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Further development of the EpiDerm™ 3D reconstructed human skin micronucleus (RSMN) assay

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ABSTRACT

The upcoming ban on testing of cosmetics in animals by the European Union's 7th Amendment to the Cosmetics Directive will require genotoxicity safety assessments of cosmetics ingredients and final formulations to be based primarily on *in vitro* genotoxicity tests. The current *in vitro* test battery produces an unacceptably high rate of false positives, and used by itself would effectively prevent the use and development of many ingredients that are actually safe for human use. To address the need for an *in vitro* test that is more predictive of genotoxicity *in vivo*, we have developed an *in vitro* micronucleus assay using a three-dimensional human reconstructed skin model (EpiDerm™) that more closely mimics the normal dermal exposure route of chemicals. We have refined this model and assessed its ability to predict genotoxicity of a battery of chemicals that have been previously classified as genotoxins or non-genotoxins based on *in vivo* rodent skin tests. Our reconstructed skin micronucleus assay correctly identified 7 genotoxins and 5 non-genotoxins, demonstrating its potential to have a higher predictive value than currently available *in vitro* genotoxicity tests, and its utility as part of a comprehensive *in vitro* genotoxicity testing strategy.

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1. Introduction

The reliance on animal assays to help determine the relevance of positive *in vitro* genotoxicity results is at odds with the growing emphasis within the scientific and regulatory communities to reduce and eventually eliminate animal use. Recent regulations in Europe, such as the 7th Amendment to the Cosmetics Directive [1], will prohibit the use of *in vivo* genotoxicity tests in safety assessments for cosmetics as of 2009. Further, programs such as Registration, Evaluation, Authorization and Restriction of Chemical Substances (REACH) in Europe will need to rely heavily on *in vitro* methods, which are less costly and time consuming than *in vivo* methods, to evaluate the enormous numbers of chemicals currently projected to require safety testing. This limits the future use of *in vivo* methods to address the relevance of results generated from first tier *in vitro* genotoxicity assays. This is a significant problem since currently used *in vitro* assays produce an unacceptably high rate of false positive results. A number of recent international meetings have addressed this topic, including those sponsored by the International Life Sciences Institute-Health and Environ-

mental Sciences Institute (ILSI-HESI) and International Working Group on Genotoxicity Test Procedures (IWGT) [2,3], the European Center for the Validation of Alternative Methods (ECVAM) [4] and The European Cosmetic, Toiletry and Perfumery Association (COLIPA) [5].

A recent analysis of over 700 chemicals demonstrated that 75–95% of rodent non-carcinogens are positive in one or more *in vitro* genotoxicity tests [6,7]. This analysis was confirmed by a study of over 1000 pharmaceutical compounds [8,9]. Thus, the problem of false positive results in current *in vitro* genotoxicity assays is a broad one that impacts both industry and regulatory agencies that rely on these assays to help evaluate the safety of new products. Currently, chemicals that are genotoxic *in vitro* are typically evaluated further in *in vivo* genotoxicity assays to determine whether the chemical is a potential hazard to humans. Therefore, the high false positive rate has led to a large number of costly and time consuming animal studies. Many useful chemicals subject to the 7th Amendment to the Cosmetics Directive will likely be discarded solely on the basis of these low-specificity assays. A number of projects are being initiated to address the possible causes of the high rate of false positive results, particularly in mammalian cell assays, which include abnormal DNA repair and cell cycle control, altered xenobiotic metabolism, and the cellular overload associated with testing high, non-physiological concentrations of chemicals and drugs [5].

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Another approach to solve the problems with the existing *in vitro* genotoxicity assays involves the development of new *in vitro* methods that utilize normal human cells [10,11]. A few years ago we began development of a micronucleus assay in the 3D human EpiDerm™ skin construct [10]. We chose a human skin model because it represents the tissue that has the highest exposure after dermal application of a cosmetic or accidental exposure to many chemicals, and would best reflect the “first pass” absorption and metabolism that occurs following these exposures in humans. Because these artificial tissues are constructed from primary human cells, 3D skin models are expected to more accurately reproduce normal DNA repair and cell cycle control, and to provide a human metabolic capability that is more relevant than the exogenous rodent metabolizing enzymes currently recommended for use with *in vitro* genotoxicity assays. Detailed investigation of the metabolic capability of the EpiDerm™ model is now underway to determine how closely it reproduces the capacity of normal human skin, and whether the model might indeed provide a better estimate of systemic genotoxicity than *in vitro* assays utilizing rodent S-9. In addition, since these tissues have a functional stratum corneum, they will presumably provide more relevant exposure conditions to the target cells, avoiding the non-physiological concentrations of chemicals and drugs that often occur in the currently available *in vitro* genotoxicity tests. We anticipate that these traits of the reconstructed skin models will improve the predictive value of a micronucleus assay relative to existing *in vitro* genotoxicity assays.

