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In vitro cytotoxicity testing of airborne formaldehyde collected in serum-free culture media

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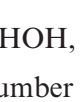
The purpose of this study was to identify a suitable sampling model for on-site toxicity assessment of soluble air contaminants such as formaldehyde, a well known industrial and indoor air contaminant. The *in vitro* cytotoxicity of formaldehyde, the selected model for soluble air contaminants, was studied using the MTS (tetrazolium salt) assay in two carcinoma cell lines, A549 epithelial lung and HepG2 hepatocarcinoma, and in skin fibroblasts. The cytotoxic effects of airborne formaldehyde were evaluated using test atmospheres in concentrations below 10 ppm (12.3 mg/m^3), generated by a dynamic diffusion method and bubbled (0.3 L/min) through serum-free culture media for one or four hours. Human cells were treated with formaldehyde air samples, and cell viability was determined after four hours incubation. In parallel, the concentration of airborne formaldehyde was monitored, using the 3500 NIOSH method. Cell viability of the HepG2 cells exposed to formaldehyde air samples (8.75 ppm $\times$ 4 h) was reduced to less than 50% (31.6 $\pm$ 1.24%). The HepG2 cell lines were found to be more sensitive (IC_{50} = 103.79 $\pm$ 23.55 mg/L) to formaldehyde than both A549 cell lines (IC_{50} = 198.36 $\pm$ 9.54 mg/L) and skin fibroblasts (IC_{50} = 196.68 $\pm$ 36.73 mg/L) ($P < 0.01$). An average of 96.8% was determined for collection efficiency of formaldehyde in serum-free culture media. The results of this study suggest that absorption of soluble air contaminants, such as formaldehyde, in serum-free culture media can be used as a suitable sampling model for on-site toxicity assessments. *Toxicology and Industrial Health* 2005; **21:** 147–154.

Key words: *air contaminants; cytotoxicity; formaldehyde;* in vitro *toxicity*; *MTS (tetrazolium salt) assay*

Introduction

Formaldehyde (CHOH,  , Chemical Abstracts Service (CAS) number 50-00-0) has a large-scale worldwide production and is a widely used industrial chemical (IARC, 2004; WHO, 2002). Its main use is in the production of resins that are inherent to the manufacture of adhesives, wood binders, plastics, textiles, leather and related industrial products. Formalin (37% formaldehyde) is also used as a disinfectant and preservative in many applications.

Common sources of formaldehyde exposure involve building materials, vehicle emissions, tobacco smoke and its use as a disinfectant. Many people are also occupationally exposed to formaldehyde including several millions in industrialized countries alone (IARC, 1995). Exposure levels are highly variable in different occupational settings.

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Formaldehyde exposure can cause a wide range of toxic effects such as formation of DNA–protein cross-links, cytotoxicity, immune activation and sensory irritation (Conolly *et al*., 2002; IARC, 1995; Lovschall *et al*., 2002; WHO, 2002). More recently, formaldehyde was classified as a proven human carcinogen on the basis of its ability to cause nasopharyngeal cancer and leukaemia (IARC, 2004). Formaldehyde can be metabolized to formate by some of the cellular enzymes, such as nicotinamide adenine dinucleotide (NAD)-dependent formaldehyde dehydrogenase. Formaldehyde dehydrogenase (FDH), the major metabolic enzyme involved in the metabolism of formaldehyde, has been detected in human liver and red blood cells and in a number of animal tissues, such as respiratory and olfactory epithelium in the rat (ATSDR, 1999; Newell, 1983; WHO, 2002).

Oral 50% lethal dose (LD_{50}) values of 800 and 260 mg/kg body weight have been reported for rats and guinea pigs, respectively (IPCS, 1989; WHO, 2002). The lethal concentration (LC_{50}) for inhalation of formaldehyde ranged from 405 ppm (497 mg/m^3) in mice to 471 ppm (578 mg/m^3) in rats after four hours of exposure (IPCS, 1989). Histopathological changes and/or increases in epithelial cell proliferation within the nasal cavity and respiratory tracts of laboratory animals have been reported to follow repeated inhalation exposure to formaldehyde up to 13 weeks (Monticello *et al*., 1991).

