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A comparison of two new *in vitro* phototoxicity methods to a published yeast phototoxicity method

S. R. RACHUI, T. BOUFAISSAL, E. A. NEWCOMBE, and R. J. ALLEN, *Stephens & Associates, Inc., 3310 Keller Springs Road, Suite 130, Carrollton, TX 75006 (S.R.R., T.B., E.A.N), and Shaklee Corporation, 1992 Alpine Way, Hayward, CA 94545 (R.J.A).*

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Synopsis

Two new *in vitro* phototoxicity methods were compared to a previously published method that utilized yeast as a phototoxicity indicator. This comparison was done with eleven materials, tested in each system, to determine assay correlation. The protocols for the first two systems were similar and measured statistical differences in cell viability between irradiated and non-irradiated cell culture as a phototoxicity endpoint. The endpoint in the yeast model was a zone of inhibition generated in response to dosed phototoxic materials. The experimental systems chosen for this work were (a) a monolayer of human neonatal fibroblasts and (b) the MatTek EPI-100 test system. UVA was used in these methods to elicit phototoxic responses from the test materials. It appears the MatTek EPI-100 system may be a more realistic predictor of phototoxicity as replicate standard deviations are smaller and statistically significant differences are more easily obtained. The MatTek system also has the advantage of being more similar to human skin than the other tested models. The monolayer system, however, may be the most sensitive of the models due to test materials directly contacting the cells and not being restricted by a stratum corneum, such as with the MatTek system. However, since statistical significance is more difficult to achieve due to greater inherent variability. The monolayer system may better be used as a screening tool. The yeast method may also be most useful when used as a rapid and inexpensive means of screening large numbers of materials prior to proceeding to more sophisticated and costly tests.

INTRODUCTION

Phototoxic materials are those that are not toxic under normal circumstances but that are chemically altered and become toxic when exposed to UV light. Evaluating phototoxicity, therefore, is an important addition to the toxicology profile of topically applied materials. A listing of materials that may stimulate phototoxic reactions is shown in Table I. In addition, many materials that are known photoallergens may also be phototoxins. These materials include topical antimicrobials, fragrances, and sunscreen ingredients.

Phototoxicity is normally evaluated in human subjects. This, however, can be costly and time-consuming when comparing multiple formulations. Using *in vitro* phototoxicity methods it is possible to evaluate materials quickly and accurately by measuring the product's effect on toxicity both before and after irradiation with UVA. UVA was selected as the irradiation source because most phototoxic materials respond to UVA (1). Research using a combination of UVA/UVB, such as with a solar simulator, was not done due to the strong cytotoxicity caused by UVB. This research compares two new assays for phototoxicity evaluation to a previously published yeast method (2).

Table I
Phototoxic Drugs and Chemicals (1)

Phototoxin	Action Spectra	Phototoxin	Action Spectra
Psoralens	UVA	Diuretics	
Porphyrins	Visible	Hydrochlorothiazide	UVA
Coal tar	UVA	Bendroflumethiazide	UVA
Antibiotics		Furosemide	Unknown
Tetracyclines	UVA	Retinoids	
Fluroquinolones	UVA	Isotretinoin	UVA/pos.UVB
Nalidixic acid	UVA	Etretinate	UVA/pos.UVB
Ceftazidime	Unknown	Antineoplastic agents	
Griseofulvin	UVA	5-Fluoruracil	Unknown
Ketoconazole	Unknown	Dacarbazine	UVA/pos.UVB
Trimethoprim	Unknown	Methotrexate	Unknown
Sulfonomides	UVB	Vinblastine	UVB
NSAIDS		Dyes	
Arylproprionic acid derivatives		Eosin	Unknown
Benoxaprofen	UVA and UVB	Fluoroscein dye	Unknown
Carprofen	UVA	Methylene blue	Unknown
Ibuprofen	UVA	Rose bengal	Unknown
Ketoprofen	UVA	Miscellaneous	
Nabumetone	UVA	Amiodarone	UVA
Naproxen	UVA	Diltiazern	UVA
Tiaprofenic acid	UVA	Fibric acid derivatives	UVB
Salicylic acids		Phenothiazines	UVA
Aspirin	UVA	Quinine	UVA
Diflunisal	UVA	Quinidine	UVA
Anthranilic acids		Sulfite food derivative	UVB
Meclofenamic acid	UVA		
Pyrazolidinediones			
Phenylbutazone	UVA		
Oxyphenbutazone	UVA		

MATERIALS AND METHODS

MATERIALS

MatTek EPI-100. The artificial tissue system selected for this study was the MatTek EPI-100. This system consists of a well-defined stratum corneum and barrier function similar to human skin and an underlying layer of epidermal keratinocytes. Reagents used with the MatTek system were supplied by MatTek.

