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The 3D reconstructed skin micronucleus assay: considerations for optimal protocol design

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Abstract

Implementation of the seventh amendment to the EU Cosmetics Directive has driven much research into suitable *in vitro* alternative assays to support satisfactory risk assessments. One such assay is the reconstructed skin micronucleus (RSMN) assay. First reported in 2006, further development occurred and a standard protocol was published in 2011. To evaluate and optimise the assay at Covance Laboratories, we tested nine chemicals [4-nitrophenol (4-NP), cyclohexanone (CH), 2-ethyl-1,3-hexanediol (2-EHD), methyl methanesulfonate (MMS), mitomycin C (MMC), ethyl nitrosourea (ENU), benzo[a]pyrene (BaP), cyclophosphamide (CPA) and vinblastine (VIN)] using the EpiDerm™ 3D skin model (MatTek Corporation®, IVLSL, Bratislava, Slovakia) and compared the data using the standard 48-h treatment regimen and also an emerging 72-h treatment protocol. The EpiDerm™ tissue has reportedly some metabolic capacity but data using 48-h treatments has provided mixed results. Our investigations demonstrate that the two chemicals requiring metabolic activation (BaP and CPA) were negative following the 48-h protocol but were clearly positive following 72-h treatment. Furthermore, Replication Index (RI) data showed higher RI values in vehicle control treatments (indicating increased cell division) across the treatment set following 72-h treatments. A general greater magnitude of micronucleus (MN) induction was also observed following test chemical treatment. These data suggest that the 72-h treatment protocol is more suitable as a standard approach for the detection of clastogenic, aneugenic and metabolically activated chemicals in the RSMN assay. For further assay optimisation, we compare the statistical power of scoring cells from duplicate or triplicate cultures per treatment concentration and provide recommendations.

Introduction

Skin is one of the four key routes of entry to the human body for xenobiotics. With an average surface area of ~1.5–2.0 m² and complex multicellular composition, our skin is constantly exposed to many environmental, as well as purposely applied, chemicals (including pharmaceuticals and cosmetics). Protection comes from the continual renewal of this biological barrier as generated by the basal keratinocytes; therefore, assessment of damage to these cells by any purposely applied chemical is an important consideration for human safety.

Hazard identification of genotoxic damage is a requirement in the regulation of xenobiotics. Guidance and strategies are provided by various organisations depending on industry sector

[the Organisation for Economic Co-operation and Development (OECD), the International Conference on Harmonisation (ICH), the Scientific Committee on Consumer Safety (SCCS), Environmental Protection Agency (EPA), European Food Standards Agency (EFSA), European Chemicals Agency (ECHA), etc.] and the strategies contained involve tiers of testing moving from *in vitro* batteries through to *in vivo* assays where allowed.

The *in vitro* micronucleus test is one of two *in vitro* tests commonly used for assessing genotoxic hazard [1] with second-tier testing, for most industries, being *in vivo* testing. However, for the cosmetics industry, the seventh amendment to EU Directive 2003/15/EC came into force in 2004 and phased in bans on testing or marketing of products containing ingredients tested on animals within the European Union. Around the same time, it was reported

that the standard genetic toxicology test battery potentially gave misleading positives [2], followed by work showing the importance of relevant cell type in the *in vitro* micronucleus assay [3, 4]. The importance of reliable, relevant follow-up *in vitro* assays for industries restricted from *in vivo* second-tier testing, coupled with the continuing effort to reduce animal use, was a high priority.

The Cosmetics Directive, in conjunction with the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) Regulation (EC no. 1907/2006) in Europe were key catalysts for driving research into non-animal alternative assays (including *in vitro* and *in silico* amongst others), which was heavily supported by the European Commission's Framework funding programmes. Additional national and international bodies also funded research programmes [e.g. National Centre for the 3Rs (NC3Rs), the European Cosmetic and Perfumery Association (COLIPA—now Cosmetics Europe), and German Federal Ministry for Research and Technology] that facilitated specific end points such as micronucleus and comet in skin *in vitro* to be developed [5, 6].

Development of genotoxicity end points in 3D human skin was identified as an important focus as early as the 1990s [7] and the advent of MatTek's *in vitro* tissues derived from human donor basal keratinocytes in 1993 opened the door to this possibility. Subsequently, in 2006, Curren *et al.* [8] published on the development of the micronucleus assay using the *in vitro* 3D reconstructed skin tissue model EpiDerm™ from MatTek Corporation® (IVLSL, Bratislava, Slovakia). Primarily intended for evaluation of the genotoxicity of dermally applied chemicals in direct response to the Cosmetics Directive and REACH, the 3D reconstructed skin micronucleus (3D RSMN) assay was further developed [9, 10] and an initial small-scale ring trial was reported [11]. Seen as a promising emerging technology at that time, the International Life Sciences Institute Health and Environmental Sciences Institute (ILSI-HESI) *In Vitro* Genotoxicity (IVGT) Working Group for Emerging Technologies supported the recommendation of evaluating genotoxicology end points in 3D skin tissue models further [12]. A standard 3D RSMN protocol for 48-h exposure was subsequently published in 2011 [5]. Around the same time, a 72-h exposure period for the detection of chemicals requiring metabolic activation was first proposed by Kaluzhny *et al.* [13] at the 47th Eurotox Congress in 2011 and Aardema *et al.* published data on four chemicals supporting that initial proposal 2 years later [14].

Our objectives were to validate the RSMN assay in this laboratory and following on from that initial phase to:

- (i) further investigate the ability of the assay to detect genotoxicants requiring metabolic activation;
- (ii) compare data from both 48- and 72-h treatment protocols and
- (iii) assess the statistical power of the assay from scoring of either two or three replicate tissues per concentration.

These investigations were designed to provide recommendations for the optimal running of the RSMN assay through testing a number of chemicals with differing modes of action (direct-acting clastogens, aneugens, metabolically activated clastogens and non-genotoxins).

Materials and Methods

Cell culture

EpiDerm™ 3D skin tissues were supplied by MatTek Corporation®, IVLSL, Bratislava, Slovakia (part number EPI-200-MNA), using Keratinocyte strains 4F1188 or 00267. Tissues were shipped on

Mondays and arrived in the laboratory on the following day. The kits contained 3D skin tissues on agar plugs and New Maintenance Medium formulated for the micronucleus assay.

Chemicals and reagents

Please refer Table 1 for details of the chemicals tested in this study.

Selection of concentrations for testing

The concentrations tested in the 48- and the 72-h regimes are given in Tables 1–5 and were based on previously published data [8, 9, 10, 11, 14]. Duplicate tissues exposed to mitomycin C (MMC) were used as a concurrent positive control in the 48-h treatment at 3.0, 4.0 and 6.0 µg/ml (data not shown). MMC and concentrations of vinblastine (VIN) that were positive in the 48-h regimen were included as positive controls for the 72-h regime. The concentrations of cyclophosphamide (CPA) tested with the 72-h regimen were amended from those used with the 48-h regimen following review of the 48-h data and consultation with other laboratories.