Although several *in vitro* methods have been reported for testing the toxic effects of airborne contaminants, most are limited to toxicity testing of urban airborne particulates. Basically, these methods involve sampling the particulate phase on filters followed by investigation of the effects of extracted and suspended particles (Alfaro Moreno *et al*., 2002; Glowala *et al.*, 2002; Hamers *et al.*, 2000; Yamaguchi and Yamazaki, 2001). Other *in vitro* methods have been developed to deal with gas-phase exposure to airborne contaminants, e.g., intermittent exposure procedures (Blanquart *et al*., 1995; Muckter *et al.*, 1998; Rusznak, 1996; Van Der Zee *et al*., 1987) and more recently, direct exposure techniques at the air/liquid interface (Aufderheide *et al*., 2003; Chen *et al*., 1993; Jabbour *et al.*, 1998; Knebel *et al*., 1998; Ritter *et al.*, 2001). However, technically these methods are more suited for laboratory-based *in vitro* toxicity investigations.

The major aim of this study was to develop an *in vitro* air sampling technique for soluble airborne contaminants such as formaldehyde that can be used for on-site toxicity assessments. In this study the cytotoxicity of formaldehyde was studied in human cells using an *in vitro* assay system. The relative susceptibility of epithelial lung carcinoma cell lines (A549), hepatocarcinoma cell lines (HepG2) and skin fibroblasts to formaldehyde cytotoxicity were assessed, using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega, USA). To evaluate the cytotoxic effects of airborne formaldehyde, as a selected model compound for soluble air contaminants, airborne formaldehyde was bubbled in serum-free culture media, and collected samples were used for *in vitro* toxicity tests. The collection efficiency of formaldehyde in serum-free culture media was determined using the 3500 National Institute for Occupational Safety and Health method (NIOSH, 1994).

Air sampling techniques for *in vitro* test systems usually require sample preparation steps, such as extraction to isolate the components of interest from a sample matrix, and ultimately, solubilization in culture media, potentially increasing experimental errors and further toxicity interactions. Hence, development of *in vitro* air sampling methods that minimize the number of sample preparation steps and reduce the use of toxic organic solvents are very important for on-site toxicity assessments. The results of the current work are presented as a suitable *in vitro* sampling method for toxicity assessment of formaldehyde and, potentially, other soluble air contaminants.

Methods

Chemical compounds

Formalin 37% solution was purchased from Chem-Supply, laboratory reagent (Australia). Chromotropic acid and sodium bisulphite for chemical analysis were purchased from Sigma (USA). *In vitro* assay reagents were purchased from Promega (USA) and Sigma (USA).

Cell types and culture conditions

Three different human cells representing different human organs of toxicological significance, A549 epithelial lung carcinoma cell lines (Lieber *et al.*, 1976), HepG2 hepatocarcinoma cell lines (ATCC No. HB-8065) and skin fibroblasts (Dr Z Wu, Westmead Hospital, Sydney) were selected for sensitivity comparisons to formaldehyde cytotoxicity.

All cells were cultured in sterile, vented 75-cm^2 cell culture flasks with Dulbecco's modified eagle's medium (DMEM/F12; Gibco, New Zealand), 5% fetal calf serum (FCS; JS Bioscience, Australia) and 1% antibiotic (200 mM L-glutamine, 10 000 U penicillin and 10 mg streptomycin per millilitre; Sigma, USA). Cultured cells were kept at 37°C in a humidified 5% CO_2 incubator.

For cytotoxicity experiments, newly confluent cell layers were enzymatically removed, using trypsin/ethylene diamine tetra acetic acid (EDTA) (Gibco, USA), and resuspended in culture medium. Cell viability was assessed by vital staining with trypan blue (Sigma, USA), and cell number was determined using a phase-contrast microscope (Leitz Wetzlar, Germany).

Appropriate cell numbers were established in preliminary studies for each cell type based on the linearity range of cell concentration versus the absorbance curve. Experiments with HepG2 and fibroblasts were conducted at cell concentrations of $(25–50) \times 10^4$ cells/mL and for A549 optimum cell concentrations were $(20–25) \times 10^4$ cells/mL.

In vitro cytotoxicity testing

The MTS cytotoxicity/proliferation assay (Promega, USA) was used to measure the toxicity of formaldehyde by determining the number of viable cells in culture. The MTS assay is based on the ability of viable cells to convert a soluble tetrazolium salt to a formazan product. The detection reagent is composed of solutions of MTS (Tetrazolium salt; Promega, USA) and PMS (an electron coupling reagent; Phenazine methosulfate; Sigma, USA). Both substances were initially dissolved in Dulbecco's phosphate buffered saline (DPBS; Gibco, New Zealand) at ratios of 2:1 (w:v; MTS:DPBS) and 0.92:1 (w:v; PMS:DPBS), filtered, sterilized (0.22 μm) and stored separately in light-protected containers at −20°C.

