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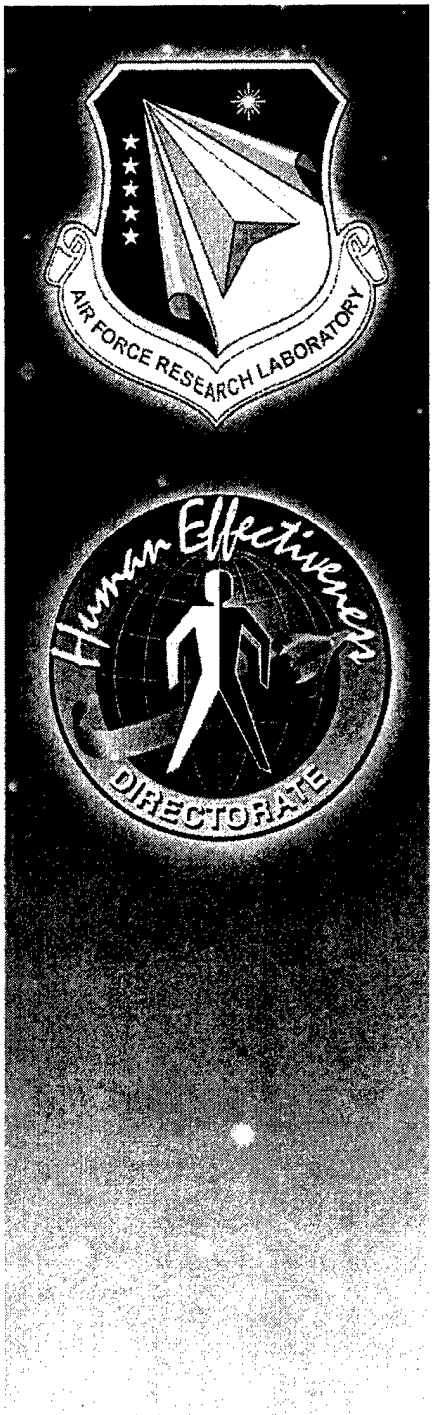

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Cytotoxicity of the murine keratinocyte line HEL30 exposed to the JP-8 jet fuel components m-xylene, 1-methylnaphthalene, and n-nonane

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This technical report has been reviewed and is approved for publication.

FOR THE DIRECTOR

//SIGNED//

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13. ABSTRACT *(Maximum 200 words)*

In vitro models are being used to evaluate the toxic and irritating effects of JP-8, a kerosene-based jet fuel. JP-8 components are volatile, which makes *in vitro* studies difficult to evaluate dose-response relationships due to changes in chemical dosimetry caused by evaporation from the exposure medium. An *in vitro* approach testing volatile chemical toxicity that we have recently developed was used to evaluate the toxicity of the JP-8 components *m*-xylene, 1-methylnaphthalene (1-MN), and n-nonane in keratinocytes. Partition coefficients were measured and used to estimate the chemical concentration in the keratinocytes. The EC_{50} for *m*-xylene and 1-MN decreased significantly ($P \leq 0.05$) at 1, 2, and 4 hr. For n-nonane, no significant decreases in the EC_{50} values were observed over time; marginal cytotoxicity of n-nonane in keratinocytes was observed at 1 hr. Within 4 hr, about 75-90% of each volatile chemical was observed to be lost from the exposure medium when tissues were exposed in unsealed 24-well plates. This decrease resulted in significantly higher medium chemical concentrations needed to obtain EC_{50} values when compared to tissues exposed in sealed vials. This study demonstrates that chemical evaporation during *in vitro* exposures can significantly affect toxicological endpoint measurements. Ultimately, relating target cell chemical concentration to cellular responses *in vitro* could be used in determining an equivalent external dose using a biologically-based mathematical model.

