Original Article

Prediction of Ocular Irritancy of 26 Chemicals and 26 Cosmetic Products with Isolated Rabbit Eye (IRE) Test*

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Abstract

Objective This study aims to establish and evaluate the methodology of isolated rabbit eye (IRE) test.

Methods IRE test was performed according to modifications of the *in vitro* toxicology (INVITTOX) Protocol No.85: Rabbit enucleated eye test by European Centre for the Validation of Alternative Methods (ECVAM), and then 26 chemicals and 26 cosmetic products were tested in both *in vitro* IRE and *in vivo* Draize tests. A statistical analysis was conducted to determine the relevance of the IRE test to the data generated in the Draize test.

Results IRE test was established successfully in our laboratory. It was shown that ranking correlation and class concordance were fairly well between the IRE test and the Draize test for 26 reference chemicals (Fisher's Exact Test χ^2=51.314, P<0.001; McNemar P=0.261; Gamma=0.960, P<0.001; Kappa=0.843, P<0.001) and 26 cosmetic products (Fisher's Exact Test χ^2=15.522, P<0.001; McNemar P=0.311; Gamma=0.967, P<0.001; Kappa=0.611, P<0.001).

Conclusion IRE test was established successfully for *in vitro* testing of eye irritation as an alternative to Draize test.

Key words: Isolated rabbit eye; IRE; Eye irritation; Alternative testing; Draize test

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INTRODUCTION

Since the Draize rabbit eye irritation test (Draize test) was developed in 1944, it has been used as a method of choice by most regulatory authorities for assessment of eye irritation hazards of cosmetic products and ingredients, and has contributed to protecting public health and eyesight. However, European Union (EU) member states have already banned testing cosmetic products and ingredients on animals. Furthermore, the 7th Amendment to the Cosmetic Products Directive (2003/15/EC) prohibited testing finished cosmetic products on animals, and a progressive ban on animal testing of ingredients was initiated in 2009 and to be followed by a marketing ban for ingredients and cosmetic products in 2013[1]. In addition, extensive interlaboratory studies have shown that animal irritation tests lack reproducibility, mainly due to the subjectivity of the grading system. Numerous reports have also cast doubts on the relevance of the extrapolation to the human response[2-3].

Adopting the 3Rs principle (the reduction,

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refinement and replacement of animal experiments)[4] has driven the development of several *in vitro* or *ex vivo* alternatives to replace the Draize test, such as *in vitro* and *ex vivo* organotypic models, chicken egg chorioallantoic membrane (CAM)-based assays, cell function-based assays, and cell cytotoxicity assays. One of the organotypic corneal models, the isolated rabbit eye (IRE) test, first proposed by Burton and his colleagues[5], measures corneal opacity, fluorescein penetration and corneal thickness, which are considered as more sensitive and objective parameters[6-7]. These endpoints demonstrate how visual ability may be adversely affected after exposure to an ocular irritant. The IRE test has been evaluated in several international validation studies, including the European Commission/British Home Office (EC/HO) study[8], The Cosmetic, Toiletry, and Fragrance Association (CTFA) phase III of the CTFA evaluation[9] and the evaluation by the Interagency Regulatory Alternatives Group (IRAG) Working Group on organotypic models[10]. Significant correlation between the IRE test and the Draize test in identifying severe eye irritants has been reported; however, the IRE test also partly distinguishes between mild and moderate eye irritants. In this study, 26 chemicals and 26 cosmetic products which had been detected by the Draize test (as "the Golden Standard") were tested by using the IRE test.