Cells were added to serial dilutions of the test chemicals in 96-well flat-bottomed microtitre tissue culture plates (Falcon, USA) in four replicates, using an appropriate dilution protocol. Backgrounds for each test concentration were also prepared separately in four replicates for considering any possible reactions induced by test chemical. Moreover, for each experiment, two internal controls were set up including an IC_0 (0% inhibitory concentration; cells only) and an IC_{100} (100% inhibitory concentration; media only), as described by Malich *et al.* (1997).

MTS/PMS reagents were thawed and mixed in a ratio of 20:1 (MTS:PMS) and immediately added to cell cultures in a ratio of 1:5. Absorbance was measured at 492 nm after four hours incubation at 37°C, using a microtitre plate reader (Multiskan MS, Labsystems, Finland) and analysed by the Genesis software program.

Calculation of cytotoxicity endpoints from dose-response curves

Dose-response curves were plotted for formaldehyde and all cell types tested. Cytotoxicity endpoints of formaldehyde, including the no observed effect concentration (NOEC), 10% inhibitory concentration (IC_{10}), 50% inhibitory concentration (IC_{50}) and total lethal concentration (TLC) values, were determined. Cytotoxicity endpoints were expressed as the mean and standard deviation (SD) of at least three repeat experiments.

Generation of formaldehyde test atmospheres and air sampling

In order to study the cytotoxicity of airborne formaldehyde, standard atmospheres of formaldehyde below 10 ppm (12.28 mg/m^3) were produced, using a dynamic diffusion method based on the diffusion rate of gas or liquid at controlled conditions (Saltzman, 1997). In this study formaldehyde was diffused from long thin glass tubes containing formalin 37% at controlled temperature and then diluted by filtered metered air to generate the required concentrations (Figure 1). The concentration of formaldehyde was continuously monitored by air sampling, using two midget impingers (SKC, USA) in series that contained absorption solution (10 mL). In parallel and in the same experimental

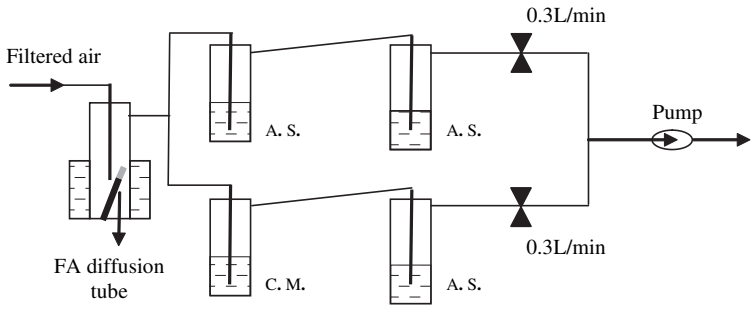

Figure 1. Production of formaldehyde test atmospheres and air sampling system (FA, formaldehyde; A.S., absorption solution; C.M., culture media.

conditions, air sampling for the *in vitro* study was carried out, using two other midget impingers in series. The first one contained serum-free culture media (10 mL) and was used for cytotoxicity assessments. The second one contained absorption solution for measuring any breakthrough of formaldehyde, i.e., for calculation of the collection efficiency of this method. Air sampling was continued for one or four hours in the same two parallel lines with constant air flow of 0.3 L/min, which was measured with a calibrated rotameter and adjusted by adjustable low flow holders (SKC, USA) in each line.

After air sampling, the first impinger containing formaldehyde in the serum-free culture media was used for the cytotoxicity tests. Prior to beginning the MTS assay, 5% FCS was added to air sample and filter sterilized. Human cells were incubated with serial dilutions of formaldehyde air samples in culture media, and cell viability was investigated after four hours. Three other air samples collected in absorption solution were subjected to chemical analysis.

Chemical analysis

The measurement of formaldehyde concentration in this study was carried out by the 3500 NIOSH method (NIOSH, 1994), with a detection limit of 0.025 ppm (0.03 mg/m^3). For air sampling, two impingers in series, containing 1% sodium bisulphite, were utilized to increase collection efficiency. Collected samples were analysed using the chromotropic acid colorimetric method (UV-VIS Pharmacia LKB, England).