Micronucleus test in the EpiDerm™ 3D tissue model

Triplicate tissues per treatment concentration (duplicates for concurrent positive control) were used. The 48h exposures were performed according to Dahl *et al.* [5]. The 72h exposures were performed according to Aardema *et al.* [14] and Dahl *et al.* [5] (see Figure 1) with some modifications as described below. In brief, tissues were transferred to six-well plates following receipt at the laboratory and either incubated overnight (48h regimen) or for at least 1 h (72h regimen) at 37°C, 5% CO₂ prior to treatment. The test chemicals were applied to the apical surface of the tissues at 24-h intervals as illustrated in Figure 1. Basal medium containing 3 µg/ml cytochalasin B (Cyto B) was refreshed immediately prior to each treatment interval. Cells were harvested from tissues 24 h after the final treatment according to Dahl *et al.* with the following modifications: 15-min trypsinisation; introduction of gentle mechanical agitation to remove cells from tissues; trypsin neutralised with Dulbecco's Minimal Essential Medium (DMEM) medium (1:1); in-house optimised fixative (7:1, methanol:acetic acid) and in-house 75-mM Potassium chloride (KCl) used in preference; increased number of fixative changes during slide preparation.

Cytotoxicity was calculated by scoring 500 cells per replicate tissue for replication index (RI). RI was calculated according to the following formula:

$$RI = \frac{\text{number binucleate cells} + (2 \times \text{number multinucleate cells})}{\text{total number of cells in treated cultures}}$$

Relative RI (expressed in terms of percentage) for each treated tissue was calculated as follows:

$$\text{Relative RI (\%)} = \frac{\text{RI of treated cultures}}{\text{RI of vehicle controls}} \times 100$$

Cytotoxicity (%) was expressed as (100 – Relative RI).

Micronucleus analysis

All slides for MN analysis were coded and scored using fluorescence microscopy under blind-scoring conditions. Prior to analysis, several drops of phosphate-buffered saline were added to the acridine orange-stained slides and the slides coverslipped. Cells were scored according to the published atlas [5]. After scoring, slides were decoded and data processed.

Table 1. Details of chemicals tested

Mode of action	Chemical	CAS number	Assay solvent*	Treatment conditions	
				48 h	72 h
Non-genotoxins	4-nitrophenol	100-02-7	Acetone	Yes	No
	Cyclohexanone	108-94-1	Acetone	Yes	No
	2-ethyl-1,3-hexanediol	94-96-2	Acetone	Yes	No
Direct-acting clastogens	Mitomycin C	50-07-7	Acetone	Yes	Yes**
	Methyl methanesulfonate	66-27-3	Acetone	Yes	No
	<i>n</i> -ethylnitrosourea	759-73-9	Acetone	Yes	Yes
	4-nitroquinoline-N-oxide	56-57-5	Acetone	No	Yes
Aneugen	Vinblastine	143-67-9	Acetone	Yes	Yes**
Metabolically activated clastogens	Cyclophosphamide	6055-19-2	Acetone	Yes	Yes
	Benzo[a]pyrene	50-32-8	Acetone	Yes	Yes

All compounds sourced from Sigma–Aldrich Chemical Company, Poole, UK. CAS, Chemical Abstracts Service.

*Chemicals formulated in suitable primary vehicles (DMSO, H₂O, etc.) and, then, subsequently diluted using stated solvent in column ensuring primary vehicle present at 1% maximum.

**Used as positive control concentrations (not tested as a full concentration range in the 72-h regime).

For the 48-h treatments, 1000 binucleate (BN) cells were initially scored from each of two tissues per concentration tested with a third tissue scored to aid the resolution of the data where necessary. For the 72-h treatments, 1000 BN cells were scored from each of three tissues per concentration and assessments conducted to the sensitivity of the statistical analysis comparing scoring of 1000 binucleated cells for MN from either two or three tissues per concentration.

For the 48-h treatments, all tested concentrations were scored. For the 72-h treatments, the highest concentration of the non-metabolically activated chemicals [*n*-ethylnitrosourea (ENU) and 4-nitroquinoline-N-oxide (4NQO)] inducing 50–60% cytotoxicity was selected for micronucleus analysis with two lower concentrations also analysed to cover a range of cytotoxicity from maximum to little or none. For chemicals requiring metabolic activation (CPA and B[a]P) multiple concentrations were analysed and covered the toxicity curve (where applicable).

The proportion of micronucleated BN (MNBN) cells at each treatment concentration was compared with the concurrent vehicle (negative) control using the Fisher's Exact Test (one-sided analysis) with probability values of $P \leq 0.05$ accepted as significant [15]. A Cochran–Armitage trend test (one sided) was applied to each treatment to assess concentration-related effects [16]. Probability values of $P \leq 0.05$ were accepted as significant. Data were also compared to the published historical control range (HCR) for the assay [5] as confirmation of a positive result.

Results

48-h treatments

Initial investigations were conducted to demonstrate the proficiency of this laboratory to perform the 3D RSMN assay on EpiDerm™ tissues using the published 48-h treatment protocol [5] by reproducing a selection of the available data [8, 9, 10, 11, 14]. The results are presented in Tables 2–4 and show the replicate data per concentration for the nine chemicals tested.

From Table 2 (48-h treatments, non-genotoxicants), no cytotoxicity was observed following exposure of tissues to cyclohexanone (CH), 2-ethyl-1,3-hexanediol (2-EHD) or 4-nitrophenol (4-NP). No

statistically significant ($P \leq 0.05$) increases in MNBN cells were observed following treatment with 2-EHD or 4-NP. A weak but statistically significant ($P \leq 0.05$) increase in MNBN cells was observed for a single concentration of CH (80.00 mg/ml). Weak but statistically significant trend tests ($P \leq 0.05$) were observed for both 2-EHD and CH; however, the MNBN cell value of all CH- and 2-EHD-treated cultures (all six concentrations analysed) fell within the published HCR of 0–0.5% MNBN cells [5]. As such, the weak statistically significant linear trend data were not considered of biological importance. Overall, CH, 2-EHD and 4-NP were concluded negative.

For the direct-acting clastogens (Table 3), concentrations of both MMS and ENU were analysed, which reached the recommended regulatory maximum cytotoxicity of $55 \pm 5\%$ [17]. For MMC, no concentration tested (up to 0.008 mg/ml) achieved cytotoxicity >31%. For the aneugenic chemical (VIN), the toxicity curve was extremely steep with 0.0006 and 0.0008 mg/ml inducing 17% and 70% cytotoxicity, respectively (Table 3). All four chemicals resulted in statistically significant increases in MNBN cells for multiple concentrations analysed with statistically significant linear trend ($P \leq 0.05$). However, in general, the magnitude of MNBN induction was weak with the majority of concentrations analysed following both MMC and MMS treatments exhibiting mean MNBN cell values that were either within or only marginally above the published HCR) of 0–0.5% MNBN cells [5]. For ENU, none of the six concentrations analysed yielded mean frequencies of MNBN cells that exceeded the published HCR. Overall, MMC and VIN were considered as clearly positive. For ENU and MMS, statistically significant increases in MNBN cells were observed, but these were considered to be of questionable biological relevance (observed mean MNBN cell values were either within or only marginally above the published HCR).