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Introduction

Chemical exposure of the skin can lead to irritant and allergic contact dermatitis. These occupational concerns have led to assessing chemicals or products to evaluate skin irritancy potential. Emphasis has been placed on *in vivo* tests such as the Draize test (Draize et al., 1944) and human patch tests (Basketter et al., 1997). Recently, *in vitro* cell culture approaches are being developed to evaluate chemical irritancy. *In vitro* systems are advantageous over *in vivo* tests by promoting high-throughput testing, reducing animal use, and comparing multiple chemicals under controlled conditions. The purpose of these *in vitro* tests is to place chemicals in irritant or non-irritant categories. Current *in vitro* skin models considered alternatives to the Draize test include normal and immortalized keratinocyte cultures, fibroblasts, and three-dimensional skin cultures (Müller-Decker et al., 1994; Sina et al., 1995; de Brugerolle de Fraissinette et al., 1999; van de Sandt et al., 1999; Eun and Suh, 2000; Lee et al., 2000; Medina et al., 2000; Fentem et al., 2001). Prevalidation tests of the three-dimensional cultures EpiDerm™, EPISKIN™, and PREDISKIN™ were conducted to determine whether these *in vitro* models could be used to accurately classify known chemicals as irritants or non-irritants (Fentem et al., 2001). Either the time to a viability decrease of 50% (ET_{50}) or the concentration that causes a 50% decrease in viability (IC_{50}) was used as the endpoint for categorizing the chemicals.

Specific cell culture tests designed for risk assessment purposes could be very useful if two problems were overcome. First, the test concentration of volatile chemicals added to the media should not decrease over time. Second, the exposure concentration should be expressed as the cellular/tissue concentration and not the exposure media

concentration. If the cellular/tissue concentrations were constant and known, it would be possible to use a biologically-based mathematical model to estimate an exposure scenario on the skin surface that leads to a specific target tissue dose that was determined to be toxic/irritating from the *in vitro* tests.

JP-8 is a kerosene-based fuel primarily used by North Atlantic Treaty Organization forces and the United States military. JP-8 is slightly irritating to the skin and a weak skin sensitizer (Kinkead *et al.,* 1992; Kanikkannan *et al.,* 2000). Petroleum middle distillates cause chronic irritation and inflammation with repeated applications (Freeman *et al.,* 1990), and may increase the incidence of skin cancer (Freeman *et al.,* 1993). There is not enough information available on skin absorption of JP-8 to determine the duration and mass of dermal exposure that would cause irritation. Aromatic and aliphatic components of JP-8 can rapidly penetrate the skin (McDougal *et al.,* 2000), which may promote mild skin irritation (Kinkead *et al.,* 1992; Kanikkannan *et al.,* 2000). Exposure to JP-8 in vapor, aerosol, and liquid forms has the potential to affect neurologic (Smith *et al.,* 1997), pulmonary (Pfaff *et al.,* 1995), and immunological (Ullrich, 1999) function. The expression of TNF-α and IL-8 mRNA and protein in keratinocytes exposed to Jet A, JP-8, and JP-8(100) have been identified as potential biomarkers of jet fuel-induced inflammation *in vitro* (Allen *et al.,* 2000). Cytotoxicity, DNA damage, oxidative stress, and cytokine release have also been observed in cultured cells (Boulares et al., 2002; Chou et al., 2002; Jackman et al., 2002; Wang et al., 2002). However, these studies utilized unsealed culture systems, which are susceptible to evaporation of chemical from the exposure medium. Such evaporation could lead to a temporal decrease

in jet fuel concentration during exposure, which may ultimately effect dose-response relationships.

Volatile organic chemicals (VOCs) are compounds with diverse consumer and commercial uses such as chemical synthesis intermediates, industrial organic solvents, degreasing agents, and fuel components. These widely used compounds present an increasing risk of occupational exposure via inhalation and dermal absorption, which can lead to local and systemic toxicological effects (Lupulescu and Birmingham, 1976; Berardesca et al., 1992; Lagorio et al, 1994; Shibata et al., 1994; Bogadi-Šare et al., 2000). Within 1-2 hours following exposure, organic solvents induce skin inflammation that is characterized by increased blood flow, vascular permeability, leukocyte infiltration into the skin, and epidermal degeneration (Steele and Wilhelm, 1966; 1970). For this study, we chose the JP-8 jet fuel components *m*-xylene, 1-methylnaphthalene (1-MN), and n-nonane as representative VOCs that are considered irritants.

