6	Cat. 2	Q	1.7	Irritating
9	n-Hexanol	111-27-3	Cat. 2	Q	1.5	Irritating
10	2-Ethoxyethyl methacrylate	2370-63-0	No Cat.	Q	0.8	Slightly irritating
11	1,6-Dibromohexane	629-03-8	No Cat.	Q	0.0	Slightly irritating
12	1-Bromo-4-chlorobutane	6940-78-9	No Cat.	Q	0.0	Slightly irritating
13	Myristyl myristate	3234-85-3	No Cat.	S	1.0	Slightly irritating
14	n-Hexyl bromide	111-25-1	No Cat.	Q	0.0	Slightly irritating
15	Ethyleneglycol diethyl ether	629-14-1	No Cat.	Q	0.3	Slightly irritating
16	4,4-Methylene bis-(2,6-di-tert-butylphenol)	118-82-1	No Cat.	S	0.0	Slightly irritating
17	p-Methylthiobenzaldehyde	3446-89-7	No Cat.	S	2	Slightly irritating
18	2,4-Difluoronitrobenzene	446-35-5	No Cat.	S	1	Slightly irritating
19	cis-Cyclo-octene	931-87-3	No Cat.	Q	0.0	Slightly irritating
20	2,4-Pentanediol	625-69-4	No Cat.	Q	0.9	Moderately irritating
21	Di-iso-butyl ketone	108-83-8	No Cat.	Q	0.0	Slightly irritating
22	3-Methylhexane	589-34-4	No Cat.	Q	0.0	Slightly irritating
23	Iso-Octyl acrylate	29590-42-9	No Cat.	Q	0.0	Slightly irritating
24	n-Octyl bromide	111-83-1	No Cat.	Q	0.0	Slightly irritating

Table 2D. Results of the EpiOcular[TM] EIT through direct test performance

No.	Test chemicals	CAS No.	UN GHS Category	Tissue viability	Prediction
1	1,2,4-Triazole, sodium salt	41253-21-8	Cat. 1	1.41 ± 0.23	No pre
2	L-Aspartic acid	70-47-3 / 56-84-8	Cat. 2	96.4 ± 3.5	No Cat.
3	Potassium cyanate	590-28-3	Cat. 2	1.0 ± 0.1	No pre
4	2-Ethoxyethyl methacrylate	2370-63-0	No Cat.	41.3 ± 2.5	No pre
5	1,6-Dibromohexane	629-03-8	No Cat.	67.2 ± 6.0	No Cat.
6	1-Bromo-4-chlorobutane	6940-78-9	No Cat.	34.9 ± 3.4	No pre
7	Myristyl myristate	3234-85-3	No Cat.	106.4 ± 6.4	No Cat.
8	n-Hexyl bromide	111-25-1	No Cat.	77.3 ± 2.6	No Cat.
9	Ethyleneglycol diethyl ether	629-14-1	No Cat.	10.5 ± 5.2	No pre
10	4,4-Methylene bis-(2,6-di-tert-butylphenol)	118-82-1	No Cat.	100.6 ± 3.8	No Cat.
11	p-Methylthiobenzaldehyde	3446-89-7	No Cat.	48.8 ± 8.6	No pre
12	2,4-Difluoronitrobenzene	446-35-5	No Cat.	68.3 ± 4.4	No Cat.
13	cis-Cyclo-octene	931-87-3	No Cat.	71.4 ± 1.2	No Cat.

No pre: No prediction can be made

and specificity were 83.3% (25/30), 93.3% (14/15) and 73.3% (11/15), respectively (Table 2A).

As a result of 4 *in vitro* eye irritation test methods, the test methods with the highest accuracy, sensitivity, and specificity were HET-CAM (96.7%), HET-CAM (100%) and STE test (100%), respectively; and the test methods with the lowest accuracy, sensitivity, and specificity were BCOP (70%), STE (75%), and BCOP (53.3%), respectively.

