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The EpiDerm™ 3D human reconstructed skin micronucleus (RSMN) assay: Historical control data and proof of principle studies for mechanistic assay adaptations



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ABSTRACT

The *in vitro* human reconstructed skin micronucleus (RSMN) assay in EpiDerm™ is a promising novel animal alternative for evaluating genotoxicity of topically applied chemicals. It is particularly useful for assessing cosmetic ingredients that can no longer be tested using *in vivo* assays. To advance the use of this test especially for regulatory decision-making, we have established the RSMN assay in our laboratory according to Good Laboratory Practice and following the principles of the OECD test guideline 487 *in vitro* mammalian cell micronucleus test. Proficiency with the assay was established by correctly identifying direct-acting genotoxins and genotoxins requiring metabolism, as well as non-genotoxic/non-carcinogenic chemicals. We also report the analysis of our historical control data that demonstrate vehicle control and positive control values for %micronuclei in binucleated cells are in the ranges reported previously. Technical issues including evaluating various solvents with both 48 h and 72 h treatment regimens were investigated. For the first time, mechanistic studies using CREST analysis revealed that the RSMN assay is suitable for distinguishing aneugens and clastogens. Moreover, the assay is also suitable for measuring cytokines as markers for proliferative and toxic effects of chemicals.

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1. Introduction

There is global interest in the *in vitro* human reconstructed skin micronucleus (RSMN) assay in EpiDerm™ for evaluating the genotoxicity of topically applied chemicals such as cosmetics and drugs. The RSMN assay is particularly useful for the safety assessment of cosmetic ingredients [1] because *in vivo* testing has been banned by the 7th Amendment of the Cosmetics Directive [2] since March 2009. The RSMN assay is one of the options discussed in the latest Scientific Committee on Consumer Safety Notes of Guidance for the Testing of Cosmetic Ingredients [3,4] for following up positive results from standard *in vitro* genotoxicity assays. A Reference Report by the Joint Research Centre of the European Commission [5] also recommends this assay as one of the alternative methods as a follow-up genotoxicity test to a positive outcome in the initial test battery.

Early reports on the RSMN assay showed good intra- and inter-laboratory reproducibility [6–8] and the assay is currently part of an international Cosmetics Europe validation project (<https://cosmeticseurope.eu>) with contributions from the European Centre for the Validation of Alternative Methods (ECVAM) [1]. The RSMN assay in EpiDerm™ has continued to demonstrate good intra- and inter-laboratory reproducibility [9], as well as utility for detection of chemicals requiring metabolic activation [10]. As a result of the promising results from the RSMN assay, there is increased interest in its use for regulatory purposes; therefore, it is important to demonstrate laboratory proficiency and the ability to conduct the assay according to Good Laboratory Practice (GLP) standards. To this end, we have tested a series of chemicals in the RSMN assay to demonstrate proficiency, and to investigate conflicting results from the literature, as well as address important protocol variables, such as treatment regimen, vehicles, and variation in tissue constructs. Based on a relatively limited dataset, a recommended standard protocol for the RSMN assay suggested the use of a 48 h treatment regimen in a first experiment and if the results are negative or questionable, use of a 72 h treatment regimen in a second experiment to increase the sensitivity of the assay to detect genotoxins (especially

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Table 1
Test chemicals, summary of existing genotoxicity and carcinogenicity data. Information was taken from the TOXNET [34], NTP [35] PubChem [36] databases, as well as from the review of Kirkland et al. [37].

True positives	False positives	True Negatives
<p>Mitomycin C (MMC) 50-07-7</p> <ul style="list-style-type: none"> • Positive Control • Carcinogenic in rats and mice • Positive in rodent <i>in vitro</i> genotoxicity assays and <i>in vivo</i> genotoxicity assays <p>Vinblastine sulfate (VB) 143-67-9</p> <ul style="list-style-type: none"> • Positive Control (aneugenic) • VB is carcinogenic in rats and mice • Positive in mouse lymphoma assay, <i>in vitro</i> MN test, <i>in vivo</i> MN test and <i>in vivo</i> chromosomal aberration assay in mice <p>Methyl methanesulfonate (MMS) 66-27-3</p> <ul style="list-style-type: none"> • Direct alkylating agent • IARC Group 2A (Probable Human) carcinogen • Haematopoietic and lung tumors in male mice • Positive in Ames assay, Mouse lymphoma assay, <i>in vitro</i> unscheduled DNA synthesis assay, <i>in vitro</i> MN assay • Induces MN <i>in vivo</i>. <p>N-Ethyl-N-nitrosourea (ENU) 759-73-9</p> <ul style="list-style-type: none"> • Direct alkylating agent • IARC Group 2A (Probable Human) carcinogen Positive in Ames assay, <i>in vitro</i> chromosomal aberration assay and <i>in vivo</i> MN assay in mice. Skin tumors in mice after dermal application <p>7,12-Dimethylbenzanthracene (DMBA) 57-97-6</p> <ul style="list-style-type: none"> • Not classified by IARC with regard to human carcinogenicity • Positive in Ames assay, Mouse lymphoma, Sister Chromatid Exchange and cell transformation assays, and <i>in vitro</i> chromosomal aberration assay • Positive in <i>in vivo</i> MN assay in mice, skin tumors in mice, hamsters and gerbils following dermal application <p>Cyclophosphamide (CP) 6055-19-2</p> <ul style="list-style-type: none"> • Caused bladder and kidney cancer in human patients with Hodgkin's disease • Positive in Ames assay, Mouse lymphoma assay, and <i>in vitro</i> chromosomal aberration assay • Positive in <i>in vivo</i> MN assay and for aneuploidy in mice 	<p>4-Nitrophenol 100-02-7</p> <ul style="list-style-type: none"> • Negative in Ames assay, HPRT, mouse lymphoma assay and UDS assay • Negative for carcinogenicity in the mouse (topical dosing) • Carcinogenicity not tested in rats • Positive in chromosomal aberration +S9 at 1500 µg/ml <p>2-Ethyl-1,3-hexanediol (EHD) 94-96-2</p> <ul style="list-style-type: none"> • Negative in Ames assay • Negative for carcinogenicity in mice 	<p>Cyclohexanone 108-94-1</p> <ul style="list-style-type: none"> • Negative for carcinogenicity in rats and mice • No <i>in vivo</i> genotoxicity data • Negative in the Ames assay and mouse lymphoma assay

for chemicals that require metabolic activation) or to confirm a negative result [10,11]. This approach is similar to that of other *in vitro* genotoxicity assays which include longer exposures in addition to a shorter exposure regimen e.g. the *in vitro* micronucleus assay [12]. This approach was evaluated further in the studies described here. The chemicals tested in these studies (Table 1) were selected to cover a range of activities [13] from: (1) true positives: including a direct genotoxic carcinogen (methyl methanesulfonate (MMS)) and chemicals that require metabolic activation (cyclophosphamide (CP) and 7,12-dimethylbenzanthracene (DMBA)), along with mitomycin C (MMC, a clastogen) and vinblastine (VB, an aneugen), which were used as positive controls; (2) false positives (genotoxic in standard *in vitro* assays but not carcinogenic): (including 4-nitrophenol and 2-ethyl-1,3-hexanediol (EHD)); and (3) a true negative (not genotoxic in standard *in vitro* assays and negative for carcinogenicity): cyclohexanone.

As an extension to the standard RSMN protocol, we have conducted mechanistic studies to distinguish aneugens from clastogens using CREST analysis (Calcinosis, Raynaud's phenomenon, Esophageal dysfunction, Sclerodactyly, and Telangiectasias). Analysis of the presence of kinetochores is used as a follow-up method to characterize the contents of micronuclei (MN), as recommended in the OECD Test Guideline 487 [12]. CREST staining is a protein based technique that relies on the binding of the CREST antibody to

the centromeric region of chromosomes. Although CREST staining is a well qualified mechanistic assay for distinguishing MN with and without a centromere, it has not yet been demonstrated using the EpiDerm™ model. As a proof-of-concept, we have evaluated the application of CREST analysis to the RSMN assay using MMC and VB as commonly used clastogenic and aneugenic test chemicals, respectively, compared to solvent control treated tissues.

An additional adaptation of the RSMN assay is to measure cytokine release into the medium as markers for proliferative and toxic effects of chemicals. Cellular interaction with chemicals may increase the expression of a number of cytokine genes [14] and subsequently cause cell proliferation, an effect which is thought to be the underlying mechanism of carcinogenicity of some non-genotoxic chemicals [15]. Indeed, TNF-α, IL-1 and IL-6 are reported to have discrete roles in tumor promotion and cell transformation [16]. Keratinocytes cultured in 2D monolayers exposed to physical or chemical stress release inflammatory, chemotactic and cellular response cytokines [17] and this response mechanism (more specifically the release of IL-1α) is incorporated in the OECD guideline for testing irritants in 3D skin models [18] as a complementary endpoint to evaluate of toxicological properties of chemicals. We conducted proof-of-concept analyses of cytokine assessment in the EpiDerm™ model in conjunction with the RSMN assay. The levels of a panel of cytokines in the media below EpiDerm™ models were

measured after treatment with direct-acting (MMS and VB), as well as bioactivated (DMBA) genotoxins.

2. Materials and methods

2.1. Chemicals and materials

The test chemicals (Table 1) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Test chemicals were dissolved in acetone and dosing solutions were prepared fresh each day. Chemicals with limited solubility were tested up to the lowest precipitating concentration, according to the OECD test guideline 487 for the *in vitro* MN assay [12]. Trypsin (0.25%)–EDTA (0.02%) was from JRH Biosciences or equivalent sources. Acridine–orange solution (10 mg/ml) and Cytochalasin B (cytoB) were from Sigma–Aldrich. Primary antibody for CREST staining was obtained from Antibody Inc. (Davis, CA, USA) and the secondary antibody was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). All other reagents were of the highest available grade.