The purpose of this study was to utilize our previously developed *in vitro* exposure method (Rogers and McDougal, 2002) in evaluating the toxicity of the JP-8 jet fuel components *m*-xylene, 1-MN, and n-nonane in keratinocytes. This study supports the need to use an *in vitro* exposure system that maintains a constant chemical dose and enables calculation of the VOC concentration in the cultured cells. Knowing the chemical concentration in the target cells during *in vitro* exposures is necessary to characterize the relationship between the chemical concentration and observed biological response(s). If the target cell/tissue dose that causes toxicity is known, biologically-based mathematical models can be used to predict toxic exposure scenarios (Ramsey and Anderson, 1984; McDougal et al., 1986).

Materials and methods

Cell culture

The murine keratinocytes line HEL30 was kindly provided by Dr. Dori Gremolec (NIEHS, Bethesda, MD). The cells were cultured in Ham's F-12/DMEM without phenol red (Sigma, St. Louis, MO), supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100μg/ml streptomycin at 37°C under 95% air and 5% CO_2.

HEL30 cells were grown on a collagen matrix to generate a three-dimensional structure to be used for chemical exposures. A stock solution (15.0 ml total volume) containing complete culture medium, rat tail collagen in 0.1% glacial acetic acid, and NaOH (to adjust the pH to 7.4) was mixed and maintained on ice. To each well of a 24-well plate, 500 μl of the collagen solution (~0.250 mg collagen) was added and allowed to congeal for 1 hour. Approximately 1.0 x 10^6 HEL30 cells (viability >95% as determined using trypan blue exclusion) were then added to each well and the plates were placed in an incubator at 37°C under 95% air and 5% CO_2. The cells were allowed to grow on the collagen matrix for 2 days before being used for *in vitro* exposures.

Partition coefficient determination

The cell culture medium:air ($PC_{m/a}$) partition coefficients for *m*-xylene, 1-MN, and n-nonane were determined as previously described (Rogers and McDougal, 2002). The $PC_{m/a}$ for *m*-xylene used for this study was previously determined to be 2.91 (Rogers and McDougal, 2002).

HEL30 cells harvested following trypsinization and centrifugation were used for determining the keratinocyte:air ($PC_{k/a}$) partition coefficients for *m*-xylene, 1-MN, and n-

nonane as previously described (Rogers and McDougal, 2002). Briefly, the cell pellet was weighed, resuspended in cell culture medium, and cell number/ml was determined using a hemacytometer. The cell number obtained corresponded to ~1 x 10^6 cells/mg pellet. Cell suspension aliquots (2 x 10^7 cells; ~0.02 g) were combined with medium in Hewlett-Packard headspace vials (12.0 ml), sealed with aluminum crimp seals containing a Teflon-coated rubber septum. Following equilibration at 37°C for 30 min on a vortex evaporator, the vials were vented and 0.1 ml (*m*-xylene and n-nonane) or 0.5 ml (1-MN) was removed from each vial. This volume was replaced with a corresponding volume of from a Tedlar gas sample bag containing either *m*-xylene, 1-MN, or n-nonane. Following incubation at 37°C for 4 hours on a vortex evaporator, the chemical concentration in the headspace of each vial was analyzed using a Hewlett-Packard 5890 Series II Gas Chromatograph containing a J&W Scientific DB-5 column (J&W Scientific Inc., Folsom, CA), and EZChrom Elite software (Scientific Software, Inc, Pleasanton, CA). The $PC_{k/a}$ for *m*-xylene was calculated using the following equation for homogenates (Gargas et al., 1989)

$$PC = \frac{Area_R(V_{vial} - V_{med}) - Area_S(V_{vial} - V_{med} - V_S) + (Area_R - Area_S)PC_{m/a}V_{med}}{Area_S(V_S)}$$

where $Area_R$ and $Area_S$ are the area counts from gas chromatography (GC) analysis for the reference and sample vials, respectively. V_{vial} is the total vial volume, and V_{med} and V_S represent the volume of the cell culture medium and cells, respectively.