MATERIALS AND METHODS

Test Materials

Twenty-six test materials (21 liquids and 5 solids) were selected from the European center for ecotoxiology and toxicology of chemicals (ECETOC) reference chemicals data bank[11] for testing. The compounds tested included inorganic salts, alkalis, acids, surfactants, alcohols, amines, ketones, esters, aromatics, and ethers (Table 1). Among the 26 chemicals, 3 chemicals were tested at 2 different concentrations, and another 3 at 3 different concentrations. Accordingly, a total of 35 tests were performed on chemicals. For the cosmetic products, 26 were obtained from the Guangdong Provincial Center for Disease Control and Prevention, consisting of 17 liquids, 1 solid and 8 paste, and were assigned arbitrary codes (A to Z). The compounds tested included shampoos, hair dyes, hair styling products and cleaning foams (Table 2). All of the compounds were coded to avoid any subjective bias.

IRE Assay

The IRE test was performed according to the INVITTOX Protocol No. 85: Rabbit enucleated eye test[12], with additional histological evaluation included. Eyes were obtained from healthy New Zealand White rabbits (Body weight=2.5 kg±0.3 kg) in laughterhouse and the ocular tissues were not expected to be affected. The rabbit eyes were enucleated immediately and carefully after the animals were euthanized, and the corneal opacity and fluorescein penetration were examined to make sure they were not damaged during the operation. The eyes were then placed in Isolated Eye Superfusion System (Manufactured by our laboratory and patented with the patent number being 200810219435.X) to maintain ideal conditions. A water jacket maintained the temperature at 32±2 °C, and a warm isotonic saline drip irrigated the corneal surfaces.

Four eyes were used per test material and 1 eye was included as a negative control. Liquid or paste test materials were tested by applying 0.1 mL topically onto the central part of the cornea for 10 s. The test material was washed from the treated cornea by rinsing the cornea with 20 mL warm isotonic saline. Solid test materials were tested by sprinkling 0.1 g over the whole surface of the cornea for 10 s. The test material was washed from the treated cornea as described above. The eyes were then placed back into the superfusion apparatus and the saline drip was repositioned as before. After 0.5 h, 1 h , 2 h, 3 h, or 4 h, corneal opacity was examined with a YZ5E slit-lamp biomicroscope (Vision Tech Inc., Suzhou, China), according to the procedures described by Draize and his colleagues[13]. Corneal thickness was measured by using an UP-1000 ultrasonic pachymeter (NIDEK Inc., Gamagori Japan); relative corneal swelling (expressed as percent swelling) at 0.5 h, 1 h, 2 h, 3 h, or 4 h was calculated by using the pre-treatment corneal thickness as the baseline. Fluorescein penetration was observed with the slit-lamp biomicroscope, and evaluated by using a graded scoring system[12]. Upon completion of the 4 h observation, the corneas were excised from the eye, fixed, stained with hematoxylin and eosin, and the histopathological changes such as the loss of the epithelial cells, pitting, mottling or sloughing of the corneas were recorded. Each material was tested by

using the IRE assay in 3 separate trials. The irritants of test materials were classed as not irritating, slightly irritating, moderately irritating or severely irritating, according to ECVAM criterion.