Two chemicals known to require metabolic activation were also tested: B[a]P and CPA (Table 4). Both chemicals induced cytotoxicity across the range of concentrations used with the highest concentrations analysed, inducing ~50% (B[a]P) or ~60% cytotoxicity (CPA). However, the cytotoxicity observed was not a linear, concentration-related trend but plateaued across multiple concentrations with the observation that RI values for the concurrent vehicle controls were on the low side (mean RI of 0.60 and 0.58 for B[a]P and CPA

Table 2. 48-h treatments: non-genotoxicants

Chemical	Treatment (mg/ml)	RI	Cytotoxicity (%)			Total BN cells			BN cells with micronuclei			Mean frequency MNBN (%)	
			Mean			A			A			Mean (SD)	
			A	B	C	A	B	C	A	B	C	A	C
CH	Solvent	0.51	0.50	N/A	N/A	1000	1000	N/A	0	2	N/A	1.00 (1.41)	0.10
	UTC	0.57	0.55	N/A	N/A	860	763	N/A	3	2	N/A	2.50 (0.71)	0.30
	10.00	0.54	0.46	N/A	N/A	1000	1000	N/A	1	1	N/A	1.00 (0.00)	0.10
	20.00	0.41	0.46	N/A	N/A	1000	831	N/A	2	2	N/A	2.00 (0.00)	0.20
	40.00	0.58	0.53	N/A	N/A	1000	1000	N/A	2	1	N/A	1.50 (0.71)	0.20
	60.00	0.61	0.62	N/A	N/A	1000	693	N/A	3	1	N/A	2.00 (1.41)	0.20
	80.00	0.38	0.47	N/A	N/A	1000	1000	N/A	7	1	N/A	4.00 (4.24)	0.40*
	100.0	0.52	0.53	N/A	N/A	1000	1000	N/A	2	2	N/A	2.00 (0.00)	0.20
						Cochran–Armitage (one-sided) Trend Test ($P \leq 0.05$)						#	
2-EHD	Solvent	0.64	0.58	N/A	N/A	1000	1000	N/A	0	1	N/A	0.50 (0.71)	0.10
	UTC	0.53	0.58	N/A	N/A	1000	1000	N/A	0	0	N/A	0.00 (0.00)	0.00
	20.00	0.65	0.63	N/A	N/A	1000	1000	N/A	1	0	N/A	0.50 (0.71)	0.10
	40.00	0.73	0.74	N/A	N/A	1000	1000	N/A	0	1	N/A	0.50 (0.71)	0.10
	50.00	0.72	0.75	N/A	N/A	1000	1000	N/A	0	2	N/A	1.00 (1.41)	0.10
	60.00	0.58	0.71	N/A	N/A	1000	1000	N/A	1	2	N/A	1.50 (0.71)	0.20
	70.00	0.69	0.68	N/A	N/A	1000	1000	N/A	1	2	N/A	1.50 (0.71)	0.20
	80.00	0.53	0.65	N/A	N/A	1000	1000	N/A	2	3	N/A	2.50 (0.71)	0.30
						Cochran–Armitage (one-sided) Trend Test ($P \leq 0.05$)						#	
4-NP	Solvent	0.52	0.53	0.53	0.53	1000	1000	1000	1	2	1	1.33 (0.58)	0.13
	UTC	0.55	0.44	0.52	0.50	1000	1000	1000	0	1	1	0.67 (0.58)	0.06
	0.0005	0.58	0.55	0.44	0.52	1000	1000	1000	0	2	0	0.67 (1.16)	0.06
	0.001	0.56	0.55	0.57	0.56	1000	1000	1000	0	1	1	0.67 (0.58)	0.06
	0.002	0.53	0.50	0.53	0.52	1000	1000	1000	1	0	1	0.67 (0.58)	0.06
	0.003	0.52	0.60	0.44	0.52	1000	1000	1000	3	3	2	2.67 (0.58)	0.26
	0.004	0.54	0.63	0.57	0.58	1000	1000	1000	2	2	3	2.33 (0.58)	0.23
	0.005	0.49	0.57	0.60	0.55	1000	1000	1000	2	0	1	1.00 (1.00)	0.10
						Cochran–Armitage (one-sided) Trend Test ($P \leq 0.05$)						NS	

NS, not significant; –, not scored; N/A, not applicable.
*Statistically significant ($P \leq 0.05$ Cochran–Armitage Trend Test, one sided).
*Statistically significant ($P \leq 0.05$ Fishers Exact Test, one sided).

Table 3. 48-h treatments: directing acting clastogens/aneugens

Chemical	Treatment (mg/ml)	RI	Cytotoxicity (%)	Total BN cells			BN cells with micronuclei			Mean frequency MNBN (%)			
				A	B	C	A	B	C				
MMC	Solvent	0.59	0.73	0.59	0.64	1000	1000	N/A	0	1	N/A	0.50 (0.71)	0.05
	UTC	0.56	0.62	–	0.59	1000	1000	1000	0	5	0	1.67 (2.89)	0.17
	0.001	0.61	0.53	N/A	0.57	1000	1000	N/A	0	0	N/A	0.00 (0.00)	0.00
	0.003	0.59	0.59	0.47	0.55	1000	1000	1000	1	2	1	1.33 (0.58)	0.13
	0.004	0.62	0.68	N/A	0.65	1000	1000	N/A	2	6	N/A	4.00 (2.83)	0.40*
	0.006	0.45	0.57	0.48	0.50	1000	1000	792	4	5	7	5.33 (1.53)	0.57*
	0.008	0.39	0.49	N/A	0.44	1000	1000	N/A	5	4	N/A	4.50 (0.71)	0.45*
	0.010	0.54	0.39	N/A	0.46	1000	1000	N/A	6	26	N/A	16.0 (14.1)	1.60*
	Cochran–Armitage (one-sided) Trend Test ($P \leq 0.05$)												
	#												
ENU	Solvent	0.64	0.60	N/A	0.62	1000	1000	N/A	2	0	N/A	1.00 (1.41)	0.10
	UTC	0.58	0.54	N/A	0.56	1000	1000	N/A	1	0	N/A	0.50 (0.71)	0.05
	1.000	0.34	0.42	N/A	0.38	1000	1000	N/A	2	3	N/A	2.50 (0.71)	0.25
	2.000	0.32	0.28	N/A	0.30	1000	1000	N/A	1	7	N/A	4.00 (4.24)	0.40*
	3.000	0.22	0.24	N/A	0.23	1000	1000	N/A	3	5	N/A	4.00 (1.41)	0.40*
	4.000	0.13	0.20	N/A	0.16	1000	1000	N/A	6	3	N/A	4.50 (2.12)	0.45*
	5.000	0.16	0.18	N/A	0.17	1000	1000	N/A	2	2	N/A	2.00 (0.00)	0.20
	7.500	0.23	0.10	N/A	0.17	1000	644	N/A	5	5	N/A	5.00 (0.00)	0.61*
	Cochran–Armitage (one-sided) Trend Test ($P \leq 0.05$)												
	#												
MMS	Solvent	0.48	0.54	0.55	0.52	1000	1000	1000	0	0	0	0.00 (0.00)	0.00
	UTC	0.46	0.55	0.49	0.50	1000	1000	1000	1	1	1	1.00 (0.00)	0.10
	0.300	0.53	0.50	0.44	0.49	1000	1000	1000	0	1	3	1.33 (1.53)	0.13*
	0.600	0.33	0.33	0.33	0.33	1000	1000	1000	3	3	1	2.33 (1.15)	0.23*
	0.800	0.10	0.32	0.33	0.25	623	1000	1000	3	2	3	2.67 (0.58)	0.31*
	0.900	0.31	0.22	0.25	0.26	1000	876	1000	1	3	5	3.00 (2.00)	0.31*
	1.000	0.22	0.27	0.26	0.25	826	1000	1000	4	5	6	5.00 (1.00)	0.53*
	1.200	0.12	0.30	0.33	0.25	825	1000	1000	3	3	5	3.67 (1.15)	0.39*
	Cochran–Armitage (one-sided) Trend Test ($P \leq 0.05$)												
	#												
VIN	Solvent	0.44	0.52	0.52	0.49	1000	1000	1000	1	1	3	1.67 (1.15)	0.17
	UTC	0.62	0.50	0.53	0.55	1000	1000	1000	0	2	1	1.00 (1.00)	0.10
	0.0001	0.45	0.49	0.50	0.48	1000	1000	1000	1	1	1	1.00 (0.00)	0.10
	0.0002	0.54	0.45	0.40	0.46	1000	1000	1000	6	4	3	4.33 (1.53)	0.43*
	0.0004	0.48	0.48	0.43	0.46	1000	1000	1000	8	1	7	5.33 (3.79)	0.53*
	0.0006	0.38	0.43	0.41	0.41	1000	1000	1000	10	14	11	11.6 (2.08)	1.17*
	0.0008	0.14	0.12	0.18	0.15	864	1000	1000	43	43	19	35.0 (13.9)	3.67*
	0.0010	0.20	0.11	0.12	0.14	1000	1000	1000	18	34	22	24.7 (8.33)	2.47*
	Cochran–Armitage (one-sided) Trend Test ($P \leq 0.05$)												
	#												