2.2. RSMN assay

The RSMN assay was conducted according to the protocol described by Dahl et al. [11] with the exception of the use of the Cytokinesis-Block Proliferation Index (CBPI) and Relative Viable

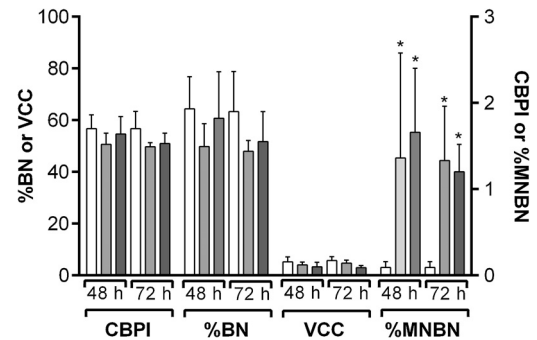


Fig. 1. Historical CBPI, % BN, VCC and %MNBN values for both 48 h and 72 h dosing regimen. Data are a mean \pm SD and include GLP as well as non-GLP studies conducted between January 2012 and December 2014. Vehicle control data (□) were from tissues treated with 10 μ l acetone, water, ethanol, PBS and saline (but not DMSO) and data for positive control values included 5 μ g/ml MMC (▒) and 0.8 μ g/ml VB (■) treated tissues. The number of experiments included in the analysis was: 48 h vehicle control = 34–36; 72 h vehicle control = 16; 48 h MMC = 16–20; 72 h MMC = 2; 48 h VB = 8–10; and 72 h VB = 2.

Cell Count (RVCC) for assessing toxicity (as per the OECD *in vitro* MN assay guideline 487 [12]), instead of using the reduction in the % binucleated (%BN) cells. For comparison purposes to previous publications on the RSMN assay, the %BN is reported in some of the analyses. EpiDerm™ EPI-200-MNA kits (donor 4F1188) and

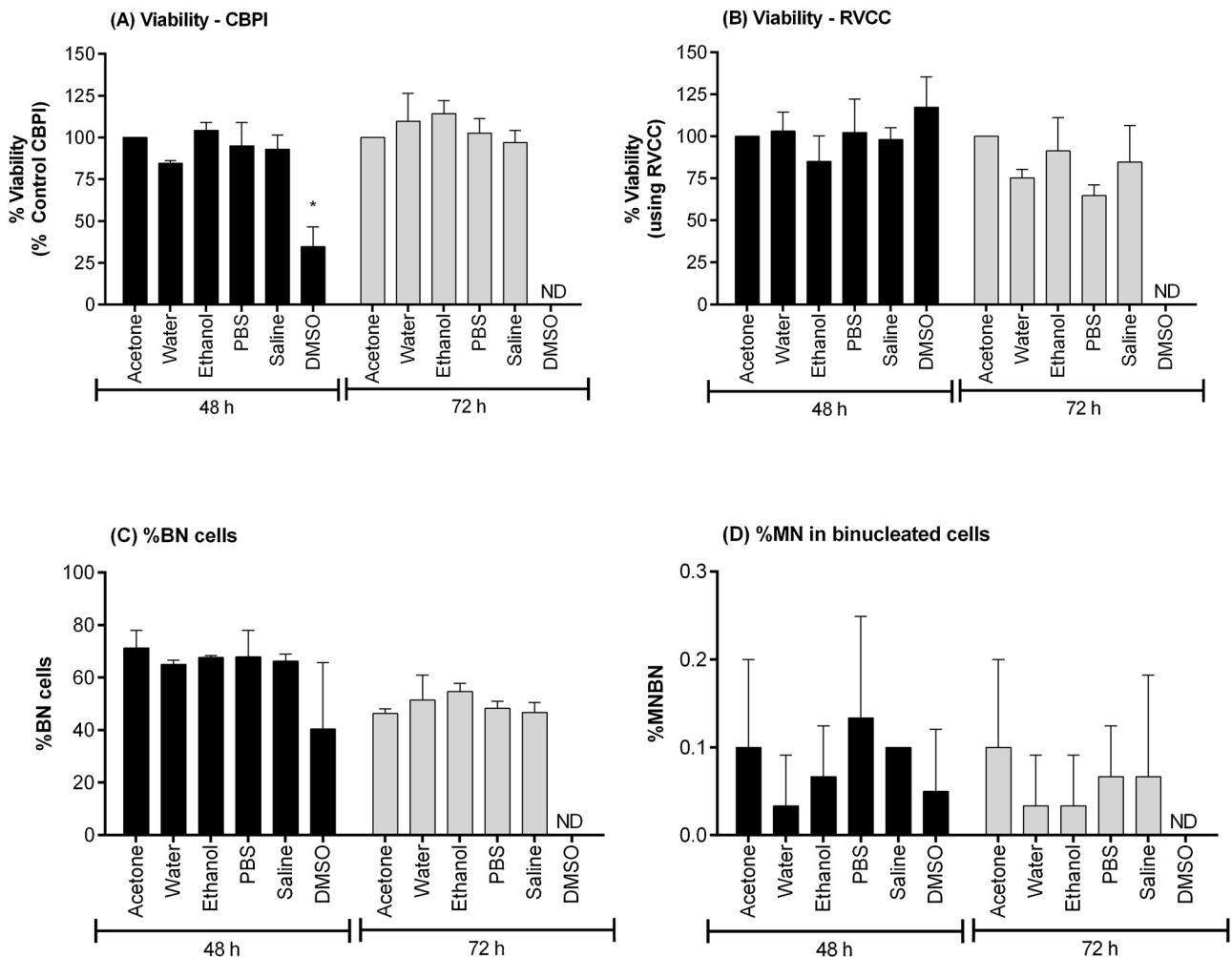


Fig. 2. Viability (A–C) and %MNBN (D) after treatment of EpiDerm™ models with different solvents for 48 h (■) and 72 h (▒). Viability was measured as the CBPI (A), RVCC (B) and % BN cells (C). Values are mean \pm SD, n = 3 tissues. An asterisk denotes a statistical difference from vehicle control values (p < 0.05).

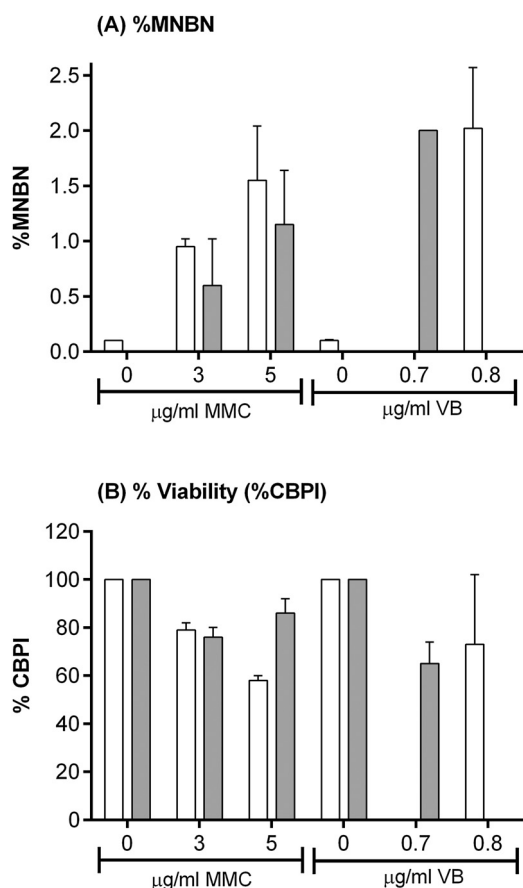


Fig. 3. A comparison of %MNBN (A) and viability (B, %CBPI) in EpiDerm™ models cultured on original (□) and new (■) inserts and treated with 3–5 μg/ml MMC or 0.7–0.8 μg/ml VB for 48 h. Values are mean ± SD, n = 6–9 for VC and 9–16 for PC treated tissues.

new maintenance medium (NMM) were shipped overnight from MatTek Corporation (Ashland, MA, USA).

The first experiment for each chemical employed a 48 h dosing regimen. If the results were negative or equivocal, a second experiment was conducted using a 72 h treatment regimen. For the 48 h dosing regimen, tissues were dosed twice, once 24 h after the tissues were received and a second time 24 h after the first dose, and cells were harvested 24 h later (48 h after the first exposure). For the 72 h dosing regimen experiments, tissues were dosed three times, the first dose was applied 1 h after tissues were received, the second 24 h later and third dose 24 h after the second dose. Cells were harvested 24 h later (72 h after the first dose). The maximum cytotoxicity for evaluation of MN was set at 60% (as defined by relative CBPI or RVCC, whichever cytotoxicity method was more sensitive (both parameters are shown in the figures)), based on the OECD 487 guideline for the *in vitro* MN assay [12].

Because there can be variability in these tissues derived from primary cells, three tissues were used for each treatment. A valid assay had at least two tissues per treatment for controls and each valid concentration. A minimum of 500 total cells per tissue are analyzed for proliferation (1N, 2N, ≥3N). A one-sided Fisher's Exact Test was used to determine the statistical significance of differences between solvent control and each of the test chemical treatments, where $p < 0.05$ was considered to be a significant positive response. A Cochran-Armitage test $p < 0.05$ was used to evaluate dose response. Assays were deemed valid if they passed the criteria described by Dahl et al. [11] and Aardema et al. [10].

