

## Evaluation of a New Alternative to Primary Draize Skin Irritation Testing Using the EpiDerm™ Skin Model

Michiru GENNO<sup>1</sup>, Ryohei YAMAMOTO<sup>1</sup>, Hajime KOJIMA<sup>2</sup>, Hiroaki KONISHI<sup>2</sup> and Mitch KLAUSNER<sup>3</sup>

<sup>1</sup>Technical Research Laboratory, Kurabo Industries Ltd. 14-5, Shimokida-cho, Neyagawa-shi, Osaka, 572-0823, Japan.

<sup>2</sup>Research Institute, Nippon Menard Cosmetic Co., Ltd., 2-7, Torimi-cho, Nishi-ku, Nagoya, 451-0071, Japan

<sup>3</sup>MatTek Corporation, 200 Homer Avenue, Ashland, MA 01721, USA

### Abstract

EpiDerm™ is a commercially available human skin model consisting of normal human-derived epidermal keratinocytes (NHEK), which have been cultured to form a multilayered, highly differentiated model of the human epidermis. We evaluated this model as an alternative to the primary Draize skin irritation test using rabbits.

Eighteen cosmetic products were tested using a new assay system which utilized the EpiDerm™ and a single 24-hour exposure at 100% concentration. The MTT assay results were compared to the results from skin irritation Draize testing using rabbits. The correlation coefficient for 18 test substances between the *in vivo* and *in vitro* results was  $r = -0.671$ . However a much better correlation was obtained provided that 2 products, both of which contained high ethanol levels (>60% (w/w)), were excluded from the analysis ( $r = -0.870$ ). As such, this method may prove useful in making *in vitro* skin irritation assays more economical and convenient.

### Introduction

Recently, to reduce the number of animals used for testing chemicals, drugs, and cosmetics

etc., many alternative *in vitro* methods have been developed. Regarding prediction of the skin irritation, monolayer cultures and 3-dimensional tissue models composed of human skin have

Correspondence : Michiru Genno, Technical Research Laboratory, Kurabo Industries Ltd., 14-5 Shimokida-cho Neyagawa-shi, Osaka 572-0823, Japan. Tel.: +(81) 720 23 8800, Fax: +(81) 720 23 7906, E-mail: genno@lab.kurabo.co.jp

Key words : skin irritation test, skin model, EpiDerm™, detergents, cosmetic products, MTT% assay, single-point assay

Received : April 17, 1998.

Accepted : September 21, 1998.

Abbreviations:

NHEK: normal human epidermal keratinocytes

Skin irritation test: primary Draize skin irritation test

MTT-50: the time of exposure that reduces mitochondrial activity by 50%

MTT%: cell viability calculated using the following formula,  $MTT\% = 100 \times \frac{[A570(\text{sample}) - A650(\text{sample})]}{[A570(\text{negative control}) - A650(\text{negative control})]}$

MDSS: maximal primary Draize rabbit skin irritation score

PBS: phosphate-buffered saline

Table 1. Alcohol contents, MDSS and MTT% values of 18 final cosmetic products.

Sample	Abbreviation	Alcohol content(%)	MDSS**	MTT%(%)***
Shampoo 1	S1	0	7.0	4.4
Shampoo 2	S2	0	4.0	5.6
Rinse 1	S3	0	3.0	67.5
Rinse 2	S4	0	1.7	51.1
Face wash 1*	S5	0	2.0	27.8
Face wash 2*	S6	0	2.0	32.0
Face wash 3	S7	0	5.7	4.0
Face wash 4*	S8	0	2.0	27.8
Cleansing cream*	S9	0	1.8	103.1
Skin lotion 1	S10	6.0	0.8	103.7
Milky lotion*	S11	0	2.2	114.5
Skin lotion 2	S12	10.0	0.5	61.8
Cream*	S13	0	1.5	111.4
Massage cream*	S14	0	0.7	110.6
Face pack 1*	S15	8.0	0.0	113.1
Face pack 2	S16	0	1.5	96.7
Hair liquid	S17	63.0	0.2	14.3
Hair tonic	S18	70.0	0.3	13.3

\*These samples were applied to the skin model as the amount of 100mg. The others applied as the volume of 100ul.

