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"A novel reconstructed human epidermis model developed by for skin irritation tests"

Fan Chen[1], Dailin Xu [2] , Yuqing Hu [2], Yijie Liu [2]and Yue Wu [2,*]

[1] *In Vitro* Research Department, Bloomage Biotechnology Corporation Limited; [2]*In Vitro* Research Department, Bloomage, Biotechnology Corporation Limited, Shanghai, China

1. Introduction

Over the past eight decades, the transition from animal testing to alternative methods in cosmetics regulation has been driven by ethical imperatives and scientific innovation, marking a transformative shift in safety assessment paradigms. Initially, the 1938 U.S. Food, Drug, and Cosmetic Act mandated animal safety testing, establishing a precedent for cosmetic safety evaluations. The European Union pioneered regulatory changes by prohibiting animal-tested cosmetic ingredients in 2009 and enforcing a full ban on animal testing for cosmetics in 2013, driven by ethical concerns and advancements in alternative methods. This catalyzed global momentum, with countries like India and Norway adopting similar bans [1]. Reconstructed human epidermis (RHE) models have been increasingly adopted as in vitro alternatives to animal testing for evaluating skin irritation, corrosion, and dermal safety of cosmetic ingredients, aligning with global regulatory shifts toward non-animal methodologies.

As the largest organ of the human body, skin acts as the primary natural barrier to the external environment, playing a crucial role in protecting against pathogenic microorganisms, regulating water loss, and preventing the penetration of solar ultraviolet radiation and chemicals [2]. The Draize rabbit skin irritation test has been used to predict the skin irritation potential of xenobiotics since 1946, which exhibits several drawbacks such as high inter-laboratory and inter-animal variability. The development of RHE models, such as Episkin™ and EpiDerm™, marked a pivotal transformation towards *in vitro* alternatives [3]. Subsequent to the adoption of the *in vitro* skin irritation RHE test method as OECD Test Guideline 439, new RHE models have been encouraged to be further developed for skin irritation testing (SIT) [4]. The OECD TG 439 were separated into an independent document in 2015, referred to as Guidance Document 220 (GD 220), which demonstrates the functioncal conditions such as cell viability, barrier function, reproducibility, the predictive performance of reference chemicals for the validation of new RHE models for skin irritation test [5],[6]. Currently, seven RHE models have been formally validated and incorporated into OECD TG 439 as reference methods for skin irritation test, including Episkin™ (SM), EpiDerm™ SIT (EPI 200), SkinEthic RHE™, LabCyte EPI-Model24 SIT, epiCS®, Skin+® and KeraSkin™ SIT [6].

In this study, we have developed an innovative RHE model at Bloomage Biotechnology Co., Ltd., which was derived from normal human primary keratinocytes. The RHE displays stratified epithelial morphology, consisted of organized basal layer, fully-differentiated stratum spinosum, granular layer, and stratum corneum. At the meantime, standard performance of the RHE was verified according to OECD TG 439, including morphology, viability, reproducibility, tissue integrity and barrier function. To evaluate the predictive capacity of the RHE,

a modified protocol for distinguish skin irritant from non-irritant chemicals, which was defined in United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) for the classifying skin irritation, was developed [7]. In the present study, the RHE was tested in three independent runs using 20 chemicals based on the protocol to evaluate inter-laboratory reproducibility, predictive capacity including sensitivity, specificity and accuracy according to OECD GD220 [6].

2. Materials and Methods

2.1 Materials

Twenty test substances listed in the minimum list in OECD GD 220 were evaluated and compared with VRMs. The test substances consisted of 10 non-irritants and 10 irritants (**Table 1**), which were obtained from Sigma-Aldrich in a purity of at least 95%. 5% (w/v) SDS and phosphate-buffered saline (PBS) were used as positive control and negative control separately.

2.2 Reconstructed human epidermis model

The reconstructed human epidermis (RHE) model were derived from normal human foreskin keratinocytes. Briefly, keratinocytes were seeded in polycarbonate cell culture inserts for one day, followed by air-liquid interface culture for another 7 days.