Studies were conducted in compliance with the US FDA GLP Regulations as published in 21 CFR Part 58 and the OECD Prin-

ciples of GLP (C(97)186/Final) in all material aspects and use of Standard Operating Procedures (SOPs). Dosing formulation analysis was not performed. Since the required cytotoxicity was achieved in these experiments, it was concluded that the test system was sufficiently exposed to the test chemicals. At least one in-lab phase, the raw data and final report(s) were audited by Quality Assurance. The Quality Audit Unit is an independent department that comprises Registered Quality Assurance Professionals in Good Laboratory Practice (RQAP-GLP) certified by Society of Quality Assurance (SQA).

2.3. CREST labeling

Slides were prepared from cell suspensions isolated from tissues treated with solvent (acetone) and positive controls (VB at 0.6 and 0.8 μg/ml, or 3 and 5 μg/ml MMC) using a Cytospin™ 4 Cytocentrifuge (Thermo scientific, West Palm Beach, FL, USA). Cells harvested from tissues were washed with Dulbecco's phosphate buffered saline (DPBS) and finally suspended in 0.25–5 ml of DPBS, depending on the size of the pellet. Approximately 70 μl cell suspension was loaded in each Shandon EZ Single Cytofunnel and centrifuged at 120 rpm for 7 min at room temperature. Slides were observed under phase contrast microscope to confirm the presence of a uniform thin layer of cells before they were immersed in ice-cold methanol for 20 min, air dried, and stored at –20 °C in moisture-free environment until labeling. Prior to labeling, slides were brought to room temperature. The slides were first soaked with DPBS containing 0.2% (v/v) Tween-20 (DPBST) for 4 min. Primary antibody was diluted with DPBST (1:1) and 30 μl of diluted antibody was applied to the slide, covered with plastic cover slip and placed in a humidified chamber for 1 h at 37 °C. After this time, slides were washed twice by submersing them for 2 min in a coupling jar containing DPBST. Secondary antibody (Fluorescein (FITC) AffiniPure F(ab')₂ Fragment Goat Anti-Human antibody) was diluted 1:200 in DPBST and 30 μl of diluted antibody was applied to the slide, covered with plastic cover slip, and further incubated in a humidified chamber for 30 min at 37 °C. Slides were washed twice with DPBST as described above and quickly air dried. DNA was counterstained with propidium iodide. The antibody blocking agent, bovine serum albumin (BSA), was used during CREST labeling in order to avoid the unspecific binding of antibodies. Slides were evaluated for MN with the presence (CREST+) and absence (CREST-) of kinetochores using a fluorescent microscope (Olympus, model-BX43), 100 × magnification.

2.4. Cytokine analysis

Following 24 h and 48 h treatment, culture media underneath the tissue was collected for cytokines analysis. The human pro-inflammatory and regulatory cytokines, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12 p70, TNF-α, IFN-γ, and GM-CSF, were quantified in the media samples using a MSD® 9-Plex Ultra-Sensitive Kit (Meso Scale Diagnostics, Rockville, Maryland, USA). The MSD® 9-Plex Ultra-Sensitive Kit works in the form of sandwich immunoassay. Each well of the plate has 10 spots pre-coated with defined capture antibody. Cytokines from the sample bind to the capture antibody on the surface of the wells and then to the detection antibodies conjugated with electrochemiluminescent labels (MSD SULFO-TAG™). Voltage applied to the plate electrode causes the capture label to emit light which quantitatively measures the cytokines present in sample [19].

Replicate wells were loaded with 25 μl of medium collected after the treatment of tissue. Plates were sealed and shaken at 150 rpm for 2 h at room temperature (the shaker was set to "Form" of 20 and amplitude of 7). The plate was washed three times with 300 μl PBS containing 0.05% Tween-20. After washing, 25 μl

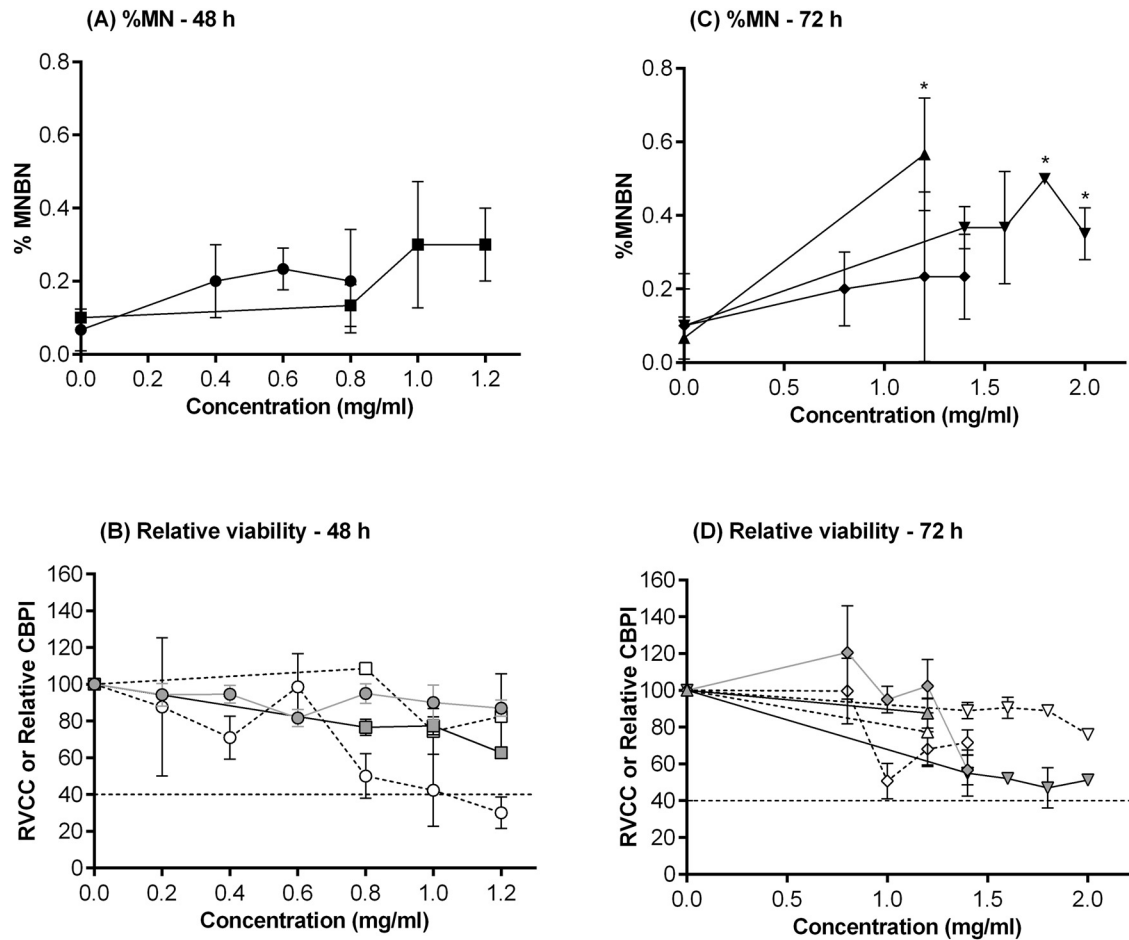


Fig. 4. Viability and %MNBN in EpiDerm™ models treated with the true positive chemical, MMS. Closed symbols denote %MNBN, open symbols denote the viability assessed using RVCC and grey symbols denote the viability assessed using CBPI. Experiment 1 = circles, Experiment 2 = squares, Experiment 3 = triangles; Experiment 4 = diamonds and Experiment 5 = inverted diamonds. Values are mean \pm SD, $n = 3$ tissues. An asterisk denotes a statistical difference from vehicle control values ($p < 0.05$).

1X “Detection Antibody solution” was added to each well of the plate and incubated for a further 2 h in a shaker incubator at room temperature. After this, the wells were washed 3 times with 300 μ l PBS containing 0.05% Tween-20. Finally, 150 μ l 2X “Read Buffer” was added to each well of plate and the samples were analyzed using SECTOR® Imager 6000 (Meso Scale Discovery, Rockville, MD, USA). The cytokine assay kit standards for IL12p70 (range 2500–0.61 pg/ml) and IFN- γ (range 2500–2.44 pg/ml) were used as positive controls, while assay buffer was used as a negative control. The 24 h and 48 h media samples were analyzed separately. Mean values of the fold increase in cytokine(s) at each time-point were reported.

3. Results and discussion

3.1. Reproducibility of control values

The OECD test guidelines for standard genotoxicity tests were recently updated, including OECD 487 for the *in vitro* micronucleus assay, which was adopted in September, 2014 [12]. Although the RSMN assay is not included in the OECD 487 test guideline at this time, the principles therein are being applied to the RSMN assay to help advance its use in regulatory decision making. The new OECD test guidelines emphasize the need to establish a robust historical control database and the use of 95% control limits of distribution for evaluating the validity of an assay, as well as in the interpretation of results. Fig. 1 provides an analysis of the historical control

data for studies conducted in our laboratory. Studies in this analysis include GLP as well as non-GLP studies. Non-GLP studies were also conducted according to a fixed protocol and SOPs but without the involvement of quality assurance department. Historical data from all 48 h and 72 h dosing studies were from vehicle control treated tissues (acetone, water, ethanol, PBS and saline (but not DMSO)) and positive control treated tissues (including MMC and VB).