Table 1. Classification of 26 Chemicals Assessed by the IRE and Draize Tests

Category	Chemicals	Corneal Opacity	Corneal Swelling (%)	Fluorescein Penetration	IRE test Classification	Draize test Classification
Inorganic	PBS (li)	0.00	0.67	0.00	Not irritating	Not irritating
Salt	Sodium perborate (s)	1.83	7.20	2.50	Moderate	Moderate
	Ammonium nitrate (s)	1.17	7.10	1.50	Slight	Slight
	Sodium oxalate (s)	1.22	5.83	1.08	Slight[d]	Moderate
	Calcium mercaptoacetate (s)	0.91	1.00	0.75	Slight	Slight
Alkali	1%NaOH (li)	1.00	26.37	2.67	Moderate	Moderate
	10%NaOH (li) [a]	—	—	—	Severe	Severe
Acid	3%trichloroacetic acid (TCA) (li)	0.61	9.07	2.17	Slight	Slight
	30%TCA (li)[a]	—	—	—	Severe	Severe
	Acetic acid (li)[b]	—	—	—	Severe	Severe
	L-Aspartic Acid (s)	1.33	4.50	2.50	Slight	Slight
Anionic	3%SDS (li)	1.43	10.20	2.16	Slight	Slight
Surfactants	15%SDS (li)	0.94	10.87	1.67	Moderate[d]	Severe
	30%SDS (li)	1.28	7.50	2.67	Moderate[d]	Severe
Cationic	Domiphen Bromide (li)[a]	—	—	—	Severe	Severe
Surfactants	1% Benzalkonium chloride (li)	2.50	35.70	2.50	Moderate	Moderate
	5% Benzalkonium chloride (li) [c]	—	—	—	Severe	Severe
	10% Benzalkonium chloride (li) [c]	—	—	—	Severe	Severe
	0.1% Cetylpyridium bromide (li)	1.42	9.97	1.58	Slight	Slight
	1% Cetylpyridium bromide (li)	2.00	18.7	2.00	Moderate	Moderate
	10% Cetylpyridium bromide (li) [c]	—	—	—	Severe	Severe
Nonionic	Tween 20 (li)	0.83	3.00	0.88	Slight	Slight
Surfactants	5%Triton-X-100 (li)	2.08	12.30	2.25	Moderate	Moderate
	10%Triton-X-100 (li)	1.67	7.30	2.00	Moderate	Moderate
	Polyethylene glycol 400 (li)	0.33	1.60	0.25	Not irritating[d]	Slight
Alcohols	Glycerol (li)	0.03	3.33	0.25	Not irritating	Not irritating
	Cyclohexanol (li)[c]	—	—	—	Severe	Severe
Amines	Triethanolamine (li)	0.75	2.10	0.25	Not irritating[d]	Slight
	Diisopropanolamine (li)	1.00	4.00	0.75	Slight	Slight
	Promethazine (li)[c]	—	—	—	Severe	Severe
Ketones	Acetone (li)	1.83	6.40	2.50	Moderate	Moderate
Esters	Ethyl acetate (li)	0.83	14.00	2.50	Slight	Slight
	γ-Butyrolactone (li)	1.67	8.20	2.50	Moderate	Moderate
Aromatics	Dimethyl benzene (li)	1.00	3.60	2.00	Slight	Slight
Ethers	Aether (li)	1.08	6.10	1.50	Slight	Slight

Note. [a]Immediate corneal opacity score 3, classified as severely irritating. [b]Corneal opacity score 4, classified as severely irritating. [c]Severe loosening of epithelium, classified as severely irritating. [d]IRE test classified the eye irritation lower relative to the Draize classification. li=liquid, s=solid.

Table 2. Classification of 26 Cosmetic Products Assessed by the IRE and Draize Tests

Category	Cosmetic Products	Corneal Opacity	Corneal Swelling (%)	Fluorescein PEnetration	IRE test Classification	Draize test Classification
Shampoo	A (li)	1.25	70.37	2.75	Moderate[a]	Slight
	B (li)	0.98	6.17	0.69	Slight	Slight
	C (li)	0.54	7.32	0.88	Slight	Slight
	D (li)	0.54	1.22	0.88	Slight	Slight
	E (li)	0.80	2.60	0.73	Slight	Slight
	F (li)	0.89	9.90	0.75	Slight	Slight
Hair-dye	G (p)	0.69	5.37	0.44	Slight	Slight
	H (p)	0.81	11.10	0.69	Slight	Slight
	I (p)	0.79	6.68	0.37	Slight	Slight
	J (p)	0.89	12.75	0.82	Slight	Slight
	K (p)	0.83	17.70	1.67	Moderate	Moderate
	L (p)	0.94	10.00	0.75	Slight[b]	Moderate
	M (p)	0.94	14.20	1.62	Moderate	Moderate
	N (p)	0.83	6.36	0.58	Slight[b]	Moderate
Marcel Agents	O (li)	0.46	0.76	0.25	Not irritating	Not irritating
	P (li)	0.67	2.69	0.25	Not irritating	Not irritating
	Q (li)	0.92	4.80	0.75	Slight	Slight
	R (li)	0.58	12.23	0.50	Slight	Slight
	S (li)	0.79	1.07	0.13	Not irritating[b]	Slight
	T (li)	0.58	8.83	0.38	Slight	Slight
Cleaning Foam	U (li)	0.54	3.02	0.13	Not irritating	Not irritating
	V (li)	1.25	6.17	0.56	Slight	Slight
	W (li)	0.25	2.09	0.25	Not irritating	Not irritating
	X (li)	1.08	4.76	1.17	Slight	Slight
	Y (s)	0.72	6.60	0.42	Slight	Slight
	Z (li)	0.50	1.35	0.25	Not irritating[b]	Slight

