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Development of in vitro methods for toxicity testing of workplace air contaminants

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Abstract
While the OECD test guidelines and mostly animal assays have been used to study the toxic effects of chemicals for many years, very little is known about the potential toxicity of vast majority of inhaled chemicals. Considering large number of chemicals and complex mixtures present in indoor and outdoor air, heavy reliance on animal test methods appear to be not adequate. Continuing scientific developments are needed to improve the process of safety evaluation for the vast number of chemicals and inhaled materials. The aim of this study was to optimise *in vitro* methods for toxicity testing of airborne contaminants. An integrated approach was designed in which appropriate exposure techniques were developed. A diversified range of *in vitro* assays using multiple human cell systems were implemented. Direct exposure of cells to airborne contaminants was developed by culturing cells on porous membranes in conjunction with a horizontal diffusion chamber system. Dose-response curves were generated allowing the measurement of toxicity endpoints. Toxicity ranking of test chemicals, based on obtained IC_{50} (50% inhibitory concentration) values, in different human cells and in vitro assays were determined. Airborne IC_{50} values were calculated for selected volatile organic compounds (xylene; 5350 ± 328 ppm > toluene; 10500 ± 527 ppm) and gaseous contaminants (NO_2; 11 ± 3.54 ppm > SO_2; 48 ± 2.83 ppm > and NH_3; 199 ± 1.41 ppm). Results of this research indicate the significant potential of *in vitro* methods as an advanced technology for toxicity assessment of airborne contaminants.

Keywords
toxicity, air, methods, contaminants, vitro, testing, development, workplace

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ORIGINAL ARTICLE

Development of *in Vitro* Methods for Toxicity Testing of Workplace Air Contaminants

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ABSTRACT

While the OECD test guidelines and mostly animal assays have been used to study the toxic effects of chemicals for many years, very little is known about the potential toxicity of vast majority of inhaled chemicals. Considering large number of chemicals and complex mixtures present in indoor and outdoor air, heavy reliance on animal test methods appear to be not adequate. Continuing scientific developments are needed to improve the process of safety evaluation for the vast number of chemicals and inhaled materials. The aim of this study was to optimize *in vitro* methods for toxicity testing of airborne contaminants. An integrated approach was designed in which appropriate exposure techniques were developed. A diversified range of *in vitro* assays using multiple human cell systems were implemented. Direct exposure of cells to airborne contaminants was developed by culturing cells on porous membranes in conjunction with a horizontal diffusion chamber system. Dose-response curves were generated allowing the measurement of toxicity endpoints. Toxicity ranking of test chemicals, based on obtained IC_{50} (50% inhibitory concentration) values, in different human cells and *in vitro* assays were determined. Airborne IC_{50} values were calculated for selected volatile organic compounds (xylene; 5350 ± 328 ppm > toluene; 10500 ± 527 ppm) and gaseous contaminants (NO_2; 11 ± 3.54 ppm > SO_2; 48 ± 2.83 ppm > and NH_3; 199 ± 1.41 ppm). Results of this research indicate the significant potential of *in vitro* methods as an advanced technology for toxicity assessment of airborne contaminants.

Keywords: *Adenosine triphosphate, Airborne contaminants, Direct exposure method, In vitro cytotoxicity, MTS tetrazolium salt, Neutral red uptake*

INTRODUCTION

People at work have a great potential for exposure to chemical contaminants. Exposure to hazardous chemicals, toxic gases, vapours of volatile organic compounds (VOCs), particulates and mixtures of these can cause both acute and chronic adverse health effects predominantly in the respiratory system [1-4]. Data derived from routine air sampling and analytical techniques do not provide direct measures of toxicity and the potential mechanisms inducing toxic effects. Conventional animal toxicity tests do not provide a satisfactory basis for risk assessment due to scientific, economic and ethical concerns [5-10]. Although an extensive background database from toxicological studies has been developed on animal models, most toxicity data are from oral and dermal exposures rather than inhalation exposure [11-12]. While, *in vitro* methods have been increasingly implemented in the multiple disciplines of toxicology, the application of *in vitro* models in inhalation toxicology is still limited. Therefore, standardized and reproducible *in vitro* exposure techniques which mimic inhalation exposure pattern *in vivo* are urgently needed [13-14].