The %BN cells in vehicle-treated tissues was 63–64% in 48 h and 72 h studies conducted with EpiDerm™ tissues derived from donor 4F1188, compared to an average of ~50% %BN cells in EpiDerm™ tissues derived from donor 254 [8,9]. The higher %BN cells observed in EpiDerm™ tissues derived from donor 4F1188 was also noted by other laboratories [8]. In our studies, the mean CBPI in vehicle control tissues was 1.7 for both 48 h and 72 h time points, indicating the tissues proliferate at the same rate over both dosing durations. Differences in the rates of proliferation between different donors of 3D skin tissues are not unexpected and emphasize the importance of qualifying new tissue constructs.

Importantly, the higher proliferation rate of tissues from donor 4F1188 had no impact on the cell recovery and viability or background frequencies of MN in BN cells (%MNBN). The mean yield of viable cells (VCC) in vehicle control treated tissues observed in our studies was the same regardless of the dosing regimen (5.2×10^5 cells per tissue (48 h dosing) and 5.7×10^5 cells per tissue (72 h dosing)); and was in the same range as that published previously ($\sim 2\text{--}5 \times 10^5$ cells per tissue [8]), indicating the process of cell isolation did not compromise the cells in these studies. There was also

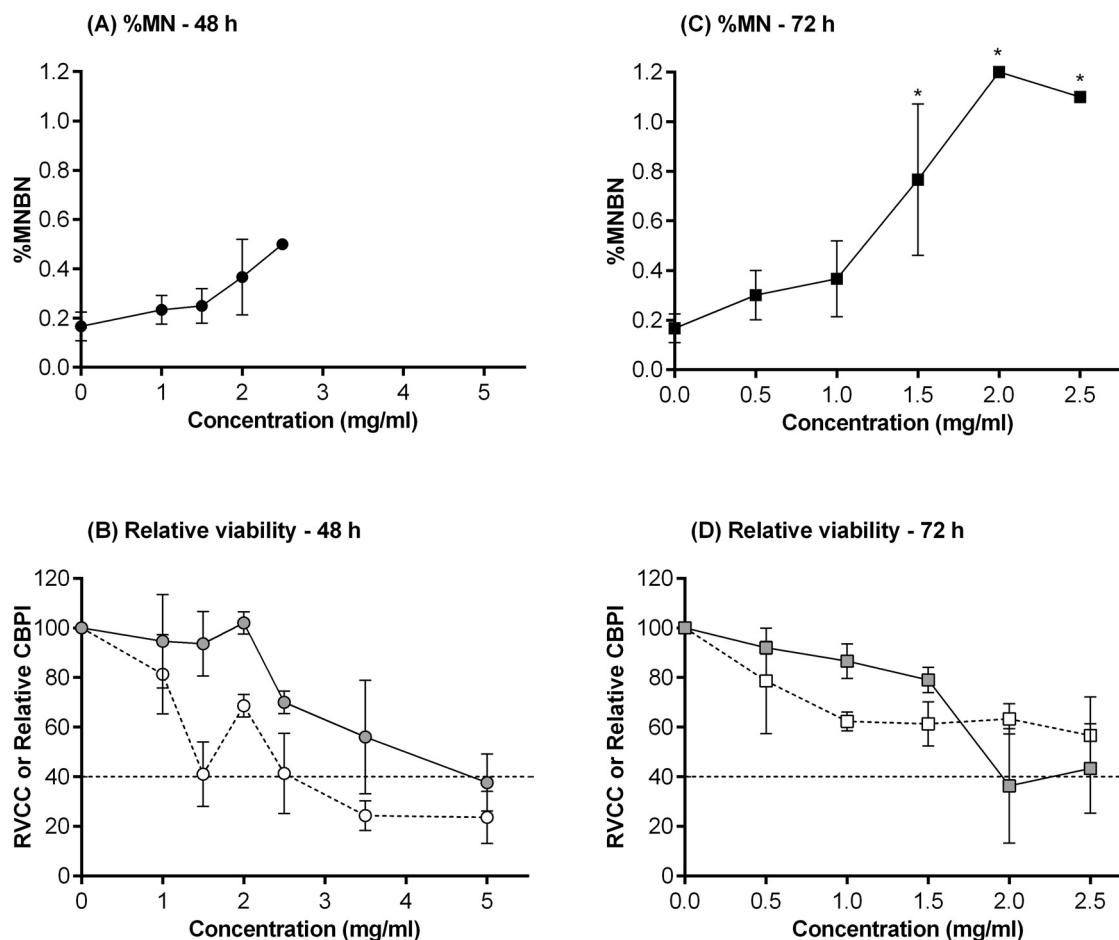


Fig. 5. Viability and %MNBN in EpiDerm™ models treated with the true positive chemical, ENU. Closed symbols denote %MN, open symbols denote the viability assessed using RVCC and grey symbols denote the viability assessed using CBPI. Experiment 1 = circles, Experiment 2 = squares. Values are mean \pm SD, $n = 3$ tissues. An asterisk denotes a statistical difference from vehicle control values ($p < 0.05$).

no detrimental effect of an extended dosing regimen on the recovery of cells or their viability (according to Trypan blue exclusion), which were both greater than 93%. The mean %MNBN of 0.09% for vehicle controls using 48 h dosing is in agreement with the average of approximately 0.08% across various laboratories, as reported by Dahl et al. [11] and Hu et al. [8]. The %MNBN in vehicle control tissues was unaffected by increasing the dosing time to 72 h ($0.09 \pm 0.07\%$). In our studies, the 95% upper control limit for MNBN for 48 h and 72 h dosing was 0.22% and 0.24%, respectively. The %MNBN was significantly increased by MMC and VB using both 48 h and 72 h dosing regimen (Fig. 1). Previous studies have employed MMC as a positive control but this is the first time VB has been demonstrated as a suitable alternative for the RSMN assay.

These findings demonstrate that this assay is reproducible across experiments and between laboratories; it is transferable and robust enough to be conducted according to GLP, and verify that the background %MNBN is in agreement with previous results in the literature.

3.2. Effect of solvents

Results published previously indicated that 10 μ l of acetone, 75%–100% ethanol and saline were acceptable solvents for the RSMN assay, since they did not induce toxicity after exposure for 48 h, while DMSO was toxic [11]. Since it is recommended that test articles that are negative in the RSMN assay after a 48 h exposure are then evaluated with an extended 72 h exposure, it was impor-

tant to verify the appropriateness of different solvents using this longer exposure time, especially since the tissues used in our studies have a higher proliferation rate. The effects of 10 μ l of 100% of each solvent (water, ethanol, PBS, saline, or DMSO) incubated over 48 h were compared to those at 72 h (Fig. 2). The proliferation rate (CPBI) of the tissues after 48 h and 72 h was unaffected by exposure to the different solvents, with the exception of DMSO, which decreased the CBPI significantly to $40 \pm 21\%$ of acetone control values after 48 h (Fig. 2A (it was not tested at 72 h)). This effect of DMSO is in agreement with previous reports [11]. Others [11] have reported cytotoxic effects of a larger volume of saline (20 μ l, evident as a decrease in the number of viable cells recovered but not %BN cells) and this effect was thought to be due to interference with the air-liquid interface of the EpiDerm™ model, decreasing differentiation and cell number in the tissues [8,11]. In our studies, none of the solvents tested at 48 h or 72 h were toxic at the volume tested (10 μ l), as determined using the RVCC (Fig. 2B). The effect of different solvents on the %BN cells in tissues is shown in Fig. 2C. As reported by Dahl et al. [11], the %BN cells in tissues was not affected by the different solvents. The exception was in two of the three tissues treated with DMSO, in which the binucleation rate was decreased to 36% and 18%, compared to a mean of $71 \pm 7\%$ in acetone control treated tissues.

Despite the decrease in viability of the EpiDerm™ models treated with DMSO, there was no statistically significant difference in the %MNBN cells in these tissues (Fig. 2D). The %MNBN in tissues treated with the other solvents tested was also unaf-

