January 19, 2011

ICCVAM Workshop Series on Best Practices for Regulatory Safety Testing
Assessing the Potential for Chemically Induced Eye Injuries
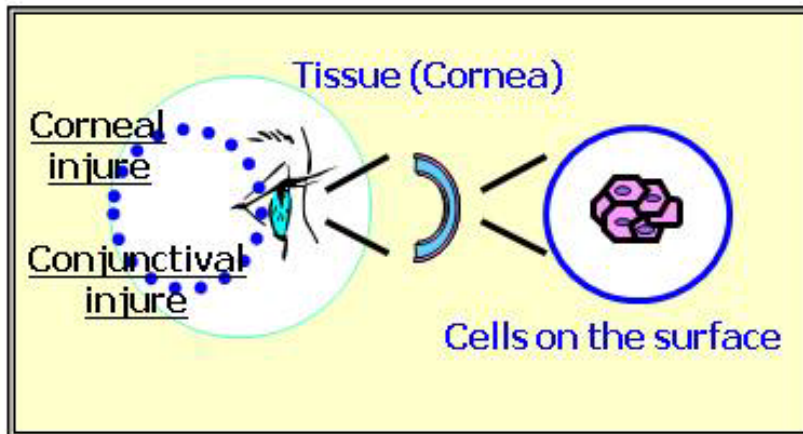
New Models in the Validation Pipeline for Ocular Safety Testing
Short Time Exposure Test: STE test

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Focus of development

Tissue (Cornea)

Corneal injure

Conjunctival injure

Cells on the surface

New in vitro test: Identifying minimal to moderate eye irritation potential

- **Short contact**
  - Eliminate time when a drop of 50mL place in the eye（Motose ea al., 1984）
  - Human : 1-2 min, Rabbit : 3-4 min (80% elimination)
  - **Retention time in eye is short (around 5 min)**
- **Contact with the surface cells of cornea**
  - **Cytotoxicity → Onset of eye irritation**

Development a short time exposure cytotoxicity test designed for the actual exposure condition
Short Time Exposure（STE）test

Procedure of Short Time Exposure (STE) test

Cell culture ➡ **Exposure** ➡ **Measurement of viability**
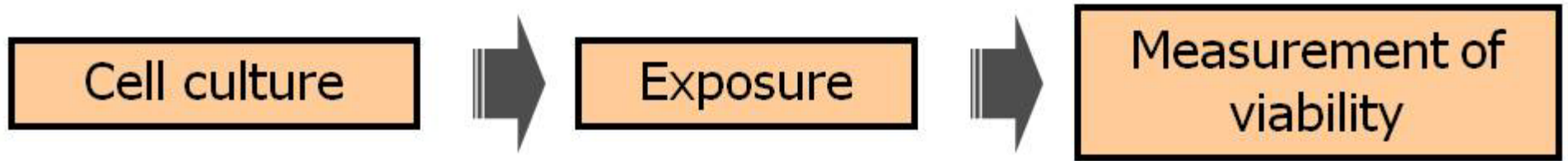
SIRC cells

rabbit corneal cell line

Seeded into 96-well plate at 3.0×10^3 cells/well

Pre-incubation (37°C, 5%CO_2) for 5 days

5 minutes

Test concentration: 5% and 0.05%

Vehicle: Saline, 5% DMSO in saline, or mineral oil
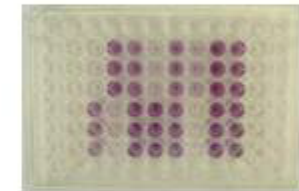
(Select vehicle based on solubility)

Measurement of viability

Measurement of Formazan formation (MTT assay)

n=3

Mean cell viability (%)

$$\frac{OD_{570}\ (\text{Test Sample})}{OD_{570}\ (\text{Vehicle Control})} \times 100$$

- **For each sample concentration, three wells were used to obtain viability**

Takahashi Y. et al., Toxicology in Vitro, 22 (2008)

Prediction models of the STE test

• Category classification

Based on viability at 5% test conc.

Criteria for classifying

	Viability (cutoff value)
non irritant	> 70 %
irritant	≤ 70 %

• Rank classification

Based on total score obtained from viabilities at 5% and 0.05% test conc.

Scoring of 5% and 0.05% test conc.

