Skin toxicity testing utilizing the RAFT™ 3D Cell Culture System
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Significant efforts have been made to better predict skin toxicity when directly exposed to compounds or chemicals with the use of *in vitro* skin models. Here we demonstrate the use of RAFT™ Skin Models in assessing skin toxicity. The model was evaluated using histology for morphology and MTT assay for viability. Results indicate that this model correctly classified the test substances as corrosive or non-corrosive.

Introduction

In vitro skin models are often used for evaluating safety of consumer products. Three-dimensional (3D) cell culture systems aim to recreate an authentic structure utilizing primary cells from barrier tissue that is grown in a more natural environment with the help of hydrogel matrixes or synthetic scaffolds.

Data on skin irritation and corrosion effects are required by several pieces of legislation, including the EU regulation on cosmetics products, the Classification Labelling and Packaging (CLP) Regulation and the REACH Regulation[1]. Dermal toxicity assessment of compounds and subsequent hazard identification typically consist of corrosion, irritation, and sensitization tests. Internationally accepted test methods for skin irritation and corrosion include the traditional *in vivo* animal test as well as *in vitro* test methods.

Significant efforts have been made to better predict skin toxicity when directly exposed to compounds or chemicals with the use of *in vitro* skin models. These models are based on the reconstructed human epidermis consisting of non-transformed keratinocytes that have formed a multilayered epidermis and a multi layered stratum corneum containing the lipid profile that functions as a barrier.

The RAFT™ (Real Architecture for Tissue) 3D Cell Culture System allows the creation of tissue-like structures with cells growing within or atop a high-density collagen scaffold[2]. In this technical note we further describe the use of RAFT™ 3D Culture System in developing a full thickness skin model with keratinocytes and fibroblasts for toxicity testing.
The RAFT™ Skin Model consists of a compressed collagen-type-I-based hydrogel that closely mimics the human dermis. Collagen, in particular collagen type I, is one of the most abundant extracellular matrix proteins in the body and therefore an often-used 3D cell culture material. This system provides a scaffold for differentiating keratinocytes, while also embedding fibroblasts in an Air liquid Interface format (ALI), thus mimicing the epidermis.

Materials

Materials	Vendor	Cat. No.
RAFT™ 24-well insert kit	Lonza	016-1R25
24-well Transwells®	Corning	3470
Primary Human Neonatal Dermal Fibroblasts (NHDF)	Lonza	CC-2509
Primary Human Neonatal Epidermal Keratinocytes (NHEK)	Lonza	00192907
T150 cell culture flasks	Corning	430825
Accutase™ *	STEMCELL Technology	07920
FGM™ 2 BulletKit™	Lonza	CC-3132
KGM™ Gold BulletKit™	Lonza	00192060
CnT-Prime Airlift Medium	CellnTec	CnT-PR-FTAL
Paraformaldehyde solution 16%, diluted to 4% final (v/v) in PBS**	Electron Microscopy Sciences	RT 15710
Phosphate Buffered Saline (PBS)	Lonza	17-5160
Water for cell culture	Lonza	17-7240
Triton-X 100	Sigma	X100-100mL
MTT assay	ThermoFisher	V13154
Lactic acid	Sigma Aldrich	L6661-100mL
Octanoic Acid	Sigma Aldrich	03907-500mL
Benzylacetone	Sigma Aldrich	B16003-5g
2-phenylethyl bromide	Sigma Aldrich	B65780-5G
Glacial Acetic Acid	Sigma Aldrich	A507-P500
0.9% NaCl	Sigma Aldrich	07982-100TAB-F
Isopropanol	Sigma Aldrich	W292907
Acetic Acid	Sigma Aldrich	04-355-85

*Any other dissociating reagent can be used
**10% Buffered Formalin can be used at RT as a substitute

Other Supplies and Equipment

- Standard cell culture incubator set to 37°C and 5% CO_2
- Plate reader
- Microscope
- Centrifuge
- Cell Counter
- 96-well plates

Methods

Fibroblasts and Keratinocyte Sub-Culture: An ampule of NHDFs was thawed according to the manufacturer's recommendation. Fibroblast growth medium (FGM™ 2 Medium) was prepared according to the manufacturer's recommendation, except Gentamicin/Amphotericin-B (GA-1000) was omitted. The cells were seeded in FGM™ 2 Growth Medium at 2,500 cells/cm2 in one or two T150 flasks. The flasks were placed in a 37°C, 5% CO_2 incubator for 5 days and medium was exchanged with fresh 30 mL FGM™ 2 Medium per T150 flask every 2 days. Upon reaching 80–90% confluence, fibroblasts were harvested from the flask(s) with Accutase™, according to the manufacturer's instructions and diluted with FGM™ 2 Medium and centrifuged in a 50 mL conical tube at 200 × g for 5 minutes at RT. The supernatant was aspirated and NHDFs were suspended in 4 mL FGM™ 2 Medium before performing cell counts.