Also, we evaluated 24 test chemicals except insoluble chemicals in the STE test to compare predictive capacities by *in vitro* method for the same materials (Table 2B). As a result, the predictive capacities of the STE test were accuracy of 87.5% (21/24), sensitivity of 75% (9/12), and specificity of 100% (12/12). In the BCOP test, the accuracy, sensitivity and specificity were 70.8% (17/24), 91.7% (11/12), and 50% (6/12), respectively; and HET-CAM test were 95.8% (23/24), 100% (12/12), and 91.7% (11/12), respectively. In the EpiOcular™ EIT, the accuracy, sensitivity and specificity were 87.5% (21/24), 100% (12/12) and 75 % (9/12), respectively.

3. Predictive capacities for eye irritation potency in each in vitro test method

In this study, the categories were subdivided and analyzed to compare the correct predictive capacities of each test method. As shown in Table 3A, in the 30 test substances, the methods accurately predicting Cat. 1 test chemicals were in the order of HET-CAM (71.4%, 5/7), BCOP (42.9%, 3/7), and STE (16.7%, 1/6) and the methods accurately predicting Cat. 2 test chemicals were in the order of BCOP (75%, 6/8), STE (50%, 3/6) and HET-CAM (50%, 4/8). The methods accurately predicting No Cat. test chemical were in the order of STE (100%, 22/22), HET-CAM (93.3%, 14/15), BCOP (53.3, 8/15). The STE test had the highest predictive capacities for No Cat. chemicals of 100% (22/22). However, it had the highest rate of predicting Cat. 1 chemicals as Cat. 2 (83.3%, 5/6). The HET-CAM test had the highest predictive capacities 71.4% (5/7) for Cat. 1 chemicals, but it showed high tendency of over-predicting Cat. 2 chemicals as Cat. 1(50%, 4/8). The BCOP test had the highest predictive

Table 3A. The predictive capacities for each test method for 30 test chemicals (Cat.1, Cat.2 vs No Cat.).

	Predictive capacity[a]					
	Accuracy		Sensitivity		Specificity	
	n	%	n	%	n	%
STE	21/24[b]	87.5	9/12	75.0	12/12	100
BCOP	21/30	70.0	13/15	86.7	8/15	53.3
HET-CAM	29/30	96.7	15/15	100.0	14/15	93.3
EpiOcular™	25/30	83.3	14/15	93.3	11/15	73.3

[a] These values of predictive capacities were calculated based on Cat. 1/Cat. 2 vs No Cat.
[b] As 6 test chemicals were not dissolved in Saline, 5% DMSO and Mineral oil, they were excluded from the STE test.

Table 3B. The predictive capacities for each test method for 24 test chemicals (excluded 6 insoluble test chemicals)(Cat.1, Cat.2 vs No Cat.).

	Predictive capacity[a]					
	Accuracy		Sensitivity		Specificity	
	n	%	n	%	n	%
STE	21/24	87.5	9/12	75.0	12/12	100
BCOP	17/24	70.8	11/12	91.7	6/12	50.0
HET-CAM	23/24	95.8	12/12	100.0	11/12	91.7
EpiOcular™	21/24	87.5	12/12	100.0	9/12	75.0

[a] These values of predictive capacities were calculated with 24 substances in order to compare their predictive capacities more precisely based on Cat. 1/Cat. 2 vs No Cat.

capacities for Cat. 2 chemicals of 75% (6/8) but, it showed high tendency of under-prediction that 42.9% (3/7) of Cat. 1 chemicals were classified as Cat. 2. Also, we analyzed 24 substances except insoluble substances in the STE test (Table 3B). Based on the above results, it was confirmed that the single *in vitro* test methods did not accurately determine the Cat. 1 and Cat. 2 chemicals.

4. Predictive capacities of two tiered approaches against 30 test chemicals

The final decisions of the eye irritation potency against 30 test chemicals by applying the tiered approach A or B are presented in Table 4. Predictive capacities of the two tiered approaches were thus calculated as shown in Table 5. The tiered approach A shows an accuracy of 83.3% (25/30), a sensitivity of 93.3% (14/15), and a specificity of 73.3% (11/15), whereas the tiered approach B shows an accuracy of 86.7% (26/30), a sensitivity of 93.3% (14/15), and a specificity of 80.0% (12/15). Prediction rates of Cat. 1 of the two tiered approaches were 71.4% (5/7) or 57.1% (4/7), respectively. Meanwhile, prediction rates of Cat. 2 of the two tiered approaches were 75.0% (6/8).