Monolayer. A monolayer of neonatal human fibroblasts, obtained from Clonetics, was grown in a serum-free medium to approximately 80% confluence and used in the assay.

Yeast. Commercial Fleischmann's yeast was selected as the indicator organism for this assay.

Light source. A bank of fluorescent UVA bulbs were used for irradiation in this assay.

MTT. The metabolic dye, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (MTT), was used as an indicator of cell viability with the MatTek EPI-100 and monolayer systems. Mitochondria within viable cells will reduce MTT from a yellow to purple color that is retained inside the cells until later extracted with isopropanol.

Positive control. The positive control used in this assay was 400 μg/ml 8-methoxypsoralin (8-MOP). This positive control was selected based on its inclusion in the published yeast method. (2–4).

METHODS

MTT compatibility. Cell viability, the endpoint for phototoxicity with the MatTek EPI-100 and monolayer systems, was measured using MTT. In some cases, test materials are capable of reducing MTT and causing its color change from yellow to purple, which would normally be indicative of cellular metabolic activity. This is controlled by combining equal volumes of test materials and MTT and incubating them in the dark for a time equal to that used for MTT exposure in the assay. At the end of the incubation period, materials that have not caused a color change to purple are considered MTT-compatible, while materials that have caused a color change to purple are incompatible. Further steps are taken for MTT-incompatible materials to ensure these materials do not cause false readings in the assay. None of the test materials used in this study were MTT-incompatible.

In the monolayer and MatTek EPI-100 systems, materials that show a significant ($p \leq 0.05$) increase in toxicity after UVA irradiation, when compared to the toxicity of the same materials without irradiation, are considered phototoxic. Statistical significance is important in the interpretation of results, as statistical measurements effectively account for biologic variability. Results that directionally appear to indicate phototoxicity may be attributable to biologic variability unless the difference between treatment and no treatment is large enough to preclude the effects of biologic variability. Although statistical significance is a more objective endpoint for measuring phototoxicity, Duffy *et al.* have established a protocol in which the cutoff for phototoxicity is based on the magnitude of difference in viability between non-irradiated and irradiated cell culture (5).

Monolayer. Since test materials cannot easily be assayed neat in monolayer systems, test materials were diluted to 1% of their stock concentration. Dilution of test materials also served to avoid cytotoxicity, which was noted with several of the materials at higher concentrations in previous assays with monolayer culture.

Fibroblasts were suspended in Dulbecco's Modified Eagles medium with 10% fetal bovine serum (DMEM). One (1) ml aliquots of this mixture were then seeded into wells of 24-well plates at a titer of 2×10^4 cells/well. The cells were allowed to fix to the plate for six hours, and then fresh DMEM containing test material, was added in triplicate using duplicate plate sets, one set for UVA exposure and one set that was not exposed to UVA. The final volume of each well was 1.5 ml, with the final concentration of test material being 1% of the neat concentration. Once dosed, both plate sets were incubated for 24 hours at 37°C and 5% CO_2 to allow for material metabolism. After 24 hours' incubation, the set for UVA irradiation was removed from incubation and placed under a bank of UVA bulbs at approximately 1.5 mw/cm^2 for 30 minutes, resulting in a UVA dose of about 3 joules. After irradiation, the plates were placed back into the incubator with the non-irradiated plates for another 24-hour period. After this second 24-hour incubation, both plate sets were removed from the incubator and the DMEM was aspirated and replaced with 1 mg/ml MTT prepared in DMEM. The plates were incubated at 37°C and 5% CO_2 in the presence of MTT for two hours. At the end of this period, the MTT solution was aspirated, the cells were rinsed with PBS, and 1 ml of isopropanol was added to each well. The plates were incubated at room temperature for one hour to extract the converted MTT from the cells. At the end of incubation, the absorbance of each well was measured at 570 nm. The viability of the culture was determined using the following formula:

$$\frac{\text{Mean absorbance density from cultures treated with test material}}{\text{Mean absorbance from untreated control cultures}} \times 100 = \%\text{viability}$$