Calculation of collection efficiency

After sampling airborne formaldehyde in serum-free culture media, any breakthrough of formaldehyde was determined by using a second impinger containing absorption solution. The average of formaldehyde lost was determined as $3.2 \pm 1.1\%$ when compared to the total amount of formaldehyde obtained by chemical analysis. Therefore, an average of 96.8% was calculated for collection efficiency of formaldehyde in serum-free culture media.

Statistical analysis

Statistical analyses were performed using Microsoft Excel 2002 and SPSS (version 11.01) Software. Analysis of variances was used to compare the mean of IC_{50} values of formaldehyde in three cell types, followed by multiple comparisons to identify which cell type was statistically significantly different. The Student's *t* test was used to compare the average cell viability of treated cells with collected formaldehyde air samples and control groups. Differences were considered significant at $P < 0.05$.

Results

Cytotoxicity of formaldehyde to human cells

The NOEC, IC_{10}, IC_{50} and TLC values of formaldehyde in three cell types with the MTS assay are summarized in Table 1. Each value represents the mean and standard deviation for at least three experimental replicates.

Table 1. Cytotoxicity endpoint values of formaldehyde in three cell types with the MTS assay (mean ± SD).

Cell types	NOEC (mg/L)	IC_{10} (mg/L)	IC_{50} (mg/L)	TLC (mg/L)
HepG2	1.36 ± 0.31	15.78 ± 3.58	103.79 ± 23.55	708.40 ± 181.07
A549	1.49 ± 0.18	30.15 ± 1.45	198.36 ± 9.54	1506.50 ± 77.25
Skin fibroblast	4.99 ± 0.86	29.90 ± 5.58	196.68 ± 36.73	1155.21 ± 278.47

IC_{50} values of formaldehyde in three human cell types are compared in Figure 2. Analysis of variances (ANOVA) showed that there was a significant difference between the IC_{50} values of formaldehyde in the cell groups ($P < 0.01$). Multiple comparisons revealed that the IC_{50} value of formaldehyde obtained with HepG2 cell lines was statistically significantly different from other cells tested ($P < 0.01$).

Cytotoxicity of airborne formaldehyde to human cells

Cytotoxic effects of airborne formaldehyde in HepG2 cell lines with the MTS assay resulting from different airborne concentrations and sampling time are presented in Figure 3. The mean and standard deviation of MTS conversions, and hence cell viability, are calculated as the percentage of controls.

Cytotoxic effects of airborne formaldehyde in HepG2 and A549 cell lines with the MTS assay resulting from identical airborne concentration and sampling time are compared in Figure 4. The mean and standard deviation of cell viabilities are calculated as the percentage of controls.

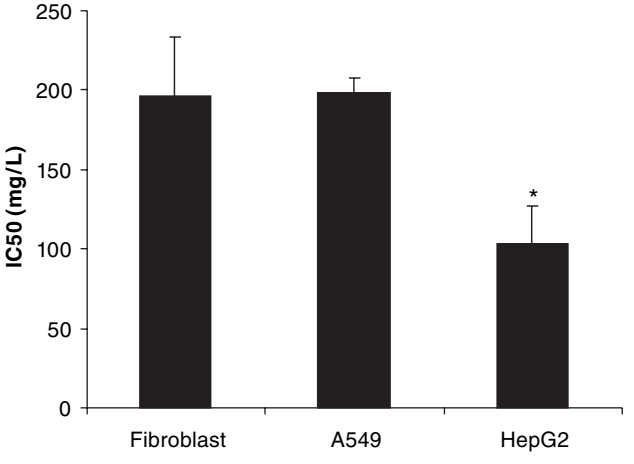

Figure 2. The mean and standard deviations of formaldehyde IC_{50} values in three cell types with the MTS assay (significant difference from other cell groups; $*P < 0.01$).

Percentage viability of HepG2 cell lines exposed to airborne formaldehyde samples are presented in Figure 5. The mean and standard deviations are calculated as percentage of controls. Statistically significant dose-related effects (concentration × time) of airborne formaldehyde were observed in HepG2 cell lines with the MTS assay.