2.3 Quality Control

2.3.1 Tissue viability

The tissue viability were evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The RHE model were incubated with 300 µL of 0.5 mg/mL MTT at 37℃ for 3 h±15 min. After that, 1 mL Isopropanol was used to extract formazan, which was incubated overnight at 4℃. The extracted solutions were transferred to a 96-well plate and the optical density (OD) was measured at 570 nm.

2.3.2 Barrier function

The barrier function of RHE were assessed by measuring the half inhibitory concentration of SDS. The tissues were treated with varying concentrations (0, 1, 2 mg/mL)of SDS for 18 h at 37℃, 5% CO_2 and the viability was measured by MTT assay. A calibration curve was generated to represent the percentage of viability relative to the 0 mg/mL. The IC_{50} value was then determined by fitting a regression curve.

2.4 Morphology and Immunofluorescence assay

For morphology detection, the RHE models was fixed with 10% neutral formalin and dehydrated with gradient ethanol, followed by paraffin embedding. Sections were cut into slices of 5 µm and stained with henatoxylin and eosin stain (H&E). The observation were performed under a microscope.

For immunofluorescence assay, the RHE models were embedded with O.C.T. and cut into slices of 5 µm, fixed with cold methyl alcohol for 15 min. Subsequently, the sections were blocked with 5% (w/v) BSA for 1 h and incubated in specific primary antibodies for P63 (ab124762,Abcam), loricrin (ab85679,Abcam), Claudin-1(51-9000, Invitrogen), e-cahderin (20874-1-AP, Proteintech) and filaggrin (ab81468,Acbam) overnight at 4℃ and incubated for 1 h with corresponding secondary antibodies. The slices were then mouted with a mounting media containing DAPI to counterstain nuclei. Photos were taken under a fluorescence microscope.

2.5 Protocol for skin irritation test

The RHE models were transferred into new 24 well plates containing 1 mL of pre-warmed culture media. Liquid chemical (8 µL ± 1 µL) or solid chemical (25 mg ± 1 mg), including the negative control (NC) and positive control (PC), were applied in triplicate to the surface of the RHE model for 45 min ±1 min at room temperature. After applying the test substances, we applied the RHE model with a sterile mesh if necessary. In subsequent, the RHE models were rinsed with 2-3 mL PBS each time for 20 times. The inserts were then transferred to a new well containing 1 mL culture media and incubated for 24 h±0.5 h. After 24 h, the inserts were transferred to a new well containing 1 mL culture media for another 18 h±0.5

h. Tissues viability were measured by MTT and optical density (OD) was measured at 570 nm.

2.6 Prediction model for SIT

The test protocol enables the prediction of skin irritation potential for test substances, in accordance with the UN-GHS criteria for classification and labeling. Substances were classified as irritants if tissue viability decreased to ≤ 50% of the negative control. Conversely, tissue viability exceeding 50% of the negative control was considered indicative of non-irritant classification.

3. Results

3.1 The RHE model presented a human epidermis–like morphology

The RHE model displays a well-differentiated stratified epithelial structure, comprising an organized basal layer, stratum spinosum, granular layer, and stratum corneum (**Fig. 1a**). Specific protein expression patterns were observed: E-cadherin and claudin-1 were localized to the cell membrane, while loricrin and filaggrin were expressed in the granular layer (**Fig. 1b**). Notably, the expression of P63 in the basal layer indicates the preservation of keratinocyte stemness (**Fig 1b**). Histological analysis and immunofluorescence assays confirm that the RHE model closely mimics the structural organization and biomarker expression profiles of native tissue.