5%	score	0.05%	score
viability > 70	0	viability > 70	1
viability ≤ 70	1	viability ≤ 70	2

5% score + 0.05% score= STE rank

Eye irritation rank

1 : Minimal irritant
2 : Moderate irritant
3 : Severe irritant

Today's presentation

- **Predictive capacity**
  - Evaluation of 109 chemicals by the STE test to compare with GHS classification
- **1st validation study data**
  - Transferability, Inter-laboratory reproducibility, Predictive capacity
  - 25 blinded chemicals were evaluated by 5 laboratories
- **2nd validation study data**
  - Predictive capacity
  - 40 blinded chemicals were evaluated by 3 laboratories

Test chemicals	GHS Class	STE 5% con. Category	STE 5% con. viability (%)	STE 0.05% con. viability (%)	Rank
Methoxyethyl acrylate	1	I	0.1	101.1	2
Butyl cellosolve	1	I	5.6	106.9	2
Cyclohexanol	1	I	1.4	104.5	2
2,5-Dimethyl-2,5-hexanediol	1	NI	73.4	99.5	1
3-Methyl-pentynol	1	I	8.3	91.0	2
Diethylethanolamine	1	I	0.2	91.5	2
Acetic acid	1	I	4.9	94.6	2
Lactic acid	1	I	4.4	87.3	2
2-Methylbutanoic acid	1	I	4.7	100.4	2
Tetrahydrofuran	1	I	35.2	103.8	2
Imidazole	1	I	1.4	95.3	2
Pyridine	1	I	6.4	101.1	2
Sodium hydroxide	1	I	1.4	-1.4	3
Promethazine hydrochloride	1	I	2.4	94.9	2
Nonylphenyl-polyethylene glycol	1	I	-0.3	3.4	3
Di(2-ethylhexyl) sodiumsulfosuccinate	1	I	2.5	3.7	3
Potassium laurate	1	I	0.8	1.7	3
Sodium lauryl sulfate	1	I	0.3	-0.8	3
Benzalkonium chloride	1	I	2.1	3.1	3
Benzethonium chloride	1	I	3.8	3.7	3
Cetylpyridinium bromide(10%)	1	I	0.6	4.2	3
Cetylpyridinium chloride	1	I	-0.1	4.2	3
Cetyltrimethylammonium bromide	1	I	0.4	2.5	3
Distearyldimethylammonium chloride	1	I	57.6	101.7	2
Domiphen bromide	1	I	3.6	4.0	3
Triton x-100	1	I	-0.1	0.7	3
Methyl acetate	2A	NI	92.6	96.2	1
Methyl cyanoacetate	2A	I	39.2	98.8	2
2,6-Dichlorobenzoyl chloride	2A	NI	83.9	98.3	1
Benzyl alcohol	2A	I	3.4	97.9	2
Butanol	2A	I	8.4	90.9	2
Cyclopentanol	2A	I	7.2	105.4	2
2-Ethyl-1-hexanol	2A	I	44.0	93.4	2
Ethanol	2A	NI	98.2	97.1	1
n-Hexanol	2A	I	-0.3	98.3	2
Isobutyl alcohol	2A	I	6.1	98.3	2
Isopropylalcohol	2A	NI	101.6	97.6	1
1-Octanol	2A	I	-0.5	96.8	2
Citric acid	2A	I	8.8	82.5	2
Acid red 92	2A	I	-0.1	12.8	3
Propylene carbonate	2A	I	67.2	96.9	2
Acetone	2A	I	9.6	101.4	2
2-Butanone	2A	I	44.7	100.7	2
Butyrolactone	2A	I	32.7	98.0	2
Calcium thioglycollate	2A	I	7.0	109.8	2
Potassium sorbate	2A	NI	100.1	100.5	1
Sucrose fatty acid ester	2A	I	-0.6	103.2	2
m-Phenylenediamine	2A-2B	I	6.4	96.7	2
Ethyl-2-methylacetoacetate	2B	I	1.7	99.4	2
2-Methyl-1-pentanol	2B	I	1.8	101.6	2
Monoethanolamine	2B	I	0.5	93.7	2
n-Lauroylsarcosine sodium salt	2B	I	-0.6	3.5	3
Glycolic acid	2B	I	3.4	85.8	2
Camphen	2B	NI	100.4	97.4	1
Sodium monochloroacetate	2B	NI	95.9	95.5	1
Di(propylene glycol) propyl ether	2B	I	0.4	100.0	2

