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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.

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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

Table 1
Cytotoxicity of MMC and VB to EPI/001 tissue followed by MTT and Cytostasis (reduction in binucleated cells) after 48 h exposure. Results from at least two independent experiments are shown. Each experiment result was obtained from three tissues per dose.

	Viability decrease (MTT)	Cytostasis	p
MMC 1 μg/ml	−14.4%	17.8%	NS
MMC 2 μg/ml	11.4%	0.2%	NS
MMC 3 μg/ml	−14.2%	38.4%	**
MMC 5 μg/ml	10.6%	37.1%	**
MMC 10 μg/ml	17.8%	52.8%	*
MMC 15 μg/ml	18.5%	55.6%	*
MMC 20 μg/ml	15.3%	72.8%	**
VB 0.1 μg/ml	−0.9%	40.9%	*
VB 0.3 μg/ml	5.6%	38.0%	**
VB 0.5 μg/ml	6.5%	47.4%	**
VB 1 μg/ml	13.3%	69.8%	**
VB 3 μg/ml	16.0%	95.5%	**
VB 5 μg/ml	22.5%	85.0%	**
VB 10 μg/ml	17.3%	ND	**

NS: non-significant.
* Significant at $p < 0.05$ (for cytostasis data).
** Significant at $p < 0.001$ (for cytostasis data).

of a test substance should not produce more than 55 ± 5% cytostasis. This method is well established and is known to give reliable results in the micronucleus test using 2D cell culture.

However, cytotoxicity to a reconstructed epidermis is usually evaluated by the MTT assay, according to established guidelines [25]. Because the micronucleus test performed on reconstructed epidermis currently has no validated guideline, we decided to test both cytotoxicity indicators in order to select the most reliable one.

Table 1 shows the viability results obtained with the MTT assays compared with the cytostasis results for treatment with VB and MMC.

The increasing cytostasis effect is clearly visible at high doses of MMC and VB, whereas the data provided by the MTT test are more dispersed. The cytostasis rate for any chemical should not exceed 60%, which is the case for the highest dose of MMC (20 μg/ml) and for the highest three doses of VB (1–5 μg/ml). However, the MTT test provided unclear results on the effect of each chemical. Thus, the application of the cytostasis rate is recommended for cytotoxicity evaluation of chemicals based on the micronucleus tests performed on reconstructed epidermis.

3.2. Selection of the optimal dose of Cytochalasin-B

Cytochalasin-B was shown to be a good binucleated cell inductor [20–24] on other reconstituted human epidermis models. Here we exposed the EPI/001 model to growth medium containing different doses of Cyt-B and evaluated the number of binucleated cells at different times in order to determine the optimal concentration of Cyt-B and the optimal exposure time to generate a suitable number of binucleated cells for the micronucleus analysis.

Fig. 2 shows the numbers of binucleated cells obtained with three concentrations of Cyt-B (1, 3, and 5 μg/ml) and without Cyt-B (control) and three exposure times (24, 48, and 72 h.) Results show an increase in the number of binucleated cells that is Cyt-B dose-dependent. In the absence of Cyt-B, a very small number of binucleated cells was detected, independent of the duration of exposure. After the 24-h exposure, a comparable proportion of binucleated cells (10–13%) was found at all Cyt-B doses. The number of binucleated cells was higher at 48 h and 3 μg/ml (20%) and at 72 h and 5 μg/ml (30%).

Based on the fact that 5 μg/ml of Cyt-B did not result in a significant increase of the number of binucleated cells obtained with 3 μg/ml, we chose a standard Cyt-B dose of 3 μg/ml and an exposure time of 48 h.

