OPTIMIZATION OF THE VITROCELL® EXPOSURE SYSTEM FOR *IN VITRO* TOXICITY TESTING OF DIESEL EMISSIONS AT THE AIR-LIQUID INTERFACE

Rebecca Greenan[1], Tak Chan[2], Mathias Könczöl[3], Julie Bourdon[1], Debbie Rosenblatt[2], Marie-Josée Poitras[2], and Paul A. White[1]

[1]Mechanistic Studies Division, Environmental Health Science and Research Bureau, Health Canada, Ottawa, ON, Canada
[2]Emissions Research and Measurement Section, Environment Canada, Ottawa, ON, Canada
[3]Department of Pharmaceutical Biology and Biotechnology, University of Freiburg, Germany

INTRODUCTION

Diesel Emissions

•Known human carcinogen (IARC Monograph 105).[1]
•Emission composition is dependent on a large number of variables, e.g., engine design, maintenance, test cycle, operating conditions, fuel formulation, and after-treatment. [2]
•Changes in exhaust composition may result in significant alterations in toxicity.

Study Purpose:

Development of an *in vitro* method for toxicological profiling of diesel emissions following exposure of mammalian cells at an air-liquid interface. Will permit assessment of changes in toxicity related to changes in fuel formulation and/or aftertreatment.

Conventional Toxicity Testing

In Vivo:

- Whole Exhaust or Gas Phase: Inhalation exposure of rodents.
- Particulate Matter (PM) or PM Extracts: Intratracheal or intrapulmonary instillation in rodents.

Expensive and time-consuming; not suitable for assessment of multiple variables.

In Vitro:

- Gas Phase: Bubbled through liquid culture medium.
- PM or PM Extracts: Exposure in liquid culture medium.
- Not a realistic model of inhalation exposure.

VITROCELL® Exposure Device

•Cultured cells are directly exposed to diluted aerosols at an air-liquid interface.
•Ideal for the assessment of complex aerosols, i.e., diluted vehicle exhaust.
•Relatively high-throughput.

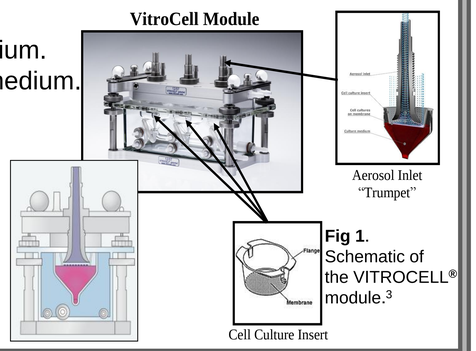

Fig 1. Schematic of the VITROCELL® module.[3]

EXPERIMENTAL DESIGN

Air-liquid interface exposures of cultured cells to diluted emissions from a light-duty diesel engine fueled with diesel, biodiesel, and diesel-biodiesel blends.

Cell Culture

•A549 (adenocarcinomic human alveolar epithelium) cells were seeded on microporous membrane inserts (Greiner Bio-One, USA).

Fuel Formulations Examined:

- **ULSD**: Ultra-low Sulfur Diesel
- **B20 Tallow**: 20% tallow biodiesel; 80% ULSD
- **B20 Canola**: 20% canola biodiesel; 80% ULSD
- **B100 Tallow**: 100% tallow biodiesel
- **B100 Canola**: 100% canola biodiesel

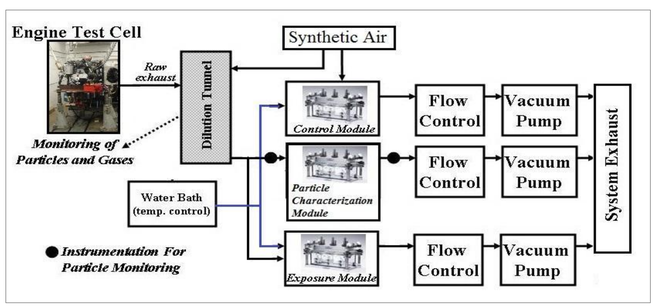

Fig 2. Schematic of the set-up for diesel exhaust toxicity testing with the VITROCELL®.