MatTek EPI-100. The MatTek tissue was transferred from its shipping tray to six-well plates containing 0.9 ml culture media. Tissue was dosed with 100 µl of test material in triplicate using duplicate plate sets, one set for UVA exposure and one set that was not exposed to UVA. Test materials were dosed neat in this assay by applying neat materials to the stratum corneum of the tissue construct. Once dosed, both plate sets were incubated at 37°C and 5% CO_2 for 24 hours to allow for material penetration and metabolism. After 24 hours' incubation, the set for UVA irradiation was removed from incubation and placed under a bank of UVA bulbs at approximately 1.6 mw/cm^2 for 30 minutes, resulting in a UVA dose of about 3 joules. After irradiation, the plates were placed back into the incubator with the non-irradiated plates for another 24-hour period. After this second incubation, both sets of plates were removed from the incubator, and the tissue was rinsed free of test material with PBS and transferred to a 24-well plate containing 300 µl MTT. At the end of three hours, the MTT solution was aspirated, the cells were rinsed with PBS, and 1 ml of isopropanol was added to each well. The plates were incubated at room temperature for two hours to extract the converted MTT from the cells. At the end of incubation, the absorbance of each well was measured at 570 nm. The viability of the culture was determined using the following formula:

$$\frac{\text{Mean absorbance from cultures treated with test material}}{\text{Mean absorbance from untreated control cultures}} \times 100 = \%\text{ viability}$$

Yeast. A culture of commercial Fleischmann's yeast was prepared by adding 200 mg of yeast to 100 ml of deionized water. This suspension was then diluted by adding 7.4 ml of the suspension to 92.6 ml of deionized water. Cultures were made by generously streaking this prepared culture onto Sabouraud Dextrose agar in six-well plates. Test material was exposed in triplicate using duplicate sets, one set for UVA exposure and one set that was not exposed to UVA. Test materials were dosed neat in this assay by applying them to circular filter pads and allowing them to dry for 15 minutes. Once dry, the filter pad was placed onto the center of the yeast culture. The plate set for UVA exposure was then placed under a bank of UVA bulbs and irradiated overnight at 0.8 mw/cm^2, while the non-irradiated plate set was placed in the dark at room temperature and incubated overnight. The following day, the irradiated plates were removed from UVA exposure and placed in the dark with the non-irradiated set of plates for another overnight period. On the third day, plates were examined for a zone of inhibition surrounding the filter disk. A material that causes a zone of inhibition of greater than 2 mm in diameter around the filter disk, when compared to the non-irradiated controls, is considered phototoxic. The zones of inhibition were measured with calipers (2).

RESULTS AND CONCLUSIONS

Phototoxicity in the monolayer and MatTek EPI-100 assays is noted when a significant ($p \leq 0.05$) difference is seen between irradiated and non-irradiated cultures. The results for all three test systems are listed in Table II and presented graphically in Figures 1–3.

Table II
Raw Data Presentation

Material	Monolayer (% viability)				MatTek EPI-100 (% viability)				Yeast—Zone of inhibition (mm)	
	No UV	Std. Dev.	With UV	Std. Dev.	No UV	Std. Dev.	With UV	Std. Dev.	No UV	With UV
A	68.1	17.35	90.5	40.91	99.7	6.94	102.0	4.48	0.3	0.3
B	79.3	18.07	67.7	7.16	82.6	4.23	87.8	1.51	1.0	3.0
C	69.7	22.47	58.9	4.10	43.5	26.98	16.7	7.35	0.3	2.7
D	65.4	4.70	60.1	8.54	91.4	2.44	77.7	19.99	0.0	1.0
E (1%)	100.5	22.26	62.7	6.76	65.3	22.76	79.1	27.56	0.3	0.8
E (3%)	97.3	23.71	79.7	8.06	91.8	2.71	98.1	4.47	0.7	5.7
E (5%)	75.5	5.27	79.1	20.23	72.1	28.84	62.4	26.72	6.3	2.7
F (1%)	65.4	4.70	89.2	19.43	102.6	8.33	107.0	5.79	0.0	0.0
G	52.1	6.02	77.8	2.69	108.3	11.82	102.8	2.70	0.0	0.0
H	60.1	11.75	93.7	8.82	104.8	6.69	89.5	5.91	0.0	0.0
I	45.2	4.93	76.6	13.55	89.4	1.70	91.2	13.42	0.0	0.0
400 μg/ml 8-MOP	101.6	19.52	83.5	5.60	99.4	6.60	48.2	0.42	0.0	9.0