Preparation of dosing solution and exposures

The dosing solutions prepared and exposures were conducted as previously described (Rogers and McDougal, 2002). Briefly, initial *m*-xylene dosing solutions were prepared by adding 5 µl *m*-xylene or 1-MN to sealed 12.0 ml Hewlett-Packard headspace vials (containing two 3.0 mm glass beads to aid mixing) completely filled with complete culture medium. No vehicle (e.g. ethanol) was used to prepare the dosing solutions.

On the day of exposure, four keratinocyte-collagen matrices were added into each of six separate 12.0 ml glass Hewlett-Packard headspace vials. Dilutions (1:10, 1:5, 1:2.5, 1:1, 0) of initial dosing solutions were prepared by combining fresh complete culture medium with the dosing solution in a gastight syringe. Each vial was filled with a separate dilution of exposure medium, immediately sealed with an aluminum crimp cap seal containing a Teflon-coated rubber septum, and incubated at 37°C for 1, 2, or 4 hours. Sham-exposed (medium only) samples were used as controls. At the end of the exposure, 20 µl exposure medium was removed from each vial using a gastight syringe, injected in duplicate into pre-weighed headspace vials, and the headspace was analyzed by GC to determine the chemical concentration in the exposure medium. The GC method for analysis was conducted as previously described (McDougal et al., 2000). Briefly, 1.0 ml of the headspace from each sample vial was injected at 140°C with a Tekmar 7000/7050 Headspace Sampler (Cincinnati, OH) onto a 0.53 mm X 30 m SPB-1 column in a Varian 3700 GC with FID (Palo, Alto, CA). The column oven temperature was programmed to hold at 50°C for 5 min and then increase at 5°C/min to 185°C. Results were processed with Perkin-Elmer Nelson integration software (Norwalk, CT). The chemical

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concentration (determined from a standard curve) was expressed as μg/ml. The keratinocyte-collagen tissues were removed from each vial and assessed for cell viability.

The chemical dosimetry for our exposure method was compared with a traditional method using unsealed 24-well culture plates. Dosing solutions were prepared as described above and each undiluted dosing solution was added in quadruplicate to unsealed 24-well culture plates (1.0 ml/well), or sealed 12.0 ml exposure vials. The vials and culture plates were incubated at 37°C under 95% air and 5% CO_2. The medium chemical concentration in the culture plates was measured by GC in duplicate at 0 and 4 hours.

Cell viability

The viability of keratinocytes exposed to *m*-xylene, 1-MN, or n-nonane was assessed by measuring reduction of the tetrazolium dye MTT in active mitochondria (Mosmann, 1983). Following chemical exposure, each collagen matrix populated with HEL30 cells was incubated in 500 μl of 0.5 mg/ml MTT in phosphate-buffered saline (pH 7.4) for 1 hour at 37°C. Following incubation, the tissues were individually placed in a 1.5 ml microcentrifuge tube containing 700 μl of acidified isopropanol. Each sample was sonicated for 5-10 sec and centrifuged at 12,000*g* for 5 min. Aliquots (200 μl) of the isopropanol supernatant were added to a 96-well plate in triplicate, and the absorbance read at 570 and 630 nm using a SpectraMAX 190 microplate reader (Molecular Devices, Sunnyvale, CA). The difference in absorbance at 570 and 630 nm was determined and the viability was expressed as a percentage of sham-exposed controls.

Additional exposures were performed to determine whether maintaining the tissues in a sealed vial for 4 hours would affect cell viability. Sham-exposed tissues were incubated for 0 or 4 hours in sealed 12.0 ml exposure vials assessed for cell viability using the MTT assay described above. The viability of dermal equivalents at 4 hours was expressed as a percentage of the 0 hr controls.