–, not scored; N/A, not applicable.
#Statistically significant ($P \leq 0.05$ Cochran–Armitage Trend Test, one sided).
*Statistically significant ($P \leq 0.05$, Fishers Exact Test, one sided).

Table 4. 48-h treatments: chemicals requiring metabolic activation

Chemical	Treatment (mg/ml)	RI			Cytotoxicity (%)	Total BN cells			BN cells with micronuclei			Mean frequency MNBN (%)
		A	B	C		A	B	C	A	B	C	
B[a]P	Solvent	0.59	0.60	N/A	0.60	1000	1000	N/A	0	1	N/A	0.05
	UTC	0.63	0.62	N/A	0.63	1000	1000	N/A	1	1	N/A	0.10
	0.125	0.36	0.43	N/A	0.40	1000	1000	N/A	1	0	N/A	0.05
	0.250	0.52	0.48	N/A	0.50	1000	1000	N/A	1	1	N/A	0.10
	0.500	0.33	0.30	N/A	0.32	1000	1000	N/A	1	0	N/A	0.05
	0.750	0.32	0.36	N/A	0.34	1000	1000	N/A	0	2	N/A	0.10
	1.000	0.47	0.33	N/A	0.40	1000	1000	N/A	0	3	N/A	0.15
	2.500	0.32	0.31	N/A	0.32	1000	1000	N/A	1	0	N/A	0.05
Cochran–Armitage (one-sided) Trend Test ($P \leq 0.05$)												
CPA	Solvent	0.50	0.57	0.67	0.58	1000	1000	1000	0	0	0	0.00
	UTC	0.43	0.44	0.51	0.46	1000	1000	1000	0	0	0	0.00
	0.040	0.43	0.35	0.42	0.40	1000	1000	1000	0	1	0	0.03
	0.050	0.27	0.34	0.26	0.29	1000	1000	1000	1	1	0	0.07
	0.060	0.40	0.33	0.27	0.33	1000	1000	1000	1	1	2	0.13*
	0.070	0.35	0.41	0.48	0.41	1000	1000	1000	0	1	1	0.07
	0.080	0.27	0.16	0.21	0.22	1000	1000	1000	0	2	1	0.10
	0.090	0.27	0.15	0.26	0.23	1000	1000	1000	1	0	0	0.03
Cochran–Armitage (one-sided) Trend Test ($P \leq 0.05$)												
NS												

NS, not significant; –, not scored; N/A, not applicable.
*Statistically significant ($P \leq 0.05$, Fishers Exact Test, one sided).

Table 5. 72-h treatments

Chemical	Treatment (mg/ml)	RI	Cytotoxicity (%)			Total BN cells			BN cells with micronuclei				Frequency MNBN (%)									
			A	B	Mean	A	B	C	A	B	C	Mean (SD)										
CPA	Solvent	0.65	0.76	0.69	0.72	0.70	1000	1000	1000	6	6	7	4	4.25 (2.36)	0.58							
	UTC	0.66		0.74	N/A	0.70	—	—	—	—	—	—	—	—	—							
	10.00	0.74		0.70	0.68	0.71	—	—	—	—	—	—	—	—	—							
	20.00	0.78		0.71	0.70	0.73	0	—	—	—	—	—	—	—	—							
	40.00	0.65		0.58	0.62	0.62	12	—	—	—	—	—	—	—	—							
	50.00	0.64		0.55	0.54	0.58	18	—	—	—	—	—	—	—	—							
	60.00	0.60		0.59	0.64	0.61	13	1000	1000	26	12	14	17.33 (7.57)	—	1.73*							
	70.00	0.47		0.49	0.47	0.48	32	1000	1000	1000	12	16	12	13.33 (2.31)	1.33*							
	80.00	0.39		0.42	0.31	0.38	47	1000	1000	1000	52	16	24	30.67 (18.90)	3.07*							
	100.0	0.17		0.34	0.27	0.26	63	1000	1000	1000	23	13	10	15.33 (6.81)	1.53*							
Cochran–Armitage (one-sided) Trend Test ($P \leq 0.05$)															#							
B[a]P	Solvent	**	**	**	**	0.70	**	**	**	**	**	**	**	**	**							
	UTC	**	**	**	N/A	0.70	—	—	—	—	—	—	—	—	—							
	0.200	0.49		0.49	0.48	0.49	31	—	—	—	—	—	—	—	—							
	0.350	0.50		0.51	0.56	0.52	26	1000	1000	15	32	19	22.00 (8.89)	—	2.20*							
	0.500	0.36		0.40	0.59	0.45	36	1000	1000	1000	28	14	10	17.33 (9.45)	1.73*							
	0.650	0.49		0.48	0.55	0.51	28	1000	1000	24	19	15	19.33 (4.51)	—	1.93*							
	1.000	0.61		0.49	0.58	0.56	21	1000	1000	1000	15	23	16	18.00 (4.36)	—	1.80*						
	2.000	0.56		0.48	0.39	0.48	32	1000	1000	1000	17	15	26	19.33 (5.86)	—	1.93*						
	Cochran–Armitage (one-sided) Trend Test ($P \leq 0.05$)															#						
	ENU	Solvent	**	**	**	**	0.70	**	**	**	**	**	**	**	**	**						
UTC		**	**	**	N/A	0.70	—	—	—	—	—	—	—	—	—							
0.125		0.66		0.69	0.64	0.67	6	—	—	—	—	—	—	—	—							
0.250		0.65		0.65	0.62	0.64	9	—	—	—	—	—	—	—	—							
0.500		0.55		0.57	0.66	0.60	15	1000	1000	22	36	24	27.33 (7.57)	—	2.73*							
1.000		0.52		0.44	0.38	0.45	36	1000	1000	827	13	8	28	16.33 (10.41)	—	1.73*						
2.000		0.33		0.34	0.32	0.33	53	1000	1000	1000	22	17	24	21.00 (3.61)	—	2.10*						
3.000		0.19		0.18	0.20	0.19	73	—	—	—	—	—	—	—	—							
4.000		0.22		0.21	0.15	0.19	73	—	—	—	—	—	—	—	—							
Cochran–Armitage (one-sided) Trend Test ($P \leq 0.05$)															#							
4-NQO	Solvent	0.62	0.57	0.54	0.54	0.58	—	—	—	—	—	—	—	—	0.44							
	0.020	0.48	0.65	0.52	0.52	0.55	4	—	—	—	—	—	—	—	—							
	0.045	0.40	0.60	0.75	0.58	0.58	0	—	—	—	—	—	—	—	—							
	0.070	0.46	0.58	0.54	0.53	0.53	9	1000	1000	1000	6	8	6	6.67 (1.15)	0.67							
	0.095	0.50	0.38	0.38	0.38	0.42	27	1000	1000	1000	0	4	3	2.33 (2.08)	0.23							
	0.120	0.32	0.29	0.19	0.19	0.27	54	1000	1000	1000	12	5	10	9.00 (3.61)	0.90*							
	0.145	0.16	0.09	0.20	0.20	0.15	74	—	—	—	—	—	—	—	—							
	0.170	0.15	0.12	0.14	0.14	0.14	76	—	—	—	—	—	—	—	—							
	Cochran–Armitage (one-sided) Trend Test ($P \leq 0.05$)															#						
	12000															53	—	—	—	—	—	—