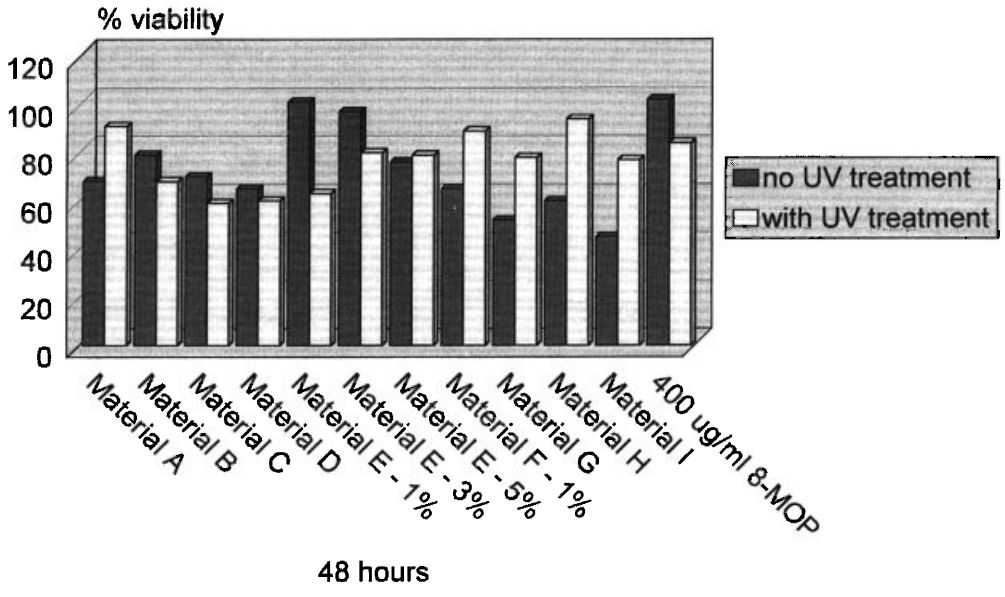

Figure 1. Monolayer phototoxicity assay.

STATISTICAL ANALYSIS

A statistical analysis of the results from the monolayer and MatTek EPI-100 assays was done using the t-test to measure differences between irradiated and non-irradiated cultures. The cutoff for statistical significance was $p \leq 0.05$. The results of the statistical analysis are presented in Table III. Phototoxicity in the yeast assay is not measured statistically (2).