Table 4A. Results of predictive capacities by category for 30 substances of each test method

UN GHS Category	Prediction	STE (24[a])		BCOP (30)		HET-CAM (30)	
		n	%	n	%	n	%
Cat. 1	Cat. 1	1/6	16.7	3/7	42.9	5/7	71.4
	No pre[b]	5/6	83.3	3/7	42.9	2/7	28.6
	No Cat[c].	0/6	0	1/7	14.3	0/7	0.0
Cat. 2	Cat. 1	0/6	0	1/8	12.5	4/8	50.0
	No pre	3/6	50.0	6/8	75.0	4/8	50.0
	No Cat.	3/6	50.0	1/8	12.5	0/8	0.0
No Cat.	Cat. 1	0/12	0	0/15	0.0	0/15	0.0
	No pre	0/12	0	7/15	46.7	1/15	6.7
	No Cat.	12/12	100	8/15	53.3	14/15	93.3

[a] As six test chemicals were not dissolved in solvents recommended from the OECD TG 491, they were excluded from the STE test so that the number of test chemicals conducted in STE test was 24. The BCOP and HET-CAM tests used 30 test chemicals for evaluating eye irritation hazard and potency identification.
[b] No pre: No prediction can be made;
[c] No Cat: No Category.

Table 4B. Results of predictive capacities by category for 24 substances (excluded 6 insoluble test chemicals) of each test method

UN GHS Category	Prediction	STE (24)		BCOP (24)		HET-CAM (24)	
		n	%	n	%	N	%
Cat. 1	Cat. 1	1/6	16.7	3/6	50.0	4/6	66.7
	No pre	5/6	83.3	2/6	33.3	2/6	33.3
	No Cat.	0/6	0	1/6	16.7	0/6	0.0
Cat. 2	Cat. 1	0/6	0	1/6	16.7	4/6	66.7
	No pre	3/6	50.0	5/6	83.3	2/6	33.3
	No Cat.	3/6	50.0	0/6	0.0	0/6	0.0
No Cat.	Cat. 1	0/12	0	0/12	0.0	0/12	0.0
	No pre	0/12	0	6/12	50.0	1/12	8.3
	No Cat.	12/12	100	6/12	50.0	11/12	91.7

Table 5. Final decision of the eye irritation potency against 30 test chemicals in applying the tiered approach A or B.

No.	Test chemicals	CAS no.	UN GHS Category	Tiered approach A					Tiered approach B				
				STE	BCOP	HET-CAM	RhCE	FD	STE	BCOP	HET-CAM	RhCE	FD
1	Sodium hydroxide (10%)	1310-73-2	Cat. 1	△	O			O	△	O			O
2	Trichloroacetic acid (30%)	76-03-9	Cat. 1	△	O			O	△	O			O
3	Quinacrine	69-05-6	Cat. 1	△	△		△	△	△	△			△
4	p-Fluoroaniline	371-40-4	Cat. 1		△	O		O		△	O		△
5	Sodium lauryl sulphate (15%)	151-21-3	Cat. 1	O				O	O	△			O
6	Lauric acid	143-07-7	Cat. 1	△	△		△	△	△	△			△
7	1,2,4-Triazole, sodium salt	41253-21-8	Cat. 1	△	O			O	△	O			O
8	Triton X-100 (5%)	9002-93-1	Cat. 2	△	△		△	△	△	△			△
9	L-Aspartic acid	70-47-3 / 56-84-8	Cat. 2		X	△	X	X		X	△	X	X
10	Potassium cyanate	590-28-3	Cat. 2	X	△		△	△	X	△		△	△
11	2,6-Dichlorobenzoyl chloride	4659-45-4	Cat. 2	X	△		△	△	X	△		△	△
12	Methyl cyanoacetate	105-34-0	Cat. 2	X	△		△	△	X	△		△	△
13	Maneb	12427-38-2	Cat. 2		△	△	△	△		△	△		△
14	2-Methyl-1-pentanol	105-30-6	Cat. 2	△	△			△	△	△			△
15	n-Hexanol	111-27-3	Cat. 2	△	O			O	△	O			O
16	2-Ethoxyethyl methacrylate	2370-63-0	No Cat.	X	X		△	△	X	X			X
17	1,6-Dibromohexane	629-03-8	No Cat.	X	X		X	X	X	X			X
18	1-Bromo-4-chlorobutane	6940-78-9	No Cat.	X	△		△	△	X	△		△	△
19	Myristyl myristate	3234-85-3	No Cat.	X	X		X	X	X	X			X
20	n-Hexyl bromide	111-25-1	No Cat.	X	△		X	X	X	△		X	X
21	Ethyleneglycol diethyl ether	629-14-1	No Cat.	X	△		△	△	X	△		△	△
22	4,4-Methylene bis-(2,6-di-tert-butylphenol)	118-82-1	No Cat.		X	X	X	X		X	X		X
23	p-Methylthiobenzaldehyde	3446-89-7	No Cat.		△	X	△	△		△	X	△	△
24	2,4-Difluoronitrobenzene	446-35-5	No Cat.		X	X	X	X		X	X		X
25	cis-Cyclo-octene	931-87-3	No Cat.	X	△		X	X	X	△		X	X
26	2,4-Pentanediol	625-69-4	No Cat.	X	△		X	X	X	△		X	X
27	Di-iso-butyl ketone	108-83-8	No Cat.	X	△		X	X	X	△		X	X
28	3-Methylhexane	589-34-4	No Cat.	X	X		X	X	X	X			X
29	Iso-Octyl acrylate	29590-42-9	No Cat.	X	X		X	X	X	X			X
30	n-Octyl bromide	111-83-1	No Cat.	X	X		X	X	X	X			X