Table 5. Continued

Chemical	Treatment (mg/ml)	RI		Cytotoxicity (%)	Total BN cells			BN cells with micronuclei				Frequency MNBN (%)																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																									
		A	B		C	Mean	A	B	C	A	B		C	Mean (SD)																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																							
VIN	Solvent† 0.0006																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																				

Shaded area: individual tissue values not presented, only mean values presented.

–, not scored; N/A, not applicable.

*Statistically significant ($P \leq 0.05$ Cochran–Armitage Trend Test, one sided).

**Statistically significant ($P \leq 0.05$, Fishers Exact Test, one sided).

**For the CPA, B[a]P and ENU treatments, both the vehicle control ($n = 4$) and untreated control tissues ($n = 2$) were shared.

[†]Solvent data pooled for all values presented ($n = 12$).

[‡]Used for HCR generation. For MMC, 24 tissues treated with vehicle (acetone) and 36 tissues treated with MMC at 0.004 mg/ml.

treatments, respectively). No statistically significant increases in MNBN cells were observed following treatment with B[a]P at any concentration tested. Following treatment with CPA, a weak statistically significant increase in MNBN cells was observed at 0.06 mg/ml; however, the mean MNBN frequency of all six CPA concentrations analysed fell within the published vehicle HCR (5) with no evidence of any concentration-related effect (non-significant linear trend tests). Therefore, both B[a]P and CPA were concluded negative in the RSMN assay when tested at 48 h.

Although the results of these initial 48-h treatments were consistent with the published data, there were areas of concern. With regards to the two chemicals requiring metabolic activation (CPA and B[a]P), although the data indicated a negative response and therefore led to a possible conclusion of lack of inherent metabolic capability in the RSMN tissues, it should also be noted that the concurrent vehicle control RI data showed low mean values (0.58 and 0.60, respectively). This indicates a relatively low cell division with the potential that insufficient cells exposed to the test chemical would be able to proceed through mitosis and present any resultant chromosome fragmentation or chromosome loss as MN, the observed plateau of cytotoxicity across the concentration range tested also indicating this as a possibility.

Overall, following 48-h treatments, the mean RI values of vehicle controls were observed to be generally low across the majority of treatments (between 0.49 and 0.66). This suggested that a longer treatment period may be beneficial in providing a more optimal treatment protocol. Further treatments were, therefore, conducted using the modified 72-h treatment regimen (Figure 1).

72-h treatments

For the two direct-acting chemicals tested at 72 h (ENU and 4-NQO), concentration-related increases in cytotoxicity were observed (Table 5). For ENU, large statistically significant increases in MNBN cells were observed for each of the three concentrations analysed. MNBN cell values of all three treated tissues at each of these concentrations analysed exceeded the laboratory's vehicle HCR (0.06–0.7% MNBN cells; see Figure 5). The result was, therefore, a clear positive.

For 4-NQO, a statistically significant increase was observed at a single concentration of 0.120 mg/ml (inducing 54% cytotoxicity), with two of the three tissues tested exhibiting MNBN cell values that exceeded the laboratory's vehicle HCR. A statistically significant linear trend test ($P \leq 0.05$) was also observed. This result was considered a weak positive.

For VIN and MMC, statistically significant increases in MNBN cells were observed at each of the concentrations analysed with all three tissues treated per concentration demonstrating MNBN cell values that exceeded the laboratory's vehicle HCR. VIN and MMC were concluded as positive.

For CPA and B[a]P, all concentrations analysed induced statistically significant increases in MNBN cells compared to the concurrent vehicle control with MNBN frequencies of all CPA and B[a]P treated tissues exceeding the laboratory's vehicle HCR (Figure 5). Both chemicals were concluded as positive.

Historical vehicle and positive control data ranges

Historical control ranges for 72-h treatments were generated, plotted and summarised in Figure 5. A total of 51 tissues were exposed to vehicle control (acetone or ethanol/water) across the 72-h treatments. These data provided a mean MNBN frequency of 0.37% [standard deviation (SD) of 0.18]. The observed range was 0.0–0.8% MNBN