The aim of this research was to explore the potential of *in vitro* test methods for toxicity assessment of workplace contaminants with respect to their applicability for hazard assessment. These methods were used to develop a practical strategy for *in vitro* toxicity testing of airborne contaminants, based on the knowledge of physicochemical properties of the test chemicals. Primarily the application and merits of *in*

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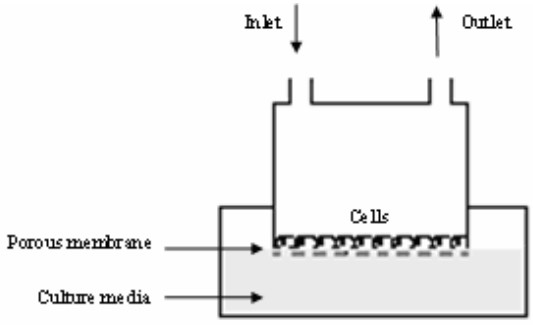

Fig 1. Dynamic direct exposure at the air/liquid interface using porous membranes.

vitro toxicity methods for prediction of toxicity of selected chemical contaminants are evaluated using multiple human cellular systems and biological endpoints. The use of human cells as bio-indicator targets in toxicity testing can potentially generate the representative data related to human chemical exposures. Appropriate *in vitro* exposure techniques were developed to predict the cytotoxic effects of airborne contaminants in human target cells.

MATERIALS AND METHODS

Test chemicals

For the preliminary study, test chemicals were selected from common workplace contaminants and included: ammonia solution (NH_3; CAS No. 7664-41-7), cadmium chloride ($CdCl_2$, $H_2O^{2\ ½}$; CAS No. 7790-78-5), cobalt chloride ($CoCl_2$, H_2O^6; CAS No. 7791-13-1), formaldehyde (CH_2O; CAS No. 50-00-0), glutaraldehyde ($C_5H_8O_2$; CAS No. 111-30-8), manganese chloride ($MnCl_2$, H_2O^4; CAS No. 13446-34-9), mercuric chloride ($HgCl_2$; CAS No. 7487-94-7), sodium dichromate ($Na_2Cr_2O_7$, H_2O^2; CAS No. 10588-01-9), sulfurous acid (H_2SO_3; CAS No. 7782-99-2), zinc chloride ($ZnCl_2$; CAS No. 7646-85-7). All selected test chemicals were of the highest analytical grade available and were soluble in culture media at test concentrations. Stock solutions were freshly prepared and used for preparation of serial dilutions of test chemicals in 96 well microtitre plates.

In the next stage a number of airborne chemicals were tested to assay airborne pollutants with diverse physicochemical characteristics. Toluene ($C_6H_5CH_3$, CAS No.108-88-3, APS Finechem, Australia, Analytical reagent) and xylene (C_6H_4 $(CH_3)_2$, CAS No. 1330-20-7, Chem-Supply, Australia, Laboratory reagent) were selected as indications of VOCs. Selected indoor and outdoor gaseous contaminants included nitrogen dioxide (NO_2, CAS No. 10102-44-0, 50 ppm), sulfur dioxide (SO_2, CAS No. 7446-09-5, 200 ppm) and ammonia (NH_3, CAS No. 7664-41-7, 200 ppm). All test gases were balanced in synthetic air and purchased from Linde Gas Pty Ltd, Australia.

Initial concentrations were estimated based on published toxicity data. For airborne test chemicals, the recommended threshold limit values were consulted [15-16]. Preliminary studies were performed to determine the appropriate chemical concentration ranges in order to obtain the complete dose-response curve representing the response at a diverse range of concentration.