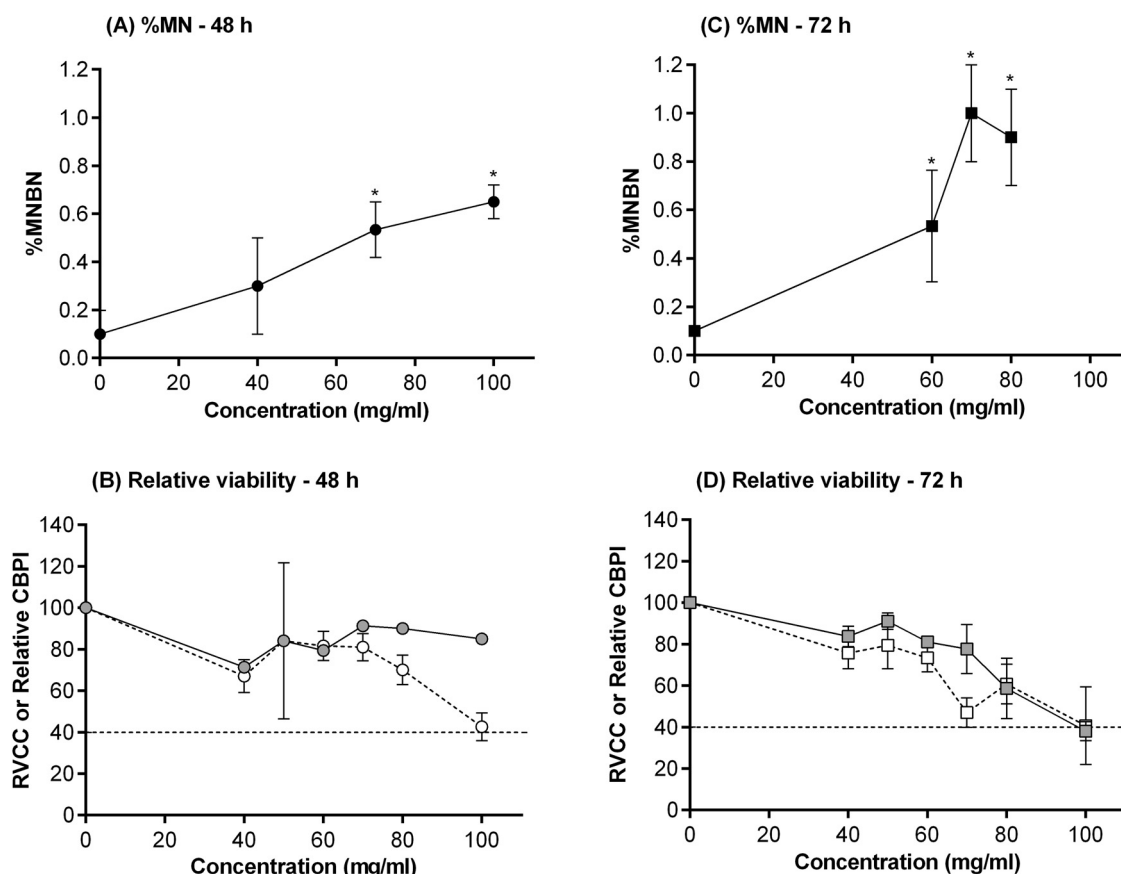


Fig. 6. Viability and %MNBN in EpiDerm™ models treated with the true positive chemical, CP. Closed symbols denote %MN, open symbols denote the viability assessed using RVCC and grey symbols denote the viability assessed using CBPI. Experiment 1 = circles, Experiment 2 = squares. Values are mean \pm SD, $n = 3$ tissues. An asterisk denotes a statistical difference from vehicle control values ($p < 0.05$).

ected compared to acetone treated tissues. Acetone and ethanol are the preferred solvents in these studies. Acetone is the primary solvent for the RSMN assay since it is commonly used in dermal rodent carcinogenicity studies and has been used in previously published RSMN studies [6–8,11]. However, other solvents such as PBS, water, and saline can be used as a solvent in this assay model when needed. Based on the extreme toxicity seen with DMSO, it is not recommended as a solvent in the assay.

3.3. Evaluation of a new tissue culture insert

At the end of these studies (but not with the test chemicals described below), there was a change in the EpiDerm™ tissue culture inserts by the manufacturer (MatTek). The change in the tissue culture insert mainly involved the manufacturing process—the material and dimensions used for the new insert remain the same. Experiments were conducted to verify that the new inserts did not affect the growth of the tissues or performance in the RSMN assay. Results with tissues grown on the new insert were evaluated using acetone vehicle control and positive control MMC and VB treatments (Fig. 3). The %MNBN from EpiDerm™ models grown on the new inserts and treated with MMC was not significantly different from the values recorded in EpiDerm™ models tested using the previous insert. As with the %MNBN, the viability of EpiDerm™ models (based on %CBPI) cultured and treated with the control reference compounds were equivalent, confirming that the new inserts had no impact on the outcome of the assay.

3.4. True positive chemicals

3.4.1. Methyl methanesulfonate (MMS)

The direct-acting genotoxicant, MMS, was first evaluated at concentrations between 0.2 and 1.2 mg/ml, using a 48 h dosing regimen (Fig. 4A and B). In Experiment 1, toxicity exceeding 60% (based on RVCC, Fig. 4B, open symbols) was observed at concentrations of 1 mg/ml and higher. There was no significant increase in %MNBN observed up to 0.8 mg/ml, and only a small increase at 1.2 mg/ml (0.40% MNBN compared to 0.07% vehicle control-treated tissues) that exceeded 60% toxicity. A second experiment using a 48 h dosing regimen was also negative up to 1.2 mg/ml (37% toxicity based on CBPI). Since small but significant increases in MMS-induced MN have been reported previously [7,8,20], we evaluated MMS with an extended dosing regimen of 72 h to determine if a higher sensitivity could be observed (Fig. 4C and D). In the first experiment with a 72 h dosing regimen, concentrations up to 2.4 mg/ml were tested, with excessive toxicity observed at 2.4 mg/ml MMS and higher. Only one concentration could be analyzed, 1.2 mg/ml MMS, which induced a statistically significant increase in %MNBN of 0.57% compared to 0.07% in control-treated tissues. In a repeat experiment with a 72 h exposure, the highest concentration of 1.4 mg/ml induced 53% toxicity based on CBPI (Fig. 4D, grey symbols), but no significant increase in %MNBN was observed (Fig. 4C). To clarify the effect of MMS with a 72 h dosing regimen, a final study with closely spaced doses of 1.4 mg/ml, 1.6 mg/ml, 1.8 mg/ml and 2 mg/ml was conducted. In this study, a statistically significant increase in MN formation was observed at 1.8 and 2.0 mg/ml. These results are in agreement with those recently reported by Chapman et al. [20] where MMS induced significant increases at

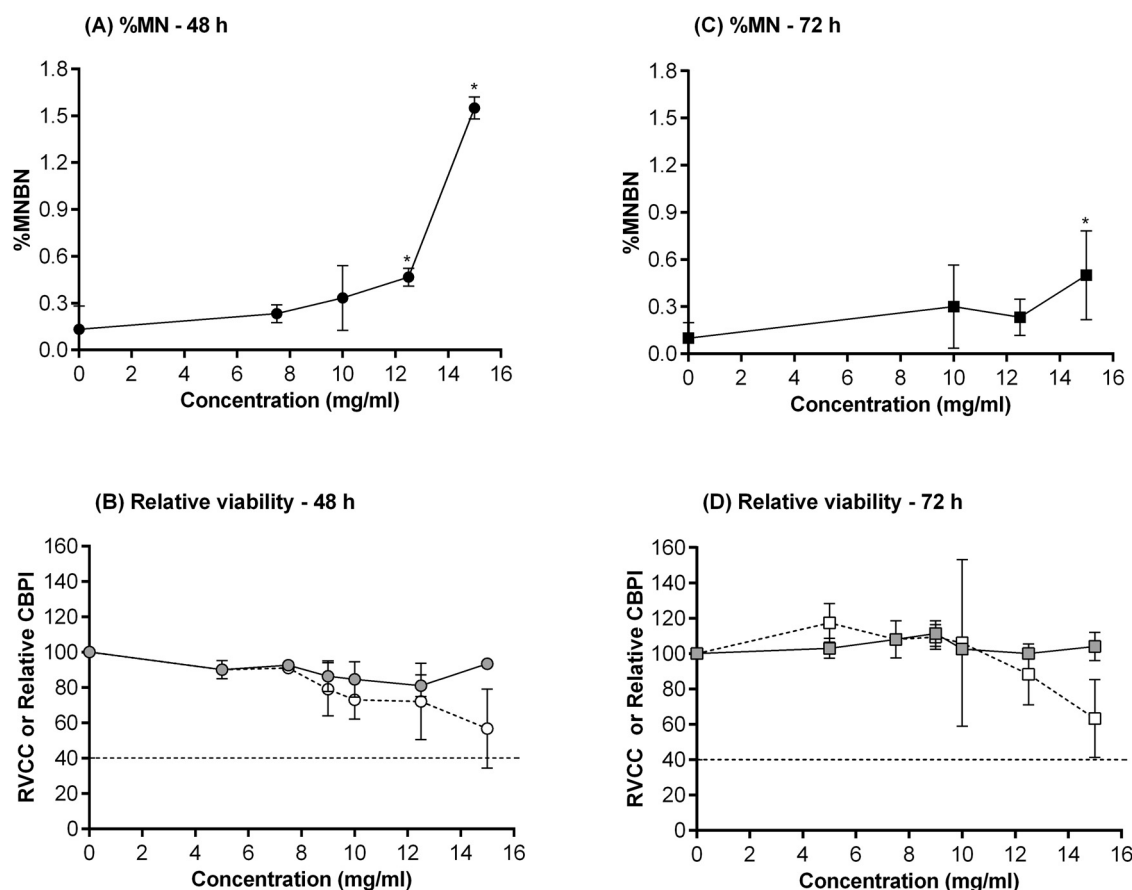


Fig. 7. Viability and %MNBN in EpiDerm™ models treated with the true positive chemical, DMBA. Closed symbols denote %MN, open symbols denote the viability assessed using RVCC and grey symbols denote the viability assessed using CBPI. Experiment 1 = circles, Experiment 2 = squares. Values are mean \pm SD, $n = 3$ tissues. An asterisk denotes a statistical difference from vehicle control values ($p < 0.05$).

concentrations of 1.7 mg/ml and above in studies conducted with EpiDerm™ from donor 4F1188. Overall, MMS was considered to be positive in the RSMN assay but it has a very narrow concentration range for inducing MN before toxicity is induced. A threshold for genotoxic effects of MMS has been reported by Doak et al. [21], which is in-keeping with the threshold-like response observed at 1 mg/ml and above in our studies. MMS is an alkylating agent and the DNA adducts formed are predominantly repaired by base excision repair and methyltransferases [22]. It is thought that the rate of repair is higher than the rate of adduct formation at lower doses of MMS but once the repair mechanisms are saturated, there is a rapid increase in DNA damage at non-toxic doses, thus possibly causing the threshold effect.