Note. [a]IRE test classified eye irritation higher relative to the Draize classification. [b]IRE test classified eye irritation lower relative to the Draize classification. li=liquid, s=solid, p=paste.

In Vivo Rabbit Eye Test

The classifications of 26 test materials come from the ECETOC reference chemicals data bank[11], and those of 26 cosmetics from the data base of Guangdong Provincial CDC. The Draize test was performed according to the OECD guidelines for chemical testing (TG 405, April 2002). Liquid (0.1 mL), paste (0.1 mL), or solid (0.1 g) test materials were placed in the lower conjunctival sac of the right eye of three New Zealand White rabbits. The eyelids of the treated eyes were held closed carefully for 1 s, and then, after 10 s' contact, were rinsed with warm saline water. The left eye remained untreated and served as a negative control. The reactions of the cornea, iris and conjunctiva were judged at 1 h, 24 h, 48 h, and 72 h after the treatment by using the scoring system[12]. The test materials were classified as not irritating [modified maximum average score (MMAS) $\leq$2.5], slightly irritating (2.5<MMAS$\leq$25), moderately irritating (25<MMAS$\leq$59), or severely irritating (MMAS>59) according to ECVAM criterion.

Statistical Analysis

The data analysis was performed with SPSS Ver.16.0 software for Windows (SPSS Inc., Chicago, USA). *In vivo* (Draize test as "the Golden Standard") and *in vitro* comparison was made by McNemar-Bowker, Gamma and Kappa test. Analysis of variance (ANOVA) was applied to evaluate the repeatability of

IRE test of 26 test materials. The sensitivity, specificity, Youden's index and agreement rate were used for describing the prediction capability of IRE test for different irritant substances.

Quality Control

All test martial were numbered with blinding methods. Four eyes were used per test material and one eye was included as a negative control. The eyes were left in the superfusion apparatus for 30 min to 45 min for stabilization and equilibration, and after that, they were examined to discard any damaged eyes if their corneal opacity and fluorescein penetration or (and) corneal swelling rate were more than 5%. In case that any corneal opacity and fluorescein penetration or (and) corneal swelling rate were found more than 7% in the negative control eye after the experimental period, the result of this test would be abandoned.

RESULTS

Development of IRE Test

No obvious changes in corneal opacity or fluorescein penetration or staining were observed in the negative control corneas throughout the testing. Furthermore, no histopathological changes were observed in the H&E stained negative control corneas after completing the testing. These findings suggested that there were no obvious adverse impacts upon the corneas resulting from the superfusion apparatus or other assay procedures. The average increase in corneal swelling for the negative controls was less than 7%, which was consistent with quality control criteria.

All 26 test chemicals were compatible with both IRE test and Draize test procedures, and could be readily tested without difficulties. The full range of corneal opacity scores (Figure 1), fluorescein penetration scores (Figure 2), and histopathological changes (Figures 3 and 4) in the IRE test were observed for the eye irritants tested.