Cell types and culture conditions

Three different human cells including pulmonary type II-like epithelial cell lines (A549, ATCC No. CCL-185), hepatoma cell lines (HepG2, ATCC No. HB-8065) and skin fibroblasts isolated from skin biopsies of healthy individuals (Cytogenetics Department, Westmead Hospital, Sydney, Australia) were used. Cells were cultured in sterile, vented 75-cm^2 cell culture flasks with DMEM/F12 (Dulbecco's modified eagle medium: Ham's F-12 nutrient mixture; Gibco, USA) supplemented with 5% (v/v) fetal calf serum (FCS; JS Bioscience, Australia), and L-glutamine (2 mM), penicillin (100 units/mL) and streptomycin (0.1 mg/mL) solution (Sigma, USA). Cultured cells were kept at 37ºC in a humidified 5% CO_2 incubator. For cytotoxicity experiments, newly confluent cell layers were enzymatically removed, using Trypsin/EDTA (Gibco, USA), and resuspended in culture medium. Cell viability was assessed by vital staining with trypan blue (0.4% (w/v); Sigma, USA), and cell number was determined using a light microscope (Leitz Wetzlar, Germany). Appropriate cell numbers were determined in preliminary studies for each cell type and *in vitro* assay, based on the linearity range of cell concentration and absorbance data [17-18].

Growing cells on porous membranes

Human cells were grown on porous membranes (0.4 µM) in Snapwell inserts (Fig 1). The Snapwell insert is a modified Transwell culture insert with a 12 mm diameter providing a growth area of 1.12 cm^2 (clear polyester Snapwell™ insert, 3801, Corning), supported by a detachable ring that was placed in a six well culture plate. Culture media supplemented with 5% FCS, 1% antibiotics and HEPES buffer (N-(2-hydroxyethyl)-piperazine-N´-2-ethanesulfonic acid; 0.01 M) was added to the membranes and the Snapwell inserts were incubated at 37ºC for 1 h as an initial equilibrium time to improve cell attachment. Culture media was then removed from the top and replaced with fresh medium (0.5 mL) containing the cell suspension (25×10^4 cells) and HEPES buffer (0.01 M). Cell cultures were incubated at 37ºC in a humidified incubator for 24 h.

Before exposure, cell confluence (75-80%) and attachment was observed using the light microscope. The medium was removed from newly confluent cells and membranes washed with Hank's balanced salt solution (HBSS; Gibco, USA). Cells were exposed to airborne concentrations of the test chemicals on their apical side while being nourished from their basolateral side.

Exposure methods

Exposure to soluble chemical contaminants

Cells were added to serial dilutions of test chemicals prepared in 96-well flat bottomed microtitre tissue culture plates in four replicates [17]. For each test concentration, blank wells were also prepared separately in four replicates in which no cell suspension were added for considering any possible reactions induced by the test chemicals and the assay reagents. For each experiment, two internal controls were set up including IC_0 (0% inhibitory concentration; cells only) and an IC_{100} (100% inhibitory concentration; media only). The MTS 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl) -2-(4-sulfophenyl)-2H-tetrazolium) *in vitro* assay was then performed as described previously [17, 19].

Exposure to volatile organic compounds

A static direct exposure technique at the air/liquid interface was developed for toxicity assessment of xylene and toluene using cultured human cells on porous membranes. In this method test atmospheres of the selected VOCs were generated in sealed glass chambers of known volumes. The airborne concentration of the generated test atmospheres were calculated as previously described [18]. Human cells were exposed to vapours of xylene and toluene at different concentrations directly at the air/liquid interface for one hour. During the exposure time, sealed glass chambers were kept in an orbital mixer incubator to equilibrate the test atmosphere (37ºC; 50 RPM; Ratek Instruments, Australia). Cytotoxicity was investigated after 24 h incubation with the MTS and NRU (Neutral red uptake; 3-amino-7-dimethyl-amino-2-methylphenazine hydrochloride) assays.

Exposure to gaseous airborne contaminants

A dynamic direct exposure technique at the air/liquid interface was developed for toxicity assessment of gaseous airborne contaminants using cultured human cells on porous membranes in conjunction with Navicyte horizontal diffusion chamber systems (Fig 1). In this method, test atmospheres of NO_2, SO_2 and NH_3 were generated using a direct dilution method. The generated test atmospheres were calibrated using appropriate sampling and analytical methods (Table 1).