3.4.2. N-ethyl-N-nitrosourea (ENU)

ENU is a direct-acting genotoxicant that was tested in a 48 h and 72 h dosing regimen (Fig. 5). The RVCC was a more sensitive measurement of viability for ENU than the CBPI in the 48 h experiment (Fig. 5B). ENU was toxic at 3.5 and 5 mg/ml according to RVCC and at 5 mg/ml according to CBPI. At the non-toxic concentrations, this chemical caused a concentration-dependent increase in %MNBN; however, none of the increases reached statistical significance (Fig. 5A). In a second experiment, this chemical was tested in the 72 h regimen with doses between 0.5 and 2.5 mg/ml. Under these conditions, there was a concentration-dependent increase in the %MNBN which was statistically significant at 1.5 mg/ml and above. The finding that ENU is positive in this assay in agreement with Aardema et al. [9] and Mun et al. [7].

3.4.3. Cyclophosphamide (CP)

CP requires metabolic activation to induce genotoxicity. CP is metabolized by a number of cytochrome P450s (CYPs), including CYP2C8, CYP2C9 and CYP3A4; of which CYP2C9 is present in EpiDerm™ models [23]. This chemical was tested over a range of concentrations (40–100 mg/ml) in a 48 h and 72 h dosing regimen (Fig. 6). There was a concentration-dependent increase in the formation of MN in the 48 h dosing regimen between 40 and 100 mg/ml, and in the 72 h dosing regimen between 60 and 70 mg/ml, with statistically significant effects induced at 60 mg/ml and above in both experiments. In a previous inter-laboratory study, Aardema et al. [10] focused on chemicals that require metabolic activation. In these studies, CP was positive with a 48 h regimen in one laboratory only but positive in both laboratories using a 72 h dosing regimen. This indicated that the longer 72 h exposure time may be more sensitive for chemicals requiring metabolism. The positive results in our studies at both 48 h and 72 h confirm that chemicals that require metabolic activation can be detected in a standard 48 h exposure.

3.4.4. 7,12-Dimethylbenzanthracene (DMBA)

DMBA is genotoxic with metabolic activation. DMBA was tested at 5–15 mg/ml using a 48 h dosing regimen (Fig. 7A and B). At these concentrations, DMBA was not toxic and 12.5 and 15 mg/ml resulted in a statistically significant increase in %MNBN. In a study with a 72 h dosing regimen (Fig. 7C and D), there was a statistically significant increase in %MNBN at 15 mg/ml only but there was more cytotoxicity observed using this longer dosing time. These results are in agreement with those reported previously [10] such

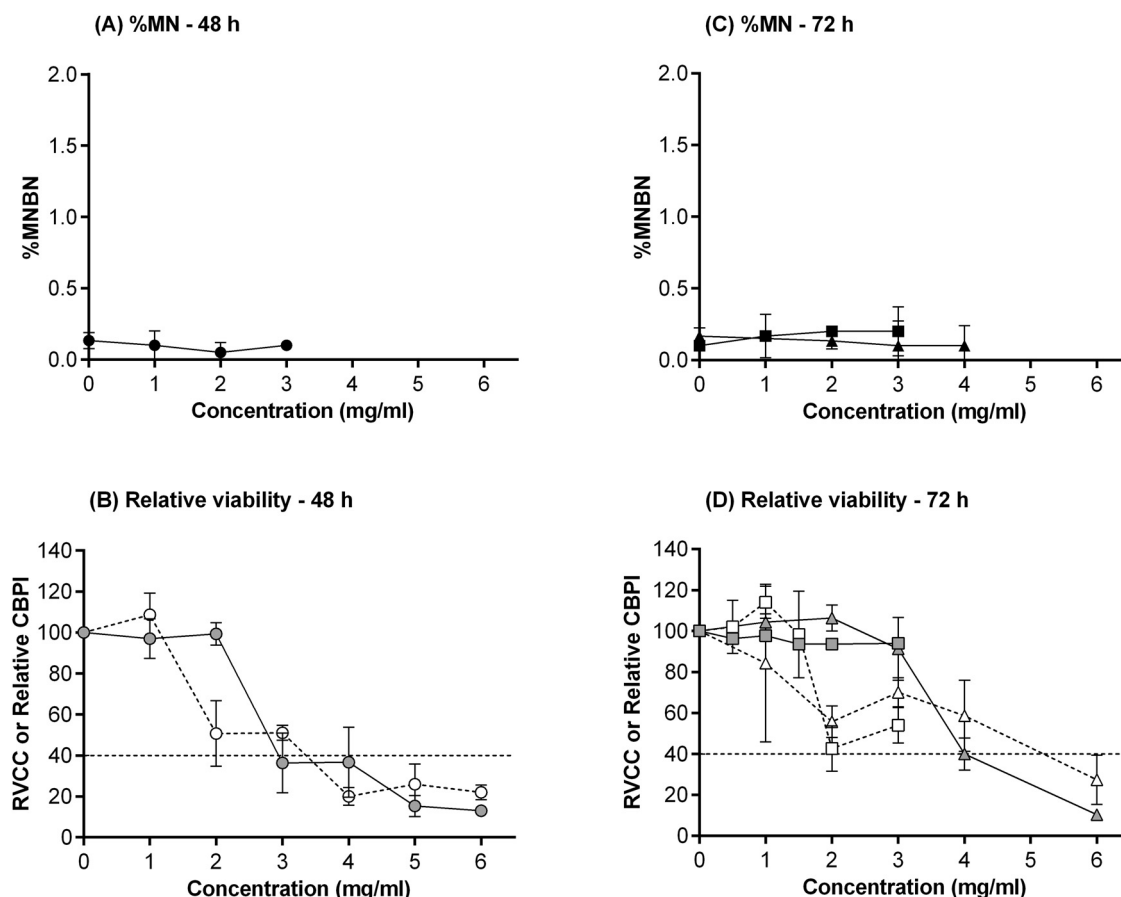


Fig. 8. Viability and %MNBN in EpiDerm™ models treated with the false positive chemical, 4-nitrophenol. Closed symbols denote %MN, open symbols denote the viability assessed using RVCC and grey symbols denote the viability assessed using CBPI. Experiment 1 = circles, Experiment 2 = squares, Experiment 3 = triangles. Values are mean ± SD, n = 3 tissues.

that DMBA is positive in the RSMN assay with both 48 h and 72 h exposures. The increase in toxicity observed with the 72 h dosing regimen compared to the 48 h dosing regimen supports the strategy of first using the 48 h dosing regimen for the RSMN assay, and then using a 72 h regimen for follow up testing.

3.5. False positive chemicals

3.5.1. 4-Nitrophenol

4-Nitrophenol was initially tested over a concentration range of 1–6 mg/ml, using a 48 h dosing regimen (Fig. 8A and B). None of the concentrations tested increased the %MNBN; however, concentrations of 4 mg/ml and higher induced greater than 60% cytotoxicity, based on CBPI and RVCC. A second experiment was therefore conducted using a maximum concentration of 3 mg/ml and the longer 72 h dosing regimen. As with the first experiment, there was no increase in %MNBN at any concentration tested but the study did not reach 50% cytotoxicity; therefore, a repeat 72 h experiment was conducted using higher concentrations up to 12 mg/ml 4-Nitrophenol was cytotoxic at concentrations of 6 mg/ml and higher (Fig. 8D shows concentrations up to 6 mg/ml). Again, 4-nitrophenol did not cause an increase in the formation of MN in the EpiDerm™ models (Fig. 8C). These results are in agreement with those published previously [7,8] and extend the data to include the 72 h treatment regimen. Recently, negative results with 4-nitrophenol were also observed in a Japanese laboratory [24].

3.5.2. 2-Ethyl-1,3-hexanediol (EHD)

EHD was tested in 3 independent experiments. The first experiment was conducted using a dose regimen of 48 h and a concentration range of 20–100 mg/ml (the maximum concentration for this assay [11] (Fig. 9A and B)). All concentrations were non-toxic, with the exception of the highest concentration, which caused >80% cytotoxicity (based on RVCC). There was no increase in the %MNBN at any concentration. These results are consistent with those reported previously [7,8], including the observation that RVCC was the most sensitive marker for toxicity, compared to CBPI (our studies) or %BN [8], which were both unaffected by EHD. This is thought to be due to interference of the air-liquid interface with liquids test articles such as EHD. To extend this analysis, EHD was evaluated using a 72 h treatment regimen over the concentration range of 40–100 mg/ml (Fig. 10C and D). Under these conditions, all but the lowest concentration was cytotoxic (>80% cytotoxicity based on RVCC). Since this experiment only had one valid dose level based on viability, a third experiment was carried out using 10–50 mg/ml EHD and a 72 h treatment duration. Under these conditions, none of the concentrations were toxic or caused an increase in %MNBN. Overall, EHD was negative in the RSMN assay. These findings are supported by those of Yuki et al. [24] who recently reported negative results for EHD in the RSMN assay (Fig. 9).