Ranking Correlation and Class Concordance between IRE Test and Draize Test

The classification of 22 chemicals determined by the IRE test (30 of the 35 chemical tests) were equal to that determined by the Draize test. However, four of the tested chemicals (solid sodium oxalate, 15% and 30% SDS, polyethylene glycol 400, and triethanolamine) were classified as one category lower by the IRE test compared to the Draize test (Table 1). The relative frequency of lower classification by the IRE test compared to the slight, moderate and severe categories as determined by the Draize test was 2/13, 1/9, and 2/12, respectively, suggesting that the predictive power was similar across the range of irritancy categories (Table 3).

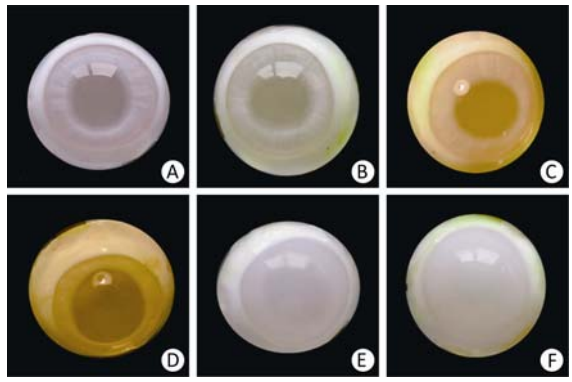

Figure 1. Corneal Opacity Scoring. A (Negative control, 0 point): No opacity. B (1%NaOH, 1 point) and C (L-Aspartic Acid, 1 point): Scattered or diffuse area, details of iris clearly visible. D (5%Triton-X-100, 2 points): Easy discernible translucent area, details of iris slightly obscured. E (30%TCA, 3 points): Nacreous area, no details of iris visible, size of pupil barely discernible. F (Acetic acid, 4 points): Opaque cornea, iris not discernible through opacity.

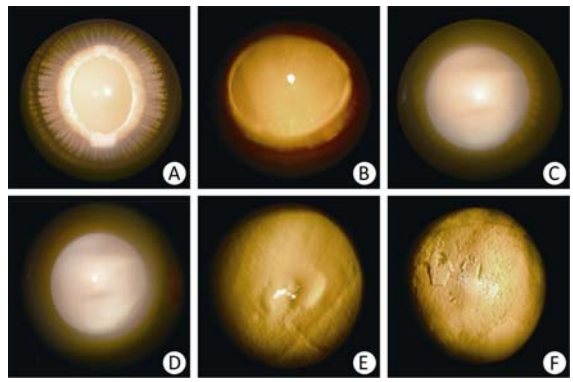

Figure 2. Pathological Changes of the Corneal Surface. (A, B, C, and D ×10; E and F ×26). A (Negative control): No damage, details of iris clearly visible. B (0.1% Cetylpyridium bromide): Scatteredly or diffusely damaged areas. C (30%TCA): Nacreous area, no details of iris visible, size of pupil barely discernible. D (Acetic acid): Opaque cornea, iris not discernible through opacity. E (1%NaOH): Bubbles. F (10%NaOH): Severe loosening of epithelium.

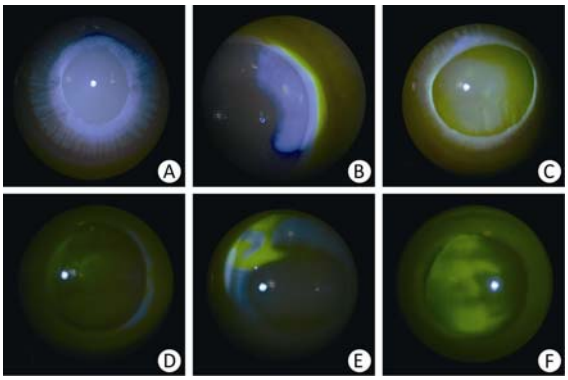

Figure 3. Penetration of Fluorescein into the Cornea (A, C, D, E, and F×10; B×26. 2% fluorescein). A (Negative control, 0 point): No staining. B (Calcium mercaptoacetate, 0.5 point): Minor single cell staining. C (Sodium oxalate, 1 point) and D (15%SDS, 1 point): Bright green staining of anterior edge of cornea but no penetration. E (30%SDS, 2 points): Focal or confluent dense small area staining. F (1%NaOH, 3 points): Bright green staining of anterior edge of cornea, gradual diffusion of stain through cornea.

