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Successful micronucleus testing with the EPI/001 3D reconstructed epidermis model: Preliminary findings

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ABSTRACT

Currently, the cosmetics industry relies on the results of *in vitro* genotoxicity tests to assess the safety of chemicals. Although the cytokinesis-block micronucleus (CBMN) test for the detection of cells that have divided once is routinely used and currently accepted by regulatory agencies, it has some limitations. Reconstituted human epidermis (RHE) is widely used in safety assessments because its physiological properties resemble those of the skin, and because it allows testing of substances such as hydrophobic compounds. Thus, the micronucleus test is being adapted for application in RHE-reconstructed tissues.

Here we investigated whether two different reconstructed epidermis models (EPI/001 from Straticell, and RHE/S/17 from Skinethic) are suitable for application of the micronucleus test. We found that acetone does not modify micronucleus frequency, cell viability, and model structure, compared with non-treated RHE. Treatment of the EPI/001 model with mitomycin C and vinblastine resulted in a dose-dependent increase of micronucleus frequency as well as a decrease of tissue viability and of binucleated cell rate, while no changes of the epidermal structure were observed. The number of binucleated cells obtained with the RHE/S/17 model was too small to permit micronucleus testing. These results indicate that the proliferative rate of the tissue used is a critical parameter in performing the micronucleus test on a 3D model.

1. Introduction

Genotoxicity and mutagenicity tests are important early steps in the regulatory process of assessing chemical safety [1,2]. The EU Cosmetic Directive 7th Amendment [6] was launched in 2009 and banned *in vivo* assays for genotoxicity testing in cosmetic ingredients and for broad chemical evaluation programs such as REACH [7]. Thus, the assessment of the genotoxic and mutagenic hazard of specific compounds and chemicals currently relies on a step-by-step strategy in which *in vitro* testing has for a long time played a relevant and well-recognized role [1–5]. With no possibility of performing *in vivo* genotoxicity assays, industry and regulators have to rely on the results of *in vitro* genotoxicity tests only.

The *in vitro* micronucleus assay detects potentially clastogenic and aneugenic chemicals [8]. Its acceptance has been supported by the development of the cytokinesis-block micronucleus (CBMN) methodology [9–11], which allows the detection of cells that have divided once. CBMN allows an accurate assessment of the appropriate cell population for micronucleus (MN) quantification, along with an easy assessment of changes in cell-division kinetics due to cytotoxicity. The *in vitro* micronucleus test has also been integrated in the global genotoxicity testing approach [12]. Although the micronucleus test is routinely used and currently accepted by regulatory agencies [13], its accuracy in predicting the *in vivo* genotoxic/mutagenic potential in mammals, and especially in humans, is still controversial [14,15].

Some of the limitations that are of special concern in the case of dermally applied compounds are its lack of toxicokinetic information and of a barrier (which leads to direct exposure of the cell to doses far higher than the corresponding physiological doses), lack of 'human-like' metabolic capacity of the cell lines (hamster ovary or lung cells and human lymphocytes), and the use of cell lines that are not relevant for predicting genetic endpoints at the target organ [16,17]. In addition, botanical extracts commonly used in cosmetic products are often lipophilic compounds that are difficult to dissolve in culture medium.

Moreover, this test has produced unacceptably high rates of positive results that are not confirmed in *in vivo* genotoxicity

Abbreviations: RHE, reconstructed human epidermis; MMC, mitomycin C; VB, vinblastine; MGG, May–Grünwald–Giemsa; H&E, Haematoxylin & Eosin; MN, micronucleus.

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Fig. 1. Standard treatment protocol. Reconstructed epidermis is allowed to recover for 24 h before the first treatment; a second treatment is applied 48 h later, and cells are collected for histological processing after a total culture time of 72 h.

and/or rodent carcinogenicity tests [14,15,18–20]. In fact, 75–90% of rodent non-carcinogens gave positive results in one or more of the standard *in vitro* genotoxicity assays [18]. Thus, relying only on results from *in vitro* genotoxicity assays may severely impact the ability to market potentially safe and beneficial new compounds.

To address these issues, the micronucleus test is being adapted for application in reconstructed human skin-tissue models [21–24]. The studies done so far have focused on a specific tissue model, Epiderm™. Herein we evaluate the possibility of performing the micronucleus test on two other reconstructed human epidermal models, EPI/001 and RHE/S/17, in an attempt to improve the safety assessment of substances that may come in contact with the skin. These two models are very similar and were not initially designed for use in the micronucleus assay. We evaluated the behavior of each of these models to determine if either one provides accurate results for this test. Our results reveal that the micronucleus test can be transferred to *in vitro* skin models, but that the outcome may depend on the model used.

2. Materials and methods

2.1. Chemicals and reagents

Phosphate-buffered saline (PBS), trypsin 0.05% EDTA.4Na, Versene 1:5000, Dulbecco's modified Eagle's medium (DMEM, high glucose, Ham's F12 medium, and foetal bovine serum (FBS) were obtained from Invitrogen (Paisley, UK). Sterile water, acetone, mitomycin C (MMC), vinblastine (VB), Cytochalasin B (Cyt-B), methanol, May–Grünwald reagent, and Giemsa stain were obtained from Sigma (St. Louis, MO, USA).