FD: Final decision; O: Severe irritant, severely irritating, Cat 1; △: Moderate irritant, mild irritant, no prediction can be made, irritating, moderately irritating; X: No Category, minimal irritant, slightly irritati

Table 6. Comparison of the predictive capacities obtained from the two tiered approaches based on the result of 30 test chemicals

	Predictive capacity		Tiered approach A		Tiered approach B	
			n	%	n	%
Hazard identification	Accuracy		25/30	83.3	26/30	86.7
	Sensitivity		14/15	93.3	14/15	93.3
	Specificity		11/15	73.3	12/15	80
	UN GHS Category	**Prediction**	**n**	**%**	**n**	**%**
Potency identification	Cat. 1	Cat. 1	5/7	71.4	4/7	57.1
		No pre	2/7	28.6	3/7	42.9
		No Cat.	0/7	0	0/7	0
	Cat. 2	Cat. 1	1/8	12.5	1/8	12.5
		No pre	6/8	75.0	6/8	75.0
		No Cat.	1/8	12.5	1/8	12.5
	No Cat	Cat. 1	0/15	0	0/15	0
		No pre	4/15	26.7	3/15	20
		No Cat.	11/15	73.3	12/15	80

Discussion

The STE test is known to show high specificity and low false positive rate (OECD, 2015; Takahashi et al., 2009; Takahashi et al., 2011; Sakaguchi et al., 2011; Kojima et al., 2013). In this study, it also showed a low false positive rate of 0% (0/22) and a false negative rate of 25% (3/12). The test chemicals predicted to be false negative were potassium cyanate, 2,6-dichlorobenzoyl chloride and Methyl cyanoacetate, which were Cat. 2 chemicals, all predicted as No Cat. The STE test is known to have a high false negative rate for some solid chemicals (OECD, 2015). In this study, potassium cyanate, a solid material, showed false negative. 2,6-dichlorobenzoyl chloride and methyl cyanoacetate were predicted as No Cat. in the previously reported studies (Sakaguchi et al., 2011; Kojima et al. 2013; Adriaens et al., 2018).

The BCOP test is well known as a high sensitivity test method (OECD, 2017b; Hayashi et al., 2012b; ICCVAM, 2010; Verstraelen et al., 2017). In this study, it also showed a high sensitivity of 86.7%, (13/15) a false negative rate of 13.3% (2/15) and a false positive rate of 46.7% (7/15). The test chemical predicted to be false negative was L-aspartic acid from Cat. 2 and it was predicted as No Cat. The test chemicals predicted to be false positive were 1-bromo-4-chlorobutane, n-hexyl bromide, ethyleneglycol diethyl ether, p-methylthiobenzaldehyde, cis-cyclo-octene, 2,4-Pentanediol, and Di-iso-butyl ketone from No Cat. and they were predicted as Cat. 1 or Cat. 2. BCOP test tends to have high over-prediction for some alcohols and ketones and is known to show high under-prediction for some solids (OECD, 2017b). In this study, 2,4-Pentanediol, alcohol chemical, of No Cat. and di-iso-butyl ketone, ketone chemical, of No Cat. were over-predicted.