Keratinocyte chemical concentrations

The medium:air and keratinocyte:air partition coefficients for *m*-xylene, 1-MN, or n-nonane were used to calculate target cellular chemical concentration. The chemical concentration in the keratinocytes was calculated with the following formula:

$$C_c = (PC_{k/m})(C_m)$$

where C_c is the chemical concentration in the keratinocytes (μg *m*-xylene/g cells), $PC_{k/m}$ is the keratinocyte:medium partition coefficient ratio for *m*-xylene, 1-MN, or n-nonane and C_m is the chemical concentration in the exposure medium (μg/ml) as determined by GC analysis at the end of the exposure. Keratinocyte viability was plotted vs. the calculated C_c. The EC_{50} was determined using non-linear regression analysis (SigmaPlot, SPSS Science, Chicago, IL) of viability curves from three independent experiments.

Statistical Analysis

Data were expressed as mean $\pm$ SEM. The one-way ANOVA (Statistica, Statsoft, Tulsa, OK) were used for data analysis to examine the effect of time on the EC_{50} for *m*-xylene, 1-MN, and n-nonane.

Results

Media chemical concentrations

Measurements of *m*-xylene, 1-MN, and n-nonane concentration in unsealed 24-well culture plates showed significant decreases in the exposure medium chemical concentrations (Figure 1). In these unsealed 24-well plates, the initial (0 hr) medium *m*-xylene, 1-MN, and n-nonane concentrations decreased by ~90, 80, and 75 percent by 4 hours, respectively. However, in sealed exposure vials, the concentration of *m*-xylene, 1-MN, or n-nonane in the exposure medium at 0 hours did not change over the observed 4-hr time period (Figure 2).

Partition coefficients

The medium:air partition coefficient for *m*-xylene was previously determined to be 2.91 (Rogers and McDougal, 2002). The medium:air partition coefficient for 1-MN and n-nonane were 46.7 $\pm$ 2.26 and 0.82 $\pm$ 0.19, respectively. The keratinocyte:air ($PC_{k/a}$) partition coefficients for *m*-xylene, 1-MN, and n-nonane were 56.44 $\pm$ 2.21, 6711.94 $\pm$ 524.38, and 63.91 $\pm$ 10.85, respectively. The keratinocyte:medium partition coefficients for *m*-xylene, 1-MN, and n-nonane were determined to be 19.4, 143.7, and 77.7, respectively, by dividing the keratinocyte:air partition coefficient by the media:air partition coefficient.

Cell viability

The 1-, 2-, and 4-hr exposures to *m*-xylene and 1-MN in sealed exposure vials led to decreasing cell viability with increasing chemical concentration in the keratinocytes.

Figure 3 demonstrates the viability curves for keratinocytes exposed to *m*-xylene and 1-MN. Using non-linear regression analysis of cell equivalent viability plotted against the calculated cellular chemical concentration, the EC_{50} for *m*-xylene in the keratinocytes at 1, 2, and 4 hours were 1248.46 $\pm$ 78.01, 1028.88 $\pm$ 11.12, and 860.8 $\pm$ 84.6 μg *m*-xylene per gram of keratinocytes, respectively. The EC_{50} for 1-MN in the keratinocytes at 1, 2, and 4 hours were 6494.3 $\pm$ 460.1, 4319.82 $\pm$ 372.61, 2201.06 $\pm$ 196.27 μg 1-MN per gram of keratinocytes, respectively. Marginal cytotoxicity was observed at 1 hr, with the lowest observed cytotoxicity being ~70% of controls. The EC_{50} for n-nonane at 2 and 4 hours were 1250.37 $\pm$ 212.31 and 1193.24 $\pm$ 136.14 μg n-nonane per gram cells, respectively. One-way ANOVA comparing the effect of time on the EC_{50} for each chemical shows that there is a significant overall decrease ($P<0.05$) in EC_{50} in keratinocytes exposed to both *m*-xylene and 1-MN. No significant difference was observed when comparing the EC_{50} at 2 and 4 hr for n-nonane. Figure 4 shows a temporal distribution of the EC_{50} for *m*-xylene, 1-MN, and n-nonane.