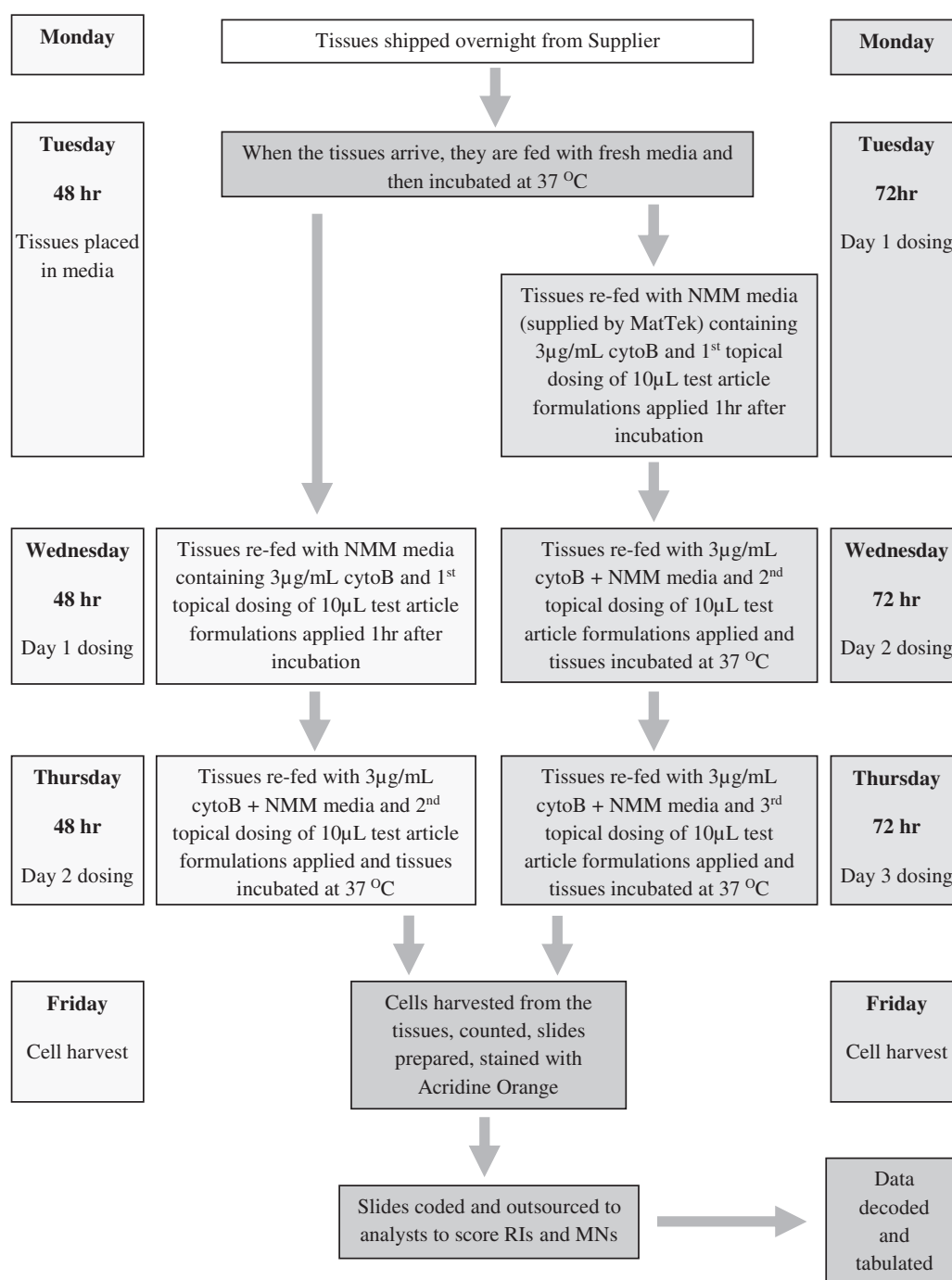


Figure 1. Schematic showing the schedule for both 48- and 72-h treatments. 48-h treatment regimen shown on left-hand side, 72-h treatment regimen shown on the right-hand side with laboratory procedures down the centre. Schematic adapted from Dahl *et al.* 2011 [5].

cells and 95% reference range was 0.06–0.70% MNBN cells. A total of 42 tissues were exposed to MMC (0.004 mg/ml) across the 72-h treatments. The mean MNBN frequency was 2.52% (SD of 0.90). The observed range was 0.6–4.7% MNBN cells and the 95% reference range was 0.7–4.4% MNBN cells.

Statistical power

The statistical power of the assay was assessed by comparison of scoring either 1000 BN cells from two tissues or 1000 BN cells from three tissues per treatment concentration (total of either 2000 or

3000 BN cells, respectively; Tables 6 and 7). Each table provides a (vehicle) control incidence and determines what MNBN cell frequency from a test chemical response would be required to be statistically significant ($P \leq 0.05$) using a one-sided chi-squared test. These data showed that, for a vehicle control incidence of 0.1%, which is the lower end of the HCR presented in Figure 5, a 4.50-fold increase in the MNBN cell value over the vehicle response would be required to be statistically significant, scoring 2000 BN cells from two tissues. However, if scoring 3000 BN cells (1000 BN from each of three tissues; Table 7), the same vehicle control incidence of 0.1% would result in a 3.33-fold increase to be detected as statistically significant.

Table 6. Statistical power calculations—1000 cells scored for two replicates per group

Control incidence [mean MNBN cell frequency (%)]	Test group (1) [mean MNBN cell frequency (%)]	Difference [test – control; mean MNBN cell frequency (%)]	Fold difference (test/control)
0/2000 (0.000)	5/2000 (0.250)	0.250	N/A
1/2000 (0.050)	7/2000 (0.350)	0.300	7.00
2/2000 (0.100)	9/2000 (0.450)	0.350	4.50
3/2000 (0.150)	10/2000 (0.500)	0.350	3.33
4/2000 (0.200)	12/2000 (0.600)	0.400	3.00
5/2000 (0.250)	13/2000 (0.650)	0.400	2.60
6/2000 (0.300)	15/2000 (0.700)	0.450	2.50

(1) Response in Test group that could be detected as statistically significant.

Assuming a control incidence of 0.100%, 1000 cells scored for two replicates would allow a difference of ~0.350% (4.50-fold increase) between groups to be detected as statistically significant at the 5% level using a one-sided chi-squared test.

Table 7. Statistical power calculations—1000 cells scored for three replicates per group

Control incidence [mean MNBN cell frequency (%)]	Test group (1) [Mean MNBN cell frequency (%)]	Difference [test – control; mean MNBN cell frequency (%)]	Fold difference (test/control)
0/3000 (0.000)	5/3000 (0.167)	0.167	N/A
1/3000 (0.033)	7/3000 (0.233)	0.200	7.00
2/3000 (0.067)	9/3000 (0.300)	0.233	4.50
3/3000 (0.100)	10/3000 (0.333)	0.233	3.33
4/3000 (0.133)	12/3000 (0.400)	0.267	3.00
5/3000 (0.167)	13/3000 (0.433)	0.267	2.60
6/3000 (0.200)	15/3000 (0.500)	0.300	2.50

(1) Response in Test group that could be detected as statistically significant.

Assuming a control incidence of 0.100%, 1000 cells scored for three replicates would allow a difference of ~0.233% (3.33-fold increase) between groups to be detected as statistically significant at the 5% level using a one-sided chi-squared test.

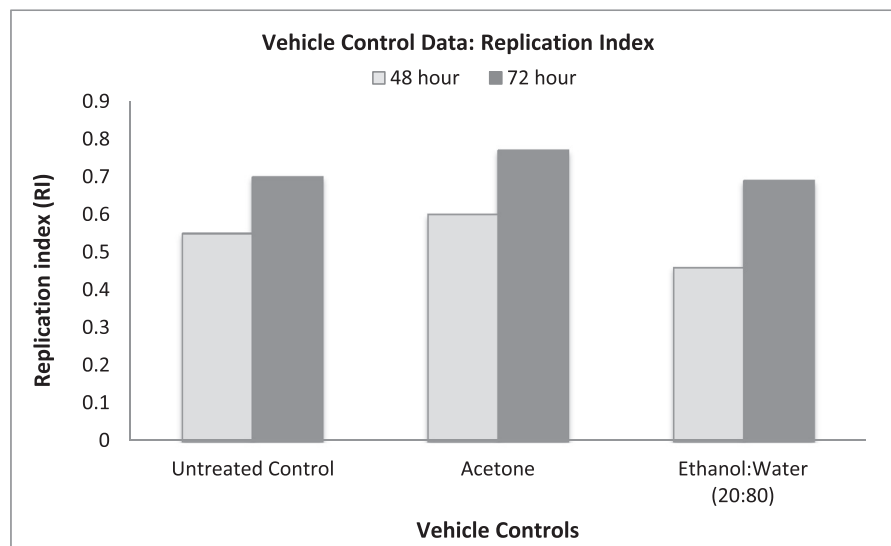


Figure 2. 48- and 72-h exposure RI data from three types of vehicle control. 48-h treatment vehicle controls (light grey) and 72-h vehicle controls (dark grey) for two common solvents used in the assay (acetone and ethanol:water [20:80]) and untreated control (unexposed tissues). RI of 0.5 or below indicates low cell division. Note that higher RI values were observed for all conditions following 72-h exposure compared to 48-h exposure.

Control data comparison

Vehicle and positive control (VIN and MMC) data from additional experiments (RI values and MN frequencies) were compared between 48- and 72-h treatments. These data are summarised in Figures 2–4.