After washing the cell monolayer with HBSS, membranes were detached from their holders and were placed into the horizontal diffusion chambers containing serum free culture media supplemented with HEPES buffer (0.01 M). The upper compartments of the diffusion chambers were closed and test atmospheres were delivered through the chambers with a low flow rate (25 mL/min) for one hour [14, 20]. The volume of culture medium in the basolateral compartment was sufficient to keep the cells hydrated. At the same time, a humidified atmosphere was supported inside the chambers by keeping exposure chambers at 37ºC. After exposure, membranes were replaced in their holders in the culture plates, fresh culture media were added to the bottom of the membranes and MTS, NRU and ATP (Adenosine triphosphate) assays were performed. For all experiments, rather than incubator media only and cell only controls, a synthetic air control was set up in which cells on membranes were placed in a horizontal diffusion chamber and exposed to a dynamic flow of synthetic air during the exposure time to consider any cell viability reduction induced by the dynamic flow of air [20].

Cytotoxicity endpoints

MTS-Tetrazolium salt assay

The Promega CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay was used to measure the cytotoxicity of test chemicals by determining the number of viable cells in culture [21]. The MTS assay is based on the ability of viable cells to convert a soluble tetrazolium salt to a formazan product. This assay has been used in our laboratory for toxicity testing of chemicals in microtitre well plates [17, 19]. The MTS assay was also adapted for toxicity testing of airborne contaminants using membranes in six well plates as previously described [18, 20]. Briefly, fresh culture medium was added to the membranes in six well plates. The MTS/PMS reagent was added and cell cultures were incubated for one h at 37ºC. After the incubation period, absorbance was recorded at 492 nm using a multiplate reader (Multiskan Ascent, Thermo Labsystems, Finland).

NRU-Neutral red uptake

The neutral red (NRU; Sigma) assay is a cell survival/viability technique based on the ability of viable cells to incorporate and bind supravital neutral red dye [22-23]. The NRU assay was adapted to measure the cytotoxicity of airborne contaminants using membranes in six well plates [18, 20]. Briefly, fresh culture media was added to the bottom and the NRU solution (0.5 mL) was added to the top part of the membranes in six well plates and cells were incubated for 3 h at 37ºC. The medium was then removed and cells fixed with fixative solution for no longer than 30 s. Membranes were rinsed with HBSS and assay solubilising solution was added. The plate was shaken for 10 min and absorbance was recorded at 540 nm using a microtiter plate reader (Multiskan MS, Labsystems, Finland).

ATP-Adenosine triphosphate

Cellular ATP content was measured using the CellTiter-Glo® Luminescent Cell Viability Assay, a homogeneous method of determining the number of viable cells present in culture [24] as previously

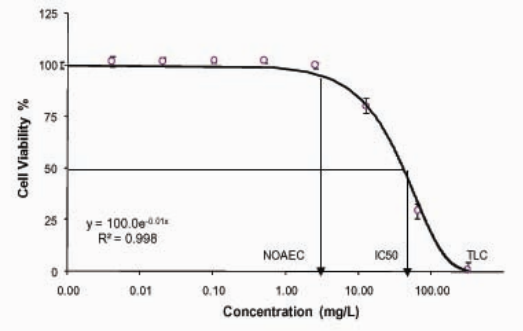

Fig 2. An example of dose response relationship for a test chemical.

described [20]. The CellTiter-Glo® Reagent is a buffered solution which contains detergents to rupture cells and release ATP immediately upon addition to cells and ATPase inhibitors to stabilise ATP once it is released from cells [25]. The reagent also contains luciferin, Mg^{2+}, and a mutant form of recombinant luciferase capable of generating a stable glow-type luminescent signal in the presence of ATP. Briefly, fresh culture media was added to the bottom and top of the membranes in six well plates. An equal volume of Cell-Titer-Glo® reagent (0.25 mL) was added to the membranes. The plate was shaken for 2 min and kept at room temperature for 10 min to stabilise the luminescence signal. Aliquots of 100 µL were transferred into 96-well opaque-walled microtitre plates and the luminescence level was recorded using a luminometer (Berthold Detection Systems, Germany).