In our previous work [10] on the reconstructed skin micronucleus assay, hereafter referred to as the RSMN assay, we described the development of methods for reproducibly isolating and processing large numbers of viable cells from the 3D EpiDerm™ skin model (MatTek Corp., Ashland, MA). We developed methods for incorporating cytochalasin B blockage of cytokinesis, to insure proper analysis of the dividing cell population. The background frequency of micronuclei (MN) was and continues to be very low and reproducible in this model, allowing for the use of robust statistical analyses. We showed that statistically significant increases in the frequency of micronucleated cells were induced by model genotoxins that act via different mechanisms, mitomycin C (MMC) a crosslinking agent that leads to chromosome aberrations, and vinblastine sulfate (VB) a mitotic poison that results in aneuploidy. MN were induced in a dose-dependent fashion either by adding genotoxins directly to the medium that resulted in exposure of the tissue from the basal surface, or by direct application of the genotoxins to the apical surface of the tissue. Based on the success of these initial studies, we have expanded our work to refine the assay procedures and build the database to allow us to begin addressing predictive capability. Since one of the primary objectives was to develop an assay that provided a more relevant assessment of potential genotoxicity, particularly a lower rate of false positive results, we felt that it was important early-on in the development process to evaluate chemicals negative in rodent dermal carcinogenicity studies. In this paper we report negative results for 5 dermal non-carcinogens. In addition, we evaluated 7 other model genotoxins including genotoxic dermal carcinogens. These genotoxins induced statistically significant increases in micronuclei in the RSMN. We also report further investigation of additional experimental parameters such as length of exposure and concentration of cytochalasin B and provide more detail to the procedures described previously.

2. Materials and methods

2.1. Chemicals and reagents

Methyl methane sulfonate (MMS), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG), 2-phenylphenol (2-PP), 4-nitrophenol, 1,2 epoxydodecane, trichloroethylene, 2-ethyl 1,3 hexanediol, beta-butyrolactone (BBL), dimethylcarbonyl chloride (DCC), n-methyl-nitrosourea

(MNU), and n-ethylnitrosourea (ENU), cell culture grade mitomycin C (MMC) with NaCl, cytochalasin B (cytoB), dimethyl sulfoxide (DMSO), methanol, acetic acid, potassium chloride (KCl), trypan blue and acridine orange (AO) were obtained from Sigma-Aldrich Corporation (St. Louis, MO). Trypsin (0.25%)-EDTA (0.02%) was obtained from JRH Biosciences (Lenexa, KS). EPI-100-NMM New Maintenance Medium (NMM) was obtained from MatTek Corporation (Ashland, MA). Dulbecco's phosphate buffered saline (DPBS), Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS) and L-glutamine were obtained from Quality Biological (Gaithersburg, MD).

2.2. Stock chemical solutions

MMC was used as the positive control and was tested concurrently with all test chemicals. Cell culture grade MMC with NaCl (Sigma Cat. No. M 4282) was used, rather than MMC without NaCl as described previously [10], to reduce possible variability in the quality of the MMC stock solutions. A stock solution of MMC (1 mg/ml) was prepared by adding sterile tissue culture grade water at room temperature (2.0 ml) to a vial of MMC (2 mg per vial). The vial was then vortexed to completely dissolve the MMC. Aliquots (100 µl) of stock of MMC were stored at −15 to −25 °C. If a precipitate was observed after thawing, the vial was vortexed and/or sonicated to achieve a uniform suspension. Fresh dosing solutions in acetone were prepared for each assay (generally 10 and 3 µg/ml) and stored at 2–8 °C for the duration of the assay. However in more recent experiments – performed subsequent to those reported in this manuscript – a 0.5 mg/ml stock solution of MMC (2 mg of MMC with NaCl in 4.0 ml water) was used to minimize precipitation during the preparation of the dosing solutions. This modified procedure is currently in use at all laboratories associated with this publication.

Cytochalasin B (cytoB) was prepared by adding either 3.3 ml of dimethylsulfoxide (DMSO) to a vial containing 10 mg of cytoB, then vortexing the vial to completely dissolve the cytoB. Aliquots of 3 mg/ml stock cytoB were stored in cryovials at −15 to −25 °C. For each experiment, an aliquot of stock cytoB was thawed and diluted in NMM to the required concentration (generally 3 µg/ml). NMM supplemented with cytoB was warmed to 37 °C before use and then stored at 2–8 °C for the duration of the assay.

2.3. Tissue constructs

EpiDerm™ tissue (EPI-200-MNA) was obtained from MatTek Corporation. The tissues were supplied on 0.64 cm² cell culture inserts. Detailed tissue handling procedures have been described previously [10]. Tissues with signs of detachment from the underlying membrane (“blisters”) or with medium pooled on the surface of the tissue (possibly indicating a compromised stratum corneum) were discarded. Upon arrival the tissues were transferred to the wells of six-well plates containing 1 ml of fresh 37 °C NMM and incubated at 37 ± 1 °C and 5 ± 1% CO₂. Culture medium was replaced every 24 h.

2.4. Treatment of tissues with test chemicals

Test chemicals (see Table 1 for more information) were administered topically as described previously [10]. In brief, tissues were equilibrated overnight in 6-well plates containing 1.0 ml NMM per well. The culture medium was then replaced with NMM containing 3 µg/ml cytoB, and 10 µl of the dosing solutions were applied topically to the tissues. Topical dosing and replacement of the culture medium with fresh medium containing cytoB was repeated after another 24 h, and 24 h after that (48 h after the first test chemical treatment) the tissues were collected for analysis. In some cases, the dosing and refeeding was repeated at 48 h and the tissues were collected at 72 h. For these studies, tissues were equilibrated in NMM for 1 h before the first dosing and addition of cytoB. Within each independent experiment, acetone alone was included as a negative or solvent control, and MMC was included as a positive control.