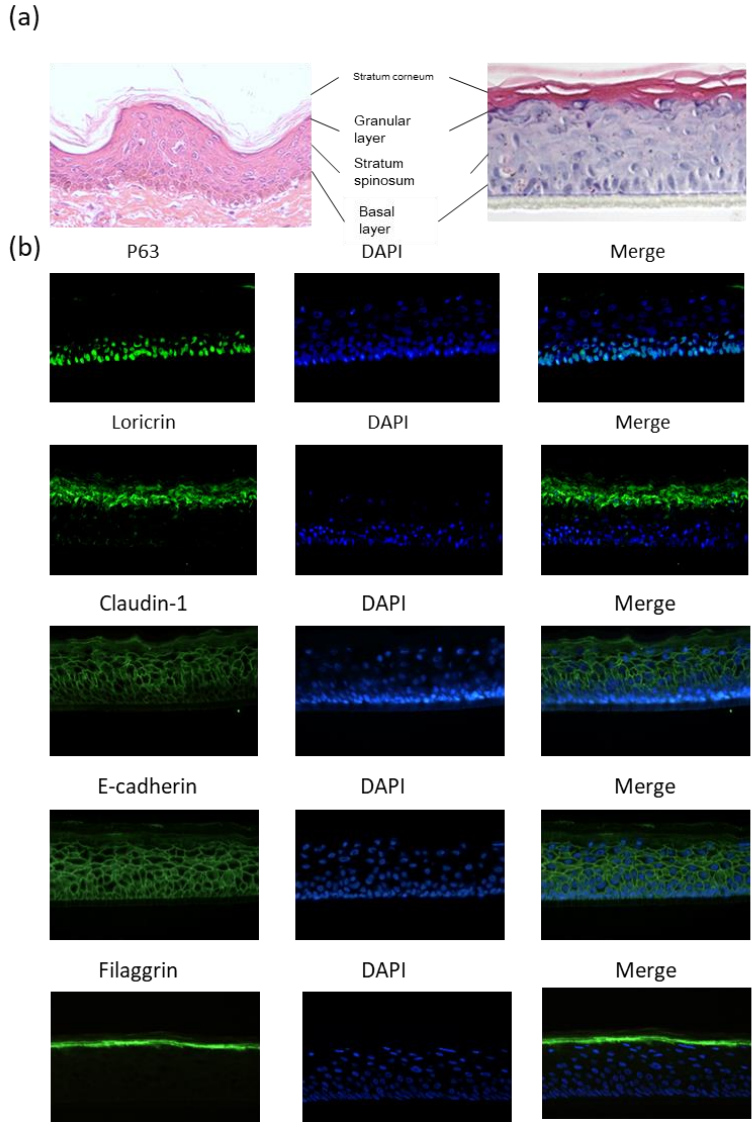

Figure 1. (a) Comparison between H&E stained paraffin-embedded sections of normal human foreskin epidermis (left) and the RHE model (right). (b) Immunofluorescence staining of P63, loricrin, claudin-1, e-cadherin, filaggrin.

3.2 Tissue viability and barrier function of the RHE model

The viability of negative control was measured by treated with PBS and post-incubated for 42 ±1 h, applied with MTT as follow. The OD of NC was 1.13±0.10 (coefficient of variation (CV)=9.42%)in the 10 batches of skin irritation tests (**Fig. 2a**). The tissue integrity and barrier function of the RHE model were determined by evaluating tissue viability resistance to SDS (1 mg/mL, 2 mg/mL) for 18 h. The half inhibitory concentration (IC_{50}) was 1.46±0.11 mg/mL, which is similar to other *in vitro* models in OECD TG 439 (**Fig. 2b**).