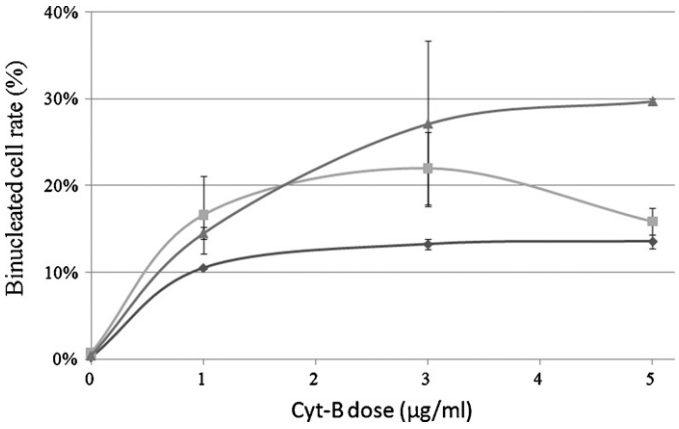

Fig. 2. Frequency of binucleated cells showing a dose-dependent increase for three different times of exposure to cytocholasin B: 24 h (diamonds), 48 h (squares), and 72 h (triangles). Results from two independent experiments are shown (except for 24 h, with one experiment). Each experimental result was obtained from three RHEs per dose.

Table 2
Binucleated cell rate and micronucleus rate on non-treated and acetone-treated EPI/001and S/17 models. Results from at least two independent experiments are shown.

	Binucleated cell rate		Micronucleus rate	
	EPI/001	S/17	EPI/001	S/17
Non-treated	34.9%	7.1%	0.9%	ND
Acetone 10 μl	33.8%	3.4%	1.0%	ND

Although lower than the number of binucleated cells produced in culture, this number remains comparable with data from previous studies with other tissue models [21], and is suitable for the micronucleus test.

3.3. Effect of acetone and basal micronucleus frequency

Application of hydrophobic substances is one of the key reasons for developing the micronucleus test with reconstructed epidermis. This model allows topical exposure of a wide range of substances including oils. Nevertheless, for some solid compounds (powder, waxes, *etc.*) solubilisation remains preferable to improve the contact surface between the tissue and the substance. Thus, we searched for a solvent that allowed a good solubilisation of hydrophobic compounds and that had no or little effect on the model. Acetone was a good candidate because it has a lower impact on the cell cycle compared with solvents such as ethanol, olive oil, or saline solvent [21]. In addition, we observed no specific effect of acetone on the reconstructed epidermis structure (Fig. 4) when compared with controls (Fig. 3). Table 2 presents the binucleated cell and background micronucleus frequencies on EPI/001.

Each treatment condition was tested in at least three independent experiments, with a total of more than nine tissues used. Non-treated and acetone-treated tissues showed very similar final binucleated cell numbers and micronucleus frequencies, indicating that the solvent had no effect on these parameters.

3.4. RHE/S/17 model

So far, only one epidermal tissue model (Epiderm) has been submitted to the micronucleus test [20,21,23,24]. Here, the micronucleus test was conducted with the EPI/001 and RHE/S/17 models. The RHE/S/17 model was assessed for its proliferative potential with Cyt-B treatment at 3 μg/ml and solvent control

Table 3
MMC was applied two times at seven different concentrations in acetone. Global binucleated cell rate reached a maximum of 33.8% with the control treatment (10 μl pure acetone). Cytostasis values as compared to controls. *P* values were calculated using replicated data (each repetition is the mean of 3 different RHEs) and were calculated with the Mann & Whitney *U* test.

MMC concentration (μg/ml)	Binucleated cell rate	Cytostasis	N	p
0	33.8%	0.0%	3	
1	27.8%	17.8%	2	NS
2	33.8%	0.2%	2	NS
3	20.8%	38.4%	3	$p < 0.001$
5	21.3%	37.1%	8	$p < 0.001$
10	16.0%	52.8%	8	$p < 0.05$
15	15.0%	55.6%	8	$p < 0.025$
20	9.2%	72.8%	7	$p < 0.001$

Table 4
VB was applied two times at six different concentrations in acetone. Global binucleated cell rate reached a maximum of 33.8% with the control treatment (pure acetone); cytostasis values as compared to controls. *P* values represent replicated data (each repetition is the mean of 3 different RHEs) and were calculated with the Mann & Whitney *U* test.

VB concentration (μg/ml)	Binucleated cell rate	Cytostasis	N	*p*
0	33.8%	0.0%	3	
0.1	20.0%	40.9%	5	$p < 0.05$
0.3	21.0%	38.0%	4	$p < 0.001$
0.5	17.8%	47.4%	11	$p < 0.001$
1	10.2%	69.8%	7	$p < 0.001$
3	1.5%	95.5%	3	$p < 0.001$
5	5.1%	85.0%	7	$p < 0.001$

(treatment with acetone). Results obtained with EPI/001 and S/17 models are shown in Table 2.