Doses were selected based on toxicity. In a typical assay, a series of concentrations of the test article were evaluated, from concentrations that induced no toxicity, up to concentrations that induced around 70% toxicity (30% survival) as suggested in the draft OECD guidelines [12] that were in place at the time of these studies for the *in vitro* micronucleus assay. Currently, updated guidelines [13] are available, which suggest evaluation only for treatments causing less than 50 ± 5% cytotoxicity.

2.5. Harvesting cells from the basal cell layer

EpiDerm™ tissues were processed for micronucleus analysis as described previously [10], with the following refinements. For the trypsinization step, each tissue insert was placed in 5 ml DPBS at room temperature for 5–15 min, then in 5 ml of EDTA (0.1%) at room temperature for 15 min, then exposed to warm (~37 °C) trypsin-EDTA solution for 15 min. Each tissue was carefully lifted from the supporting membrane with fine forceps. Both the detached tissue and the supporting membrane were transferred to a new well containing 1 ml of 37 °C trypsin-EDTA. The supporting membrane was thoroughly rinsed (4–6 times) with trypsin-EDTA to collect any remaining basal cells, and then discarded. The tissue was agitated to release additional attached cells. Any cell clumps were disrupted by repeatedly drawing the cell suspension into a pipette and gently expelling the

Table 1

Historical data describing the genotoxicity of compounds tested in the reconstructed skin micronucleus assay.

Chemical	CAS No.	Dermal bioassay reference	<i>In vitro</i> genotoxicity results	<i>In vivo</i> genotoxicity results
Mouse skin carcinogens				
β-Butyrolactone	3068-88-0	[19]	+Ames ^a [20]	+MN ^d [21]
DimethylcarbamyI chloride	79-44-7	[22]	+Ames [23] +CA ^b [24] +MLA ^c [25]	+MN [26]
EthylNitronitrosoguanidine (ENNG)	4245-77-6	[27]	+Ames [28,29] +CA [29,30]	+MN [31] +Skin MN [32]
n-EthylNitrosourea	759-73-9	[33]	+Ames [23] +CA [24,34] +MLA [35] +MN ^d [36]	+MN[31]
MethylNitronitrosoguanidine (MNNG)	70-25-7	[37]	+Ames [23] +CA [24] +MLA [25] +MN [36]	+MN [31] +skin MN [32]
n-Methyl-N-nitrosourea	684-93-5	[33]	+Ames [23] +CA [24] +MLA [25,38] +MN [39]	+MN [40,41] I MN [26]
Mouse skin non-carcinogens				
2-Ethyl-1,3-hexanediol	94-96-2	[42]	–Ames [43,44] –HGPRT ^e [44] +CA [44]	–Skin MN (Procter & Gamble, unpublished)
4-Nitrophenol	100-02-7	[45]	–Ames [45] +CA [45] –SCE ^f [45] –Drosph SLRL ^g [45] I MLA [25]	–Skin MN (Procter & Gamble unpublished)
2-Phenylphenol	90-43-7	[46]	+Ames [46] +MLA [46] –CA [46]	
1,2-Epoxydodecane	2855-19-8	[47,48]	– Ames [20]	–skin MN (Procter & Gamble unpublished)
Trichloroethylene	79-01-6	[27,49–52]	–Ames [50] –CA [50] +MLA [25]	–CA [50] +MN [50] –Skin MN (Procter & Gamble, unpublished)

I = Inconclusive.

^a Ames: Ames bacterial mutagenesis assay.^b CA: Mammalian chromosomal aberration assay.^c MLA: Mouse Lymphoma Assay.^d MN: Micronucleus test.^e HGPRT: Hypoxanthine-guanine phosphoribosyltransferase gene mutation test (mammalian).^f SCE: Sister chromatid exchange assay.^g Drosph SLRL: Drosophila sex-linked recessive lethal assay.

solution. The single cell suspension (~1.5 ml) was transferred to a 15 ml conical tube containing 1.0 ml of warm DMEM with 10% FBS (as opposed to 8.5 ml described in our previous manuscript) to neutralize the trypsin. Cells were exposed to the trypsin for no longer than 5 min in this final step. A sample of this cell suspension (0.1 ml or less) was diluted with trypan blue solution and counted using a hemocytometer.

2.6. Fixation and slide preparation

The cell suspension was centrifuged (100 × g) and the supernatant was carefully removed. The cell pellet was loosened by gently flicking the base of the centrifuge tube, and 1 ml of warm (~37 °C) 0.075 M KCl solution was carefully added down the side of the tube while gently shaking the cell suspension. After ~3 min, 3 ml of fresh (prepared on day of use), cold (stored at –15 to –25 °C) methanol/acetic acid (3:1) fixative were added slowly to fix the cells, and the cell suspension was centrifuged at 100 × g for 5 min. Each “slow” addition process took ~10 s. All but ~100–200 µl of the supernatant was removed and the cell pellet was gently resuspended in this smaller volume. A second fixation was used in several experiments since the above method sometimes resulted in salt crystals on the slide which interfered with the microscopic slide evaluation. In these cases the fixed cell preparation was centrifuged, the supernatant removed, the cell pellet loosened and 4 ml of cold fresh methanol/acetic acid (99:1) fixative was added. The cells were then centrifuged and resuspended as described above.