The HET-CAM test is known to show a high false positive rate (ICCVAM, 2006). In this study, the false positive rate was 6.7% (1/15). The test chemical predicted to be false positive was 2,4-Pentanediol in the No Cat. chemicals.

The EpiOcular™ EIT is known to show high sensitivity and low false negative rate (Kolle et al., 2011; EURL ECVAM, 2014; OECD, 2019a;). In this study, the EpiOcular™ test showed high sensitivity of 93.3% (14/15), and false negative rate was 6.7% (1/15), and false positive rate 26.7% (4/15). The test chemicals predicted to be false negative was L-Aspartic acid. The test chemicals predicted to be false positive were 2-Ethoxyethyl methacrylate, 1-Bromo-4-

chlorobutane, Ethyleneglycol diethyl ether and p-Methylthiobenzaldehyde. Among these, 2-Ethoxyethyl methacrylate and 1-Bromo-4-chlorobutane were predicted to be false positive in the EpiOcular™ EIT validation study (EURL ECVAM, 2014).

While a sensitivity of tiered approach A (93.3% (14/15)) was higher than that of tiered approach B (93.3%, 47/49), an accuracy and a specificity of tiered approach B (86.7% (25/30) and 80% (12/15), respectively) were higher than those of tiered approach A with 83.3% (25/30) and 73.3% (11/15), respectively. The prediction rate of Cat. 2 of the two tiered approaches was equal to 75.0% (6/8), but the prediction rate of Cat. 1 in the tiered approach A (71.4% (5/7)) was higher than that of tiered approach B (57.1% (4/8)). The prediction rate of Cat. 1 chemicals using two-tiered approach proposed by Hayashi et al., (2012a) was 90.9%, while that of Cat. 2 chemicals was 50%. The prediction rate of Cat. 1 chemicals using two-tiered approach proposed by Hayashi et al., (2012b) was 94%, while that of Cat. 2 chemicals was 31%. In 2018, Top-Down or Bottom-Up approaches combining the BCOP LLBO, and RhCE tests as two-tiered approach or the BCOP OP-KIT, SMI assay, RhCE test as three-tiered approach were proposed (Adrianes et al., 2018). The prediction rates of the tiered approaches to identify among Cat. 1 or Cat. 2 chemicals were 71.7～82.9% or 64.8～68.5%, respectively.

Additionally, when predictive capacities of the two tiered approaches by combining final decisions against 30 test chemicals in our study and 47 test chemicals in Ko et al., (2020) in applying the two tiered approaches were calculated, an accuracy, a sensitivity and a specificity of tiered approach A were 90.9% (70/77), 98.0% (48/49), and 78.6% (22/28), respectively. Meanwhile, an accuracy, a sensitivity and a specificity of tiered approach B were 92.2% (71/77), 95.9%, (47/49), and 85.7% (24/28), respectively. The prediction rates of Cat. 1 and 2 of the tiered approach A (72.7% (16/22), 66.7% (18/27), respectively) were higher than those of the tiered approach B (68.2% (15/22), 63.0% (17/27), respectively. Because the two tiered approaches were low prediction rates of Cat. 1, 2, further studies for developing test methods which can classify between Cat. 1 and Cat. 2. are required. As for eye irritation, Adverse Outcome Pathways (AOP) is not currently suggested such as skin sensitization. The alternative methods of skin sensitization which have been developed for each key event of the AOP, can predict skin sensitization by evaluating substances that change through metabolic processes, such as haptenization through a combination of test methods. However, in the case of the alternative methods of eye irritation, since the eye irritation is evaluated by evaluating the cell viability of corneal cells and the degree of damage to the cornea, it has a limitation that it cannot evaluate substances that change through metabolic processes *in vivo*. In conclusion, the two tiered approaches may be used to distinguish irritants (both Cat. 1 and Cat.2) from non-irritants (No Cat.) as alternatives of the *in vivo* Draize rabbit eye test.

Disclosure

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