Table 1. Engine Parameters

Manufacturer:	Volkswagen
Engine Model:	1998-2003 ALH (from 2001 VW beetle)
Engine Type:	4 cylinder, turbocharged, direct injection diesel
Displacement:	1.9 liter
After-Treatment:	300 ppmS diesel oxidation catalyst (DOC) – OEM
Nominal Engine Speed:	2000 rpm
Engine Power:	19 kW
Engine Torque:	72 ft lb
Emission Dilution to VITROCELL®:	40x

Endpoints Examined:

- **Cytotoxicity**
  - **WST-1** (Water-soluble tetrazolium salt-1)
    - Measures mitochondrial function.
  - **Neutral Red**
    - Measures lysosomal integrity.
  - **Caspase III/VII**
    - Measures presence of executioner caspases 3 and 7 as an indicator of apoptosis.
- **Oxidative Stress**
  - **TBARS** (Thiobarbituric acid reactive substances)
    - Measures malondialdehyde (MDA) as a marker of lipid peroxidation.

RESULTS & DISCUSSION

Characterization of Emissions

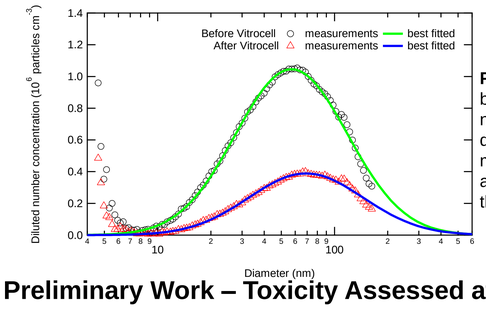

Fig 3. Comparison between the average number size distributions measured upstream and downstream of the VITROCELL®.

Table 2. Average emissions of priority pollutants, measured anterior to the VITROCELL® and corrected to reflect concentrations at the VITROCELL® (ULSD fuel).

CO (ppm):	3.46
CO_2 (%):	0.40
NO_x (ppm):	16.66
NO (ppm):	9.70
NO_2 (ppm):	7.15
Total hydrocarbon (ppm):	3.58
Total concentration (particles/ml):	4.85 x 10^6

Preliminary Work – Toxicity Assessed at a Single Endpoint

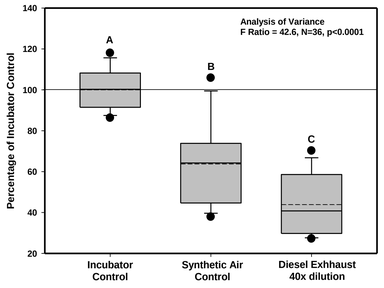

Fig 4. Effect of cell treatment on cell viability as determined by WST-1 assay. All values are expressed relative to the incubator control. Post-hoc comparisons (Duncan) showed significant differences between all means at p<0.05.

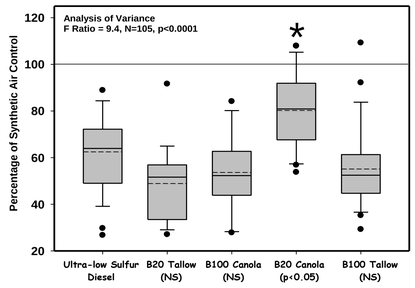

Fig 5. Effect of fuel formulation on cell viability as determined by WST-1 assay. All values are expressed relative to the synthetic air control. Post-hoc comparisons (Dunnett) showed that only B20 canola is significantly different (less toxic) than ULSD (p<0.05).

- Results indicated that optimization of the exposure system (i.e., reduction of toxicity not attributable to diesel exhaust) was necessary to increase sensitivity for toxicity assessment.
- More endpoints were needed to fully characterize the biological response of A549 cells.

Optimization of the Exposure System:

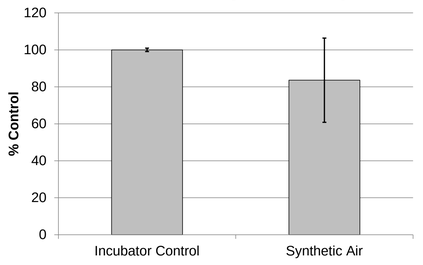

Fig 6. WST-1 assay for mitochondrial function. Cells were exposed to 5 ml/min synthetic air for 1 hour. (NS, two-tailed t-test).