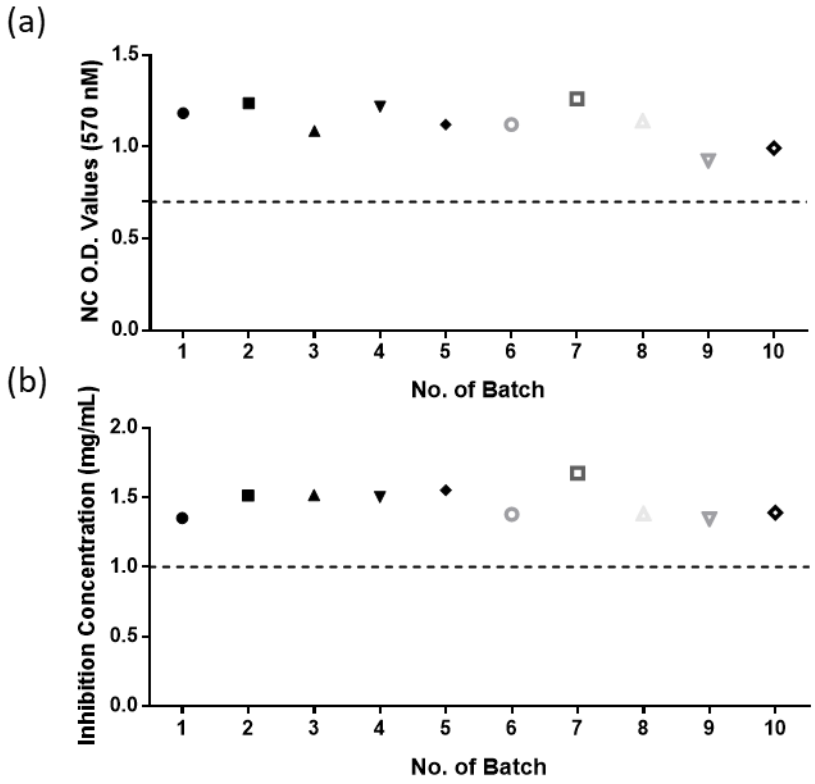

Figure 2. Quality control of the RHE model. (a) Negative control O.D values; (b) IC_{50}.

3.3 Predictive capacity of the *in vitro* skin irritation test of the RHE model

The performance of the *in vitro* skin irritation test using the RHE model was evaluated by analyzing 20 reference chemicals in accordance with OECD GD220. As a result, the specificity and sensitivity of the RHE model were determined to be 70% (7/10) and 90% (9/10), respectively, based on the UN GHS classification categories (**Table 2, Fig. 2**). The overall accuracy of the RHE model was found to be 80% (16/20).