For keratinocyte culture, Keratinocyte Growth Medium (KGM™ Gold Medium) was prepared per the manufacturer's protocol by supplementing KBM™ Gold Basal Medium with KGM™ Gold SingleQuots™ growth supplements and omitting GA-1000. An ampule of NHEK was thawed per manufacturer's instructions. Note: Keratinocytes were cultured four to five days prior to the addition of NHEK to the RAFT™ Dermis Layer (as described in **Figure 1**). The cells were seeded in KGM™ Gold Medium (without GA-1000) at 2,500 cells/cm2 in one or two T150 flasks. The flasks were placed in a 37°C, 5% CO_2 incubator for 4–5 days and medium was exchanged with fresh 30 mL KGM™ Gold Medium per T150 flask every 2 days. The cells were harvested from the flask(s) with Accutase™, according to the manufacturer's instructions, diluted with KGM™ Gold Medium and centrifuged in a 50 mL conical tube at 200 × g for 5 minutes at RT. The supernatant was aspirated gently and NHEKs were suspended in 10 mL KGM™ Gold Medium before performing cell counts. All cultures were grown without antibiotics.

Construction of dermis and epidermis layers with the RAFT™ 3D Culture System

To construct the RAFT™ 3D culture system, the dermis layer was first built using the NHDF cells. A stock of NHDF was prepared at 1.64×10^6 cells/mL concentration and mixed with RAFT™ Reagents as per the guidelines described in the manufacturer's instructions for RAFT™ Protocol and adjusted for the desired number of transwells. Instead of the full cell-collagen solution volume (240 µL) recommendation, half volume (120 µL) was pipetted gently in each transwell. The final concentration of NHDFs was at 8.25×10^3 per transwell (25,000 cells/cm2). The hydrogels were formed and compressed according to RAFT™ Kit Instructions. A quarter volume (60 µL) of the cell-collagen solution was added on top of the compressed RAFT™ Cultures. This addition enables a better barrier formation utilizing fibroblasts. The top-layer hydrogel was allowed to form without compression. FGM™ 2 Medium was added to the chambers, 100 µL to the apical and 600 µL to the basal chamber. The dermis layer was allowed to develop for 5 days at 37°C, 5% CO_2, exchanging the medium in both chambers every other day.

Once the dermis layer was developed, the epidermis layer was added to the RAFT 3D culture system using NHEK cells. A stock of NHEK suspension at 3.5×10^5 cells/mL was prepared in KGM™ Gold Medium. FGM™ 2 Medium was aspirated gently from the apical well of the prepared RAFT™ Cultures containing embedded NHDF. A volume of