Calibration of gaseous test atmospheres

Generated concentrations of gaseous airborne contaminants were determined by appropriate analytical methods (Table 1). Calibration of test atmospheres indicated a high correlation between calculated and measured test concentrations for all test gases (R^2 = 0.99). Identical distribution of generated test gas in the three different *in vitro* sampling lines was also validated [20].

Statistical analysis

Statistical analyses were performed using Microsoft Excel 2002 and SPSS (version 12.0) Software. Experimental results were expressed as mean ± standard deviation (M ± SD) for at least three different replicates. For airborne contaminants the percentage of cell viability at each test concentration was calculated [18]. Linear regression analysis was used for all correlation studies. One way analysis of variance (ANOVA) was used to compare the mean IC_{50} values of test chemicals in three cell types, followed by multiple comparisons (Post Hoc tests, Tukey HSD) to identify which cell type was statistically significantly different. Differences were considered statistically significant at $p<0.05$.

RESULTS

Cytotoxicity of soluble chemical contaminants

Complete dose-response curves were plotted for all test chemicals and cytotoxicity endpoints including the NOAEC (no observed adverse effect concentration),

Table 1. Analytical methods used for calibration of generated test atmospheres

Test Gas	Method Number	Sampling Method	Analytical Method
NO_2	6014 NIOSH	triethanolamine-impregnated molecular sieve sorbent tubes	spectrophotometry
SO_2	ID-104 OSHA	midget impinger containing hydrogen peroxide	ion chromatography, conductivity detector
NH_3	6015 NIOSH	sulfuric acid-treated silica gel solid sorbent tubes	reflectometry

NIOSH, US National institute for occupational safety and health; OSHA, US Occupational safety and health administration.

Table 2. The IC_{50} values of test chemicals in human cells with the MTS assay

Test Chemicals	IC_{50} values (M ± SD, mg/L)		
	Skin fibroblasts	HepG2-liver cells	A549-lung cells
Ammonia	612.2 ± 136.8	291.1 ± 30.5	192.8 ± 73.3
Cadmium chloride	808.7 ± 100.1	595.1 ± 30.3	651.1 ± 36.3
Cobalt chloride	12.0 ± 2.3	24.5 ± 3.3	26.9 ± 0.9
Formaldehyde	196.7 ± 36.7	103.8 ± 23.6	198.4 ± 9.5
Glutaraldehyde	99.9 ± 17.2	147.2 ± 37.9	201.7 ± 23.0
Manganese chloride	10.5 ± 1.7	15.6 ± 2.9	15.0 ± 1.2
Mercuric chloride	9.5 ± 1.8	9.3 ± 1.6	6.5 ± 1.0
Sodium dichromate	66.8 ± 16.0	138.9 ± 15.3	32.2 ± 0.7
Sulfurous acid	4.3 ± 0.7	6.3 ± 0.5	4.9 ± 0.4
Zinc chloride	418.3 ± 39.6	360.0 ± 27.8	197.1 ± 16.9

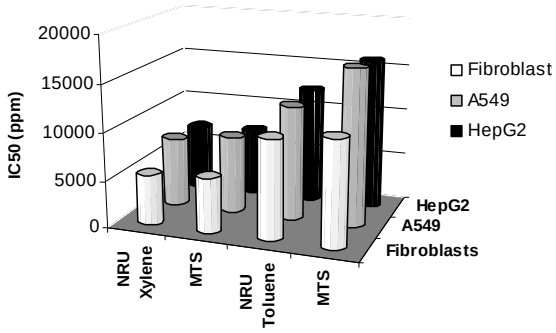

Fig 3. Airborne IC_{50} values of selected VOCs in different human cells.

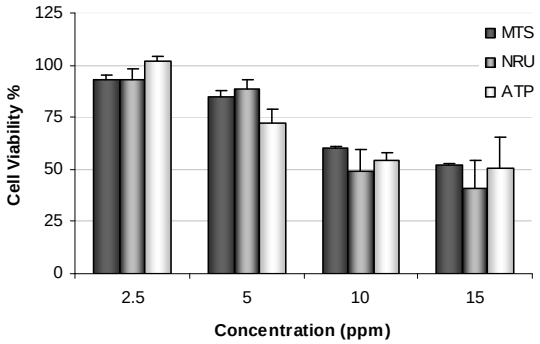

Fig 4. Cytotoxicity of NO_2 in human lung cells with three *in vitro* assays.