Test chemicals	GHS Class	STE 5% con. Category	STE 5% con. viability (%)	STE 0.05% con. viability (%)	Rank
Physiological saline	NI	NI	92.0****	92.0	1
2-Ethoxyethyl acetate	NI	NI	102.0	98.8	1
Ethyl acetate	NI	I	7.8	109.6	2
Methyl trimethyl acetate	NI	NI	104.3	105.1	1
Iso-octyl acrylate	NI	NI	90.3	99.3	1
2,2-Dimethyl-3-pentanol	NI	NI	111.6	99.9	1
3-Methoxy-1,2-propanediol	NI	NI	93.6	98.1	1
2,4-Pentandiol	NI	NI	84.4	103.1	1
3,3-Dimethylpentane	NI	NI	92.6	102.4	1
Diisopropanolamine	NI	I	0.6	95.7	2
Triethanolamine	NI	NI	101.6	99.9	1
EDTA di-potassium	NI	NI	88.6	99.3	1
Betaine monohydrate	NI	NI	102.2	98.7	1
1,3-Di-isopropylbenzene	NI	NI	97.3	93.6	1
Styrene	NI	NI	88.5	99.1	1
Xylene	NI	NI	100.3	104.4	1
Methyl cyclopentane	NI	NI	102.2	104.4	1
Buthyl acetate	NI	NI	103.2	106.7	1
Ethyl trimethyl acetate	NI	NI	99.2	98.3	1
Isopropyl myristate	NI	NI	106.0	97.1	1
1,9-Decadiene	NI	NI	98.0	98.7	1
Isopropyl bromaide	NI	NI	105.6	109.4	1
n-Octyl bromide	NI	NI	94.6	98.4	1
Petroleum ether	NI	NI	99.3	105.8	1
1,2,3-Trichloropropane	NI	NI	101.1	102.7	1
1,2,4-Trimethylbenzene	NI	NI	95.8	101.2	1
Dodecane	NI	NI	96.3	102.3	1
1,5-Hexadine	NI	NI	95.3	104.1	1
Hexane	NI	NI	88.1	93.8	1
2-Methylpentane	NI	NI	90.6	95.7	1
3-Methylhexane	NI	NI	99.6	97.0	1
Toluene	NI	NI	101.3	99.5	1
Silicic anhydride	NI	NI	79.5	110.0	1
Cyclohexanone	NI	I	17.1	106.6	2
Diisobutyl ketone	NI	NI	101.0	96.3	1
Methyl amylketone	NI	NI	91.7	101.7	1
Methyl iso-butyl ketone	NI	NI	88.5	107.3	1
2,4-Pentanedione	NI	I	9.4	101.4	2
Gluconolactone	NI	NI	88.2	91.0	1
n,n-Dimethylguanidine sulfate	NI	NI	78.6	101.0	1
3-Glycidoxypropyltrimethoxysilane	NI	NI	77.3	97.6	1
2-Ethylhexyl p-dimethylamino benzoate	NI	NI	106.4	98.3	1
Mineral oil	NI	NI	97.1****	97.1	1
Glycerin	NI	NI	95.7	100.2	1
Polyethylene glycol 400	NI	NI	92.1	85.9	1
Propylene glycol	NI	NI	90.6	100.6	1
Cyclopentasiloxane	NI	NI	106.2	105.0	1
Polyoxyethylene 23 lauryl ether	NI	NI	72.3	105.5	1
Polyoxyethylene 8 stearate	NI	I	33.5	103.7	2
Polyoxyethylene hydrogenated castoroil (60E.O.)	NI	NI	117.9	101.2	1
Tween20	NI	I	21.1	99.5	2
Tween80	NI	NI	114.1	104.6	1
Dimethyl sulfoxide	NI	NI	95.3	94.8	1

□ : Irritant by GHS (category 1 or 2) or STE test   □ : Non irritant by GHS or STE test

Predictive capacity of 109 chemicals data

(Correspondence between STE eye irritation and GHS)

Category classification

STE test GHS		Not irritant 55	Irritant 54
Not irritant	53	47	6
Iritant	56	8 *	48

*: 3 alcohols and others

Sensitivity: 86% (48/56)
Specificity: 89% (47/53)
Positive predictivity: 89% (48/54)
Negative predictivity: 85% (47/55)
Accuracy: **87% (95/109)**

