Prevalidation of EpiDerm RhE Model in the EpiSensA Assay for *in vitro* Skin Sensitization

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Abstract: Since the publication of the "The Adverse Outcome Pathway (AOP) for Skin Sensitization Initiated by Covalent Binding to Proteins" in 2012, several methods based on the modelling of AOP Key events have been validated and integrated into TG 442C, D and E. In 2023, JacVam scientifically validated the epidermal sensitization assay (EpiSensA) developed by Kao Corporation for integration into the OECD TG 442D for *in vitro* skin sensitization. This method addressing the key event 2 of the AOP is based on the use of a reconstructed human epidermis (RhE) model as an experimental system. The validation showed the performance of this method for pre/pro-haptens, thanks to metabolic activity of RhE models, as well as for lipophilic substances which can overcome some of the limitations of existing in chemico and *in vitro* 2D cells culture methods.

The objective of this work was to evaluate a similar method using the EpiDerm™ RhE model (EPI-200, MatTek). The EpiDerm™ model is readily available worldwide and already validated for the OECD TG431, TG439 and TG498 for corrosion, irritation and phototoxicity. This similar method using EpiDerm™ for EPiSensA is called **Epi2SensA**. To prepare the OECD me-too validation, the reference method was adapted to the EpiDerm™ model and implemented in two laboratories, MatTek, Ashland, MA, USA and VitroScreen, Milano, Italy.

Materials & Methods

The **Epi2SensA** is a similar method to the EpiSensA developed by Kao Corporation. The Epi2SensA uses the reconstructed human epidermal model EpiDerm™ (EPI-200, MatTek, Ashland, MA, USA and MatTek Europe, Bratislava, Slovakia) instead of the LabCyte RHE model. The EpiDerm™ model is reconstructed from normal human primary keratinocytes in a chemically defined medium and is cultured on a polytetrafluoroethylene (PTFE) membrane to form a multilayered highly differentiated model of the human epidermis.

The principle of the test is to expose topically triplicate EpiDerm™ tissues to different concentrations of a test chemical. After 6 hours exposure or 1 hour exposure and 5 hours post-incubation, the gene expression of four markers of the keratinocyte response to the early phases of skin sensitization (induction of cytoprotective gene pathways and inflammatory responses) are evaluated by RT-PCR (**Table 1**). The fold induction of genes of interest in the treated tissues are calculated by comparing the relative gene expression with that in vehicle treated tissues. When the mean fold induction of at least one out of the four marker genes exceeds the respective cut-off value (ATF3: 15-fold; GCLM: 2-fold; DNAJB4: 2-fold; IL-8: 4-fold), the chemical is judged as a positive skin sensitizer.

With the EpiDerm model, the volume of chemical applied was increased from 5 µL in the original protocol to 10 µL. Indeed, the surface area of EpiDerm™ is twice that of Labcyte and requires a larger volume to be fully covered during the exposure stage (Fig.1 and Table 2).

	ATF3	GCLM	DNAJB4	IL-8
Name	Activating Transcription Factor 3 gene	Glutamate-Cysteine Ligase Modifier Subunit gene	Dnaj homolog subfamily B member 4 gene	Interleukin 8 (CXCL8)
Function	Role in cellular stress response, inflammation, and immune response. Negatively modulates inflammatory cytokines and chemokines	Role in the body's defense against oxidative stress by regulating the synthesis of glutathione (GSH).	Part of the heat shock protein (HSP) 40 family. In skin sensitization might suppresses protein misfolding induced by oxidative stress.	Role in recruiting and activating neutrophils, contributing to inflammation, and possibly influencing the severity of the allergic reaction
Regulation	Regulated by ATP and NFκB. Haptens induce ATP release from keratinocytes that express the ATP receptor (P2X7)	Regulated by both the Nrf2/ARE and the activator protein-1 (AP-1)pathways	Regulated by Nrf2/ARE pathway and the heat shock transcription factors HSF-1/HSE pathway.	Regulated by the ATP-P2X7 and p38MAPK pathways
Positive if	15 fold	2 fold	2 fold	4 fold

Table 1: 4 genes of interest their function and potential involvement in the inflammatory responses and gene expression of antioxidant response elements identified in the Key event 2 of the adverse outcome pathway (AOP) for skin sensitization.