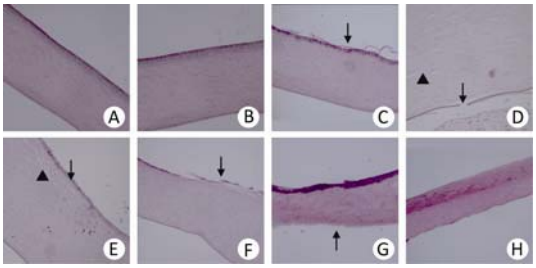

Figure 4. Histopathological Changes of the Cornea (A, B, C, E, F, G, and H ×200; D ×400 H & E). A (The fresh isolated cornea of healthy rabbit): The epithelial layer, Bowman membrane, stroma, Descemet's membrane and endothelial layer were undamaged. B (The negative control cornea maintenance in the superfusion apparatus for 4 h): No obvious adverse changes were seen. C (3%TCA): The outermost layer of squamous epithelial cells was eroded (as shown by the arrow). D (30%TCA): Interruption of Descemet's membrane (as shown by the arrow), stroma swelling (as shown by ▲). E (15%SDS): The squamous epithelium and wing cell layers were eroded (as shown by the arrow), stroma swelling (as shown by ▲). F (30%SDS): The basal layer of epithelium was eroded (as shown by the arrow). G (1%NaOH): The endothelial layer and Descement's membrane were eroded completely (as shown by the arrow). H (10%NaOH): Most of the corneal structure was destroyed, with only the stroma remaining.

While the test chemicals induced a full range of eye irritation responses in the IRE and Draize tests, the cosmetic products were relatively mild and resulted in no more than moderate eye irritation. The IRE test identified 21 of the 26 cosmetic products in the same eye irritation classification as the Draize test. Shampoo A was classified as one category higher, and hair styling product S, cleaning foam Z, hair-dye L and N were classified as one category lower (Table 2). Since the cosmetic products were generally only mild eye irritants, the results from this study would be inappropriate for evaluating the predictive power of the assay across all irritation categories. The correlation analysis between the IRE test and the Draize test are presented in Table 4.

Table 3. IRE and Draize Classifications of 26 Test Chemicals (*n*=35)

IRE Test Classification	Draize Test Classification				
	Not	Slight	Moderate	Severe	Total
Not	4	0	0	0	4
Slight	0	10	1	1	12
Moderate	0	0	8	2	10
Severe	0	0	0	9	9
Total	4	10	9	12	35

Note. Fisher's Exact Test χ^2=51.314, *P*<0.001; McNemar *P*=0.261; Gamma=0.960, *P*<0.001; Kappa=0.843, *P*<0.001.

Table 4. IRE and Draize Classifications of 26 Cosmetic Products (*n*=35)

IRE Test Classification	Draize Test Classification			
	Not	Slight	Moderate	Total
Not	4	2	0	6
Slight	0	15	2	17
Moderate	0	1	2	3
Total	4	18	4	26

Note. Fisher's Exact Test χ^2=15.522, *P*<0.001; McNemar *P*=0.311; Gamma=0.967, *P*<0.001; Kappa=0.611, *P*<0.001.

Reliability of IRE Test

ANOVA indicated that there was no statistical significance among the classifications of IRE tests for

26 chemicals, which repeated 3 times (F=0.439, P=0.646), demonstrating that the IRE test was reliable, repeatable and stable.

Validity of IRE Test

The validity of IRE test is shown in Table 5 for different irritant substances.