Discussion

The cytotoxicity of formaldehyde, as a selected model compound for soluble air contaminants, was studied in three different cell types, HepG2 liver-derived, A549 lung-derived and skin fibroblasts, using the MTS assay (Promega). Cytotoxicity endpoints of formaldehyde, including NOEC, IC_{10}, IC_{50} and TLC values, were determined for each cell type, using dose-response curves. These analyses indicated that formaldehyde is toxic to human cells (Table 1). Statistically significant differences between IC_{50} values of formaldehyde in three different cell types were identified. HepG2 cell lines were

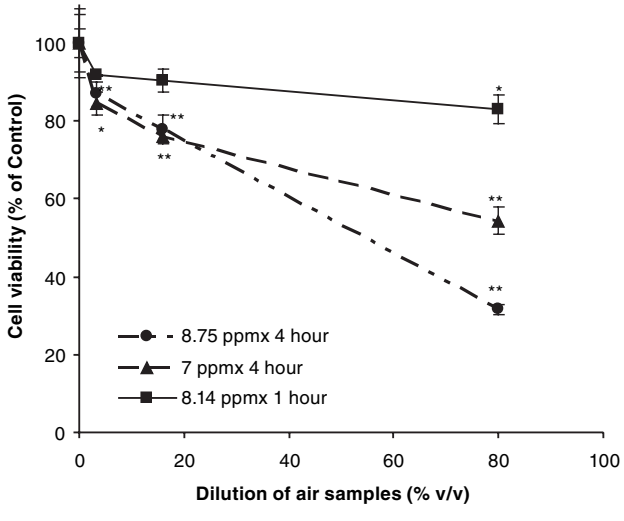

Figure 3. MTS conversion hence cell viability reduction induced by formaldehyde air samples collected in serum-free culture media in HepG2 cell lines (significant difference from control values; $*P < 0.01$; $**P < 0.0001$).

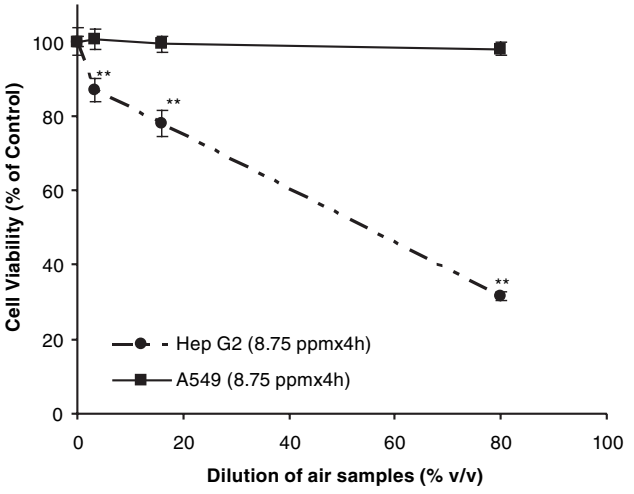

Figure 4. Comparison between MTS conversion hence cell viability of HepG2 and A549 cell lines exposed to formaldehyde air samples collected in serum-free culture media (significant difference from control values; $^{**}P<0.0001$).

found to be more sensitive ($IC_{50}=103.8\pm23.6$ mg/L) to formaldehyde than both the A549 cell lines and skin fibroblasts, when analysed by multiple comparisons ($P<0.01$) (Figure 2). This sensitivity may relate to the presence of cellular enzymes in liver cells, as compared with the other cell types used in this study. As discussed previously, formaldehyde can be metabolized to formate by NAD-dependent formaldehyde dehydrogenase, which has been detected in human liver cells (Newell, 1983; WHO, 2002). Our findings confirmed that the cytotoxicity of formaldehyde was influenced by the sensitivity of the cell types. This compared favourably to the work of Lovschall *et al*. (2002), who identified that the cytotoxicity of formaldehyde was consistently related to the choice of both cell type and endpoint assay, and that differential sensitivity of cell lines may reflect functional differences between cell types.

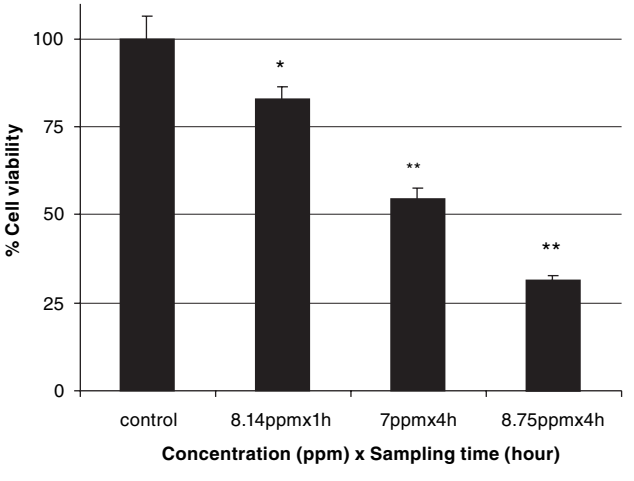

Figure 5. The mean and standard deviations of cell viability of HepG2 cell lines after incubation with formaldehyde air samples (significant difference from control values; $^{*}P<0.01$; $^{**}P<0.0001$).