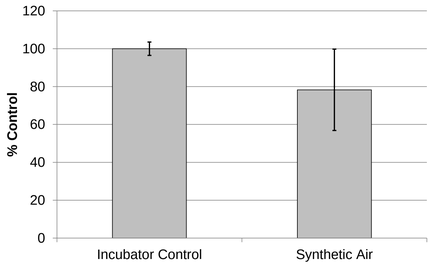

Fig 7. Neutral Red assay for lysosomal integrity. Cells were exposed to 5 ml/min synthetic air for 1 hour (NS, two-tailed t-test).

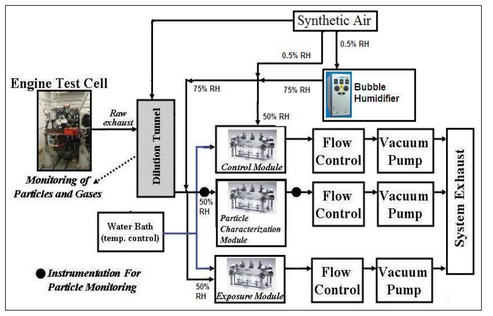

Fig 8. Proposed schematic for the incorporation of a bubble humidifier into the set-up for diesel exhaust toxicity testing, to reduce observed toxicity not attributable to diesel exhaust.

- Handling and exposure protocols were revised to increase viability of the cells *(Figure 6, Figure 7).*
- The synthetic air control was determined to have a relative humidity of 0.5%. This likely reduced viability due to dehydration. A bubble humidifier with computer-controlled gas delivery system was purchased from TesSol.[4] Work is underway to incorporate this instrument into the diesel exposure set-up *(Figure 8).*

Incorporation of Additional Endpoints

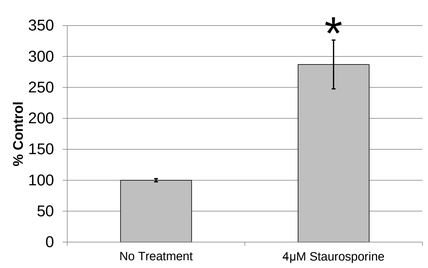

Fig 9. Caspase III/VII assay for apoptosis. Cells cultured on microporous inserts were exposed to 4μM staurosporine in media for 4 hours. (p<0.05, two-tailed t-test).

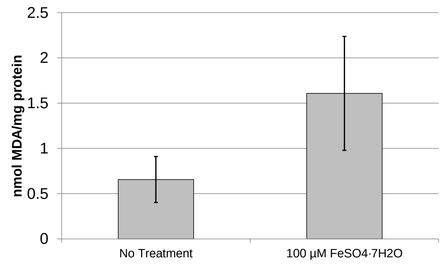

Fig 10. TBARS assay for lipid peroxidation. Cells cultured on microporous inserts were exposed to 100 μM ferric sulfate heptahydrate in media for 4 hours. (NS, two-tailed t-test).

- Assays are being validated with known positive controls (*Figure 9, Figure 10)* before use for aerosol assessments.

NEXT STEPS

- Continued optimization of assays for aerosol exposures, e.g., determining optimal post-exposure incubation for assessment of apoptosis.
- Incorporation of the humidification system into the diesel exposure set-up.
- High-throughput screening of diesel emissions collected under multiple conditions, starting with a repeat of preliminary exposures (i.e., diesel, biodiesel, and biodiesel blends).
- Incorporation of more endpoints for use with the VITROCELL®, i.e., oxidative stress and genotoxicity assessment.

REFERENCES

[1] Benbrahim-Tallaa, *et al*. 2012. *The Lancet Oncology* **13**(7): 663-664. [2] U.S. EPA, 2002. *Health Assessment Document for Diesel Engine Exhaust* **EPA/600/8-90/057F**. [2] VITROCELL Systems, 2011. http://www.vitrocell.com
[3] Fideris TesSol, Incorporated, 2012. http://www.fideris.com

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