A single drop of the concentrated cell suspension was gently pipetted onto a flat or slightly tilted clean, dry microscope slide (Gold Seal®, Becton Dickinson & Co.).

Two slides were prepared from each tissue, whenever possible. After the slides were completely dry (and on the same day as the slide preparation), they were immersed in freshly prepared AO solution (final concentration 40 µg/ml in DPBS) for 2–3 min, immediately rinsed 3 times with DPBS, and allowed to dry. Stained slides were stored in the dark at 2–8 °C. If needed, slides were restained with AO for 10 to 15 s. In some cases coverslips were sealed with clear fingernail polish.

2.7. Micronucleus scoring

For microscopic analysis, a drop of DPBS and a coverslip were placed over the fixed samples, and the slides were scored using a fluorescent microscope equipped with a blue filter (e.g. Opelco, item code CT-11001V2, EX470 BS495 EM515) and 40X or 60X objective. AO stained cells typically contained bright yellow nuclei and micronuclei, and red cytoplasm, as described previously [10]. A low percentage of cells with green staining cytoplasm (Fig. 1), also seen in rodent skin [14] and possibly representing more highly differentiated cells, were not included in any of our analyses for cytotoxicity or micronuclei. Slides can be restained, if necessary due to fading, using sequential 10–15 s exposures to the AO stain, with microscopic examination for desired intensity between exposures.

All slides were coded and scored blind, first to determine the percentage of binucleated (and multinucleated) cells, and then to determine the percentage of binucleated cells containing micronuclei. At least 500 cells per slide were counted to determine the percentage of binucleated, and tri-(or greater) nucleated cells. The relative percentage of binucleation was calculated as the ratio of percentage

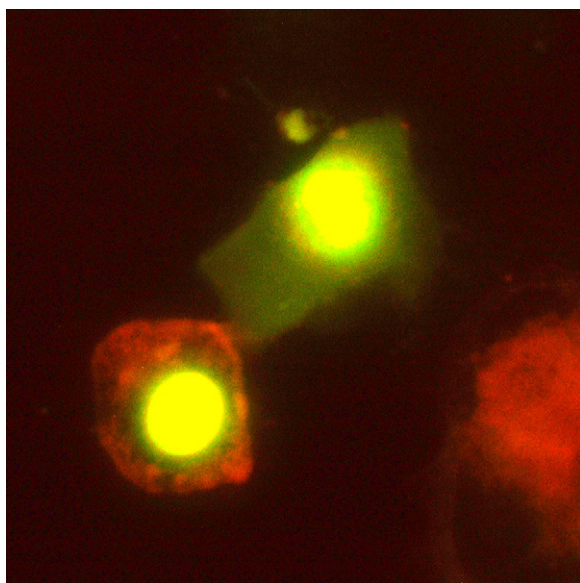


Fig. 1. A keratinized cell and a basal cell stained with acridine orange. Cells collected from the EpiDerm™ tissues were fixed and stained with the DNA stain acridine orange (AO). Cells from the rapidly dividing basal layer are identified by their round shape and red cytoplasm, which reflects AO staining of cytosolic RNA. More differentiated keratinized cells from the upper layers are irregularly shaped and have lost much of their RNA; the cytoplasm appears green following AO staining.

of binucleated and tri-(or greater) nucleated cells in treated cultures relative to the percentage of binucleated and tri-(or greater) nucleated cells in the solvent control cultures. We report the relative percentage of binucleation at each concentration of test article as a measure percent survival. Only tissues showing greater than approximately 30% survival were scored to determine the percentage of binucleated cells that contained micronuclei (at least 1000 binucleated cells per slide if possible).

Slides with fewer than 500 countable cells were considered to have 100% cytotoxicity unless it could be established that the low cell yield was due to a reason other than treatment with the test material. Only micronuclei meeting the criteria described by Fenech [15] were counted. If fewer than 500 scorable BN cells were present, the tissue was considered “not countable” and was not included in the mean calculation for that dose. All counts for MN frequency were conducted using coded slides. Three slides were counted for each test chemical concentration, and the results are presented as the mean and standard deviation of the three slides within each experiment. At least two independent experiments were performed for each test chemical. Statistical significance ($p \leq 0.05$) was determined using a one-sided Fisher's Exact Test, comparing each dose of each test chemical to the concurrent negative (solvent treated) control.

3. Results

Before the start of these experiments, procedures for the RSMN assay were successfully transferred from the Institute for In Vitro Sciences, Inc. to both the Procter and Gamble Company and MatTek

Corporation (see accompanying paper by Hu et al. [53]). Data from all three of these laboratories are contained in this report.

