The 3D Reconstructed Human Skin Comet Assay: Transferability and Reproducibility Within and Between Laboratories

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Introduction

- Currently used in vitro cytogenetic assays show a high rate of false positive results. In order to improve the in vitro prediction, we adapted the Comet assay procedure to 3D skin models that mimic exposure route and potential metabolism of cosmetic ingredients [1].
- The assay reflects now the first-site-of-contact for dermally applied ingredients and considers the species (human) and organ-specific metabolism of the skin.
- The 3D Skin Comet assay is intended to be used as a follow-up for positive results from the current in vitro genotoxicity test battery for compounds that are in direct contact with the skin [2].
- Here, we provide an update from five labs of phase I of the 3D Skin Comet assay validation using the Phenion ® Full-Thickness (FT) Skin Model.
- The work was funded both by Cosmetics Europe (CE) and the German Ministry of Education and Research (BMBF).

Methods

3D Skin project: 3 Phases of validation

Phase 1 Optimization and transferability with 2 model genotoxins

Phase 2 Intra- and inter-lab reproducibility with 8 coded compounds

Phase 3 Validation with 30 coded compounds

Selection of compounds

- The validation includes testing of 30 chemicals, selected by external experts (Rafaella Corvi, ECVAM, and David Kirkland, consultant), in an incomplete block-design.
- The coded test chemicals are equally balanced i.e. 15 with an expected negative outcome and 15 with an expected positive outcome. The negatives include true negatives and chemicals that generally yield false positive results in the in vitro genotoxicity battery.
- The coding and shipment was performed by the BfR.

Experimental design

Phenion® FT | **Dosing regime for the 3D Skin Comet assay**

The skin tissues were treated topically 3 times (48, 24, and 3 h) to allow for the detecting of pro-mutagens which are bioactivated by dermal xenobiotic metabolising enzymes [3] and to ensure the detection of acute DNA damage. See box above for an example of the aphidicolin (APC) design.

Based on a review of the data of a preceding transfer phase, the final experimental design was determined and applied in this validation study:

- 3 tissues per dose group
- Inclusion of negative, solvent and positive controls in each experiment
- Preparation of 3 slides per tissues, of which at least two were evaluated
- Evaluation of 50 comets per slide

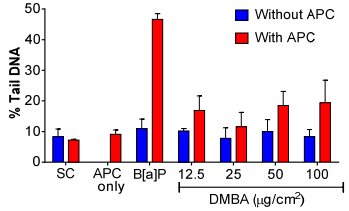
The cytotoxicity was measured using ATP content and adenylate kinase (AK) leakage. Strong cytotoxicity leading to exclusion of the respective test concentration was defined as:

- >50% reduction of intracellular ATP concentration vs solvent control
- >200% in AK release compared to solvent control

Results

Aphidicolin (APC protocol)

Results of phase 1 showed that the standard Comet protocol was generally not robust enough for detecting pro-mutagens. A proof-of-concept study showed that APC, a DNA-polymerase inhibitor, could increase the sensitivity of the assay without compromising its high specificity [4]. Negative and equivocal findings are now confirmed by additional APC experiments before finalizing its classification. An example with 7,12-dimethylbenz(*a*)anthracene (DMBA) is shown. The % tail DNA in solvent controls (acetone) was not increased by APC, confirming that it generally does not increase the background DNA damage.



Comparison of the % tail DNA in keratinocytes from Phenion® FT skin models treated with DMBA in the absence or presence of APC (5 µg/mL). SC= solvent control, B[a]P = 12.5 ug/cm² benzo*[a]*pyrene (positive control for APC).