Scoring of binucleated cells from non-treated or acetone-treated S/17 tissues yielded a very low binucleated cell frequency (5.3%), which was lower than the minimum rate required for the micronucleus test, suggesting that the S/17 model is hypoproliferative.

3.5. Micronucleus induction

For micronucleus induction, topical exposure, which most closely replicates physiological conditions, was used. A total volume of 10 μl of solubilised chemicals was applied on a 0.63-cm^2 area of the epidermis by pipetting. This represents a dose of 16 μl/cm^2, which is comparable with the dose used in *in vivo* studies in rats and mice (200 μl/12 cm^2, equal to 17 μl/cm^2) [21,28].

3.5.1. Mitomycin C

The cytotoxicity effect for MMC exposure is shown in Table 3. We observed a dose-dependent decrease of binucleated cell frequency, leading to an increase of cytostasis up to 72.8%. Thus, the subsequent micronucleus evaluation for the 20-μg/ml MMC treatment was not considered. Statistical analysis with a non-parametric test indicated that a cytotoxic effect starting at 3 μg/ml MMC was statistically significant.

Fig. 5 shows the dose-dependent induction of micronuclei following treatment with MMC (except for the 20-μg/ml results, due to cytotoxicity). We observed a basal micronucleus frequency of 1% of the binucleated cells, which is considered as a low value compared with micronucleus data obtained with cultured cells. Results showed a quite high variability, especially for the MMC concentrations that induce a statistically significant cytotoxicity (3–15 μg/ml). All results were statistically significant ($p < 0.001$) compared with the control and by use of a non-parametric statistical test, except for the lowest concentration of MMC (1 μg/ml).

The lowest observable effect (LOEL) was around 2 μg/ml, which is comparable to results obtained by Curren et al. [21] and Aardema et al. [20] in the well-described reconstructed skin micronucleus assay (RSMN), using another reconstructed epidermis model.

3.5.2. Vinblastine

The cytotoxic effect of vinblastine is shown in Table 4. As for MMC, a dose-dependent decrease of the binucleated cell frequency and a concurrent increase of cytostasis were observed. Cytostasis reached a maximum of 95.5% at 3 μg/ml, and the maximum usable dose regarding the cytotoxicity was 0.5 μg/ml with a cytostasis of 47.4%. All cytotoxicity levels were significant ($p < 0.001$, except for 0.1 μg/ml). Based on this, the final VB concentrations evaluated for the micronucleus frequency were from 0.1 to 0.5 μg/ml. Fig. 6 shows the dose-dependent increase in micronucleus frequency after treatment with vinblastine. All results were statistically significant ($p < 0.001$).

4. Discussion

We have shown here the possibility of performing the micronucleus test on a specific 3D reconstructed epidermis model, EPI/001. This kind of test has already been carried out with the Epiderm™ model [21–24]. Here we show that it is possible to perform the same test with other skin models, but the choice of which model to use depends mainly on the proliferative potential of the tissue.

The interest in 3D skin models to perform the micronucleus test is justified by the need to test a broader range of substances, including hydrophobic compounds like oils, waxes, or even tar or tallow, which are not suitable for testing in cell cultures. The 3D models have the advantage of allowing direct application of test substances on the epidermal surface. In addition, the epidermal 3D model partially mimics the metabolism of the epidermis, which leads to many toxicity effects. Finally, the 3D structure allows the model to provide a skin-barrier-like effect [29]. Further studies on how to control the accumulation of these substances (*e.g.*, specific washing procedures) must be conducted.

Cytochalasin B is commonly used in the micronucleus test to inhibit cytokinesis: after treatment, all cells undergoing mitosis are blocked in a binucleated state. The binucleated cell frequency is a key parameter in the micronucleus assay, as it is directly linked to the cytotoxicity observed, and because the micronuclei are scored only in binucleated cells.

We selected the same amount of Cytochalasin B as the dose chosen by Curren et al. [21] in developing their reconstructed skin micronucleus assay (RSMN) with primary keratinocytes. Because of the similarity with the cell type used in our studies, a similar response to Cytochalasin B was expected. Moreover, the dose used in the 3D micronucleus assay performed here is also close to that used in the 2D-cell-based micronucleus assay [13]; this may be due to the fact that the models are exposed to Cytochalasin B through the medium and that the filter of the insert does not induce any diffusion matter for this type of molecule. In addition, in the 3D models, the dividing layer of keratinocytes, which is targeted by Cyt-B, is in fact attached to the filter and thus not protected from the Cyt-B by the 3D structure of the tissues.