A comparison between sham-exposed keratinocyte tissues at 0 and 4 hr in sealed vials showed no significant difference in viability. Keratinocyte viability at 4 hours was 98.6 $\pm$ 5.4% of 0 hr controls.

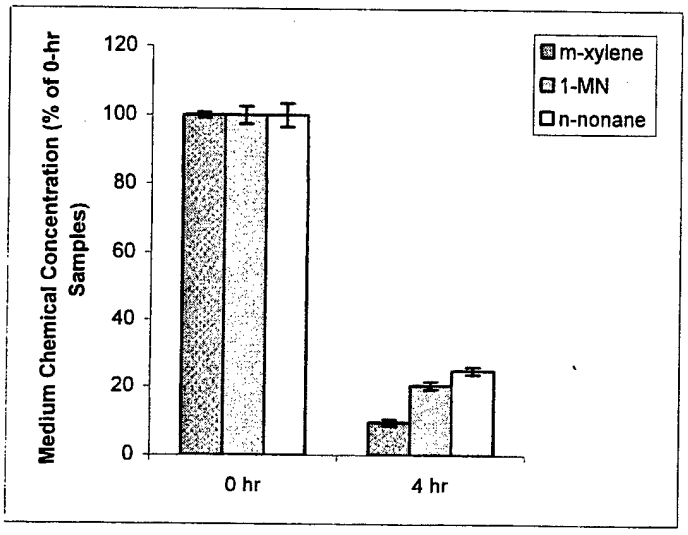

Figure 1. Mean ± SEM exposure medium *m*-xylene, 1-MN, or n-nonane concentration in 24-well plates at 0 and 4 hours. Data are expressed as percentage of 0-hr exposure medium chemical concentration.

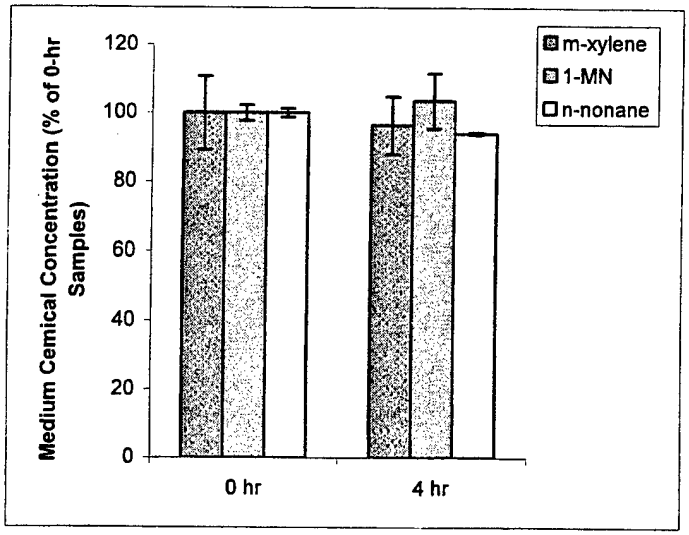

Figure 2. Mean ± SEM exposure medium *m*-xylene, 1-MN, or n-nonane concentration in sealed vials at 0 and 4 hours. Data are expressed as percentage of 0-hr exposure medium chemical concentration.