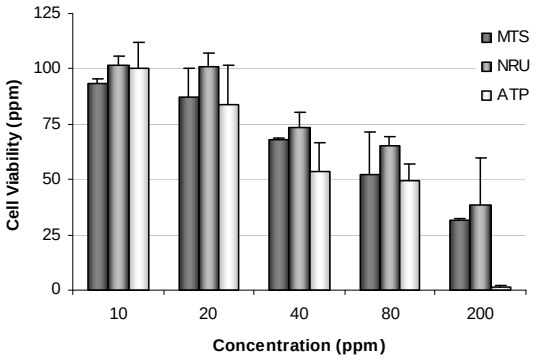

Fig 5. Cytotoxicity of SO_2 in human lung cells with three *in vitro* assays.

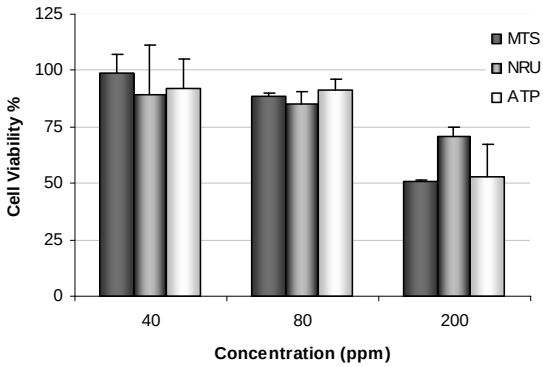

Fig 6. Cytotoxicity of NH_3 in human lung cells with three *in vitro* assays.

IC_{10} (10% inhibitory concentration), IC_{50} (50% inhibitory concentration) and TLC (total lethal concentration; IC_{100}) values were determined (Fig 2). The average IC_{50} values of test chemicals in selected human cells with the MTS assay are summarised in Table 2. While sulfurous acid (IC_{50} = 4.3-6.3 mg/L) and mercuric chloride (IC_{50} = 6.5-9.5 mg/L) were found to be the most toxic chemicals among selected soluble test chemicals, cadmium chloride appeared to be the least toxic chemical to all human cells tested with the MTS assay (IC_{50} = 595.1-808.7 mg/L).

Toxicity ranking of test chemicals

Based on obtained IC_{50} values, relative toxicity ranking of test chemicals in different human cells and both *in vitro* assays are summarized in Table 3.

Cytotoxicity of selected VOCs

The airborne concentration-effect curves of xylene and toluene in human cells were determined. Each point was generated from three separate experiments in which human cells on membranes were exposed to airborne concentrations of test chemical produced in a single glass chamber. The airborne IC_{50} values of test chemicals in three different human cells with two *in vitro* assays are presented in Fig 3. For airborne IC_{50} extrapolations both experimental and exponential curves were considered. The mean and standard deviations are calculated as a percentage of controls. Using *in vitro* test systems, xylene was found to be more toxic than toluene in all cell types tested with both MTS and NRU assays.

The lowest airborne IC_{50} values were measured for skin fibroblasts for test chemicals with both MTS and NRU assays. No statistical significant difference was observed between sensitivity of cell types except for toxicity of xylene with the MTS assay (one way ANOVA; $p<0.01$). In this case, multiple comparisons revealed that the sensitivity of skin fibroblasts was significantly different from A549 cell lines ($p<0.05$).

Cytotoxicity of selected gaseous contaminants

Airborne concentration-dependent effects of selected gaseous contaminants were observed in human cells with all selected *in vitro* assays. Cytotoxic effects of test gases in human A549 lung derived cell lines with three different *in vitro* assays are presented in Figs 4-6. Cell viability was significantly reduced after exposure of human cells to gaseous contaminants.