RAFT™ Skin Model configuration

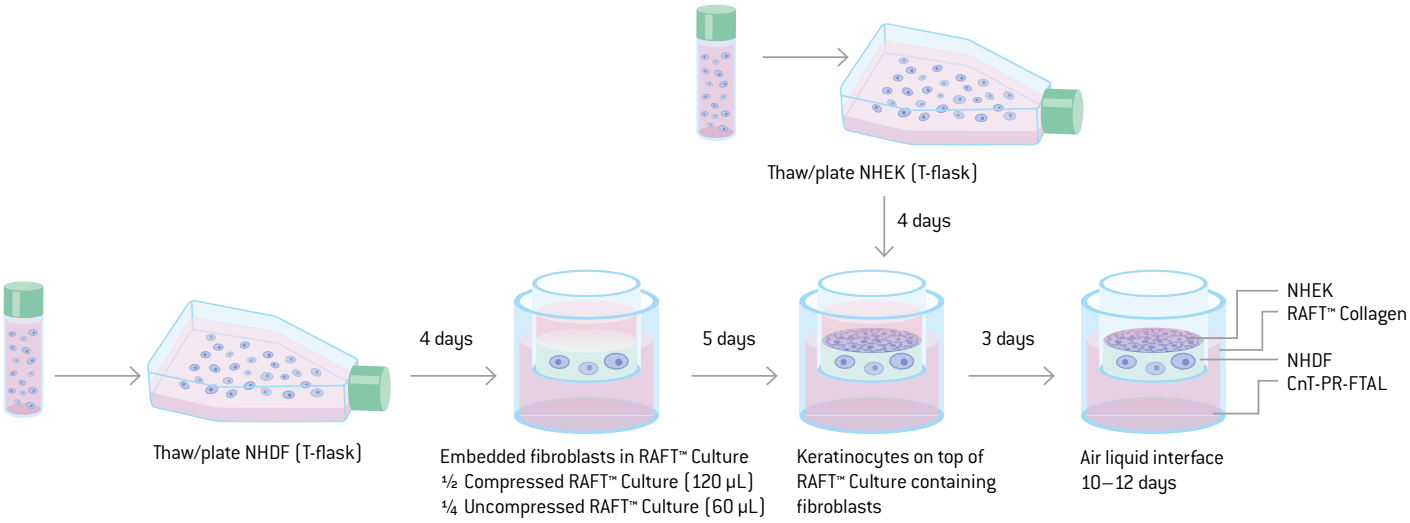

Figure 1
Using the RAFT™ Cell Culture System. NHDF were embedded in compressed/uncompressed RAFT™ Cultures. NHEK were seeded on top of the NHDF followed by air liquid interface (ALI) after 3 days for submerged culture. Top view of cultured NHEK on RAFT™ Cell Culture in a transwell after 2 days in culture macroscopically.

100 µL NHEK cell suspension was added to the apical well. The final concentration of NHEKs was at 3.5×10^4 per 24-well transwell (1.06×10^5 cells/cm2). FGM™ 2 Medium from the basal chamber was removed and replaced with 600 µL of KGM™ Gold Medium.

The RAFT™ Skin Models had KGM™ Gold Medium replaced every other day for 2 days. Differentiation medium, CnT-PR-FTAL, was prepared as per manufacturer's instructions. The medium in the apical and basal chambers of the transwells were replaced with CnT-PR-FTAL after 24 hours. Thereafter, the medium from the apical chamber was removed to allow NHEK to differentiate at the Air-Liquid Interface (ALI). The RAFT™ Skin Models were fed with 600 µL of differentiation medium in the basal chamber on alternate days for 10–12 days[2]. RAFT™ Protocol download.

Note: All cultures were grown without antibiotics.

Treatment of RAFT™ Skin Model with noncorrosive and corrosive substances

As shown in **Figure 2**, the RAFT™ Skin Model was cultured for 10–12 days with the the Air-Liquid Interface. The skin models were used to perform the corrosion test based on the Guide 431 from OECD (2016)[3]. RAFT™ Skin Model integrity and barrier function was assessed by treating the cells with 1% Triton-X 100 (in PBS) for 4 or 10 hours. The exposure time at 50% viability (ET50) was determined by a MTT assay. The RAFT™ Skin Models were tested with two corrosives Lactic acid and Octanoic Acid and two non-corrosives 2-Phenylethyl Bromide and Benzylacetone, negative control (0.9% Sodium Chloride) and a positive control (Acetic Acid). Twenty-five µL of the stock substance mentioned above were applied to the RAFT™ Skin Models for 3, 60, and 240 minutes. Cell viability was measured with a MTT assay and OD was measured as a readout for viability relative to controls. Histology was measured on select compounds to assess the ability of this RAFT™ Skin Model to reflect the effect of substances on cell morphology.