Although several oral LD_{50} values for formaldehyde have been reported in the literature, those identified in the Registry of Toxic Effects of Chemical Substances (RTECS) are 42 and 100 mg/kg body weight in mice and rats, respectively (NIOSH, 2002). In comparison, based on our results using human target cells, IC_{50} values of 103.8 ± 23.6, 196.7 ± 36.7 and 198.4 ± 9.5 mg/L were identified for HepG2 cell lines, skin fibroblasts and A549 lung-derived cells, respectively. The Multicenter Evaluation of *In Vitro* Cytotoxicity (MEIC) programme, organized by the Scandinavian Society for Cell Toxicology, has identified a relatively good correlation between rodent LD_{50} values and human acute lethal doses ($R^2=0.61$, rat; $R^2=0.65$, mouse), yet, human cell line *in vitro* assays were determined as the best predictor of human acute toxicity ($R^2=0.74$) (Clemedson and Ekwall, 1999; Ekwall, 1999). One important outcome of the MEIC study was that a test battery of *in vitro* assays can be used directly to predict human acute lethal toxicity (Ekwall, 1999). Therefore, once disposition information (absorption, biotransformation, elimination) is taken into account, *in vitro* assays have a potential to be used as a surrogate for single dose toxicity, as usually obtained from LD_{50} and similar animal toxicity tests.

To study the cytotoxic effects of airborne formaldehyde, as a selected model compound for soluble air contaminants, MTS conversion and, hence, cell viability, was investigated after treating human cells with formaldehyde air samples collected in serum-free culture media. Based on our preliminary toxicity studies, HepG2 and A549 cell lines were selected for exposure to these samples. While the viability of HepG2 cells was significantly decreased after exposing with formaldehyde air samples ($P<0.01$ and $P<0.0001$), no statistically significant differences were measured between cell viability of treated A549 cells at the concentrations tested, compared with controls (Figures 3 and 4). In particular, the concentrations of airborne

formaldehyde tested were toxic to HepG2 human liver-derived cells. In the literature, the lethal concentration (LC_{50}) for inhalation of formaldehyde was reported to range from 405 ppm (497 mg/m^3) in mice to 471 ppm (578 mg/m^3) in rats following four hours' exposure (IPCS, 1989). In comparison, in this study exposing the HepG2 cells to formaldehyde air samples (8.75 ppm $\times$ 4 h) reduced cell viability to less than 50% ($31.6 \pm 1.24\%$) following a four-hour incubation.

In vivo published data suggest that the cytotoxic effects of formaldehyde in laboratory animals appears to be more closely related to the exposure level of formaldehyde than to the time of exposure or total dose (CxT) (Conolly *et al*., 2002; IARC, 1995; Wilmer *et al*., 1989). Moreover, a lack of cytotoxic effects of formaldehyde has been reported at 2 ppm or below. In our study, dose-dependant effects (CxT) of airborne formaldehyde were observed in HepG2 cell lines at test concentrations (Figure 5). However, because increasing the sampling time consequently increased the concentration of formaldehyde absorbed in serum-free culture media, it is not possible to separately identify the effect of time. Direct exposure of the target cells at the air/liquid interface using porous membranes currently are being conducted in our laboratory and may be a key answer for this question.

Based on the results of chemical analysis, an average of 96.8% was calculated for the collection efficiency of airborne formaldehyde in serum-free culture media, suggesting the potential application of this method for sampling the airborne formaldehyde. In addition, use of serum-free culture media as a collection solution for soluble airborne contaminants is a simple technique, without any specific sample preparation or extraction steps. In this method, any potential toxic interactions of the test chemical with other toxic organic solvents during preparation are omitted. It is suggested that this sampling method may potentially be used for *in vitro* toxicity assessment of soluble workplace air contaminants such as formaldehyde, glutaraldehyde, ammonia and sulphur dioxide, some particulates like chromium (VI), cobalt and cadmium compounds, and possibly mixtures of such air contaminants that may occur in workplace environments.

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