STE irritation category (NI or I) was well correlated with GHS

Rank classification

		STE rank		
		1	2	3
GHS rank	NI	47	6	0
	Cat 2	7	21	2
	Cat 1	1	13	12

Accuracy: 73% (80/109)

STE rank (1, 2 or 3) almost correlated with GHS rank respectively

Good predictive capacity was confirmed

1st validation study

- Organization by the Validation Committee of the Japanese Society for Alternative to Animal Experiments (JSAAE) （Validation Executive Committee）

- Test Laboratories：5 labs.
  （Kanebo Cosmetics Inc., Kose Corp., POLA Chemical Industries Inc., Pias Corp., Lion Corp.)

- Duration： April, 2008 – March, 2009

- Purpose and experimental design

  1. **Confirmation of transferability**
     With 3 standard chemicals （Sodium lauryl sulfate、Calcium Thioglycollate、Tween 80）

  2. **Evaluation of inter-laboratory reproducibility**
     With 25 blinded test chemicals at each labs.

  3. **Evaluation of predictive capacity**
     With 25 blinded test chemicals at each labs.
     Evaluate the correspondence with Globally Harmonized System（GHS）

Transferability

Laboratory	Test conc.	Viability (%)		
		SLS	calcium thioglycollate	Tween 80
Lab 1	5%	2.2 ± 0.7	18.5 ± 6.8	110.3 ± 6.2
	0.05%	3.1 ± 2.2	107.1 ± 10.5	103.8 ± 3.3
	Rank	3	2	1
Lab 2	5%	2.1 ± 0.9	12.8 ± 3.6	101.7 ± 1.9
	0.05%	1.6 ± 0.3	106.8 ± 7.7	99.2 ± 3.6
	Rank	3	2	1
Lab 3	5%	0.0 ± 0	13.7 ± 4.3	102.5 ± 15.1
	0.05%	0.2 ± 0.4	99.0 ± 11.7	99.0 ± 5.4
	Rank	3	2	1
Lab 4	5%	0.0 ± 0	14.9 ± 1.3	117.1 ± 4.7
	0.05%	0.4 ± 0.6	118.0 ± 3.0	102.2 ± 1.9
	Rank	3	2	1
Lab 5	5%	4.5 ± 4.0	17.5 ± 8.2	103.4 ± 14.0
	0.05%	6.3 ± 2.7	87.9 ± 2.0	95.7 ± 2.0
	Rank	3	2	1
Lead Lab. (Kao)	5%	0.2 ± 0.3	10.8 ± 2.2	101.3 ± 8.0
	0.05%	0.4 ± 0.3	101.1 ± 5.2	98.7 ± 5.6
	Rank	3	2	1

mean ± SD (n=3) ☐ 1: minimal irritant ☐ 2: moderate irritant ☐ 3: severe irritant

- Cell viability of 3 standard chemicals at all labs and each conc were almost equivalent compared to that of lead lab.
- Ranking of all chemicals were completely-consistent between labs.

Good transferability was confirmed

Inter-laboratory reproducibility

(Consistency of category and rank classification)