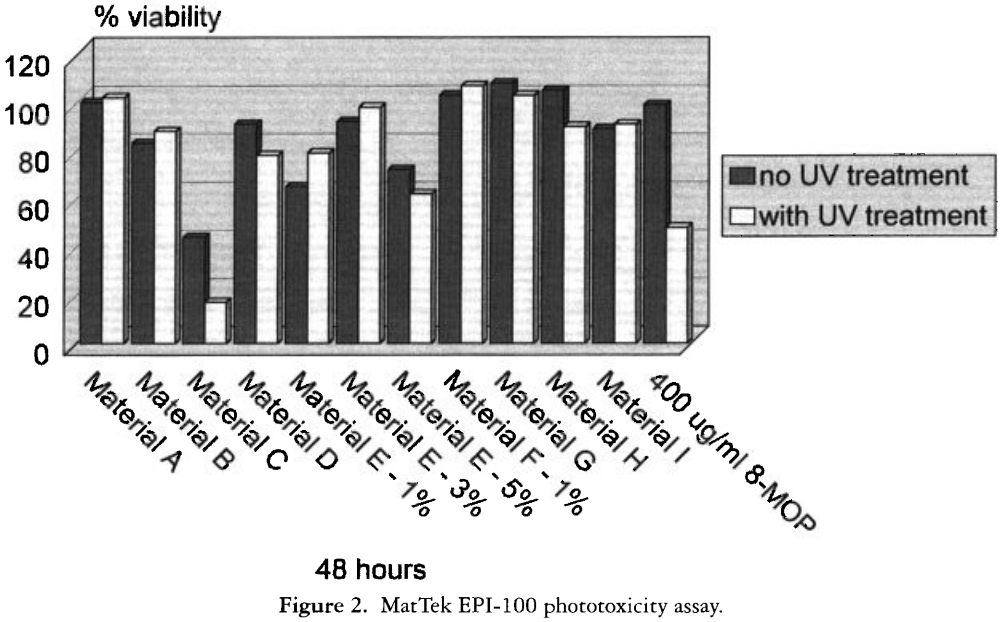

Figure 2. MatTek EPI-100 phototoxicity assay.

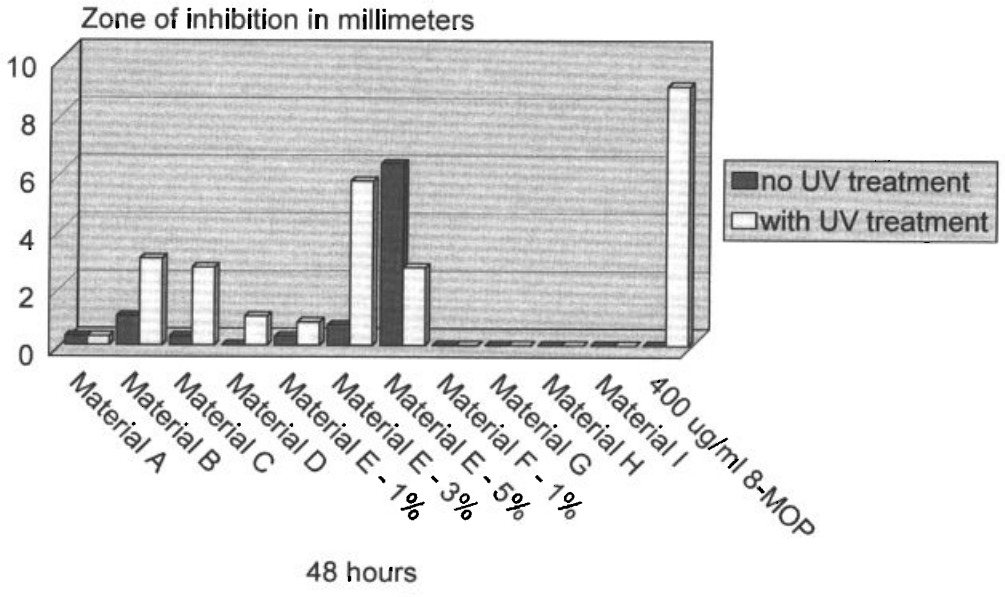

Figure 3. Yeast phototoxicity assay.

Table III
Statistical Analysis of Phototoxicity Results

Test material	Monolayer fibroblasts	MatTek EPI-100	Yeast method[b]
A	NST	NST	Not phototoxic
B	NST	NST	Not phototoxic
C	NST	NST	Phototoxic
D	NST	NST	Not phototoxic
E (1%)	NST	NST	Not phototoxic
E (3%)	NST	NST	Phototoxic
E (5%)	NST	NST	Not phototoxic
F (1%)	NST	NST	Not phototoxic
G	NST[a]	NST	Not phototoxic
H	NST[a]	NST	Not phototoxic
I	NST[a]	NST	Not phototoxic
400 μg/ml 8-MOP	NST	STN	Phototoxic

NST: No significant ($p \leq 0.05$) increase in toxicity due to UVA irradiation.
STN: A significant ($p \leq 0.05$) increase in toxicity was noted due to UVA irradation.
[a] A significant ($p \leq 0.05$) increase in viability was noted after UVA irradiation.
[b] No statistical analysis was done for the yeast assay.

