In Vitro Cytotoxicity Test on Human Cells for Assessment of Fire Combustion Products Using a Tube Furnace

F. Lestari[1], A.R. Green[2], A.J. Hayes[1], G. Chattopadhyay[3], C.A Khalil[1], and B. Markovic[1]

[1]*Chemical Safety and Applied Toxicology (CSAT) Laboratories, School of Safety Science, The University of New South Wales, Sydney 2052, Australia*
[2]*School of Safety Science, The University of New South Wales, Sydney 2052, Australia*
[3]*School of Civil and Environmental Engineering, The University of New South Wales, Sydney 2052, Australia*

Abstract

It is known that the exposure to toxic gases and smoke is the major cause of injury and death in fires. This research investigates *in vitro* cytotoxicity test for toxicity assessment of fire effluents. Three different human cells and cultures were used: liver (HepG2), lung (A549) and skin fibroblasts. Two different exposure modes were developed: impinger and direct method for exposure of human cells to fire combustion products. The later technique utilized a membrane (Snapwell^{TM} inserts) for growing the human cells, which were placed on the Harvard/Navicyte Horizontal Diffusion Chamber. The MTS *in vitro* cytotoxicity assay (Promega) was adopted for cell viability assessment. A laboratory small-scale fire test using a vertical tube furnace was developed for the generation of combustion products. Preliminary studies using carbon dioxide gas showed a reduction in cell viability using the two exposure modes. These results indicate the potential to determine the fire hazard of materials and products under different fire conditions and the impact of these toxic emissions on human cells.

1. Introduction

Smoke inhalation of toxic compounds has often been cited as the cause of death in fires, and approximately 80% of fire deaths reported are attributed to inhalation of toxic smoke and hot gases [1, 2].

Corresponding author-Tel.:Fatma Lestari
+61-2-9385-4746; *Fax*:+61-2-9385-6190.
Email address:fatma@student.unsw.edu.au

Although safety regulations and codes contribute to reduced health threats during fires, there exists an increasing incidence of death and injury, particularly from exposure to toxic smoke [3]. There are a wide variety of fire combustion products that may be generated during a fire, which comprise of gases, organic vapours, and

particulate matter. Of these compounds, a wide range of toxicological effects may be produced from sub-lethal effects to acute effects, which can appear within minutes [2,4]. To date, the assessment of toxicological hazards of fire involves the exposure of animals to fire combustion products [1], and the current international standard still relies on this method [5]. However, this method involves a large number of animals, lengthy procedures (two or three weeks) accompanied by high discomfort to animals [6]. There has been pressure to reduce and stop the use of animals in experimentation especially from animal welfare groups. Indeed, the significance of LD_{50} values for toxicity evaluation has been questioned [7] as it provides poor correlation to human data.

The use of *in vitro* cell culture techniques as an alternative to predict acute lethality *in vivo* has been studied for almost 50 years [8]. This technique has been widely used particularly in the pharmaceutical industry [9] and has broadened into other areas such as: environmental, cosmetic industrial chemical toxicology [10, 11]. Many studies have shown a strong correlation between cytotoxicity *in vitro* and animal lethality *in vivo* [12, 13]. Indeed, this technique offers several advantages such as: cost-effectiveness and sensitivity, and can be used to measure the toxicity of mixtures [14, 15].

Research in the area of *in vitro* toxicity testing of fire combustion products has been limited, especially in the use of human derived cells. The aim of this study was to develop a rapid toxicity method using human cells for determination of fire hazard materials and products under different fire conditions.

2. Methodology

2.1 Tube Furnace

The tube furnace arrangement is shown in Figure 1. It consists of three different parts: vertical furnace, load cell connected to the computer for mass loss measurement, and polycarbonate box for mixing and measurement of combustion products (Figure 1). A sample (10-20 g) is mounted vertically on a load cell in the furnace. A gas stream (air, in this procedure) of 1 L/min is passed over the sample during the test. The fire products that are released during the test are then diluted with a secondary gas stream (air, in this procedure) to a total flow of 10-50 L/min. The combustion products are then sampled using charcoal and Tenax solid adsorbent for chemical analysis using ATD-GC/MS (Automatic Thermal Desorption-GC/MS) and for cytotoxicity testing as explained below.

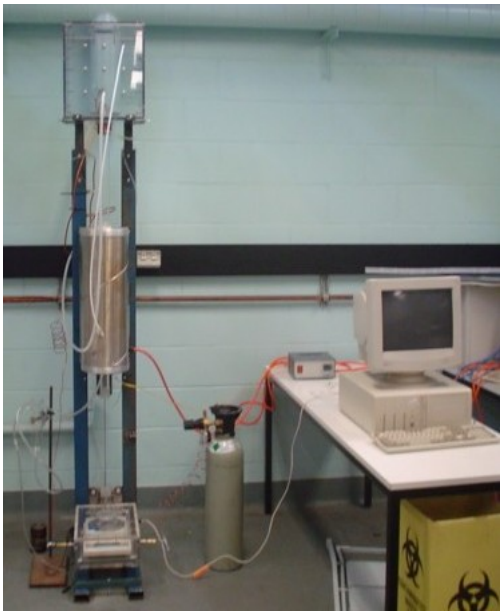

Figure 1. Tube Furnace arrangement

2.2 Materials

In the preliminary study, carbon dioxide (CO_2) gas (CAS No. 124-38-9; BOC gases) was used as a model gas for method

development and refinement. Selected materials including PMMA (Polymethlmetacrylate) are currently being studied at different decomposition conditions.