\*\*MDSS : Maximal primary Draize rabbit skin irritation score.

\*\*\* $MTT\% = 100 \times \frac{[A570(\text{sample}) - A650(\text{sample})]}{[A570(\text{negative control}) - A650(\text{negative control})]}$

been used to predict the irritancy of substances (Triglia *et al.*, 1989, Torishima *et al.*, 1990, Naughton *et al.*, 1989, Bell *et al.*, 1988, Cannon *et al.*, 1994). In this report, a new method of using the 3-dimensional skin model, EpiDerm™ which consists of normal human-derived epidermal keratinocytes (NHEK), was evaluated as an alternative to the primary Draize skin irritation test by MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Mosmann, 1983). It is reported that EpiDerm™ has a structure which is very similar to real skin and it contains many protein markers of mature epidermis-specific differentiation (Cannon *et al.*, 1993). Good correlation with the skin irritation test has also been reported (Cannon *et al.*, 1994, Doyle *et al.*, 1994, Perkins *et al.*, 1996). These results are mainly given by calculation of MTT-50 values that is the time of exposure that reduces mitochondrial activity by 50%. When the toxicity of substances is evaluated, tests are often conducted at many concentrations or exposure times. This is a reasonable strategy to determine the poten-

tial dermal irritancy or toxicity of new cosmetic products. However, it is also important to put alternative methods to practical use. If *in vitro* tests can be made simpler, more cost effective, and less labor intensive, more examiners will use them. With these goals in mind, we evaluated the ability of EpiDerm™ to predict the skin irritation potential of final cosmetic products using a single point assay that employed a single concentration and exposure time.

## Materials and Methods

### Materials

#### Test Substances

The 18 test substances used for this study were cosmetic products (Table 1). They were chosen for this study because they had a wide range of dermal irritancy. They were supplied by Nippon Menard Cosmetics Co.,Ltd. (Nagoya, Japan). All substances were applied to the EpiDerm™ test at 100% concentration.

**Fig. 1** A schematic depiction of dosed EpiDerm™.

#### Skin Model

The EpiDerm™ Skin Model (part # EPI-100a) was imported by Kurabo Industries (Osaka, Japan) from MatTek Corporation (Ashland, MA, USA) for use in this study. Note: EpiDerm™ consists of NHEK, which have been cultured to form a multilayered, highly skin-like structure on specially prepared permeable cell culture inserts (Fig. 1).

#### Methods

##### Draize skin irritation test

Six Japanese white female rabbits weighing from 2.9 to 3.5kg were used for the *in vivo* portion of this study. The rabbits were shaved on the trunk and lateral areas. Test substances at 100% concentration (0.15 ml) were applied to intact skin under 1.7cm diameter gauze patches. After 24hr the patches were removed and the skin response was scored for erythema and oedema for each rabbit at 1 and 24 hr after the removal (Draize *et al.*, 1944, FDA officials, 1959). The average value was calculated at each time for each test substance and the maximum value between two time periods was used as the observed value of MDSS (maximal primary Draize rabbit skin irritation score)(Kojima *et al.*,1995).

##### MTT% assay (Single point assay)

The cytotoxicity on EpiDerm™ skin-model was evaluated by MTT, which was purchased from Dojindo Laboratories, Kumamoto, Japan. MTT solution was used at 1mg/ml in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY). The test substances were applied to the apical surface of the skin model for 24 hours at 37C° in a humidified atmosphere (95% air and 5%CO<sub>2</sub>). For highly viscous test substances that cannot be pipetted, applicator pins were used to provide a reproducible dose. They were put on the head of a pin as the amount of 100mg, and applied to the skin model. After removing test substances by washing with 1ml phosphate-buffered saline (PBS) twice, the skin model cups were transferred to new 24-well multi-plates filled with 300µl MTT solution. The skin model cup (Millicell CM, Millipore Corp., Bedford, MA, USA) contacted with the MTT solution only at the bottom. After reaction for 3 hours at room temperature, they were washed with 1ml PBS twice. Then the tissues were transferred to new 24-well multi-plates and were submerged in 2ml of extraction solution (isopropanol). Extraction of the MTT was allowed to proceed overnight at room temperature in the dark in 24-well plates which were sealed in plastic bags. Prior to assaying, the extract was homogenized. Then the absorbance of the solution was measured at 570nm using spectrophotometer (U-3210,

**Fig. 2** Correlation between MTT% and MDSS for 18 cosmetic products.

The #S1 to S18 are abbreviations for cosmetic products shown in Table 1.

