The Comet Assay in the Phenion® Full Thickness Skin Model as a putative tool for improved in vitro genotoxicity testing

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Introduction

Due to the 7th Amendment to the Cosmetic Directive animal tests for acute toxicological endpoints (i.e. genotoxicity) are banned for cosmetic ingredients within the EU starting March 2009. However, the currently used in vitro genotoxicity tests are of low quality. One strategy to maintain safety assessment after March next year is to improve existing test batteries with test systems of higher biological relevance as compared to currently used monolayer cultures. For the skin, which is the first site of contact with max. exposure for most of the cosmetics skin models might be appropriate test systems. Since our previous data (Wiegand *et al.,* Megat 2008) revealed that beside epidermis also the dermis is mandatory to mirror xenobiotic metabolism we started to establish a Comet Assay with the Phenion® Full Thickness Skin Model (Phenion® FT-SM) which comprises dermis and epidermis (**Fig. 1A**).

Method

The Comet Assay is a widely used and scientifically well accepted method which accounts for several persisting and transient DNA damages (i.e. single strand breaks, double strand breaks, alkali labile sites). Guidelines for methodological standards are published by international expert groups.

Cell Preparation

A protocol for isolating keratinocytes and fibroblasts from the Phenion®-FT-SM was established. Briefly, dermis and epidermis were separated by thermolysin (2h at 4°C) (**Fig. 1C**). The cells/nuclei of one compartment were mechanically isolated by mincing in cold buffer (20mM EDTA in HBSS). They were dispersed onto two glass sildes in 0.5% low melting agarose. After electrophoresis (0.83V/cm, keratinocytes 30min, fibroblasts 45min) nuclei were stained with SYBR Gold and analyzed (100 nuclei/tissue) with Comet Assay IV software (Perceptive Instruments) regarding fluorescence intensity in the tail (% tail DNA).

Topical Application

Skin models were treated with varying concentrations of test compounds (see **Fig. 3**) dissolved in acetone. 25µl per tissue were applied with a mesh to guarantee uniform dispersion of the liquid.

Results

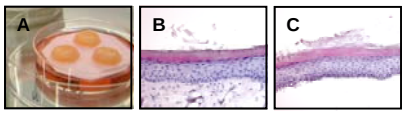

Fig.1 A) Phenion® FT-SM culture; **B**) cross section (cryo preserved, H&E stained); **C**) Epidermis after thermolysin treatment and separation from dermis.

Controls

First, the fluorescence in the tail of untreated tissues were investigated. Low levels of % tail DNA (<10%) in keratinocytes obtained from 4 different donors could be observed (**Fig 2**). Acetone was identified as an appropriate solvent inducing no DNA damages.

Exp.	Donor	Neg Ctrl. (%tail DNA)	Acetone (%tail DNA)
1	06-04	2.25 (4.69)	1.93 (7.24)
2	06-04	6.33 (10.32)	2.23 (7.09)
3	07-01	8.37 (2.16)	10.41 (3.56)
4	07-01	5.54 (2.13)	5.59 (2.67)
5	07-06	8.02 (2.57)	12.31 (3.35)
6	07-06	7.69 (1.22)	7.27 (1.10)

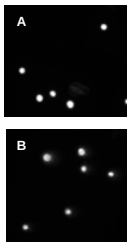

Fig.2 Table) Overview of % tail DNA of keratinocytes from different cell donors in control groups. Data shown as mean within group of slides (triplicates/group). SD in brackets. **A**) Representative photo of nuclei of untreated tissues or **B**) the solvent control (acetone).

Test compounds

Second, Phenion® FT-SM were treated with direct acting genotoxic substances for 3h (**Fig.3**). A 3h treatment should provide a protocol for the detection of both DNA damages which are repaired rapidly and those which are slowly accumulating i.e. by substances which need to be metabolized before they interfere with DNA integrity.

Methyl methanesulfonate (MMS) (**Fig. 3A, B**) and 4-Nitroquinoline 1-oxide (4NQO) (**Fig. 3C**) induced a clear dose-dependent increase of % tail DNA in keratinocytes. Since % tail DNA in control groups were below 15% a great dynamic range (15-90%) for effects of testcompounds is provided by the test system.

Furthermore, MMS induced a similar increase in DNA migration in fibroblasts as well.

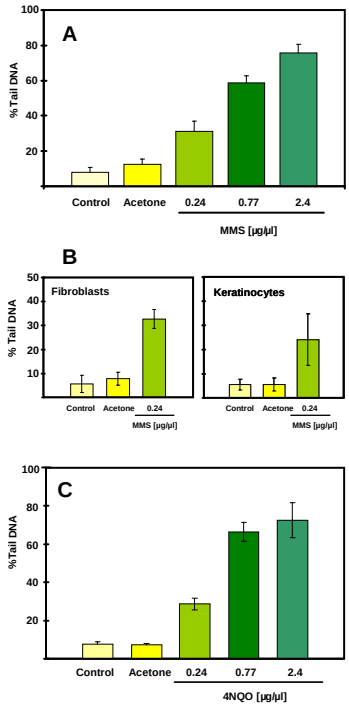

Fig.3 Phenion®-FT-SM were treated with 25µl MMS (**A**, **B**) or 4NQO (**C**) dissolved in acetone with indicated concentrations (triplicates/conc.). After 3h, dermis and epidermis were separated with thermolysin and minced separately. Cells from one compartment were spread onto 2 slides. % tail DNA of keratinocytes (**A, B, C**) or fibroblasts (**B**) are displayed as a mean within one group of slides.

Conclusion

Phenion® Full Thickness Skin Model is suitable for Comet Assay:

- Low background in control groups provides a high dynamic range for effects of test compounds.
- Robust data with different cell donors.
- Allows investigation of both keratinocytes and fibroblasts.
- Similar protocol for both cell types provides good comparability.
- Positive controls for further studies identified.

For a verification of the test system additional studies with standard genotoxic substances, especially those undergoing metabolization, are ongoing.

The work was supported by