RAFT™ Skin Model configuration

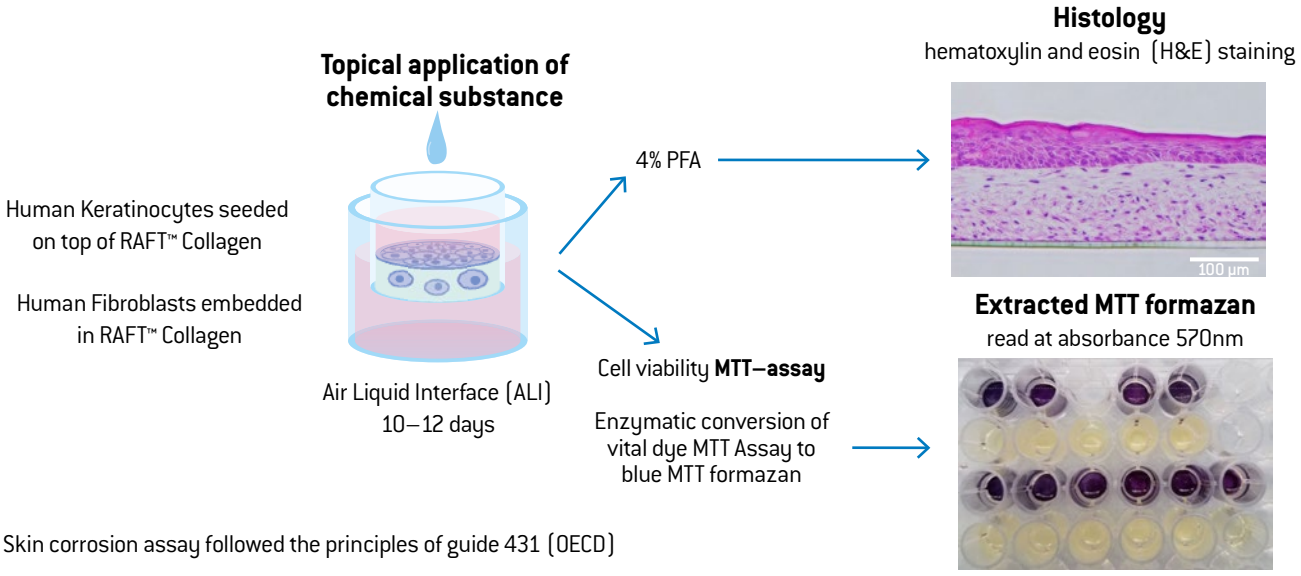

Figure 2

Human keratinocytes and fibroblasts were used to construct the RAFT™ Skin Model. After 10–12 days of culture, following the principles of Guide 431 from OECD,2016 (3), the RAFT™ Skin Models were exposed to chemical substances for multiple time points. The health of the skin models were evaluated using histology for morphology and MTT assay for viability.

Determining cell viability with MTT Assay

To determine the effect that test compounds had on cell viability, the MTT protocol as recommended by ThermoFisher (V13154) was performed. Tests were performed following the guidelines as described from OECD , 2016[3]. Media was carefully removed from the RAFT™ Skin Model plate , and replaced with 100 µL of fresh CnT-Prime Airlift Medium. 10 µL of the 12-mM MTT stock solution was added to each well. A negative control was included by adding 10 µL of the MTT stock solution to 100 µL of medium alone. The plate was incubated at 37°C for 4 hours. After incubation, 100 µL of the SDS-HCl solution was added to each well, and pipeted up and down thoroughly to mix. The plate was incubated at 37°C for 4–18 hours in a humidified chamber. The samples were pipeted up and down to mix again, then the absorbance was read at 570 nm in the transwells.

Fixation of RAFT™ Skin Models for histological examination

Post treatment of substances, the medium in the basal chambers of the transwells was aspirated followed by the addition of 4% PFA in PBS to the models: 100 µL in the apical chamber and 600 µL in the basal chamber. To allow for complete fixation, the models were stored at 4°C overnight for 24 hours. Using a sharp scalpel, the transwell membrane was cut out and with the help of forceps the models were transferred to a 15 mL conical tube containing 70% ethanol. Histological processing, embedding, sectioning and Hematoxylin-Eosin staining was contracted to histology services.

Results

Barrier function study:

In accordance with the OECD guidelines[3] the ability of a multi-layered skin cell system to produce a robust functional barrier that resists rapid penetration can be tested with cytotoxic benchmark chemicals, e.g. sodium dodecyl sulfate (SDS) or Triton X-100. The RAFT™ Skin Models integrity and barrier function was assessed with Triton X-100 by determining the exposure time required to reduce cell viability by 50% (ET50) upon application of the test material. Cell viability was measured with MTT assay after 4 hours and 10 hours and serves as an indirect indicator of barrier integrity.
As shown in Figure 4 , when tested with Triton X-100, the cell viability ET50 (50% viability) of this model is between 4 and 10 hours. This demonstrates good barrier formation and the values are comparable to the SkinEthic RHE model as described within OECD guide[3].