	volume	Surface of the RHE	Ratio volume /surface
EpiDerm™	10 µL	0.63 cm2	15.9 µL/cm²
LabCyte	5 µL	0. 32 cm2	15.6 µL/cm²

Table 2: Surface comparison between the two RHE models and impact of the volume exposure to use

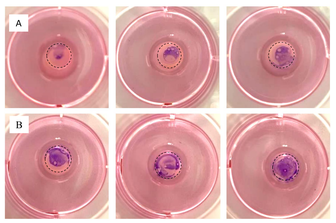

Fig. 1: Visualization by addition of crystal violet dye in acetone/olive oil solvent (4:1) of the distribution on the RHE surface of a 5µL (A) or 10µL (B) application.

Viability assays:

- The **LDH** (Lactate Dehydrogenase) cell viability assay is based on the measurement of LDH released from cells. LDH is a stable cytoplasmic enzyme which is rapidly released into the tissue culture medium upon damage to the plasma membrane. The quantity of LDH released by 100% killed control tissues is used to calculate cell viability of the tissues exposed to test samples. In the reference protocol, Killed controls are exposed 6 hours to 10 µl of 10%Triton-X applied topically.

- The **MTT** assay measures the colorimetric change of the MTT dye to an insoluble formazan product by mitochondrial enzymes of living cells. Cell viability is calculated by comparing the amount of formazan in the tissues exposed to test samples with a control tissue treated with solvent only.

Results

With 6 hours exposure duration, all extreme, moderate and non skin sensitizers were correctly classified. However, two weak skin sensitizers, eugenol and methyl methacrylate, were misclassified as non sensitizers. Because of the acceptance criteria of ≥80% cell viability, the maximum tested concentration for these weak sensitizers was well below the concentration required to induce a positive response in animals.

We evaluated a shorter duration of exposure, 1 hours, followed by 5 hours post-incubation to reduce cytotoxicity effects. Even though eugenol was correctly classified, cytotoxicity still limited the concentrations tested.

Based on our experience with the EpiDerm™ model, we questioned the protocol for measuring cell viability. In particular, the measurement of the maximum quantity of LDH released by the killed controls.

Chemical name	CAS	Log Kow	EC3 (%)	Pre/pro	Potency (Ecetoc)	VRM protocol (6h)	Shorter exposure (1h+5h)	Maximum concentration tested
4NBB	100-11-8	2.7	0.05		Extreme	S (4/4)	S (4/4)	0.10
Clotrimazole	23593-75-1	**6.26**	4.8		Moderate	S (4/4)	S (3/4)	0.78%
DNCB	97-00-7	2.27	0.054		Extreme	S (2/2)	*nt*	0.2%
Tetrachlorosalicylanilide	1154-59-2	**5.87**	0.0265		Extreme	S (1/1)	*nt*	0.39%
Isoeugenol	97-54-1	3.04	1.3	X	Moderate	S (1/1)	S (2/2)	1.56%
Eugenol	97-53-0	2.73	11.6	X	Weak	NS (2/3)	S (1/1)	0.78%
α-Amyl cinnamaldehyde	122-40-7	**4.35**	>25%		Weak	S (1/1)	*nt*	25%
Imidazolidinyl urea	39236-46-9	-0.86	24		Weak	*nt*	NS (1/1)	0.78%
Methyl methacrylate	80-62-6	1.38	75		Weak	NS (1/1)	NS (1/1)	1.56%
Benzyl butyl phthalate	85-68-7	**4.84**	NS		Negative	NS (1/1)	NS (1/1)	6.25%

Table 2: List of the 10 chemicals, including the 2 positive controls, 4-NBB and Clotrimazole, tested in the Epi2SensA method with EpiDerm™ model. In addition to 6 hours exposure of the original protocol (VRM), we evaluated a shorter 1 hour exposure followed by rinsing and 5 hours post-incubation. (*nt* = not tested)

- Cell viability and LDH release by killed control tissues

In the LDH method, the maximum value of LDH released in the medium from killed tissue is a key parameter for calculation of cell viability:

$$\text{Cell viability \%} = 100 - \frac{\text{LDH test chemical} - \text{LDH non treated}}{\text{LDH killed control} - \text{LDH non treated}} \times 100$$

Underestimating LDH release from killed controls reduces the calculated cell viability of tissues exposed to the test sample

The kinetics of LDH release after exposure to Triton-X showed that maximum release was not reached before 48 hours (**Fig. 2**). This explains why 6 hours after topical application of 10 µl of 10%Triton-X, cell viability measured by MTT was still around 50% (**Fig. 3**), whereas it's supposed to be 0% according to the EpiSensA method.