MTT% was calculated from following formula:  $100 \times \{[A570(\text{sample})-A650(\text{sample})]/[A570(\text{negative control})-A650(\text{negative control})]\}$  using skin model EpiDerm™. MDSS means maximal primary Draize rabbit skin irritation score.

Hitachi Ltd., Tokyo, Japan). The absorbance at 650nm was used as reference. The cell viability (MTT%) was calculated using the following formula:

$$\text{MTT\%} = 100 \times \{[A570(\text{sample})-A650(\text{sample})]/[A570(\text{negative control})-A650(\text{negative control})]\}$$

Distilled water was used as the negative control. In the case of using applicator pins, the negative control was determined by applying water with a pin on the skin model. Each MTT% value was determined from triplicate assays.

## Results

### *MDSS and MTT% assay (Single point assay)*

The MDSS values and MTT% values for 18 test substances are shown in Table 1 and the relationship between them is shown in Fig. 2. As a

**Fig. 3** Effect of ethanol on EpiDerm™.

MTT% was calculated from following formula:  $100 \times \{[A570(\text{sample})-A650(\text{sample})]/[A570(\text{negative control})-A650(\text{negative control})]\}$  using skin model EpiDerm™. MDSS means maximal primary Draize rabbit skin irritation score.

result, the coefficient of correlation between MTT% values and the MDSS values in 18 test substances was  $r = -0.671$ . A relatively good correlation between them was observed with exception of the hair liquid (S17) and the hair tonic (S18) ( $r = -0.870$ ). S17 and S18 showed relatively strong toxicity to the skin model compared with the result from *in vivo*. Because S17 and S18 contain high concentrations of ethanol (>60%), the cytotoxicity of ethanol on the skin model was examined (Fig. 3). It was found that ethanol above 30% decreased skin model viability.

## Discussion

The 18 test substances which were all cosmetic products, were tested by the MTT% assay (Single point assay) to evaluate the possibility of using EpiDerm™ as an alternative method to the rabbit skin irritation test.

The single point assay for the cosmetic prod-

ucts gave a relatively good correlation with the *in vivo* results (Fig. 2). Good correlation ( $r = -0.870$ ) with the *in vivo* results was observed with the exception of the two cosmetics containing high concentrations of ethanol. The two cosmetic products (S17 and S18) were relatively toxic to the skin model in relation to the *in vivo* results. In an effort to understand why the skin model was overpredicting the irritation potential of these substances, we confirmed the toxicity of ethanol alone on the skin model (Fig. 3). In a dose-response test, it was found that ethanol concentrations above 30% affected the skin model, but had minimal effects *in vivo* (Fig. 3). The explanation for this difference is believed to be due to the higher permeability of ethanol through the skin model versus true skin. In a related tissue model produced by MatTek Corporation (EpiOcular™), ocular irritation is overpredicted for ethanol containing substances and requires special handling as a separate group of substances (Klausner et al., 1997).

Furthermore, some test substances representing approximately score 2 in MDSS, which are estimated as mild toxicants in MDSS, were not clearly discriminated by the MTT% values (e.g. S4, S5, S6, S8, S9 and S11 in Fig.2). However moderate or severe toxicants (S1, S2 and S7) and non toxicants (S10, S12, S14 and S15) were predicted by the MTT% values. These facts show that this *in vitro* method can discriminate between non toxicants and moderate or severe ones except for cosmetic products containing high concentrations of ethanol. If we try to discriminate mild toxicants, or to predict the toxicity of

cosmetic products containing high concentrations of ethanol, it seems that other detection methods are needed. We applied only the single point MTT% assay on the skin model. It is possible that other assay methods will give different results, for example, cytokine release or the leakage of enzymes. Recently, some cytokines and chemical mediators related to skin inflammation have been analyzed in this skin model (Kubilus et al., 1996). The combination of cell viability assay and cytokine release assay may offer a more accurate prediction for skin irritation.