2.3 Controls

Two internal controls were set up for each experiment: (1) an IC_0 consisting of cells only; and (2) an IC_{100} consisting of medium only. Background absorbance due to the non-specific reaction between test compounds and the MTS reagent was deducted from exposed cell values as described by Hayes and Markovic [16]. Instrumental grade synthetic air (BOC gas) was used as a control for comparison against exposure with CO_2.

2.4 Experimental conditions

Exposure of cells to carbon dioxide was performed at ambient temperature. Oxidative non-flaming conditions were applied to study the combustion products released from PMMA: temperature: 350^{o}C, primary air flow-rate: 1 L/min, and secondary air flow-rate: 10-50 L/min.

2.5 Human cell lines and cell cultures

Primary cell cultures of human skin fibroblasts were obtained from fresh skin biopsies taken from the arm of healthy individuals (Cytogenetics Department, The New Children's Hospital, Westmead, Australia). Permanent cell lines included those of HepG2 liver cells derived from a human hepatocarcinoma (ATCC No. HB8065) and A549 lung cells derived from a human epithelial lung carcinoma (ATCC No. CCL-185).

2.6 Culture methods

All cultures were maintained in a phenol red free medium DMEM/F12 (Gibco Invitrogen), supplemented with 5% (v/v) serum (JSBioscience, Australia) and 0.01% (v/v) of antibiotic (Sigma, USA). Cultures were maintained at 37^{o}C in a humidified 5% CO_2 incubator.

2.7 Exposure system

Two different exposure systems were developed for the exposure of fire combustion products in our laboratory (Chemical Safety and Applied Toxicology Laboratories, UNSW, Sydney, Australia) using an impinger method and a direct method.

2.7.1 Impinger

Gas and fire combustion products were bubbled through 5-10 ml of serum free culture medium in the impinger for 30 minutes at a flow rate of 200 ml/min. After completion of the exposure time, 5% (v/v) fetal calf serum (JSBioscience, Australia) was added. This mixture was then transferred into 96 well plates, where serial dilution was applied as described by Malich *et al*. (14,15) directly on the 96 well microplates (Cellstar, greiner bio-one).

2.7.2 Direct exposure

Figure 2 displays the cross section and schematic diagram of the individual Horizontal Harvard/Navicyte Diffusion Chamber for the direct exposure. This chamber consisted of two parts: top part for gas inlet/outlet, and bottom part for culture medium inlet/outlet, with the membrane placed between these parts. The complete arrangement is shown in Figure 3 where the six chambers are connected with the thermal regulator. The human cells were grown on a porous membrane (Snapwell^{TM} insert) 24 hrs before exposure. During exposure time, the serum free culture medium was added only to the bottom part, so the cells on the top part were exposed directly to the combustion products. After the exposure time, the membrane was removed from the chamber, and placed into six well plates, which contained culture medium, supplemented with serum and antibiotic. Plate was then incubated for 24 hrs prior to performing

the *in vitro* cytotoxicity assay .

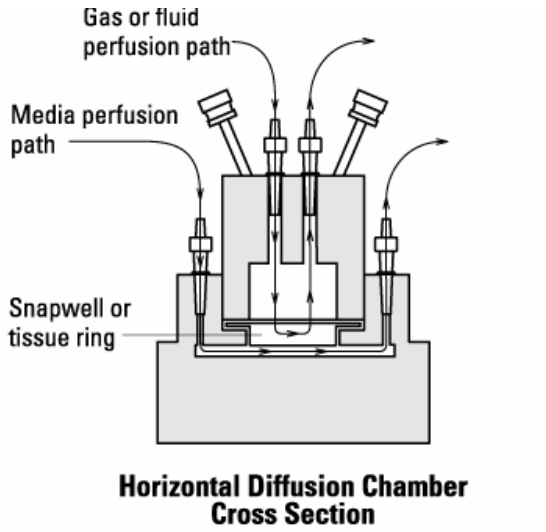

Figure 2. Schematic diagram of the *in vitro* Horizontal Diffusion Chamber for direct exposure

Figure 3. *In vitro* chamber arrangement for direct exposure

2.8 MTS Assay Cytotoxicity testing

The cytotoxicity testing was performed using the Promega CellTiter 96AQ$_{ueous}$ Non-Radioactive Cell Proliferation (MTS) Assay in conjunction with the addition of electron coupling reagent (PMS, Sigma) [17]. Evaluation of cytotoxicity of carbon dioxide involved test protocols as described by Malich *et al.* [14, 15].