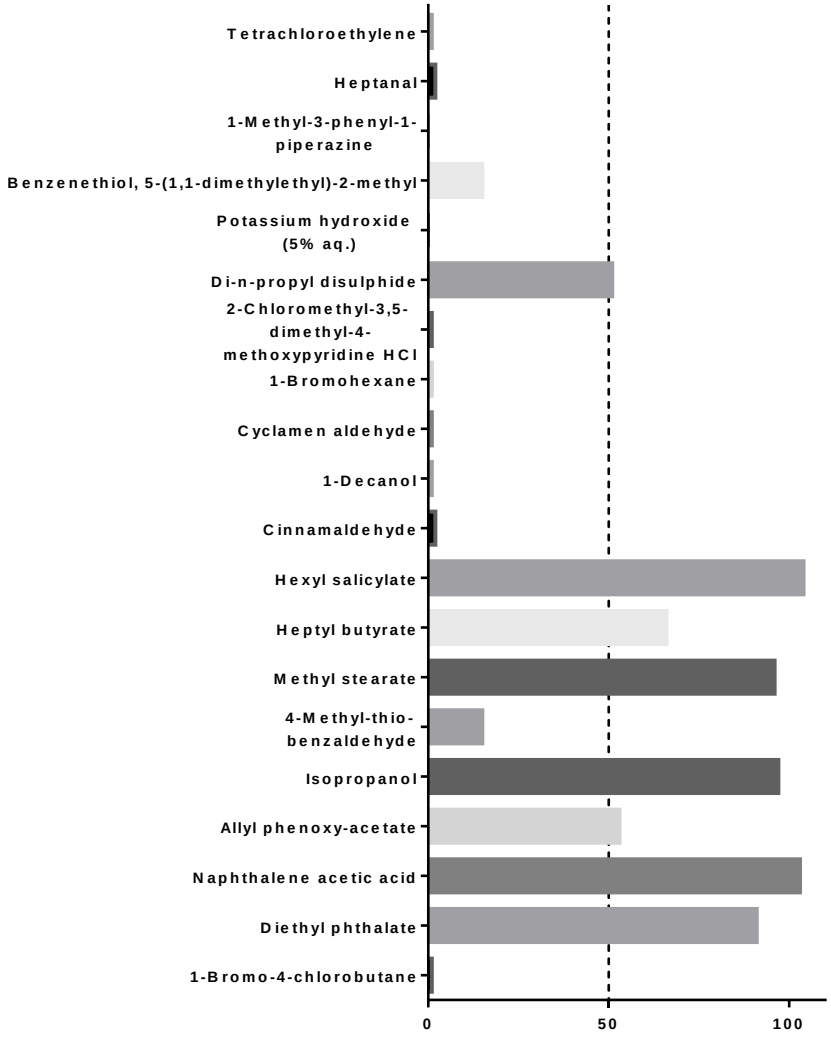

Figure2. Skin irritation results of 20 reference chemicals

Table 1. Test Substance

	Test substance	Cas No.	Solid/Liquid	UN GHS Cat.	Supplier
1	1-Bromo-4-chlorob-tane	6940-78-9	Liquid	No Cat.	Sigma
2	Diethyl phthalate	84-66-2	Liquid	No Cat.	Sigma
3	Naphthalene acetic acid	86-87-3	Solid	No Cat.	Sigma
4	Allyl phenoxy-acetate	7493-74-5	Liquid	No Cat.	Sigma
5	Isopropanol	67-63-0	Liquid	No Cat.	Sigma
6	4-Methyl-thio-benzaldehyde	3446-89-7	Liquid	No Cat.	Sigma
7	Methyl stearate	112-61-8	Solid	No Cat.	Sigma
8	Heptyl butyrate	5870-93-9	Liquid	No Cat. (Optional Cat.3)	Sigma

9	Hexyl salicylate	6259-76-3	Liquid	No Cat. (Optional Cat.3)	Sigma
10	Cinnamaldehyde	104-55-2	Liquid	No Cat. (Optional Cat.3)	Sigma
11	1-Decanol	112-30-1	Liquid	Cat.2	Sigma
12	Cyclamen aldehyde	103-95-7	Liquid	Cat.2	Sigma
13	1-Bromohexane	111-25-1	Liquid	Cat.2	Sigma
14	2-Chloromethyl-3,5-dimethyl-4-methoxypyridine HCl	86604-75-3	Solid	Cat.2	Sigma
15	Di-n-propyl disulphide	629-19-6	Liquid	Cat.2	Sigma
16	Potassium hydroxide (5% aq.)	1310-58-3	Liquid	Cat.2	Sigma
17	Benzenethiol, 5-(1,1-di-methylethyl)-2-methyl	7340-90-1	Liquid	Cat.2	·Sigma
18	1-Methyl-3-phenyl-1-pipera-zine	5271-27-2	Solid	Cat.2	Sigma
19	Heptanal	111-71-7	Liquid	Cat.2	Sigma
20	Tetrachloroethylene	127-18-4	Liquid	Cat.2	Sigma

Table 2. Test result for 20 chemicals

	Test substance	RHE Prediction	UN GHS Cat.
1	1-Bromo-4-chlorob-tane	FP	No Cat.
2	Diethyl phthalate	TN	No Cat.
3	Naphthalene acetic acid	TN	No Cat.
4	Allyl phenoxy-acetate	TN	No Cat.
5	Isopropanol	TN	No Cat.
6	4-Methyl-thio-benzalde-hyde	FP	No Cat.
7	Methyl stearate	TN	No Cat.
8	Heptyl butyrate	TN	No Cat. (Optional Cat.3)
9	Hexyl salicylate	TN	No Cat. (Optional Cat.3)
10	Cinnamaldehyde	FP	No Cat. (Optional Cat.3)
11	1-Decanol	TP	Cat.2
12	Cyclamen aldehyde	TP	Cat.2