An advantage of skin model cultured at the air-interface is that it presents a useful dosing surface to apply test substances in a manner similar to topical application to human skin (Osborne et al., 1994). EpiDerm™ consists of organized basal, spinous, granular, and cornified layers analogous to those found *in vivo* (Appa et al., 1996). Creams can be applied directly to the apical surface which has stratum corneum. The 3-dimensional and differentiated structure is a distinct advantage of this *in vitro* method and may give a good correlation with *in vivo*.

Thus, with some exceptions, this single point assay using EpiDerm™ is likely to be applicable for cosmetic products and become a good alternative to the primary Draize skin irritation test because it is simple, cost effective, and less labor intensive.

We conclude that EpiDerm™ appears to be a useful pre-clinical tool which can assure the safety of volunteers used to test new cosmetic products.

## References

- Appa, Y., Asuncion, A.G., Podda, M., Duke, M. A., Rachui, S. R. (1996) An *in vitro* method to evaluate the antioxidant efficacy of topical formulations, Presented at American Acad. of Dermatol. Annual Meeting.
- Bell E., Parenteau N.L., Haimes H.B., Gay, R.J., Kemp, P.D., Fofonoff, T.W., Mason, V.S., Kagan, D.T. and Swiderek, M. (1988) Testskin: a hybrid organism covered by a living human skin equivalent designed for toxicity and other testing, In alternative methods in toxicology, vol. 6, 15-25.
- Cannon, C.L., Klausner, M., Kubilus, J., Neal, P.J. (1993) Initial characterization of a new model for Dermal irritancy, Presented at Tissue Culture Association Annual Meeting, San Diego, CA USA.
- Cannon, C.L., Neal, P.J., Southee, J.A., Kubilus, J. and Klausner, M. (1994) New epidermal model for dermal irritancy testing, Toxic. in Vitro, 8, 889-891.
- Doyle, J.M., Dressler, W., Rachui, S.R. (1994) Evaluation of two *in vitro* human skin equivalents (the EpiDerm and Skin<sup>2</sup> model ZK1300) for assessing the skin irritation potential of personal care products and chemicals, Presented at symposium, alternatives in the assessment of toxicity; theory & practice, Aberdeen,

MD USA.

- Draize, J., Woodard, G. and Calvery, H. (1944) Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes, *Journal of Pharmacology and Experimental Therapeutics*, 82, 377-390.
- Food and Drug Administration (1959) Appraisal of the safety of chemicals in foods, drugs and cosmetics by the staff of the division of Pharmacology, Food and Drug Administration, Department of Health, Education and Welfare, 48-52.
- Kojima, H., Hanamura, A., Miyamoto, S., Sato, A., Konishi, H. and Yoshimura, I. (1995) Evaluation of seven alternative assays on the main ingredients in cosmetics as predictors of Draize eye irritation scores, *Toxic. in Vitro*, 9, 333-340.
- Klausner, M., Kubilus, J. (1997) EpiOcular prediction model, a reproducible in vitro means of predicting Draize scores, *The Toxicologist*, 36 (1), 44.
- Kubilus, J., Cannon, C., Neal, P., Sennott, H. and Klausner, M. (1996) Response of the EpiDerm skin model to topically applied irritants and allergens, *In Vitro Toxicology*, 9, 157-166.
- Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay, *Journal of Immunological Methods*, 65, 55-63.
- Naughton, G.K., Jacob, L. and Naughton, B.A. (1989) A physical skin model for in vitro toxicity studies, In *alternative methods in toxicology*, vol.7, 183-189.
- Osborne, R. and Perkins, M.A. (1994) An approach for development of alternative test methods based on mechanism of skin irritation, *Fd. Chem. Toxic.*, 32, 133-142.
- Torishima, H., Arakawa, H., Matsui, S. and Watanabe, M. (1990) Application of normal human epidermal keratinocytes in serum free medium as an alternative to the Draize ocular irritating test, *AATEX*, 1, 20-26.
- Triglia, D., Wegener, P.T., Harbell, J., Wallace, K., Matheson, D. and Shopsis, C. (1989) Interlaboratory validation study of the keratinocytes neutral red bioassay from Clonetics Corporation, *Altern. Methods Toxicol.*, 7, 357-365.