Phase 2 results from coded compounds using Phenion®FT skin models

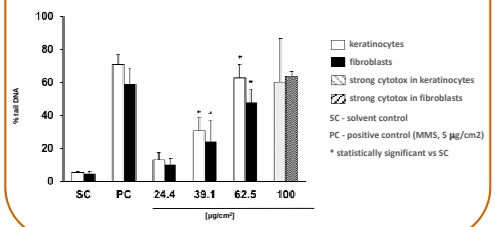
Test chemical	Type	Interpretation at: Lab 1	Lab 2	Lab 3	Lab 4	Lab 5
Mitomycin C	TP - Direct - cross-linker	positive	positive		Inconclusive	
Cadmium chloride	TP - Direct			positive	negative	positive
N-Ethyl-N-nitrosurea	TP - Direct	positive	positive	positive		
7,12-Dimethylbenz(*a*) anthracene	TP - Bioactivated		positive	positive		positive
Eugenol	FP	negative	negative			negative
Propyl gallate	FP			negative	negative	negative
Cyclohexanamone	TN	negative		negative	negative	
Di-(2-thylhexyl)phthalate)	TN		negative		negative	negative
	Predictivity	100%	100%	100%	80%	100%

Chemical types tested:
TP = True positive, FP = False positive, TN = True negative

Results

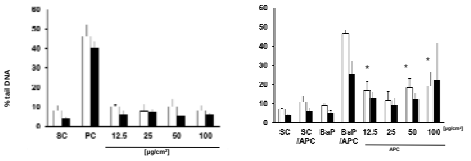
Cadmium chloride

Direct acting mutagen without APC protocol

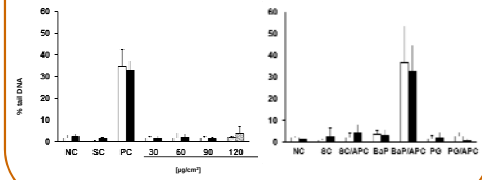


DMBA and Propyl Gallate

DMBA: Difficult positive compound in protocol +/- APC



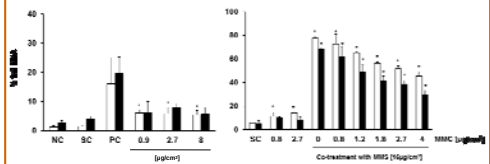
Propyl Gallate: True negative compound in protocol +/- APC



Mitomycin C

MMC: Difficult positive compound (crosslinker)

Mitomycin C (MMC) was judged inconclusive by Lab 3, since this chemical caused an increase in % tail DNA at low doses but a decrease at higher doses (the statistical tests indicated a positive finding in both experiments). Nevertheless, MMC was correctly classified as positive by Labs 1 and 2. MMC is a DNA cross-linker that intercalates between DNA strands, leading to covalent binding affecting also fragmented DNA which suppresses positive Comet signals at higher doses [5]. The suppression of of the MMS-induced DNA damage by MMC is demonstrated. This modified protocol might help to efficiently detect cross linkers.



Comet assay cross-linker protocol: MMC-induced DNA damage in keratinocytes and fibroblasts in Phenion® FT models after co-treatment with MMS

Conclusions

- Data support use of the Phenion® FT 3D skin model in the Comet assay since the predictivity for 8 coded chemicals tested across 5 labs was >90%.
- When APC was included in the protocol, predictivity of the assay was improved since it enabled more efficient detection of pro-mutagens like BaP and DMBA.
- MMC, a DNA cross-linker, caused borderline, but significant increases in the standard and APC protocols. A cross-linker specific protocol clearly confirmed MMC cross-linking activity.
- Phase 3 testing (data base development) is continuing with the Phenion® FT model to obtain a complete data set for 30 chemicals.
- Once validated, the 3D Skin Comet assay is envisioned to be used as a follow-up test for positive results from the current in vitro genotoxicity test battery 2.
- **These results support the use of 3D reconstructed skin models as a direct replacement for animal testing of dermally exposed chemicals.**

• References: (1) Reus et al. Mutagenesis. 2013, 28(6):709-20; (2) Pfuhler et al. Reg. Tox. Pharm. 2010, 57(2-3):315-24; (3) Wiegand et al. Skin Pharmacol Physiol. 2014, 27(5):263-75; (4) Brinkmann et al. Tox. Sci. 2013, 131(2):351-9; (5) Pfuhler et al. Environ. Mol. Mutagen. 1996;27(3):196-201.

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