13	1-Bromohexane	TP	Cat.2
14	2-Chloromethyl-3,5-dimethyl-4-methoxypyridine HCl	TP	Cat.2
15	Di-n-propyl disulphide	FN	Cat.2
16	Potassium hydroxide (5% aq.)	TP	Cat.2
17	Benzenethiol, 5-(1,1-dimethylethyl)-2-methyl	TP	Cat.2
18	1-Methyl-3-phenyl-1-piperazine	TP	Cat.2
19	Heptanal	TP	Cat.2
20	Tetrachloroethylene	TP	Cat.2

4. Discussion

Since the OECD introduced an *in vitro* SIT protocol utilizing the RHE model in 2010, seven RHE models have been validated as VRMs and incorporated into OECD TG 439 [6].The OECD GD 220 provides performance standards for validating newly developed RHE methods for skin irritation testing, as outlined in OECD TG439.

The novel reconstructed human epidermis (RHE) model developed by Bloomage Biotechnology Co., Ltd. is derived from human foreskin keratinocytes, consists of multilayered structure including organized basal layer, well-differentiated stratum spinosum, granular layer and stratum corneum similar to native human skin. Beyond that, the RHE model demonstrated expression of stemness maker P63 in the basal layer, cell-cell junction markers E-cadherin and claudin-1, and cornified envelope-associated proteins like loricrin, filaggrin, which is further suggested correspondence with human skin.

To assessed the quality control criteria of the RHE model according to OECD TG439 and GD 220, tissue viability, barrier function were examined. The negative control O.D. in the 10 batches of skin irritation test was 1.13±0.10 at 570 nm, which is comparable to Epskin™ (SM) and other seven VRMs. The barrier function estimated with IC_{50} was 1.46±0.11 mg/mL, which is comparable to Epskin™ (SM). The RHE model was demonstrated to be an effective reconstructed human epidermis, exhibiting comparable performance and morphological features as required for VRMs in OECD TG439.

In the skin irritation test of the minimum list of 20 reference chemicals, diethyl phthalate, naphthalene acetic acid, allyl phenoxy-acetate, isopropanol, methyl stearate, heptyl butyrate, hexyl salicylate were recognized as non-irritants, which is aligned with UN GHS Category. 1-bromo-4-chlorobutane, 4-methyl-thio-benzaldehyde and cinnamaldehyde were recognized as false positive, which is similar in other VRMs [8]–[10]. 1-Decanol, cyclamen aldehyde, 1-bromohexane, 2-chloromethyl-3,5-dimethyl-4-methoxypyridine HCl, potassium hydroxide (5% aq.), benzenethiol, 1-methyl-3-phenyl-1-piperazin, heptanal, tetrachloroethylene were recognized as irritants correctly. Interestingly, di-n-propyl disulphide exhibited various responds in different studies utilizing VRMs [1],[8]. It was identified as a false negative in our research and aligns with certain VRMs outlined in OECD TG 439.Overall, the prediction capacity of the RHE model of Bloomage Biotechnology Co., Ltd. was estimated to be 70%, 90% and 80% for specificity, sensitivity and accuracy, which meets the OECD TG439 PS recommendation.

The creation of new skin models and testing methods is crucial for the worldwide adoption of *in vitro* alternatives to animal testing. Based on our study's findings, we are currently un-

dertaking an OECD TG439 PS-based validation study. This study involves both intra- and inter-laboratory validation, with participation from three laboratories. We expect to share the results of this PS-based validation study in the future.

5. Conclusion

In conclusion, this study evaluated a novel reconstructed human epidermis (RHE) model developed by Bloomage Biotech Co., Ltd., in accordance with OECD Test Guideline 439. The RHE model demonstrated strong performance, with 80% accuracy, 90% sensitivity, and 70% specificity, alongside excellent within-laboratory reproducibility. Histological and physiological analyses revealed characteristics akin to native human epidermis, including a multilayered structure and key protein expression (e.g., P63, filaggrin, loricrin). These findings suggest the RHE model is a reliable alternative for skin irritation testing and holds potential for cosmetic ingredient efficacy screening.

6.References

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