3.6. True negative chemical: cyclohexanone

Cyclohexanone was initially tested over a concentration range of 20–100 mg/ml using a treatment period of 48 h (Fig. 11A and B). There was no increase in the %MNBN at concentrations between 20

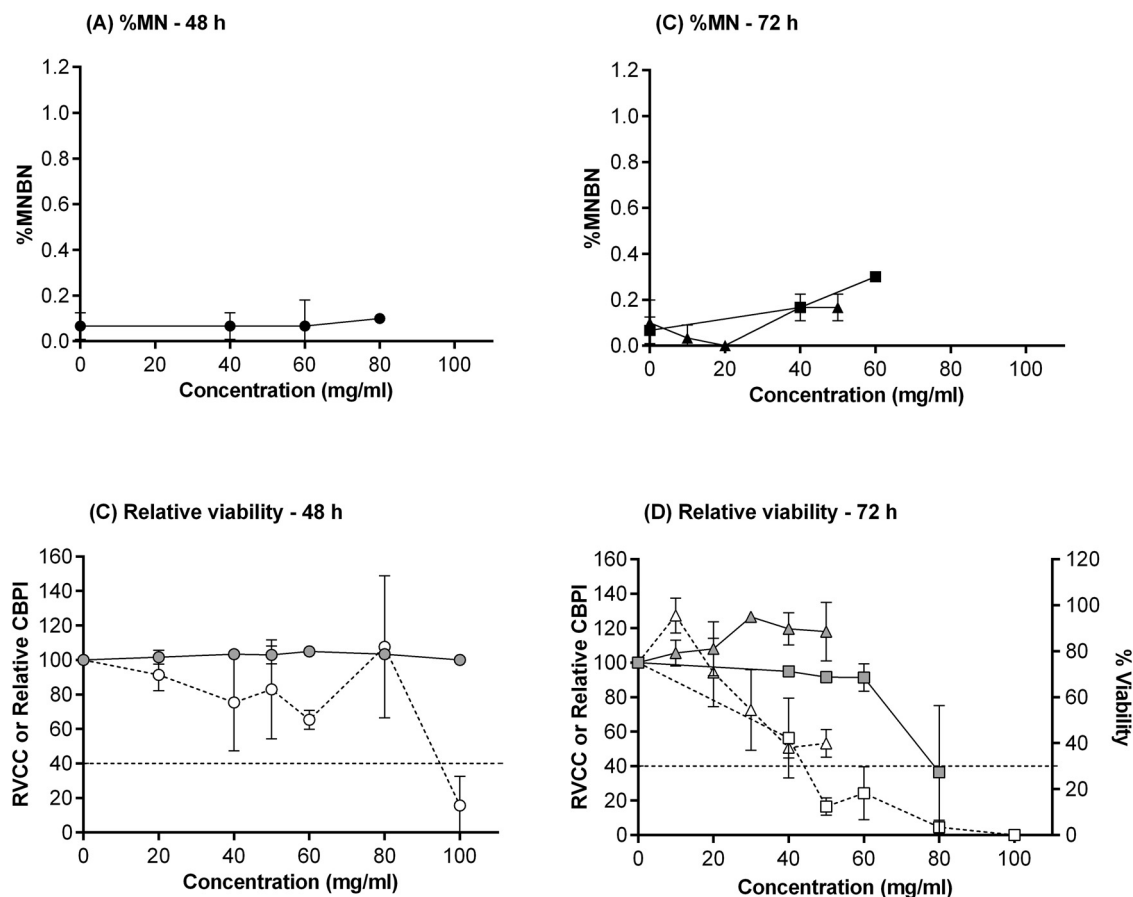


Fig. 9. Viability and %MN formation in EpiDerm™ models treated with the false positive chemical, EHD. Closed symbols denote %MN, open symbols denote the viability assessed using RVCC and grey symbols denote the viability assessed using CBPI. Experiment 1 = circles, Experiment 2 = squares, Experiment 3 = triangles. Values are mean \pm SD, $n = 3$ tissues.

and 80 mg/ml with 100 mg/ml being excessively toxic according to RVCC. In a second experiment with a 48 h treatment regimen, 80 and 100 mg/ml cyclohexanone both induced 0.43% MNBN, which was significant without causing marked cytotoxicity (less than 20% toxicity). Since there was no concentration-dependent effect and the increase was small, a third experiment was carried out with the same concentration range but with a treatment time of 72 h (Fig. 10C and D). In this experiment, cyclohexanone was not toxic at any concentration up to 100 mg/ml and did not cause an increase in the %MNBN. Based on these experiments, cyclohexanone was judged to be negative in the RSMN assay since there was no reproducible increase in %MN. These results are in agreement with those published by Aardema et al. [9]

3.7. CREST labeling

MMC, a potent DNA crosslinker is a well known clastogen in *in vitro* studies. VB is a model aneugen that binds tubulin thereby inhibiting the assembly of microtubules leading to missegregation of chromosomes and formation of MN [25]. It has been shown that MMC primarily induces kinetochore negative MN and VB induces kinetochore positive MN in various cell types [26,27,28] but this has not yet been investigated in the 3D skin model. We optimized standard methods and used them to compare MN from MMC and VB-treated EpiDerm™ tissues with MN in acetone-treated tissues to determine the applicability of CREST analysis in the RSMN assay. We also tested MMC in this assay as an example of a clastogen. Examples of CREST-positive MN resulting from aneugenic mode of action and CREST-negative MN resulting from clastogenic

mode of action are shown in Fig. 11. Of the cells isolated from EpiDerm™ models exposed to acetone, 29% of MN (11 MN in total) contained kinetochores, compared to 71% that did not contain kinetochores. This is in agreement with background levels in different cell types [29,30]. The percentage of MN that contained kinetochores in MMC-treated tissues (23% kinetochore positive and 77% kinetochore negative) was similar to that in acetone control tissues, confirming that this is a clastogen and not an aneugen and that the assay did not produce a false positive result. By contrast, the percentage of MN containing kinetochores was greatly increased in cells from EpiDerm™ models exposed to 0.6 μ g/ml VB to nearly 90%. Although additional chemicals will need to be tested in order to confirm the applicability, these initial data support that the EpiDerm™ model is suitable for use of CREST labeling of the MN to determine whether a genotoxic chemical causes aneugenecity or clastogenicity.

3.8. Cytokine expression

Fig. 12 shows how the levels of cytokines in the medium were affected in EpiDerm™ models treated with VB (0.4 μ g/ml), and a range of concentrations of DMBA and MMS in the 48 h treatment regimen. Both VB and DMBA were non-cytotoxic according to CBPI and RVCC measurements and this was reflected in the minimal changes in most cytokine levels (Fig. 12A and B). Interestingly, while none of the other cytokines were increased by DMBA, IL-6 was increased in a dose-dependent manner up to 3.8-fold. VB also increased the release of IL-6 by 2.7-fold but its main affect was to increase IL-10 by 64.1-fold. These findings suggest

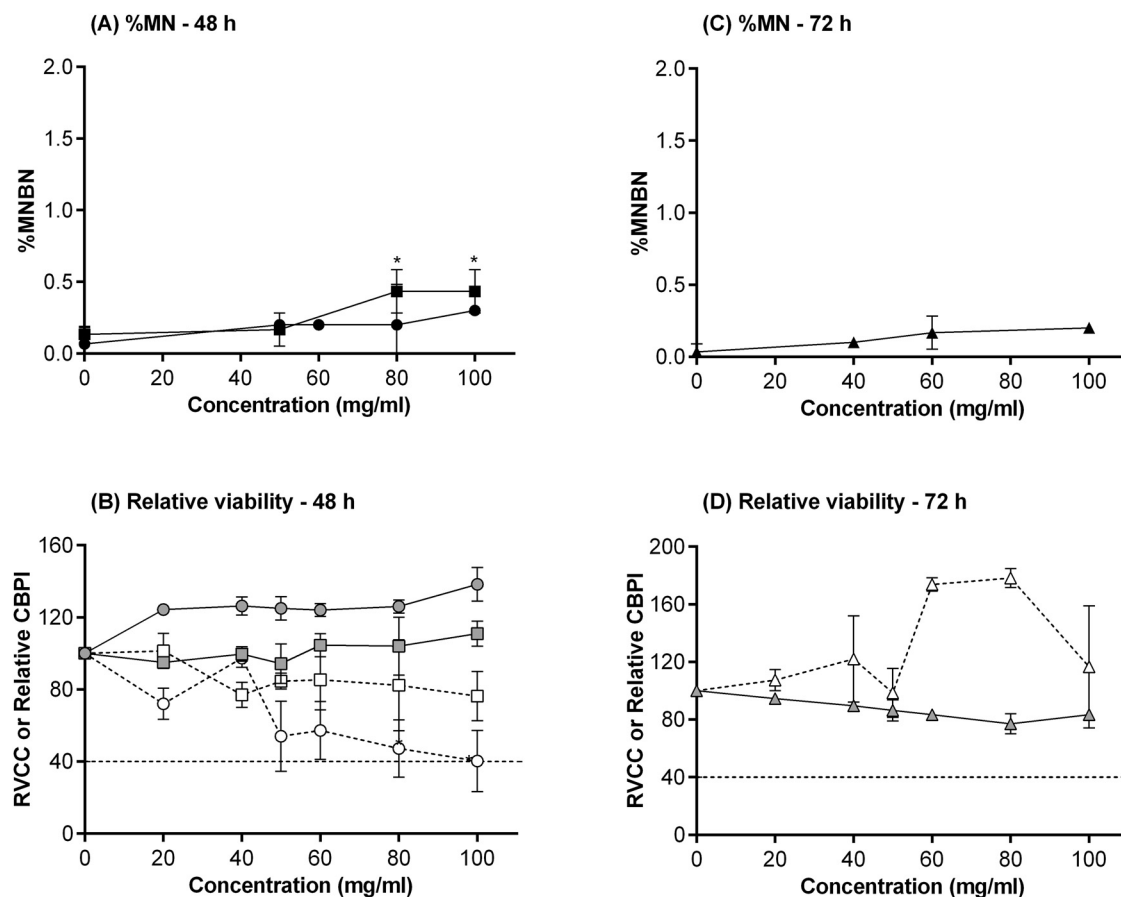


Fig. 10. Viability and %MNBN in EpiDerm™ models treated with the true negative chemical, cyclohexanone. Closed symbols denote %MN, open symbols denote the viability assessed using RVCC and grey symbols denote the viability assessed using CBPI. Experiment 1 = circles, Experiment 2 = squares, Experiment 3 = triangles. Values are mean \pm SD, $n = 3$ tissues. An asterisk denotes a statistical difference from vehicle control values ($p < 0.05$).

both compounds stimulate proliferation but VB also suppresses inflammation. Cytokines such as TNF- α , IL-1, IL-6 and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) are all involved in cell proliferation [31]; whereas, IL-10 is an immunoregulatory cytokine which down-regulates the immunoresponsive cytokines and allows transformed cells to grow [32]. MMS was much more toxic to EpiDerm™ models and caused them to release almost all cytokines we measured into the medium (with the exception of GM-CSF) (Fig. 12C). The increased toxicity (CBPI) correlated very well with the release of IFN- γ ($R^2 = 0.93$), IL-12 p70 ($R^2 = 0.99$), IL-1 β ($R^2 = 0.82$), IL-8 ($R^2 = 0.90$) and TNF- α ($R^2 = 0.96$) over the concentration range 0–3.6 mg/ml MMS, suggesting that these cytokines would be good additional indicators of toxicity in this model. Higher concentrations of MMS (4.8–7.2 mg/ml) caused 100% toxicity measured by CBPI and this was reflected in a decrease in the release of cytokines into the medium but a continued increase in TNF- α . The continued release of TNF- α may be expected since it has been shown to be released systemically after skin injury; whereas, other cytokines are only released locally within the skin [33]. These initial findings demonstrate the use of the measurement of cytokines for additional mechanistic information in the RSMN assay indicating both proliferative and cytotoxic effects of test chemicals.