Table 5. Validity of IRE Test for Different Irritant Chemicals and Cosmetic Products

	Classification	Sensitivity (%)	Specificity (%)	Youden's Index	Agreement Rate (%)
Chemicals	Not	100.0	93.9	0.939	94.3
	Slight	84.6	95.5	0.801	94.3
	Moderate	88.9	87.5	0.764	91.4
	Severe	81.8	100.0	0.818	94.3
Cosmetics	Not	84.6	69.2	0.538	76.9
	Slight	44.4	88.2	0.326	73.1
	Moderate	100.0	95.5	0.955	96.2

DISCUSSION

In this study, all the test materials (26 chemicals and 26 cosmetic products) were examined by the IRE test regardless of their physical state, such as liquid, solid or paste. High correlations between the *in vivo* classifications and the IRE classifications were obtained, especially for the chemical material compounds. Some severely irritating chemicals, such as alkaline and acid materials, and anionic and cationic surfactants, were clearly classified; however, several other compounds, including sodium oxalate (solid, MMAS=61), 5% SDS (MMAS=59), and 30% SDS (MMAS=60.5), with MMAS scores near the threshold for moderate/severe eye irritation predictions (MMAS ≥ 59) were underestimated. These differences may be explained, in part, by the facts that the IRE test parameters do not reflect the conjunctival damage occurring *in vivo*, and that the IRE test may be not sensitive enough to distinguish these boundary compounds.

The IRE test offers multiple endpoint alternatives to the Draize test: corneal opacity and fluorescein penetration which represent the most important aspects of ocular irritation, and corneal swelling, In this study, the additional endpoint of histological evaluation of various corneal tissue layers was a useful and sensitive endpoint for corneal damage, since microscopic evaluation detected slight histopathological changes, even in the absence of obvious corneal opacity or fluorescein penetration changes. However, a standardized histopathological scoring scheme and a means to combine the evaluations with other endpoints for eye irritation classification require investigation and refinement in future studies. The *ex vivo* IRE test does not include inflammatory response elements, as in the *in vivo* Draize test, nor is the assay a model for corneal recovery under these test conditions. However, the histopathological endpoints may aid in the assessment of the depth and degree of corneal injuries, which are predictive of the potential for corneal recovery. Therefore, the IRE test is a promising alternative method to the Draize test.

For the cosmetic products, slightly lower but still significant correlation was found between the IRE test classification and the Draize test classification. Surfactants contained in some cosmetic products (e.g., shampoo, cleaning foam) may induce corneal opacity *in vivo* by damaging the corneal epithelium, as the result of corneal swelling and disruption of the normal corneal epithelial and stromal elements[14]. However, numerous studies demonstrated that the milder surfactants used in personal care products induced limited opacity in isolated corneas, even when damage was observed[15]. In this study, 4 cosmetic products were classified as less irritating by the IRE test, although one shampoo (shampoo A) was classified as more irritating due to increased fluorescein penetration and more than 70% corneal swelling. Most of the corneal opacity scores of these products were less than 1, and less obvious corneal damage was seen in the IRE test than in the Draize test. Overall, the most irritating cosmetic products resulted in less eye irritation than the most irritating reference

chemicals, and the majority of the cosmetic products were classified as not irritating or slightly irritating.

These results demonstrate that the IRE test results correlate well with the *in vivo* test. Where the IRE classified chemicals as less irritating than the Draize classification, the difference was only one category, and the predictive power was generally similar across the irritancy categories. However, as noted by other studies, the IRE assay was originally designed to detect severe eye irritants. Within the context of a tiered eye irritation strategy and according to widely held principles[16], we believe that the IRE test will be useful as a prescreening test for severe irritants and will reduce the use of experimental rabbits in eye irritation testing. Furthermore, modifications of the test procedure and further mechanistic research and refinement are warranted, to achieve greater efficacy for potential use as a replacement for *in vivo* methods.

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