For either untreated or vehicle (acetone or ethanol:water) treated tissues, a clear increase in mean RI was observed using the 72-h treatment, indicating that a higher proportion of cells had passed through mitosis compared to the 48-h treatment (Figure 2). The importance of suitable cell turnover for the ability of cells to express

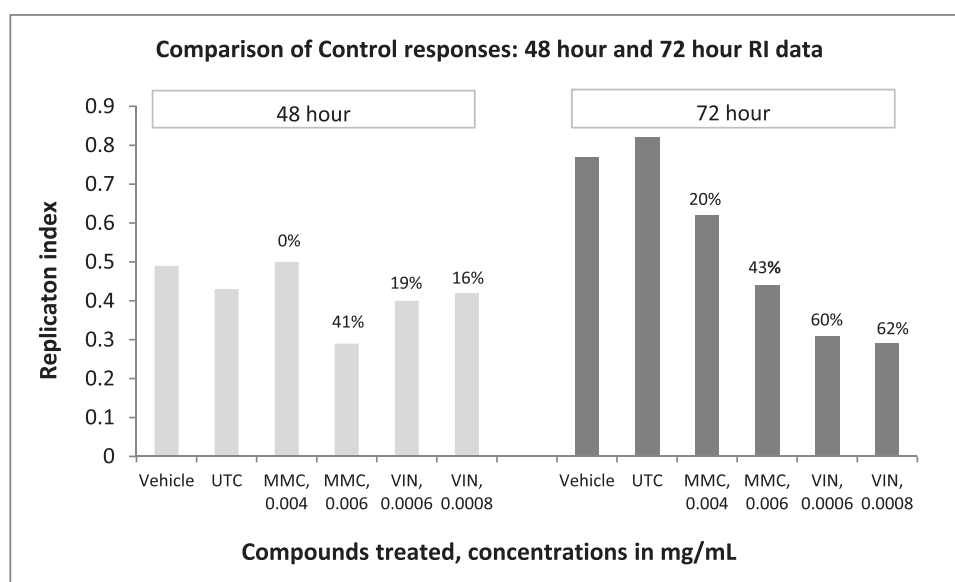


Figure 3. RIs following 48-h (light grey columns) and 72-h (dark grey columns) exposures to vehicle and positive controls (MMC/VIN). % toxicity—calculated as percentage reduction in RI from test article dose to concurrent vehicle control. Reduced RI observed for VIN at 72 h matches expected profile for an aneugenic response, whereas this was not observed after 48 h.

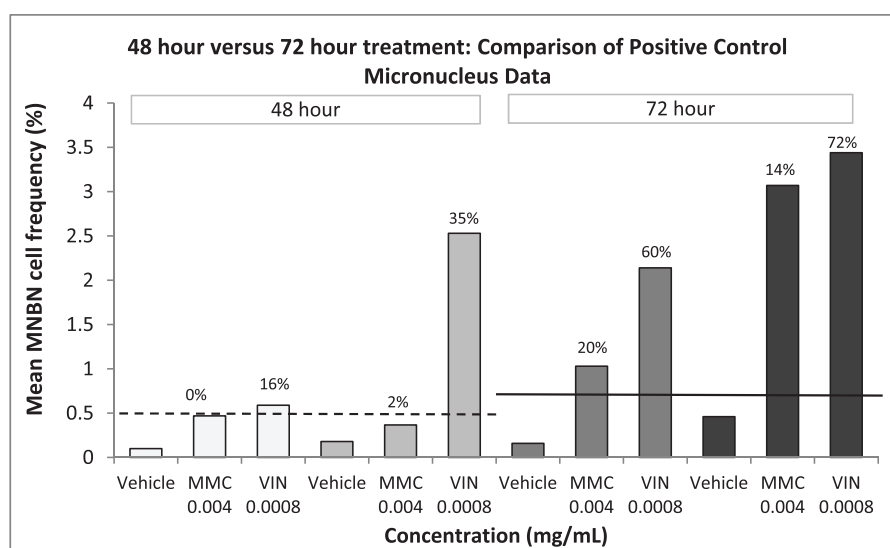


Figure 4. 48-h versus 72-h treatment: comparison of positive control micronucleus data (MMC/VIN). Dashed line—48-h vehicle MNBN range upper limit as published by Dahl *et al.* 2011; solid line—upper 95% confidence interval of Covance 72-h positive control HCR (MMC 0.004 mg/ml); both positive controls exceed the upper limit after 72 h; % toxicity—calculated as percentage reduction in RI from test chemical dose to concurrent vehicle control.

induced damage as MN can be seen from the positive control data comparisons (Figures 3 and 4), where higher MN frequencies were observed with MMC and VIN following 72-h treatment. With VIN, higher cytotoxicity was observed following 72-h treatment, but this was associated with a far higher magnitude of MNBN cell induction. These data were not unexpected as this well-known aneugenic chemical targets mitotic spindle microtubule assembly, inducing cell-cycle delay and chromosome loss.

Discussion

Validation of the assay at this laboratory by replicating the published protocol [5] and published data [8, 9, 10, 11] for testing chemicals

on EpiDerm™ 3D skin tissues using the 48-h treatment regimen was successful. However, our data gave rise to questions regarding the 48-h treatment regimen as low vehicle control and untreated control (UTC) RI values were observed indicating suboptimal cell division rate during this treatment period. This was further reflected by the range of MNBN observations across the genotoxic chemicals. For the direct-acting clastogens (MMC, ENU and MMS) a positive induction of MNBN cells was observed (coupled with statistically significant trend tests) but the magnitude of response was relatively low compared to the published HCR. This was unexpected given the nature of the genotoxic chemicals tested. For the metabolically activated chemicals (B[a]P and CPA), no biologically relevant increases in MNBN cells and negative trend tests were observed, questioning

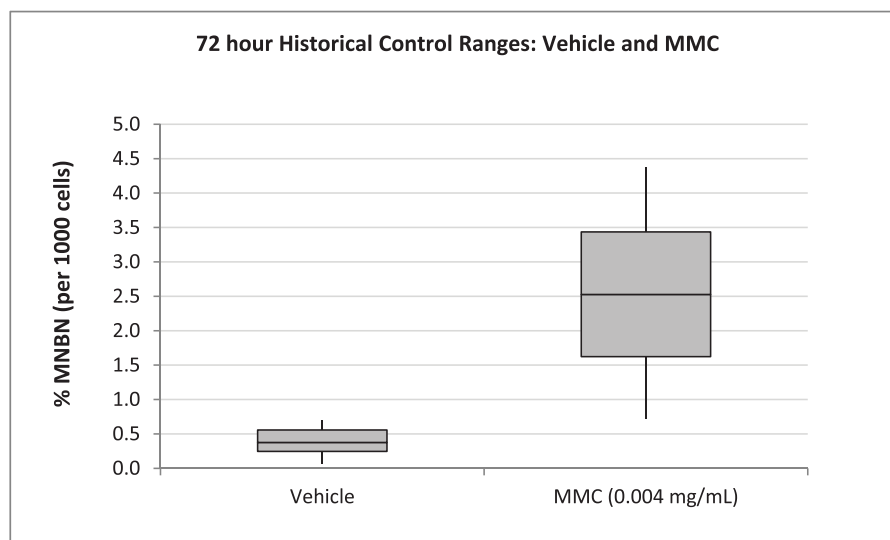


Figure 5. Box and whisker plots for vehicle control and positive control (MMC) following 72-h exposure. 95% reference range shown for pooled vehicles (acetone and ethanol:water) and MMC (whiskers) with the box showing the calculated mean and SD. The vehicle range was 0.06% to 0.70% with a mean of 0.37% and SD of 0.18. MMC's range was 0.71% to 4.37% with a mean of 2.52% and SD of 0.9.

whether this was due to a lack of inherent metabolic capability or suboptimal treatment conditions. For the aneugen (VIN), a positive response was observed but, again, the magnitude of MNBN cell induction for concentrations inducing <70% cytotoxicity was low.