3.1. Optimizing cytochalasin B concentration

In previous studies [10], we determined that tissues treated for 48 h with 3 $\mu\text{g}/\text{ml}$ cytoB yielded an optimal number of binucleated cells from negative control EpiDerm™ EPI-200 tissues. To investigate how cytoB concentration affects binucleation in the presence of genotoxins, we tested 3, 5, and 6 $\mu\text{g}/\text{ml}$ of cytoB in the presence or absence of treatment with 3 $\mu\text{g}/\text{ml}$ MMC. Increasing the cytoB concentration to 5 or 6 $\mu\text{g}/\text{ml}$ cytoB did cause a slightly higher percentage of binucleated cells; however, this was accompanied by an increase in relative toxicity (the difference in the % binucleated cells in MMC treated cells compared to controls within each cytoB treatment group) from 17% (3 $\mu\text{g}/\text{ml}$ of cytoB) to 25% (5 $\mu\text{g}/\text{ml}$ cytoB) and 33% (6 $\mu\text{g}/\text{ml}$ cytoB). Increasing concentrations of cytoB also caused a dose-dependant decrease in the total number of cells (mononucleated and binucleated), which could be isolated from each tissue, but had no effect on either the consistently low level of background micronuclei or the induction of micronuclei by 3 $\mu\text{g}/\text{ml}$ MMC. Since 3 $\mu\text{g}/\text{ml}$ cytoB provided the highest total number of binucleated cells that could be recovered from these tissues and the lowest level of toxicity with MMC, we conclude that 3 $\mu\text{g}/\text{ml}$ is the optimal concentration of cytoB for the RSMN assay.

3.2. Effect of extending exposure time of direct acting genotoxins from 48 to 72 h

In our previous paper [10], we presented our rationale for selecting a 48 h exposure procedure (2 treatments, 24 h apart). To further investigate whether a longer exposure time would affect micronucleus induction by genotoxic agents, we exposed EpiDerm™ tissues to MMC and MNNG for 48 (2 treatments, 24 h apart) and 72 h (3 treatments, 24 h apart). We observed no obvious increase in the micronucleus frequency or percent survival for either MMC (Fig. 2A) or MNNG (Fig. 2B) if the exposure was increased to 72 h. An additional assay (data not shown) was performed with MMC to evaluate essentially non-cytotoxic doses between 0.5 and 1 $\mu\text{g}/\text{ml}$, and similar, statistically identical responses (significant MN inductions at 0.75 and 1 $\mu\text{g}/\text{ml}$) between a 48 h and 72 h exposure were found. In subsequent experiments using direct acting genotoxins, tissues were exposed topically for 48 h.

3.3. Evaluating sensitivity and specificity of the RSMN assay using a panel of known genotoxins and non-genotoxins

To begin characterizing the predictive capacity of the RSMN, we identified a number of compounds that had previously been tested for dermal carcinogenicity and genotoxicity both *in vitro*

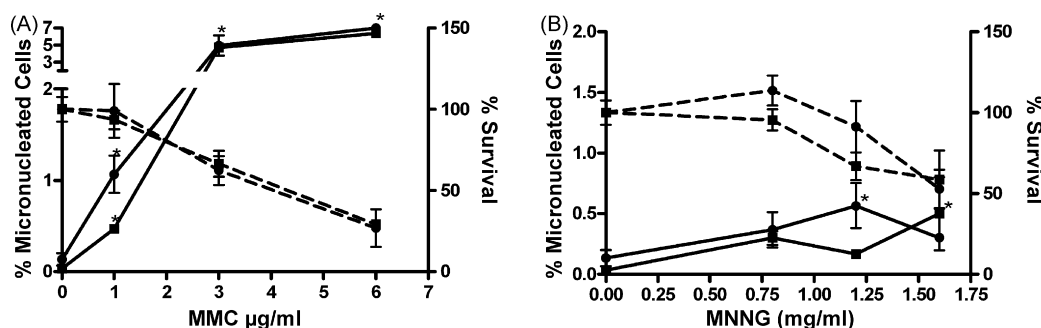


Fig. 2. Effects of extending exposure times beyond 48 h. EpiDerm™ tissues were exposed to (A) mitomycin C (MMC) or (B) N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) for 48 (circles) or 72 (squares) in the RSMN assay. Solid lines indicate % micronucleated cells (left axis), dashed lines indicate % survival (right axis). * Significant increase in micronuclei compared to negative control ($p \leq 0.05$).