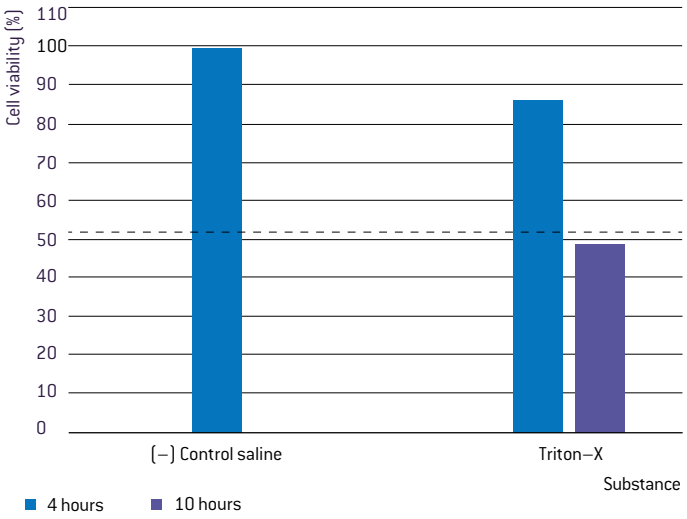

Figure 3
The barrier function and penetration of cytotoxic marker chemicals in RAFT™ Skin Model were evaluated by assessing cell viability using 1% Triton X-100. Negative control was 0.9% sodium chloride.

Cytotoxic substance penetration study

The cell viability of RAFT™ Skin Models following exposure to corrosive and noncorrive substances was assessed following the guidelines from the OECD guidelines[3].

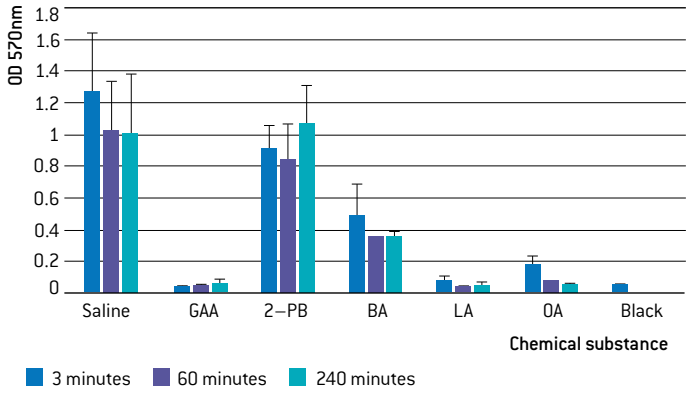

Figure 4
MTT absorbance (OD) values of RAFT™ Skin Model indicating cell viability following application of chemical substances for 3, 60 and 240 minutes. The viability of the samples was analyzed relative to the negative control at 3 minutes.

As observed in Figure 4, non-corrosive test reagents (saline, 2-phenylethyl bromide (2-PB), benzylacetone (BA)) showed higher cell viability and maintenance of barrier over a period of 4 hours, while corrosive reagents such as glacial acetic acid (GAA), lactic acid (LA) and octanoic acid (OA) as expected were negatively impacted and unable to hold the integrity of the barrier.

Cellular Integrity study

To demonstrate the cellular integrity of the skin model in response to non-corrosive and corrosive substances, RAFT™ Skin Model were analyzed histologically.