The use of Cytochalasin B was consistent with the goal of obtaining as many binucleated cells as possible. As explained before, this is a very important requirement for the feasibility of the micronucleus test. Nevertheless, the proportion of binucleated cells induced by Cytochalasin B depends on the proliferative potential of the model. Here we showed that the proliferative potential of the two test models were very different. For instance, the proportion of binucleated cells obtained with the S/17 model was only 5.3%, which is considered to be too low for the micronucleus test. Thus, not all epidermal models are suitable for this genotoxicity assay.

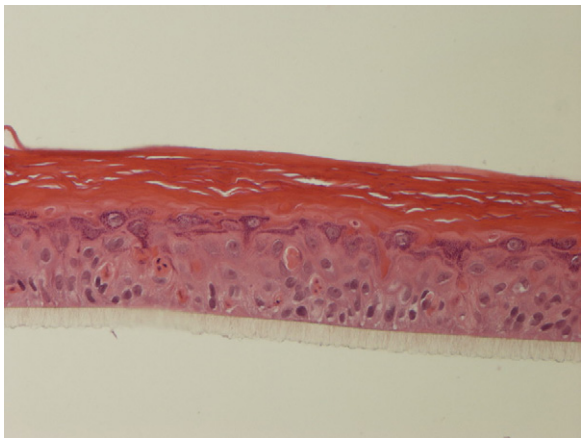

Fig. 3. H&E staining of a cross-section representative of an EPI/001 model after 72 h of culture without any treatment.

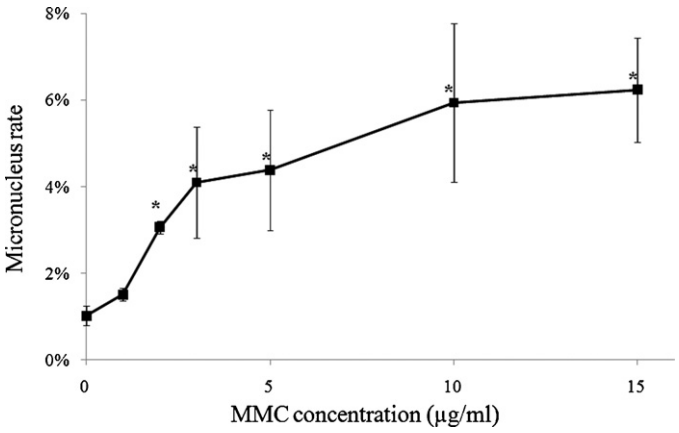

Fig. 5. Micronucleus induction in the EPI/001 model after topical exposure to MMC. Results from at least three independent experiments are shown. Each experimental result was obtained from three tissues per dose. *Significant at $p < 0.001$.

This finding is in fact not surprising as the S/17 model was initially designed for skin irritation testing. The S/17 model was produced with low hormonal stimulation in order to avoid a high basal level of cytokine release, as required for the irritation assay [30]. This leads to a non-stressed and hypoproliferative model, which is less suitable for micronucleus testing. On the other hand, the EPI/001 model produces a percentage of binucleated cells comparable to that obtained in previous studies on Epiderm™ models, indicating that this model is suitable for the micronucleus assay [20–24].

Apart from binucleated cell production, the type of solvent used in these models is also relevant for micronucleus tests. In this study, acetone was used because it had no effect in the epidermal micronucleus test developed with the Epiderm™ model [21]. In addition, it is very volatile, it is easy to spread on the epidermal surface by pipetting, and it is suitable for solubilisation of many substances. Finally, acetone has been used as a common dosing vehicle for rodent carcinogenicity studies with dermal exposure. Other solvents should also be tested to allow the application of substances that are not soluble in acetone. We observed that acetone had no effect on the epidermal structure (Figs. 3 and 4), and it was the standard solvent in our tests. It had no effect either on the very low (<1%) basal micronucleus frequency observed in the EPI/001 model (Table 2).