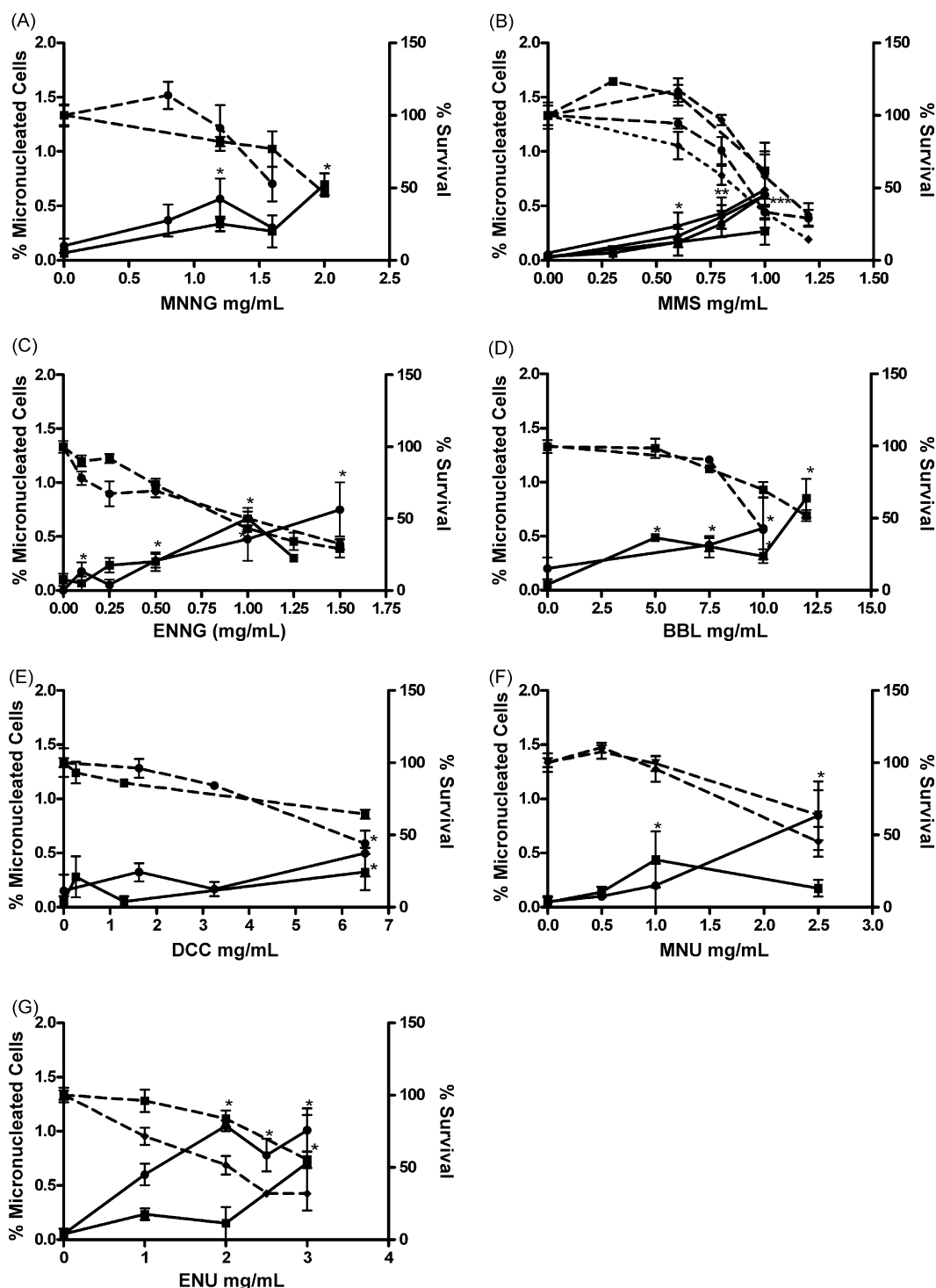


Fig. 3. Cytotoxicity and micronucleus induction in EpiDerm following treatment with known genotoxic rodent carcinogens. Solid lines indicate % micronuclei (left axis), dashed lines indicate % survival (right axis). Two independent experiments are shown on each graph. Error bars indicate standard deviation of three tissues per experiment. *** Significant increase in micronucleus induction compared to untreated tissues ($p < 0.05$). (A) N-methyl-N'-nitro-N-nitrosoguanidine (MNNG); (B) methyl methane sulfonate (MMS); (C) N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG); (D) beta-butyrolactone (BBL); (E) dimethylcarbamy chloride (DCC); (F) n-methyl-nitrosourea (MNU); (G) n-ethylnitrosourea (ENU).

and *in vivo* (Table 1). We tested seven compounds (N-methyl-N'-nitrosoguanidine, methyl methane sulfonate, N-ethyl-N'-nitro-N-nitrosoguanidine, beta-butyrolactone, dimethylcarbamy chloride, N-methyl-nitrosourea, and N-ethyl-nitrosourea), classified as genotoxic dermal carcinogens in at least two independent RSMN assays (Fig. 3). MMC was used as a positive control in all experiments. For all of these compounds, we found statistically significant increases in MN, generally in all experiments. One exception was

MMS (Fig. 3B) where only 3 of 4 experiments showed a statistically significant increase in MN induction. All chemicals induced a positive response in at least one treatment resulting in >50% survival.