Test Chemicals		Eye irritation category						Eye irritation rank					
		GHS	STE					GHS	STE				
			Lab. 1	Lab. 2	Lab. 3	Lab. 4	Lab. 5		Lab. 1	Lab. 2	Lab. 3	Lab. 4	Lab. 5
A	3-Methoxy-1,2-propanediol	NI	NI	NI	NI	NI	NI	NI	1	1	1	1	1
B	Polyethylene glycol 400	NI	NI	NI	NI	NI	NI	NI	1	1	1	1	1
C	Glycerol	NI	NI	NI	NI	NI	NI	NI	1	1	1	1	1
D	Tween20	NI	NI	NI	NI	NI	NI	NI	1	1	1	1	1
E	Ethanol	I	NI	NI	NI	NI	NI	2	1	1	1	1	1
F	Sodium hydroxide	I	I	I	I	I	I	1	3	3	3	3	3
G	Triton X-100	I	I	I	I	I	I	1	3	3	3	3	3
H	Cetylpyridinium bromide	I	I	I	I	I	I	1	3	3	3	3	3
I	Benzalkonium Chloride	I	I	I	I	I	I	1	3	3	3	3	3
J	Methyl amyl ketone	NI	NI	NI	I	NI	NI	NI	1	1	3	1	1
K	2-Methyl-1-pentanol	I	I	I	I	I	I	2	2	2	2	2	2
L	n-Hexanol	I	I	I	I	I	I	2	2	2	2	2	2
M	3,3-Dimethylpentane	NI	NI	NI	NI	NI	NI	NI	1	1	1	1	1
N	Methyl cyclopentane	NI	NI	NI	NI	NI	NI	NI	1	1	1	1	1
O	Methyl isobutyl ketone	NI	NI	NI	NI	NI	NI	NI	1	1	1	1	1
P	Toluene	NI	NI	NI	NI	NI	NI	NI	1	1	1	1	1
Q	1-Octanol	I	I	I	I	I	I	2	2	2	2	2	2
R	2-Ethyl-1-hexanol	I	I	NI	I	I	NI	2	2	1	2	2	1
S	Acetone	I	NI	NI	NI	NI	NI	2	1	1	1	1	1
T	Cyclohexanol	I	I	I	I	I	I	1	2	2	2	2	2
U	n,n-Dimethylguanidine sulfate	NI	NI	NI	NI	NI	NI	NI	1	1	1	1	1
V	2-Ethylhexyl p-dimethyl-amino benzoate	NI	NI	NI	NI	NI	NI	NI	1	1	1	1	1
W	Gluconolactone	NI	NI	NI	NI	NI	I	NI	1	1	1	1	2
X	Methyl ethyl ketone	I	NI	I	I	I	I	2	1	2	2	2	2
Y	Propylene glycol	NI	NI	NI	NI	NI	NI	NI	1	1	1	1	1

Legend:
- ☐: non irritant
- ☐: irritant
- ☐: GHS NI or STE 1: minimal irritant
- ☐: GHS 2 or STE 2: moderate irritant
- ☐: GHS 1 or STE 3: severe irritant

- Most estimated eye irritation category and ranking of test chemicals were consistent between the five labs (except J, R, W and X)

Inter-laboratory reproducibility is good

Category classification for 25 chemicals

GHS	STE (5%)		Sum
	NI (CV>70)	I (CV≤70)	
NI	13 (12[*1])	0 (1[*1])	13
I (Cat. 1 and 2)	3 (2[*2])	9 (10[*2])	12

*1: Lab 3, 5   *2: Lab 3, 4

	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	MEAN
Sensitivity	9/12	9/12	10/12	10/12	9/12	(78.3)
	(75.0)	(75.0)	(83.3)	(83.3)	(75.0)	
Specificity	13/13	13/13	12/13	13/13	12/13	(96.9)
	(100.0)	(100.0)	(92.3)	(100.0)	(92.3)	
Accuracy	22/25	22/25	22/25	23/25	21/25	(88.0)
	(88.0)	(88.0)	(88.0)	(92.0)	(84.0)	

- **STE (5% data) irritation category (NI or I) showed good correlation with GHS category (NI or I: category 1 and 2)**
- **All most same results in all labs**

Rank classification for 25 chemicals

GHS Rank	STE Rank			Sum
	1	2	3	
NI	13 (12[*1])	0 (1[*2])	0 (1[*3])	13
Cat. 2	2 (3[*4])	5 (4[*4])	0	7
Cat. 1	0	1	4	5

*1: Lab 3, 5, *2: Lab 5, *3: Lab 3 *4: Lab 1, 2, 5

	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	MEAN
Accuracy	21/25	21/25	21/25	22/25	20/25	(84.0)
	(84.0)	(84.0)	(84.0)	(88.0)	(80.0)	

- **STE rank (1, 2 or 3) showed good correlation with GHS rank (NI, Cat. 2, Cat. 1)**
- **All most same results were obtained among all labs**

- **Predictive capacity**
  - Accuracy: 87% (109 chemicals)
  - Good predictive capacity was confirmed
- **1st and 2nd validation study data**
  - Good transferability was confirmed
  - Inter-laboratory reproducibility was good
  - Good predictive capacity was confirmed
  - Some alcohols and organic salts were false negatives
- The next step: **Make further progress toward peer review**

| Method Development | Pre Validation | Validation | Peer Review | OECD Test Guideline |