3D Skin Comet assay: Update on the ongoing validation

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Cosmetics Europe
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Introduction

Currently used *in vitro* cytogenetic assays show a high rate of false positive results. Therefore, we are following a new approach in which skin tissues (mimicking the exposure route and potential metabolism of ingredients) are combined with the Comet assay[1]. This assay reflects the first-site-of-contact for dermally applied ingredients and considers the species (human) and organ-specific metabolism of the skin. The aim is to provide a relevant assay 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 on the progress of the validation of the 3D Skin Comet assay, funded by Cosmetics Europe (CE) and the German Ministry of Education and Research (BMBF). As a result of a comparison of three skin models, the Phenion® and EpiDerm™ Full-Thickness (FT) Skin Models were selected to enter the validation phase. We present here the results of the initial validation phase using Phenion® FT tissues tested in four laboratories.

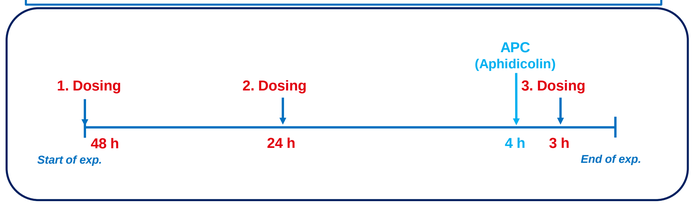
Methods

The validation includes testing 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 initial testing phase is now complete and focused on inter- and intra-laboratory reproducibility using the Phenion® FT model first.

Eight coded chemicals were each evaluated in three laboratories using the following dosing regimen:

Phenion® Full Thickness Skin model

Dosing regimen for the 3D Skin Comet assay



The tissues are treated 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.

If the outcome of the first experiment was negative, a subsequent experiment included aphidicolin (APC) to increase sensitivity of the assay (see Results below). APC inhibits the final step of specific DNA-repair processes and induces an accumulation of single strand breaks, which increases the Comet signal.

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

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

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

Results

Results of initial validation

The Phenion® FT model was well suited to the comet assay since:

- There was a good overall predictivity of the expected genotoxicity. Of the laboratories, 3 correctly identified all 5 chemicals and the fourth correctly identified 80% of the chemicals (Table 1). Data from a fifth laboratory are pending.
- Comets in negative and solvent control tissues were small.
- Results on the solvent and positive control (MMS or B[*a*]P) were comparable among laboratories.
- There was a good reproducibility within and between laboratories.

The outcome for Mitomycin C (MMC) was inconclusive in Lab 3, although this chemical did cause an increase of % tail intensity (% tail DNA) at low doses, it decreased at higher doses (in addition, the statistical tests indicated a positive finding in both experiments). Nevertheless, MMC was correctly classified as positive by Lab 1. MMC is a DNA cross-linker that intercalates between DNA strands, leading to covalent binding. This activity also affects fragmented DNA such that positive Comet signals can be suppressed at higher doses[4]. The suppression of the MMS-induced DNA damage by MMC was also demonstrated using the Phenion® FT model (Figure 1). This modified protocol might help to efficiently detect crosslinkers.

Effect of APC

Results of the transfer phase showed that the standard Comet protocol was not robust enough for detecting pro-mutagens. Therefore, the protocol was amended with APC after a proof-of-concept study showed that the DNA-polymerase inhibitor could increase the sensitivity of the assay without compromising its high specificity[5]. As a result, every negative finding is now being confirmed by additional APC experiments before finalizing its classification. An example using 7,12-dimethylbenz(*a*)anthracene (DMBA) is shown in Figure 2. APC was added 4 h before cell isolation and helped to identify the genotoxic potential of this pro-mutagen. The % tail DNA in solvent controls (acetone) was not increased by APC, showing that this compound generally does not increase the background DNA damage in our assay (B[*a*]P is used as positive control in the APC experiments).

Conclusion

- These data support the use of the 3D skin model, Phenion® FT, in the Comet assay since the predictivity for 8 coded chemicals was good in all laboratories.
- The experimental design performed well in terms of incubation time, maximum concentration used and the sensitivity.
- The suppression of MMS-induced Comet signal by the cross-linker, MMC, was clearly demonstrated. MMC also caused borderline increases in the standard protocol.
- When APC was included in the protocol the predictivity of the assay was improved.
- Testing will be continued with the Phenion® FT model to obtain a complete data set for all 30 chemicals. The remaining compounds will be tested in one laboratory only.
- Once validated, the 3D Skin Comet assay is envisaged to be used as a follow-up test for positive results from the current *in vitro* genotoxicity test battery[2].

Figure 1

Effect of co-treatment of MMC on the % tail DNA caused by MMS in keratinocytes and fibroblasts from Phenion® FT models. SC= solvent control.

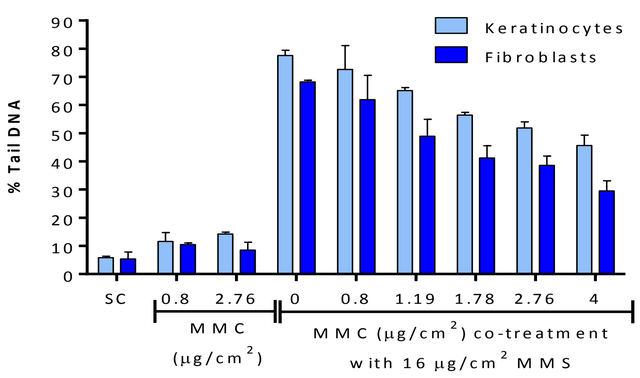


Figure 2

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

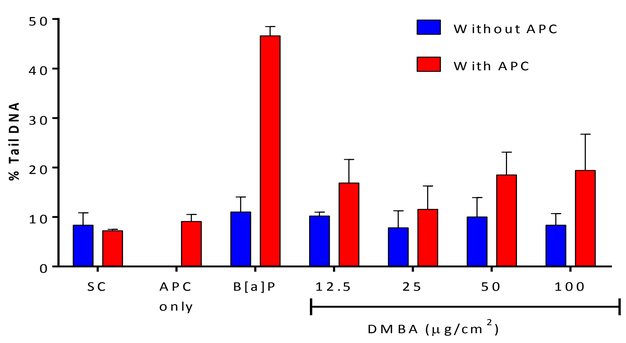


Table 1

Outcome of experiments by different laboratories. TP = true positive, FP = false positive, TN = true negative; + = positive call, - = negative call, ± = inconclusive

Chemical	Type	Lab 1	Lab 2	Lab 3	Lab 4
Mitomycin C (MMC)	TP – Direct – cross-linker	+		±	
Cadmium chloride	TP – Direct		+	-	+
N-Ethyl-N-nitrosurea	TP- Direct	+	+		
7,12-Dimethylbenz(*a*)anthracene	TP- Bioactivated	+	+		+
Eugenol	FP	-			-
Propyl gallate	FP		-	-	-
Cyclohexanamone	TN		-	-	
Di-(2-thylhexyl)phthalate)	TN	-		-	-
	Predictivity	**100%**	**100%**	**80%**	**100%**

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

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