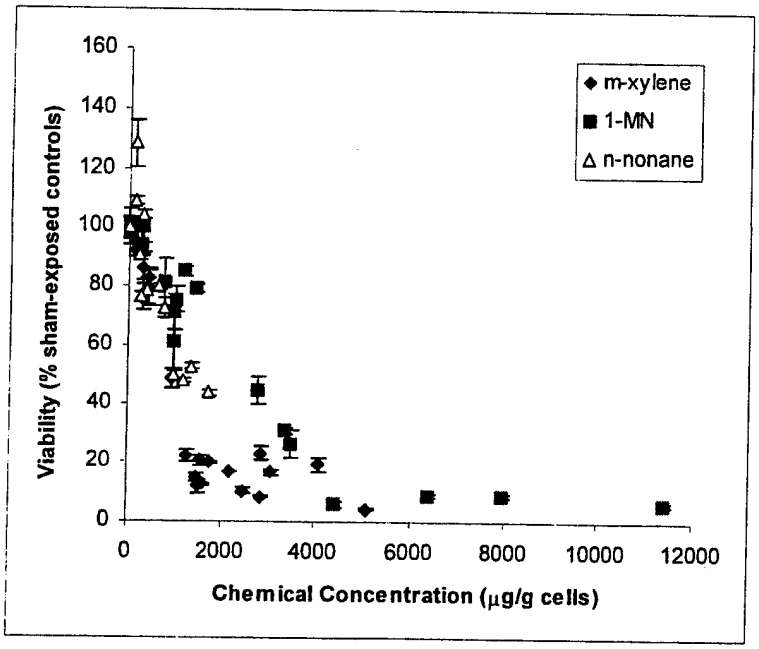

Figure 3. Mean viability ± SEM (n=4 per sample) of keratinocytes following a 4-hr exposure to *m*-xylene and 1-MN determined using the MTT assay. Values are expressed as a percentage of the sham-exposed controls.

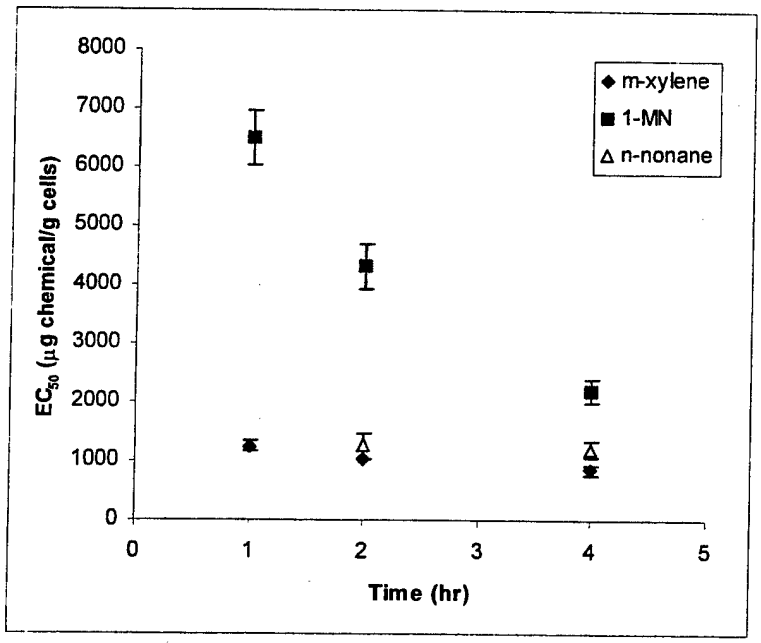

Figure 4. Plot of EC_{50} values for *m*-xylene, 1-MN, and n-nonane in keratinocytes exposed for 1, 2, and 4 hours.

Discussion

Various *in vitro* exposure systems, ranging from sealed and unsealed containers to open-lid cultures maintained in a larger closed chamber, have been developed to evaluate the toxicity of volatile chemicals in a variety of tissues (Kedderis and Held, 1996; Mückter et al., 1998; DelRaso and Channel, 1999; Geiss and Frazier, 2001; Gülden et al., 2001; Lantz et al., 2001; Croute et al., 2002; Rogers and McDougal, 2002). Studies exposing cultured cells to VOCs have provided data for describing the mechanistic and cytotoxic effects of these chemicals. *In vitro* dosimetry with VOCs is influenced by the headspace and culture medium volume, chemical solubility, and partitioning of the chemical into the culture medium and cells. Moreover, aromatic hydrocarbons can interact with the polystyrene of tissue culture flasks and plates (Croute et al., 2002). Since most VOCs possess low solubility in aqueous solution, maintaining these chemicals in solution is important to achieve a uniform exposure. In unsealed *in vitro* systems, evaporation of the VOC may occur from the culture medium, resulting in decreasing chemical concentration in the exposure medium. Croute and co-workers (2002) suggested that adding VOCs directly to culture medium in unsealed containers results in transient chemical concentrations that decrease within 30 min due to evaporation into the surrounding atmosphere. Such decreases can lead to a non-uniform or unknown chemical dose throughout the exposure period. We have previously observed such a temporal decrease in *m*-xylene from the culture medium in unsealed 24- and 48-well culture plates (Rogers and McDougal, 2002). This temporal decrease in chemical concentration resulted in viability values that were 4 to 6-fold higher than viability values determined using our sealed system (Rogers and McDougal, 2002). In