We also tested five compounds that had previously been classified as non-carcinogens or non-genotoxins (4-nitrophenol, 1,2 epoxydodecane, trichloroethylene, 2-ethyl-1,3-hexanediol, and 2-phenylphenol) in at least two independent RSMN assays (Fig. 4). MMC was used as a positive control for micronucleus induction in

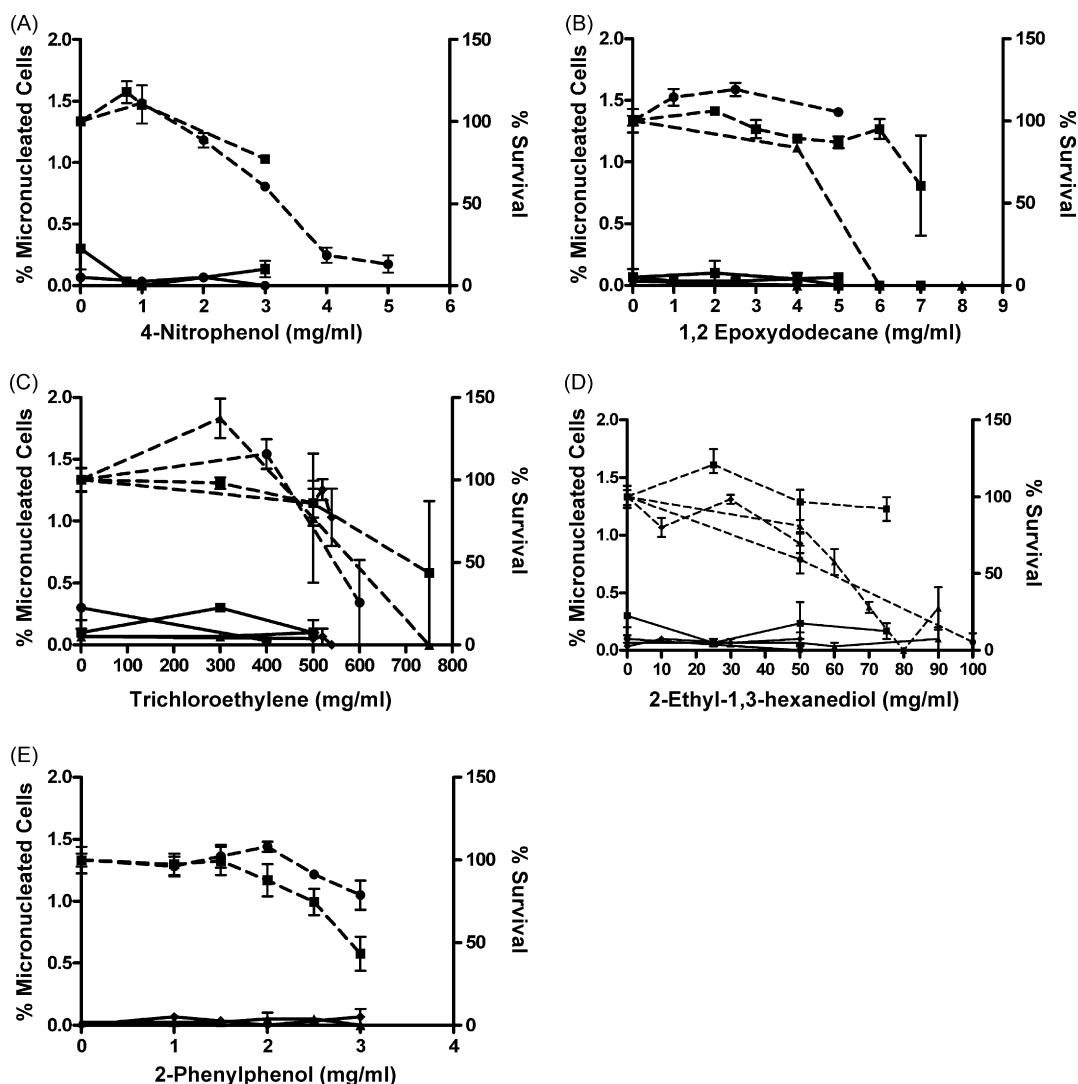


Fig. 4. Cytotoxicity and micronucleus induction in EpiDerm following treatment with rodent dermal non-carcinogens. Solid lines indicate % micronuclei (left axis), dashed lines indicate % binucleation relative to untreated tissues (right axis). At least two independent experiments are shown on each graph. Error bars indicate standard deviation of three tissues per experiment. (A) 4-nitrophenol; (B) 1,2 epoxydodecane; (C) trichloroethylene; (D) 2-ethyl-1,3 hexanediol; (E) 2-phenylphenol.

all experiments. None of these compounds produced a significant increase in micronuclei with treatments causing at least 50% survival. Therefore, the RSMN assay correctly identified seven genotoxic dermal carcinogens as positive and five non-genotoxic, non-dermal carcinogens as negative, and shows promise as a sensitive and specific *in vitro* genotoxicity assay for dermally exposed chemicals.

4. Discussion

Traditionally, positive results in *in vitro* genotoxicity assays have been followed up with attempts to confirm the potential genotoxicity in a second tier animal test. For example, a positive *in vitro* micronucleus or chromosome aberration assay would be followed by an *in vivo* test for micronuclei or chromosomal aberrations in mouse bone marrow. There are several rationale that justify such retesting, e.g. the concern that the high doses of test material used during the *in vitro* test might have caused artifactual induction mutations through osmotic effects, or that the high xenobiotic metabolizing capacity contributed by the addition of Aroclor-induced rat hepatic enzymes might overpredict the activity of the normal phase I and phase II metabolic pathways present in the intact animal. With such a strategy, chemicals that appear to

be genotoxic after first tier *in vitro* testing may be found to be safe for use after the subsequent animal assessment [3].