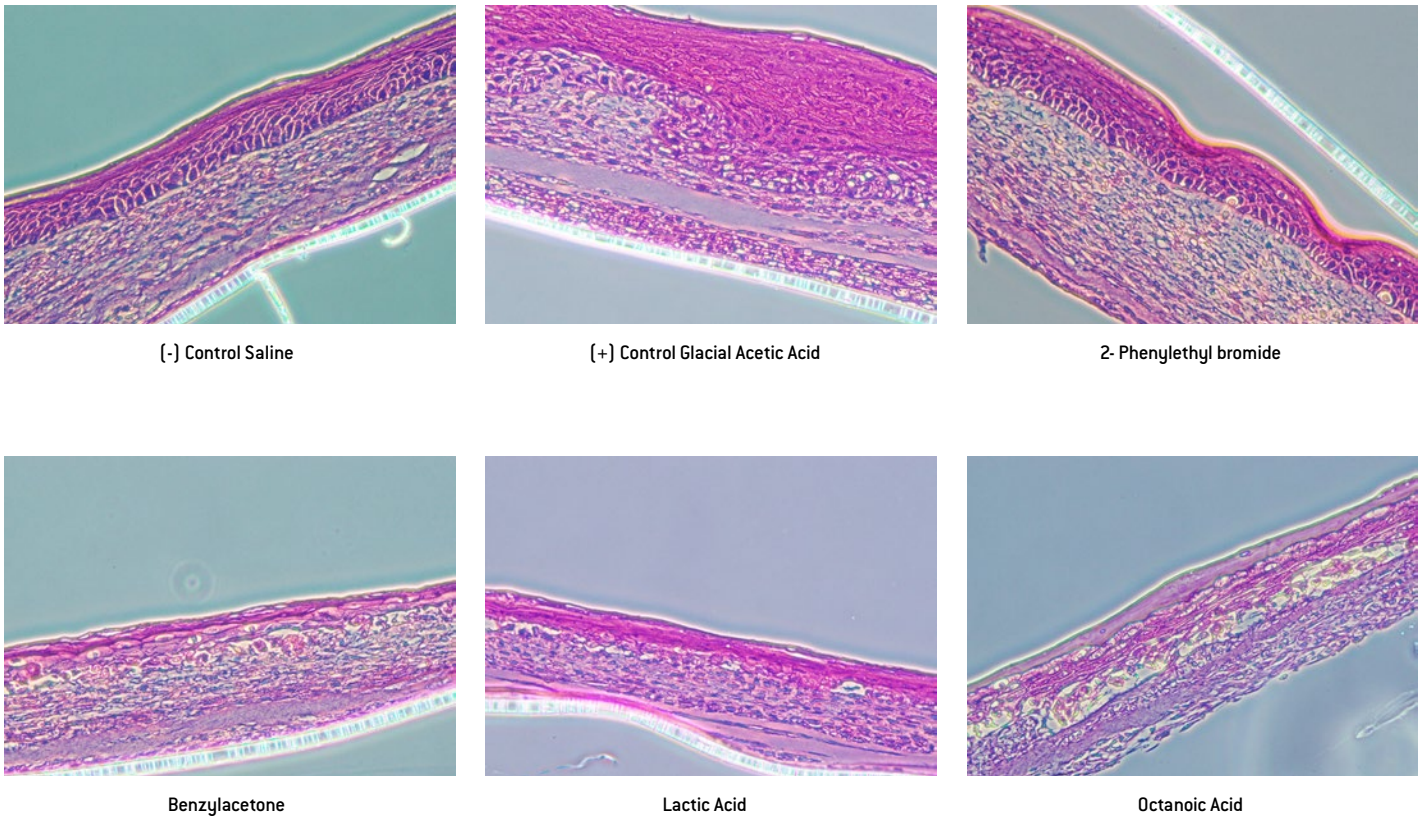

Figure 5
Histological Analysis of RAFT™ Skin Model after 240 minutes exposure to chemical substances.The morphology of the RAFT™ Skin Models following application of negative control and chemical substances for 240 minutes were evaluated using hematoxylin and eosin (H&E) staining of transverse sections.

As observed in histology sections shown in Figure 5, the RAFT™ Skin Models exposed to Saline and 2-Phenylethyl Bromide maintained integrity but RAFT™ Skin Models did not maintain dermal and epidermal features when treated with glacial acetic acid, octanoic acid and lactic acid as would be expected with these reagents. Thus, the RAFT™ Skin Models ability to maintain a barrier as assesed by cell viability (Figure 4) was also confirmed by the histological analysis (Figure 5).

Conclusions

Skin models are widely used in chemical, cosmetic, and to a somewhat lesser extent, pharmaceutical industries. Several complex *in vitro* models of skin such as 3D tissues, organ-on-a-chip, organoids have been developed and assays validated for regulatory purposes. Most of these systems are pre-made, thus limiting the flexibility sometimes needed in assay development.

Here we demonstrate the use of RAFT™ Skin Models in assessing skin toxicity. The model was evaluated using histology for morphology and MTT assay for viability. Results indicate that this model correctly classified the test substances as corrosive or non-corrosive as indicated by cell viability studies. The barrier function studies suggests that this model is in line with other best in class models that are used for regulatory purposes for distinguishing corrosive from non-corrosive substances. Moreover, the health of the RAFT™ Constructed Skin models with morphological analysis using H&E staining validated the corrosive or non corrosive classification data.

Several gaps exist for screening of systemically administered pharmaceutical agents. The total duration to obtain the full thickness skin models can vary from 28–30 days as determined from seeding the fibroblasts, and keratinocytes through formation of a differentiated skin model. The RAFT™ 3D Cell Culture System could provide an advantage of shortening the culture period to 22–24 days. One gap is the availability of skin models and validated assays with functional immune cells[4]. The RAFT™ Skin Model is versatile and could be further adapted to include incorporation of other cells types such as immune cells.

Here we demonstrate that the RAFT™ System can be used by researchers to create full thickness skin model in their own hands for toxicity testing. This model has been shown to deliver results similar to the best in class commercially available premade models. Time to results is also shortened with this system. Thus RAFT™ System supports researchers to lower cost of assay development as well as provide them the flexibility to adapt the system to their development needs.

References

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