the present study, we observed similar decreases in the exposure medium concentrations of *m*-xylene, 1-MN, and n-nonane in 24-well plates. For these chemicals, at least 75% of the initial chemical concentration (0 hr) was observed to be lost by 4 hr. In contrast, the medium concentration of *m*-xylene, 1-MN, and n-nonane in sealed vials changed little (~5%) between the 0-hr and 4-hr samples. These results suggest that our sealed system prevents VOC evaporation from the exposure medium, resulting in a uniform chemical dose throughout the exposure period.

The viability of keratinocytes exposed to *m*-xylene, 1-MN, and n-nonane at 1, 2, and 4 hours decreased with increasing chemical concentration. Moreover, an overall decrease in the EC_{50} was observed for each chemical over the 1, 2, and 4 hour exposure period. For n-nonane, marginal cytotoxicity (~30% maximal observed decrease) was observed at 1 hr, while the EC_{50} at 2 and 4 hours were similar. For *m*-xylene and 1-MN, the results suggest a dose and temporal effect exists in for the cytotoxicity of keratinocytes. Previous studies have examined the cytotoxic effects of similar VOCs, including xylene and toluene, in which the cells were exposed while seeded in unsealed culture plates (Malich et al., 1997; Yang et al., 2000). For xylene, the IC_{50} determined for cells exposed for a similar period of time as presented in the present study (~1-4 hours) ranged from ~40 to 67 mM xylene (Malich et al., 1997). Converting the data in our study to molar concentration *m*-xylene in the exposure medium, our values would range from 0.42 to 0.61 mM. Moreover, for a 24-hr exposure period ~80-100% (v/v) toluene was needed during exposure of cells before cytotoxicity was observed (Yang et al., 2000). The effects of VOC exposure to cultured cells observed in these studies

demonstrates that evaporation of the VOC from the exposure medium is probably occurring, thus leading to inaccurate estimation of VOC-induced cytotoxicity.

Similar to using a tissue:blood partition coefficient, the fibroblast:medium partition coefficient can be an important factor in relating observed biological responses to target cellular dose *in vitro*. Partition coefficients (i.e. blood:air, tissue:air, and tissue:blood) are used when developing physiologically-based pharmacokinetic models to assess uptake, distribution, and elimination of VOCs *in vivo* (Andersen, 1982; Ramsey and Andersen, 1984; McDougal et al., 1986). In our sealed exposure system, which lacks a headspace, the culture medium, collagen, and keratinocytes represent the individual compartments into which *m*-xylene, 1-MN, or n-nonane can be distributed during exposure. The tissue culture media is in contact with both the collagen matrix and the keratinocytes and therefore the keratinocyte:media partition coefficient for *m*-xylene and 1-MN can be used to determine the cellular chemical concentration. Since the keratinocyte:air partition coefficients for *m*-xylene, 1-MN, and n-nonane are higher than the media:air partition coefficient, each chemical can be expected to be in higher concentration in the keratinocytes compared to culture medium. Using the keratinocyte:medium partition coefficient, we calculate that the *m*-xylene, 1-MN, and n-nonane concentration (μg chemical/g cells) in the keratinocytes would be 19.4, 143.6, and 77.7 times higher than the media concentration, respectively.

Summary

Dose- and time-response relationships for cellular toxicity can be investigated using this improved cell culture method for volatile chemicals. This method could

provide an effective approach to relate dosimetry to cellular responses, leading to the assessment of VOC-induced dermal irritation and/or toxicity. Ultimately, a biologically-based mathematical model could be developed for predicting the toxicity of VOCs.

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