2.2. Reconstructed human epidermis and specific culture medium

EPI/001 RHEs were obtained from Straticell (Gembloux, Belgium) and S/17 RHEs from Skinethic (Nice, France). These two models are multilayered, differentiated tissues containing all physiological epidermal layers (basal, spinous, granular, and cornified). They were obtained from a pool of primary normal human epidermal keratinocytes (foreskin) isolated by the manufacturers and then cultivated in a cell-culture insert. Epidermal differentiation was obtained by cultivating the keratinocytes at the air–liquid interface and by adding specific medium complements. The tissues were delivered by express shipment (24 h) in 24-well plates filled with agar as feeding support and with a refreshment system. The plates were kept dry with an absorbent tissue and were sealed in a sterile bag. After arrival, the tissues were evaluated for macroscopic defaults and transferred into 6-well plates filled with fresh, warm growth medium (1 ml/well) provided by the manufacturers. The tissues were cultivated at 37 °C in 5% CO_2 for approximately 24 h before use and culture medium was renewed every 24 h. Both manufacturers produced the tissues from the same cell batch by extracting the cells from the original tissue (foreskin) to expand them and to store the batch of keratinocytes by cryopreservation. For each production run, one vial was used to obtain the keratinocytes required [25–27].

2.3. 3D micronucleus assay

2.3.1. Chemical preparation

Cytochalasin B ready-made solution in DMSO at 10 mg/ml was used to prepare Cyt-B medium by diluting the ready-made solution directly into the culture medium.

MMC and VB powders were diluted in water at 1 mg/ml by vortexing and then diluted in acetone for topical application. MMC at 1 mg/ml in water was diluted in acetone to 1, 2, 3, 5, 10, 15, and 20 μg/ml. VB at 1 mg/ml in water was diluted in acetone to 0.1, 0.3, 0.5, 1, 3, 5, and 10 μg/ml. Pure acetone was used as a negative control.

The 3D micronucleus assay is focused on three different end points: tissue viability, tissue histology, and micronucleus frequency (Fig. 1).

2.3.2. Treatment conditions

Upon arrival, the RHEs were treated topically with MMC or VB. A total of 10 μl of each chemical dilution were added directly to the surface of the tissue and spread by pipetting (4 RHEs per condition were used). The RHEs were transferred to 6-well plates containing 1 ml of warmed maintenance medium (also provided by the manufacturers) with 3 μg/ml of Cytochalasin B. Treatment was repeated once after 24 h and the RHEs were incubated for 24 h at 37 °C and 5% CO_2. Cells were treated for 72 h.

2.3.3. MTT test

Fresh MTT medium (1 mg/ml) was prepared and 0.3 ml pipetted into each well of a 24-well plate. The RHEs were transferred to the 24-well plate containing the MTT medium and incubated for 3 h (5% CO_2, 37 °C, saturated humidity). The RHEs were rinsed twice with PBS, transferred to a new 24-well plate with 2 ml isopropanol/well and incubated for 2 h at room temperature. Next, 200 μl of the isopropanol extract was transferred to a 96-well plate (in duplicate for each RHE) and the optical density was read at 570 nm with isopropanol as blank. The reduced cell viability in treated tissues was compared with the negative control and expressed as percentage (%) [25].

2.3.4. Histology sampling

One epidermis was used to check the histological structure of the tissues. Just before proceeding to cell harvest, one RHE was cut off from its insert and immersed in 4% formaldehyde. The fixed sample was then sliced, mounted, and stained following the Haematoxylin–Eosin (H&E) staining procedure [31] to allow verification of the structure.

2.3.5. Cell harvest and slide preparation

The RHEs were processed to extract the basal layer of keratinocytes 72 h after their arrival. RHEs were removed from their treatment medium, blotted to remove any remaining medium and transferred to a new 12-well plate for a PBS wash (1 ml out, 1 ml in) for 10–15 min. The RHEs were then transferred to a new 12-well plate containing 500 μl/well of Versene. Next, the wells were filled up with an additional 500 μl of Versene and the tissues incubated at room temperature for 15 min. After that, the tissues were transferred to a new 12-well plate containing 500 μl/well of warm (37 °C) trypsin–EDTA, filled up with an additional 500 μl/well of trypsin–EDTA, and incubated at 37 °C for 5 min and at room temperature for 10 min. Following the dissociation step, the tissues were removed from their insert and agitated in a trypsin solution to obtain keratinocytes in suspension. The cell suspension was mixed with complete culture medium (DMEM with 10% FCS) to neutralize the trypsin. In order to allow a good visualization, cells in suspension were then deposited on histological slides by use of a Cytospin (900 rpm 100 g/5 min) and the slides were then stained with the May–Grünwald staining method: slides were immersed in May–Grünwald reagent for three min, transferred to diluted May–Grünwald (1/10 in water) reagent for 45 s, transferred to diluted Giemsa reagent (1/10 in water) for 20 min, rinsed thoroughly with tap water, and mounted.

2.4. Micronucleus counting

Micronucleus scoring was performed manually by use of the NIS Element taxonomy software (Nikon). For all treatment conditions, at least 1000 cells were considered to determine the final percentage of multinucleated cells and then to determine the frequency of micronucleated cells in the binucleated cell population. According to the MTT test, cell survival was at least 30% for all treatment conditions, compared with the control. All treatment conditions were also evaluated for toxicity with the CBPI and cytostasis calculation [13]. Micronucleus detection criteria were as defined by Fenech et al. [10,11]. Each treatment condition was carried out at least in triplicate (a total of 3× 1000 cells counted for each condition) and the final result was expressed as mean and standard error. Significance of differences obtained was evaluated with the Mann & Whitney non-parametric *U*-test.

3. Results

3.1. Cytotoxicity

The cytotoxicity of the Cyt-B-based micronucleus test can be determined with the Replicative Index (RI) or cytostasis (OECD Guideline 487 (13)). According to this index, the cytotoxicity is associated to the cell proliferation and the highest concentration