Although our data from the 48-h protocol produced the same conclusions for all nine chemicals tested as presented in the literature, our cytotoxicity data suggested that the observed basal keratinocyte division rate in the tissues may have been suboptimal for the micronucleus assay (i.e. low vehicle control and UTC RI values). The initial assay development publications [8, 9, 10, 11] utilised a cut-off of at least 25% binucleated cells in the vehicle controls but, following completion of that work, use of Cytokinesis-Block Proliferation Index (CBPI) or RI were added into the published protocol [5], thus, bringing the assay in line with the then recently published OECD Test Guideline 487 (2011, re-issued in 2016) [17]. This Guideline states that the target cells should be exposed to the test chemical for 1.5–2 cell cycles. Having observed the low RI in our control tissues following 48-h treatments and aware that only Aardema *et al.* [14] investigated a 72-h treatment protocol, we too investigated the longer 72-h treatment protocol using five of the chemicals originally tested plus 4NQO.

Following the 72-h treatments, higher RI values for vehicle and UTC tissues were apparent. Data illustrated in Figure 2 show RI values of 0.55, 0.60 and 0.46 (48-h treatment) compared to 0.70, 0.77 and 0.69 (72-h treatment) demonstrating that the longer treatment time allowed an increased number of cells to pass through division. The importance of this result is that the increased treatment time will have facilitated an increased opportunity for damage to occur and subsequently be expressed as MN—an effect that was observed in our data as an increase in the magnitude of MNBN cell induction for all genotoxins tested. This included the two chemicals requiring metabolic activation (B[a]P and CPA; Table 3) where negative responses were observed following the 48-h treatment protocol but positive induction of MN was observed following the 72-h treatment. For the two positive control chemicals tested in multiple experiments (MMC and VIN), the MN responses following 72-h treatments were as expected (Figure 4) and generally consistent with micronucleus data using 2D test systems [18, 19, 20].

These observations and data demonstrate the importance of treatment time in this *in vitro* micronucleus assay. Andres *et al.* [21] commented that ‘proliferative rate’ of the tissue is a ‘critical parameter’ when performing the micronucleus assay on a 3D tissue and the OECD Test Guideline 487 [17] also highlights the importance of allowing a sufficient period of time for the cells to grow so that chromosome damage or other effects on the cell cycle or cell division, which can lead to the formation of micronuclei following mitosis, can occur. Our data support those comments.

As previously discussed, the longer 72-h treatment appears to facilitate a more robust expression of any resultant genotoxic damage due to the increased RI observed. Aardema *et al.* [14] reported that extending the treatment period to 72 h increased the reliability of detection for CPA and enabled detection of 4-NQO (negative after 48 h); however, they also reported that two other chemicals requiring activation [dimethylbenzanthracene (DMBA) and dimethylnitrosamine (DMN)] were negative and concluded that this was due to the limited metabolic capacity of the tissue.

Our results for B[a]P have shown that it was negative when tested for 48 h (Table 2) but, following the 72-h treatment protocol (Table 5), all five analysed concentrations resulted in statistically significant increases in MNBN. Both concentration mean and all individual tissue MNBN cell values clearly exceeded the laboratory vehicle HCR such that the conclusion was positive. B[a]P is a polycyclic aromatic hydrocarbon and potent carcinogen that requires metabolism in order for the genotoxic metabolite to form and covalently bind to DNA [22] and, without that activation, the chemical does not elicit a genotoxic response. CPA did not induce biologically relevant increases in MNBN cells across the six concentrations analysed following 48-h treatment (Table 4) but was clearly positive following 72-h treatment. Metabolism of CPA preferentially requires CYP2B6 but other CYPs (including members of the CYP3A family) are also utilised [23]. The metabolic profiles of native skin versus 3D tissue models have been previously examined [23] and, although the profiles exhibited some differences, key CYP enzymes were found to be present in the EpiDerm™ 3D tissue model (although at reduced levels), including the CYP3A family. This supports Aardema

et al.'s [14] conclusion of limited metabolic capacity, a result also seen in this laboratory under a different project [24]. Together, these data add weight to the argument that EpiDerm™ tissue has some inherent metabolic capabilities and that detection of chemicals that require metabolic activation is possible following the longer treatment (72 h) schedule, an important factor when testing unknown chemicals in the RSMN.

For the direct-acting genotoxin ENU, comparison of the 48- and 72-h treatment data showed a far higher magnitude of MNBN cell induction across the concentrations analysed from the 72-h treatment. The lowest concentration inducing a statistically significant increase was 2.000 mg/ml following 48-h treatment (mean MNBN frequency of 0.4%) and 0.500 mg/ml following 72-h treatment (mean MNBN frequency of 2.73%). This increase in sensitivity was also observed following treatment with the DNA cross-linking chemical MMC where a concentration of 0.004 mg/ml resulted in a mean MNBN frequency of 0.4% following 48-h treatment (Table 3) versus a mean MNBN frequency of 2.61% following 72-h treatment (Table 5). The two treatment protocols differ by facilitating a further treatment application as part of the 72-h protocol (i.e. two versus three treatments for the 48- and 72-h protocols, respectively). Whether it is a combination of additional treatment application and time that increases the sensitivity of the assay is open to question. However, the key observation of a general increase in RI (due to increased cell division) seems to be linked with a higher magnitude of MNBN cells following genotoxic insult.

A comparison of statistical sensitivity of either two or three tissues per treatment concentration, where 1000 BN were scored per tissue, demonstrated a greater ability to detect a lower fold increase over the vehicle control as statistically significant where triplicate tissues were used (see Tables 6 and 7). For the 48-h protocol, a higher incidence of zero counts was observed in vehicle tissues, but the inclusion of scoring cells from a third tissue increases the chance of scoring at least one MNBN cell. Zero counts are known to be problematical for statistical analyses [25] and data should be treated with caution where they occur [5]. HCR data from this laboratory showed that only 1/41 vehicle tissues exhibited a zero count for MNBN, whereas following 48-h treatments, 11/22 tissues scored a zero count for MNBN.

Conclusions

From the data generated in this present series of experiments, we would conclude the following:

- The published 48-h protocol [5] is reproducible without any cross-training in an independent laboratory.
- The extended 72-h treatment is a more suitable protocol for testing unknown chemicals as the extended treatment period facilitates greater cell division in the tissues.
- The EpiDerm™ tissues do exhibit some inherent metabolic capacity with the longer 72-h treatment period able to detect both of the metabolic activation requiring chemicals B[a]P and CPA as clearly positive.
- Analysis of 1000 MNBN cells from three and not two replicate tissues per treatment concentration improves the statistical sensitivity of the assay.

These data provide valuable information for investigators using the RSMN assay with EpiDerm™ tissues to optimise protocol design.

Acknowledgements

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