For the impinger system, the plates were incubated at 37°C for 4 hrs with MTS/PMS mixture, the plates were placed on a mechanical plate shaker of a computerized microwell plate reader (Multiskan MS, Labsystem, Finland), shaken for 10 sec and absorbance read at 492 nm. Each experiment was repeated a minimum of three times. A typical MTS assay plate is displayed in Figure 4.

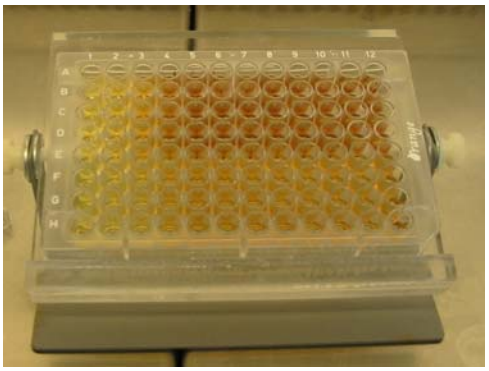

Figure 4. MTS Cytotoxicity test

In the direct exposure system, culture medium was added on top of the membrane and an aliquot of 100 µl of MTS/PMS mixture was added to the inside of the snapwell membrane. The plates were placed in the incubator at 37^{o}C and humidified for 1-hr. After the incubation period, aliquots of 40 µl x 4 of the liquid from the top part of the membrane was transferred to the 384 well plates for absorbance reading with the Multiskan reader. Each experiment was repeated a minimum of three times.

2.9 Results and Discussion

Preliminary studies exposing carbon dioxide to cells using the impinger method resulted in a 5% reduction of HepG2 cell viability, 20% reduction of A549 and 44% of skin fibroblast cell viability compared to controls. While in the direct exposure mode, a 24% reduction of HepG2, 34% reduction of A549 and 50% reduction of fibroblast cell viability compared to controls was achieved (Figure 5 and 6). Carbon dioxide is non-toxic at levels normally observed in fires. However, exposure to high levels of CO_2 stimulates the breathing rate and causes the

accelerated uptake of toxicants and irritants from combustion products. The *in vitro* exposure of carbon dioxide to human cells caused a reduction of cell viability as observed on the three human cells. The mechanism underlying this cytotoxic response is unclear, since the effect of exposure to carbon dioxide *in vitro* is poorly researched. However, it may be assumed that there is hypoxia condition as a result of acidosis in the cell culture.

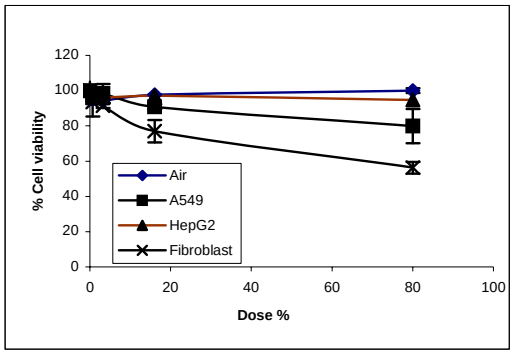

Figure 5. Cytotoxicity of carbon dioxide on human cells using impinger method

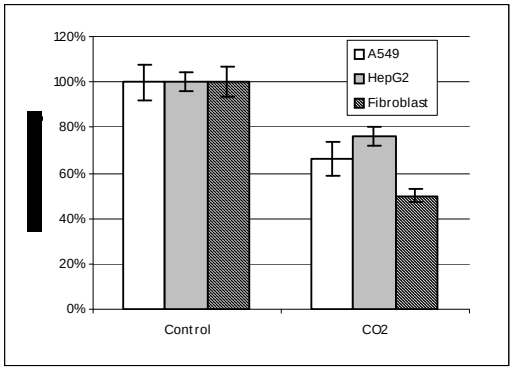

Figure 6. Cytotoxicity of carbon dioxide on human cells using direct exposure method

The system involving direct exposure was chosen for the exposure of human cultures to the fire effluents, as it simulates the exposure mechanism as would occur in humans. Direct exposure overcomes the problem of the impinger method as it eliminates the solubility problem of insoluble combustion products in the culture medium.

A small-scale tube furnace was adopted as the fire test method as it has the potential to model a wide range of fire conditions by using different combinations of temperature, non-flaming and flaming decomposition conditions and different fuel/oxygen ratios in the tube furnace [19]. It is widely used and recommended in several international and regional standards such as German standard (DIN 53436), UK standards [18, 19] and ISO standards [20] for the generation of thermal decomposition products and toxicological evaluation. However, in these standards, the furnace orientation is horizontal and no mass loss measurement is made on the test sample. In our experiment, the vertical orientation was chosen as it enabled the use of a simple system for mass loss measurement; which allowed data from the toxicity measurements to be quantified more readily and correlated to other fire material data such as that derived from cone calorimetry. Several materials are currently being studied under different decomposition conditions using this furnace.

3. Conclusion

The results of CO_2 studies and preliminary trials with chemicals typical of fire products (data not shown), strongly suggest that the method can be used to determine the fire hazard of materials and products under different fire conditions and the impact of the toxic emissions from fire effluents on human cells.

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