Toxicity ranking of airborne chemicals

Toxicity ranking of selected airborne chemicals in human A549-lung cells were determined (Table 4). The airborne IC_{50} values of test chemicals in human lung cells were compared to inhalational LC_{50} values for rat taken from the registry of toxic effects of chemical substances; RTECS [26]. Comparable toxicity ranking of selected VOCs (xylene > toluene) and gaseous contaminants (NO_2 > SO_2 > NH_3) were achieved using both *in vitro* and published *in vivo* data.

Table 3. Toxicity ranking of test chemicals in multiple human cell systems and *in vitro* assays

Chemicals	Fibroblasts-MTS	HepG2-MTS	A549-MTS	A549-NRU
Ammonia	9	8	6	7
Cadmium chloride	10	10	10	3
Cobalt chloride	4	4	4	9
Formaldehyd	7	5	8	6
Glutaraldehyde	6	7	9	2
Manganese chloride	3	3	3	10
Mercuric chloride	2	2	2	1
Sodium dichromate	5	6	5	4
Sulphureous acid	1	1	1	8
Zinc chloride	8	9	7	5

Table 4. Toxicity ranking of airborne chemicals in human A549-lung cells

Airborne Chemicals	*In Vitro* Toxicity Data		*In Vivo* Toxicity Data	
	IC_{50} (A549) m ± SD; ppm/h	*In vitro* assay	LC_{50} (Rat) ppm/h	Reference
Gases				
NO_2	11 ± 3.54	NRU	117	RTECS
SO_2	48 ± 2.83	ATP	2520	RTECS
NH_3	199 ± 1.41	MTS	2000*	RTECS
Vapours				
Xylene	7400 ± 1389	NRU	5000*	RTECS
Toluene	12100 ± 2257	NRU	13000*	RTECS

* After 4 h exposure

DISCUSSION

Exposure to air pollutants is significantly associated with both short-term and long-term health effects. While an extensive background database from *in vivo* toxicological studies have been developed, most toxicity data is from oral and dermal chemical exposures rather than inhalation exposure [11-12]. An integrated approach was designed to optimise *in vitro* methods as an alternative model for toxicity testing of airborne contaminants. The MTS, NRU and ATP assays were adapted to measure the *in vitro* effects of selected air pollutants in human cells.

Primarily the application and merits of *in vitro* methods for prediction of toxicity of selected soluble chemical contaminants including ammonium hydroxide, cadmium chloride, cobalt chloride, formaldehyde, glutaraldehyde, manganese chloride, mercuric chloride, sodium dichromate, sulfurous acid and zinc chloride were investigated. *In vitro* cytotoxicity data including the NOAEC, IC_{10}, IC_{50} and TLC values of test chemicals were calculated from plotted dose response curves for all test chemicals. The average IC_{50} values of test chemicals in human cells with the MTS assay are summarised in Table 2. Linear regression analysis was used to characterise the relationship between cytotoxicity of test chemicals in human cells *in vitro* and published *in vivo* acute toxicity data. Human HepG2 cells appeared to be the best predictor for acute *in vivo* toxicity ($r = 0.99$, $p<0.001$) followed by skin fibroblasts (data not shown; $r = 0.97$, $p<0.001$). Similarly, it has been reported that acute human toxicity could be more accurately predicted using human hepatocytes than using rat hepatocytes or murine nonhepatic 3T3 cells [27-28].

Toxicity ranking of test chemicals, based on obtained IC_{50} values, in different human cells and both *in vitro* assays are summarized in Table 3. Among selected workplace chemicals, sulphureous acid (e.g. $IC_{50} = 4.89 \pm 0.38$ mg/L, in A549 cells) and mercuric chloride (e.g. $IC_{50} = 6.51 \pm 1.01$ mg/L, in A549 cells) were found to be the most toxic chemicals to all human cells tested with the MTS assay. Mercuric chloride was also appeared to be the most toxic chemical to A549 cells with the NRU assay ($IC_{50} = 7.32 \pm 0.61$ mg/L). In contrast, while cadmium chloride was appeared to be the least toxic chemicals among selected workplace contaminants using the MTS assay (e.g. $IC_{50} = 651.09 \pm 36.30$ mg/L, in A549 cells), Manganese chloride was shown the least toxicity ranking among selected test chemicals with the NRU assay ($IC_{50} = 3080.66 \pm 666.98$ mg/L, in A549 cells). However, different results of biological endpoints may provide an indication of the possible mechanisms responsible for the cytotoxic effects of test chemicals. For example, chemicals which have a direct effect on lysosomes or interfere with mitochondrial enzymes may exhibit greater toxicity in the NRU and MTS assays, respectively.