In the future, however, the ability of manufacturers, especially those in the cosmetics industry, to further assess a new product or ingredient in an animal model will be severely constrained by requirements of the 7th Amendment to the Cosmetics Directive. This Amendment prohibits the use of whole animal genotoxicity tests to ascertain the safety of cosmetics ingredients after 2009. This restriction could significantly hamper the ability of cosmetics companies to develop new products since it has been shown that the current battery of *in vitro* genotoxicity tests leads to an extremely high number of false positive results [6]. In an attempt to find a solution to this problem, we developed an *in vitro* MN assay using engineered human skin (RSMN) and described preliminary results with two known genotoxic agents in our previous paper [10].

Before any new *in vitro* test can gain general acceptance and eventual regulatory approval, it must undergo a validation process which has been described by several different international bodies (OECD, ECVAM, ICCVAM) [16–18]. One important requirement that we begin to address in this paper is the development of a standardized protocol. We address that requirement in this manuscript by: 1) providing detailed procedures for preparing cytoB and the positive control MMC to attain greater reproducibility, 2) describ-

ing a refined process for isolating the basal and supra basal cell populations that will be scored for micronuclei, and 3) detailing the procedures for fixing and staining the epidermal cells. Recommendations for extending the types of measurements in the cytokinesis-block micronucleus assay to more fully assess DNA damage and toxicity have recently been proposed by Fenech [15]. Future studies with the RSMN assay will explore these endpoints.

We have also begun to investigate the applicability domain and predictive capacity of the assay. It is imperative when characterizing a new assay – whether an *in vitro* or an *in vivo* assay – to carefully define the set of test materials against which the performance of the assay is being judged. Often a limiting factor in this process is simply the amount and type of reference data – in this case animal data – that are available. Although ideally we would like to judge the assay based on its ability to predict known human carcinogens, the set of such materials is small. Consequently we have decided to first investigate those materials with well-established animal data for genotoxicity and carcinogenicity via the route of exposure that we are trying to model with the RSMN protocol. Since we are primarily interested in developing a test with better specificity for non-genotoxins and non-carcinogens than the current set of *in vitro* genotoxicity assays, we have chosen chemicals which are rodent skin non-carcinogens via dermal exposure and are also rodent non-genotoxins after dermal application. Results for five such materials (2-phenylphenol, 4-nitrophenol, 1,2-epoxydodecane, trichloroethylene, and 2-ethyl-1,3-hexanediol) are reported in this manuscript, all of them negative in the RSMN assay. Importantly, all of these chemicals were positive in one or more *in vitro* genotoxicity assays with the exception of EDD which has only been evaluated *in vitro* in the Ames assay and was negative. Thus, for this set of chemicals, the RSMN in the EpiDerm™ model has greater specificity than the standard *in vitro* genotoxicity assays. We have also expanded our database using seven chemicals known to be positive in dermal carcinogenicity and genotoxicity assays. These chemicals (MNNG, ENNG, MMS, BBL, DCC, MNU and ENU) have given positive results in the RSMN assay.

It is of interest to compare results presented in this manuscript with results from an *in vivo* assay developed by Nishikawa, et al. [12,19] which detects MN after direct application of test materials to rodent skin. MN induction by MNNG is observed in both systems at around the same range (~1 mg/ml), but MN induction by MMS is already detectable at concentrations approximately 1 log lower in the RSMN than in the rodent assay. The RSMN is even more sensitive in detecting ENNG and MMC. MN induction with ENNG is found at concentrations approximately 1 1/2 logs lower in the RSMN, and MMC activity is found at concentrations approximately 2 logs lower in the RSMN than in the *in vivo* rodent assay. Whether these differences can be attributed solely to the different species involved (human versus rat or mouse) will be explored in future studies.

Although our current report deals with only direct acting carcinogens/genotoxins, we recognize that the acceptance of this novel MN assay will also depend on demonstrating its reliability in predicting potential genotoxicity of chemicals that require metabolic activation. Recently, our laboratories have shown a positive response in the RSMN using cyclophosphamide, a chemical known to require metabolic activation (data not shown). However, this positive result appears to require a 72 h exposure rather than the 48 h exposure we have described for direct acting genotoxins. Experiments to further evaluate this new 72-h procedure with additional chemicals requiring metabolic activation, along with investigations of the overall xenobiotic metabolizing capacity of EpiDerm, are currently underway.

In conclusion, we have further refined an *in vitro* reconstructed skin micronucleus assay (RSMN assay) that has significant advantages over the currently available *in vitro* genotoxicity tests. Our initial results indicate that it is sensitive and specific for dermal

carcinogens, and may possess a higher predictive value for dermal exposures than most *in vitro* mammalian cell assays. The three-dimensional structure and the use of normal human cells make the reconstructed skin model particularly relevant for testing cosmetics, and we believe it will be a valuable replacement for animal models to address safety concerns in light of the upcoming animal testing ban in Europe.

Conflict of interest statement

Three co-authors (Yulia Kaluzhny, Mitchell Klausner and Viktor Karetsky) of this manuscript are employed by MatTek Corporation, the manufacturer of the reconstructed human tissue model used in the experiments.

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