MONOLAYER

No significant phototoxicity ($p \leq 0.05$) was seen in this assay with any of the test materials, including the 8-MOP positive control. The lack of significant phototoxicity with the positive control is attributed to high standard deviations that are inherent in monolayer systems. HTe directional results, however, do indicate phototoxicity of the positive control.

Although not statistically significant, directional trends suggest Material C and Material E at 0.01% and 0.03% were phototoxic. At 0.05%, however, Material E was cytotoxic, and reliable phototoxicity measurements were not possible. These findings are consistent with trends seen in the MatTek EPI-100 system. Materials C and E (at 3%) were also phototoxic in the yeast assay.

Since no stratum corneum is present to restrict test material contact with cells, the monolayer method is a sensitive means of assessing phototoxicity. In addition, since mammalian cells are used, phototoxicity of the test compound and potential metabolites can be assessed. Due to the nature of the test system, however, materials cannot easily be assayed neat. In this test, cytotoxicity was more of an issue than with the MatTek EPI-100 and yeast methods. Also, the absence of a stratum corneum and barrier function does not allow for a realistic evaluation of phototoxicity on intact skin. While no statistical differences were noted with the materials, directional data correlated with the results in the yeast assay.

MatTek EPI-100

A significant ($p \leq 0.05$) increase in toxicity as a result of the 8-MOP positive control was noted in tissue irradiated with UVA, indicating phototoxicity. No significant phototoxicity ($p \leq 0.05$) was seen in this assay with any test material. Although not significant, directional trends indicate Material C was phototoxic. This directional trend was also noted in the monolayer and yeast assays.

YEAST

This assay was the benchmark used to evaluate results obtained in the monolayer and MatTek EPI-100 assays. Materials that caused a zone of inhibition of greater than 2 mm were defined as phototoxic (2–4).

Phototoxicity was noted with the 8-MOP positive control and Materials C and E (3%) in this assay. The phototoxicity associated with Material C in this assay is consistent with the trends noted in both the MatTek EPI-100 and monolayer systems. Although Material E at 3% showed phototoxicity, the same material was not phototoxic at 1%. The difference between 1% and 3% may be a simple concentration effect. At 5%, Material E was cytotoxic, as indicated by the large zone of inhibition surrounding the non-irradiated filter disk, and reliable phototoxicity assessments were not possible.

DISCUSSION

The results seen across the three test systems were similar. Directional trends indicate that Material C was phototoxic in both the MatTek and monolayer systems. Results with the yeast model confirmed this phototoxicity. The two lowest concentrations of Material E were phototoxic in both the yeast and monolayer assays, but, because of cytotoxicity, phototoxicity was not noted at the highest concentration. The lack of response with Material E in the MatTek EPI-100 system may be due to the presence of the stratum corneum, which serves as a protective barrier and limits material penetration to the underlying epidermis.

Each of these systems is useful, depending on the type of application and specific needs. The MatTek EPI-100 system is the most sophisticated system tested, as it is constructed to be similar to human skin, including a well-defined stratum corneum and barrier function. Because the MatTek system is constructed to be similar to human skin, it is considered to most closely mimic *in vivo* test conditions. As the monolayer system is limited by the fact that (a) materials cannot easily be tested neat, (b) there is no protective stratum corneum, and (c) the variability of monolayer systems is inherently high, it may be most useful as a screening tool for phototoxic effects. Finally, the yeast method is a useful and inexpensive means to assess phototoxicity on a large number of materials prior to more expensive testing. The yeast method, however, does not measure the phototoxic potential of test material metabolites in mammalian cells and tests on a substrate dissimilar from the conditions of use.

With a good understanding of the limitations and advantages of the different systems, any one of these assays may be used effectively to screen materials for phototoxic effects. Since statistical measurements are more meaningful, the MatTek EPI-100 shows the most promise in this research as a final test for phototoxicity. The data presented here with the monolayer and MatTek EPI-100 systems indicate a good degree of correlation to data obtained using the published yeast assays. Any lack of correlation may be due to differences in the systems as described previously.

Finally, it should be noted that the use of the monolayer and MatTek EPI-100 systems for evaluating the phototoxic potential of metabolites from topical preparations is promising. Evaluating metabolites from oral products, however, is more difficult, due to the increased complexity associated with the metabolism of oral products.

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