The underestimation of LDH levels for killed control led to a bias in the calculation of cell viability, limiting the range of applicable concentrations for a tested chemical. We have shown that to achieve the maximum level of LDH released in 6 hours, Triton-X should be applied basolaterally rather than topically (**Fig. 4**).

- Kinetic of LDH release and cell viability

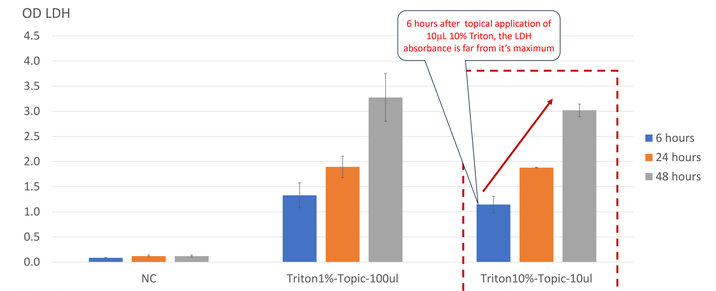

Fig. 2: LDH absorbance measured in the culture medium of EpiDerm™ tissues after 6, 24 or 48 hours exposure to 10 or 100µL of 1 or 10% Triton-X applied topically or in the basolateral medium.

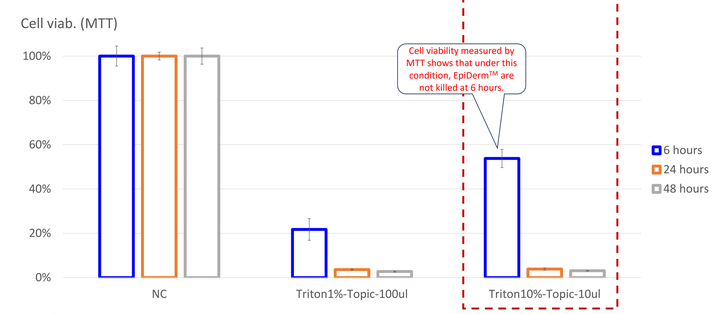

Fig. 3: Cell viability of the EpiDerm™ measured by MTT method after 6, 24 or 48 hours exposure to 10 or 100µL of 1 or 10% Triton-X applied topically or in the basolateral medium.

- Topical versus basolateral exposure

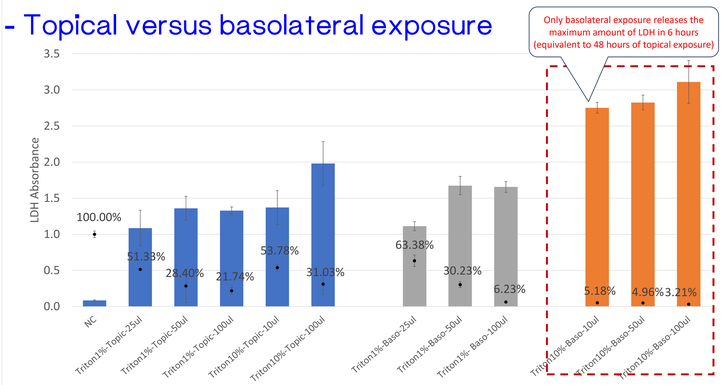

Fig. 4: LDH release by the EpiDerm™, 6 hours after topical or basolateral exposure to different volumes and concentrations of Triton-X. Bars represent LDH absorbance (OD) and black dots cell viability measured by MTT (%)

Conclusions

- To prepare the OECD me-too validation, the method has been transferred to two naïve laboratories: MatTek Corporation (USA) and VitroScreen (Italy).

- The method has been optimized for improved cell viability calculation, to enable higher concentrations to be tested.

- The impact of this refinement, particularly for weak sensitizers, will be assessed in the coming weeks.

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