The study of the toxic effects of inhaled chemicals is typically more challenging due to the technology required for the generation and characterisation of test atmospheres, and the development of effective and reproducible techniques for exposure of target cells to airborne chemicals [9, 13, 29]. Although inhalation studies are technologically more complicated, proper hazard characterizations are essential for comparable risk assessment of inhaled materials. Therefore, in the next phase, direct exposure techniques were established to study the toxicity of airborne pollutants.

A static direct exposure technique was developed for toxicity assessment of VOCs as an important group of occupational and environmental airborne contaminants. While exposure to volatile chemicals is a significant contributor to human health problems, toxicity testing of VOCs has always faced significant technological problems [30-33]. Apart from high volatility, many of these are slightly soluble or insoluble in water. These physicochemical properties may produce technical challenges during the course of *in vitro* experiments [18]. In the static method, test atmospheres of vapours of xylene and toluene were generated in small glass chambers with known volumes. Human cells were exposed to vapours of test chemicals at different concentrations directly at the air/liquid interface and cytotoxicity was investigated using the MTS and NRU *in vitro* assays. Airborne concentration-dependent effects of volatile test chemicals were observed in all human cells tested. Using the static direct exposure method enabled the establishment of airborne IC_{50} values for selected VOCs (Fig 3). Comparable toxicity ranking of test chemicals (xylene > toluene) was achieved using both *in vitro* and published inhalational *in vivo* data (Table 4). The static direct exposure method proved to be a practical and reproducible technique for *in vitro* inhalation study of xylene and toluene.

The cytotoxicity of well known indoor and outdoor air pollutants including NO_2, SO_2 and NH_3 was investigated in human A549-lung derived cells using the dynamic direct exposure method (Figs 4-6). Airborne IC_{50} values were calculated based on the most sensitive *in vitro* assays for each test gas (Table 4). All three *in vitro* assays revealed similar toxicity ranking for selected gaseous contaminants. Comparable toxicity ranking of selected gases (NO_2 > SO_2 > NH_3) were achieved using both *in vitro* and published inhalational *in vivo* data (Table 4).

It is anticipated that in the future, more and more emphasis will be placed on *in vitro* toxicity test methods to study toxicity [34]. Although *in vitro* data is not a direct substitute for whole animal studies [9], comparable *in vitro* exposure techniques can supplement *in vivo* toxicity tests for conventional quantitative risk assessment of inhaled chemicals. Even when the target cells are in the respiratory system, and therefore potentially directly exposed to an air/tissue interface, the relationship between inspired airborne concentrations and target cell concentrations is still going to be influenced by toxicokinetic factors, such as respiratory tract absorption and/or deposition. Therefore, the application of predictive tools such physiologically based toxicokinetic (PBTK) models may provide a scientific basis for extrapolation of *in vitro* concentrations which produce cellular toxicity *in vitro*, to equivalent *in vivo* dosages [9].

CONCLUSION

Although studying the toxic effects of inhaled chemicals is more challenging, promising *in vitro* exposure techniques have been developed in this research that may offer new possibilities to test biological activities of inhaled chemicals under biphasic conditions at the air liquid interface. An optimal *in vitro* exposure system for studying the cellular interactions upon chemical airborne exposures needs to meet several requirements such as direct exposure of target cells to unmodified airborne chemicals, continuous nourishment of cells during exposure and duplication of *in vivo* parameters as closely as possible [9, 20, 35, 36]. Considering these criteria, our findings demonstrate that both static and dynamic direct exposure of human cells to airborne pollutants have a potential to be applied more extensively for investigating the toxic effects of inhaled chemicals.

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