We also checked the possibility of conducting the MTT, in addition to the CBPI calculation. The MTT assay did not give a reliable cytotoxicity indicator for the micronucleus test. Thus we kept the CBPI as the cytotoxicity indicator for the 3D micronucleus test.

The EPI/001 model responds well to two known genotoxicants (mitomycin C and vinblastine) in a dose-dependent manner, following topical application (Figs. 5 and 6). Tests done with CBPI revealed a strong cytotoxic effect of some of the doses used, especially of the potent genotoxicant vinblastine, which led us to test additional lower doses. Topical application was preferred, as the final goal of the test is to mimic cosmetic application on human skin.

The micronucleus induction was comparable with results obtained by Curren et al. [21], who used the Epiderm™ model and ethanol as solvent, or with the results obtained in the international pre-validation study with the same model, but with acetone as the solvent. However, for the results obtained with mitomycin C, some differences should be mentioned. In the EPI/001 model, MMC was tested in acetone up to 15 μg/ml for an acceptable cytostasis of 55.6%, whereas in the Epiderm™ model the maximum testable dose was around 10 μg/ml [20]. Regarding the micronucleus formation, the EPI/001 model showed a 6% micronucleus frequency at 15 μg/ml, whereas the Epiderm™ model showed a 2–3% frequency at 10 μg/ml. The EPI/001 model allowed testing of vinblastine up to 0.5 μg/ml, whereas the Epiderm™ model allowed testing up to 1 μg/ml; in this case, the EPI/001 model showed a maximum micronucleus frequency of around 5% whereas the Epiderm™ model showed a maximum between 1 and 1.5%. These differences,

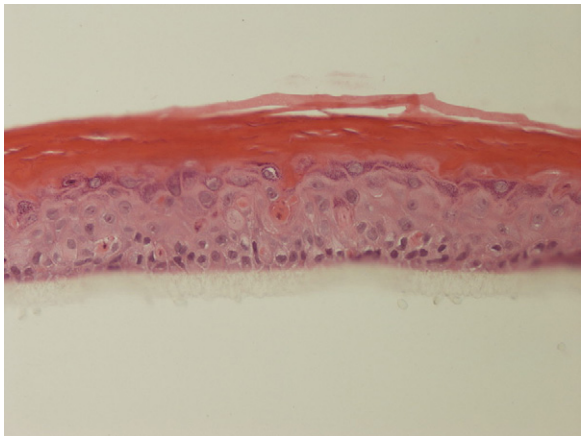

Fig. 4. H&E staining of a cross-section representative of an EPI/001 model, after 72 h of culture, treated two times (24 and 48 h) with 10 μl of acetone.

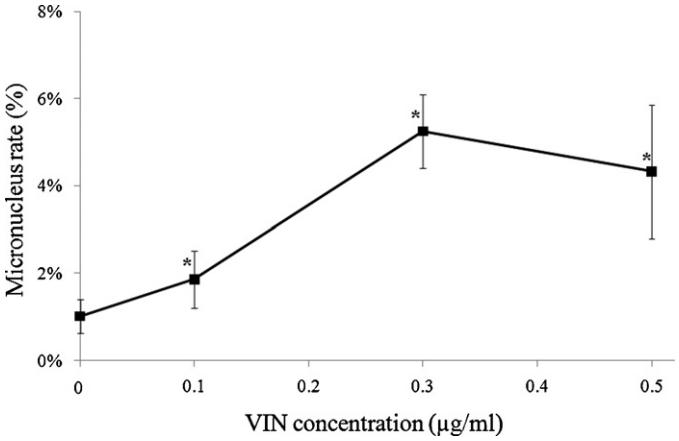

Fig. 6. Micronucleus induction in the EPI/001 model after topical exposure to VB. Results from at least three independent experiments are shown. Each experimental result was obtained from three tissues per dose. *Significant at $p < 0.001$.

although not significant, suggest that the EPI/001 model exhibits a higher sensitivity to genotoxicants, which may be explained by its higher proliferative rate.

It would be interesting to investigate whether this type of model is capable of reducing the false positive results obtained with testing in cultured cells [18,19]. The metabolic activity of the reconstructed epidermis deserves further investigation to better understand the xenobiotic metabolism in the epidermis. This remains a key feature that enables the model to detect a broad range of genotoxic substances.

Conflict of interest statement

None declared.

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