4. Conclusions

We have established the RSMN assay in our laboratory and now routinely conduct this assay. Overall, these studies demonstrate the transferability of the RSMN assay to a GLP setting with SOPs. Our robust historical control dataset for vehicle and positive control

values for %MNBN and viability data (RVCC, CBPI and %BN) indicates a good reproducibility of the assay.

The studies reported here demonstrate correct identification of both direct-acting and bioactivated genotoxins as positive, and negative results for chemicals considered to be false positive and true negatives. Although the binucleation rate of the EpiDerm™ donor we tested was higher than that of other donors, the vehicle control values for %MNBN were in the ranges reported by others using different donors. This supports the use of the EpiDerm™ model to provide consistent results, regardless of the donors employed. In order to confirm the outcome of an evaluation, we support the recommendation to use a 72 h treatment in cases where the first assay conducted using a 48 h dosing treatment is negative or inconclusive. An example of this was MMS, which was negative using the 48 h regimen but positive using the 72 h regimen. In addition, although CP was positive in the 48 h regimen, the response was higher using the 72 h dosing. Importantly, the chemicals predicted to be negative, were negative in both the 48 h and 72 h regimens demonstrating that the longer exposure does not compromise the performance of the RSMN assay.

Technical details, such as the effects on the %MNBN and tissue viability of different solvents and the new MatTek inserts, are described; as well as use of VB as a positive control for 48 h and 72 h experiments. Investigations into the effect of different solvents on cell viability and %MNBN revealed that DMSO compromised the viability of EpiDerm™ models, evident as a decrease in the CBPI after 48 h, in agreement with previous reports. Our studies support the use of acetone, ethanol, PBS, water and saline (but not DMSO) as suitable vehicle control solvents, since these had no effect on

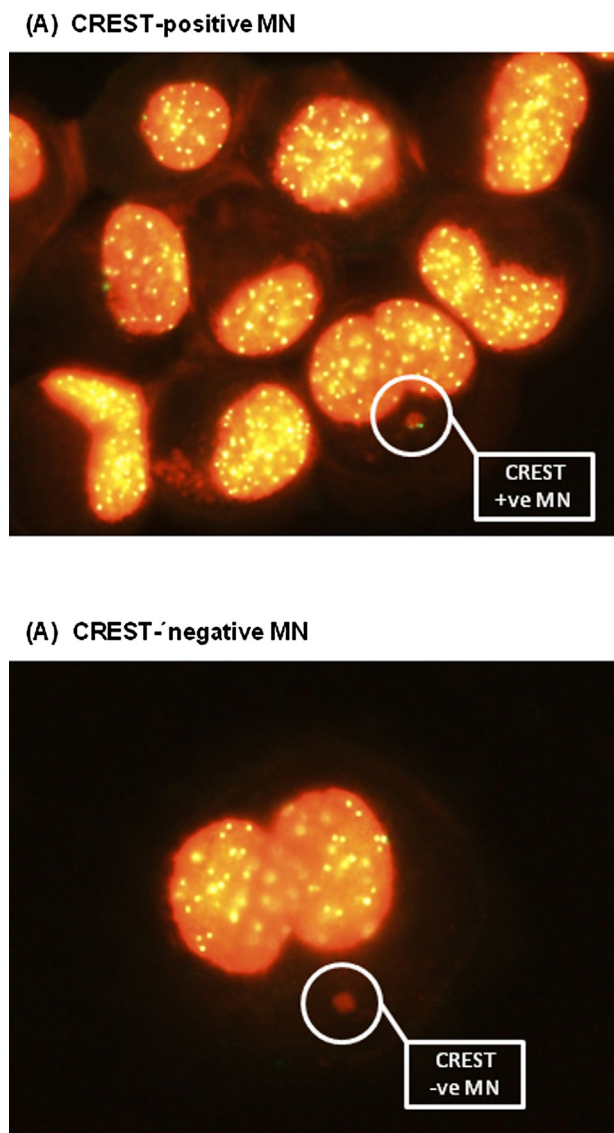


Fig. 11. CREST-positive (A) and CREST-negative MN from cells from the RSMN assay. Cells were labeled with CREST-antibodies showing MN with centromeric signal (CREST-positive MN) (aneugenic mode of action) and CREST-negative MN (clastogenic mode of action). Cells were stained with Acridine orange (orange color). CREST-antibodies are FITC labeled (green color).

viability or the %MNBN in EpiDerm™ models in the 48 h or 72 h dosing regimens.

Initial proof-of-concept studies were run to investigate the incorporation of additional endpoints into the RSMN assay protocol, making the assay more informative without the need for additional tissue samples. The inclusion of CREST analysis of the MN demonstrate for the first time the application of this technique in the RSMN assay to gain information on the mechanism of genotoxicity. In addition, measurement of a panel of proinflammatory and regulatory cytokines in the medium is possible in the RSMN assay to obtain information on markers for proliferative and toxic effects of the chemical.

Overall, the results reported here indicate that the RSMN assay is an exciting novel *in vitro* method that is easily transferable and robust for conduct in a GLP setting. The RSMN assay provides a biologically relevant approach for testing dermally applied chemicals and drugs.

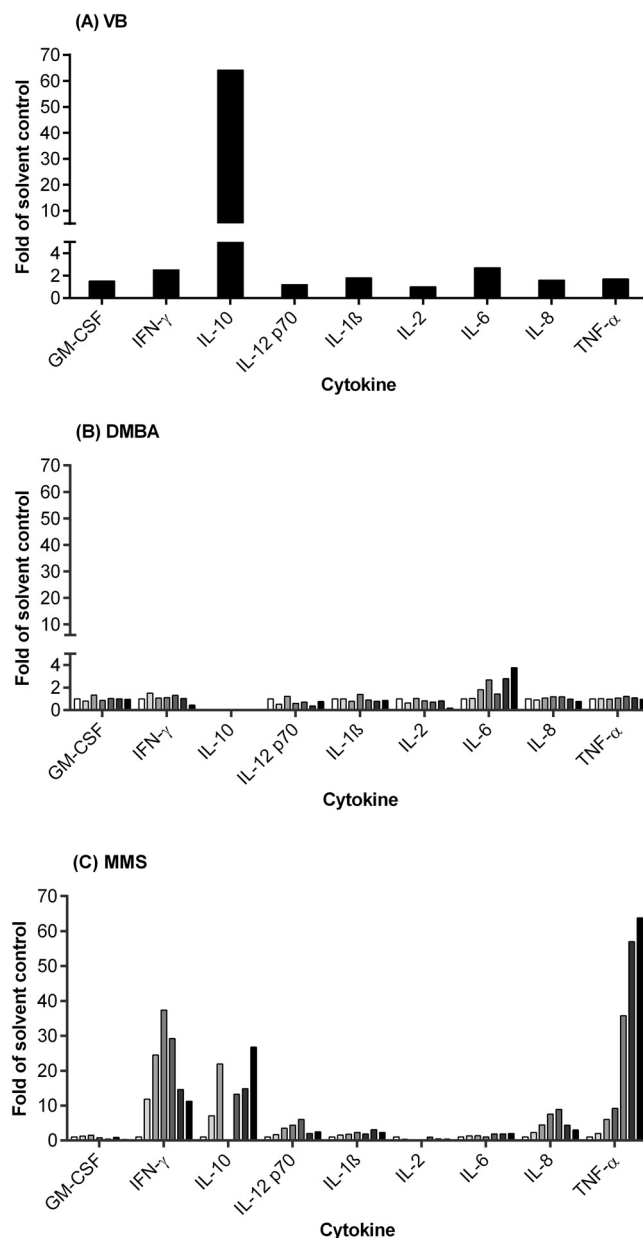


Fig. 12. Cytokine profiles of media from EpiDerm™ models treated with VB (A), DMBA (B) and MMS (C). Levels of cytokines in the media are expressed as the fold of control (acetone) treated model media. Values are a mean of 2 tissues. The concentration of VB was 0.4 mg/ml. DMBA was tested at 5, 7.5, 9, 10, 12.5 and 15 µg/ml and MMS was tested at 1.2, 2.4, 3.6, 4.8, 6 and 7.2 mg/ml (increasing concentrations are represented by increasing darkness of the bars). Values are from pooled media from 3 tissue incubations and two time points.

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