ASSESSING THE CYTOTOXICTY OF BLACK CARBON AS A MODEL FOR ULTRAFINE ANTHROPOGENIC AEROSOL ON HUMAN EPITHELIAL LUNG CELLS AND MURINE MACROPHAGES

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ABSTRACT

Combustion-derived nanomaterials or ultrafine (<1 µm) atmospheric aerosols are primarily products of anthropogenic activities, such as the burning of fossil fuels. Ultrafine particles (UFPs) can absorb other noxious pollutants including volatile organic compounds (VOCs), polycyclic aromatic hydrocarbons (PAHs), toxic organic compounds, and heavy metals. The combination of high population density, meteorological conditions, and industrial productivity brings high levels of air pollution to the metropolitan area of El Paso, Texas, USA/ Ciudad Juarez, Chihuahua, Mexico, comprising the Paso del Norte air basin. A study conducted at Research Triangle Park, NC analyzed sites adjacent to heavy-traffic highways in El Paso and elucidated higher UFP concentrations in comparison to previously published work exploring pollution and adverse health effects in the basin.

UFPs can penetrate deep into the alveolar sacs of the lung, reaching distant alveolar sacs and inducing a series of immune responses that are detrimental to the body: evidence suggests that UFPs can also cross the alveolar-blood barrier and potentially endanger the body's systemic immune response. The properties of UFPs and the dynamics of local atmospheric and topographical conditions indicate that emissions of nanosized carbonaceous aerosols could pose significant threats to biological tissues upon inhalation by residents of the Paso del Norte.

The research conducted will ultimately help identify constituents of the Paso del Norte ultrafine particles or aerosols that specifically contribute to increased incidence of respiratory ailments and to help strategically develop therapies for local residents adversely impacted by these. Future work will investigate how chronic exposure to BC induces oxidative stress and activates cytokine production, processes concomitant with diseases of the lung, and ultimately improve understanding of the mechanisms of impact of nanosized PM on human health.

INTRODUCTION & PURPOSE

This study utilizes Black Carbon (BC) as a model for environmental UFPs and its effects on pulmonary cellular response. BC is formed via incomplete combustion of fossil fuels and is emitted directly into the atmosphere. Due to its size it has access to the deeper recesses of the lungs presenting a unique biological hazard (Figure 1). An *in vitro* approach is used to assess BC biological activity upon long-term exposure due to its feasibility, reproducibility, low-cost of overall laboratory needs and ability to access biochemical processes. The two cells utilized are epithelial and macrophage in derivation. Epithelial lung cells help maintain epithelial moisture and trap particulate material and pathogens transported through the airway. Macrophages are specialized cells of the immune system that recognize, engulf, and destroy foreign materials.

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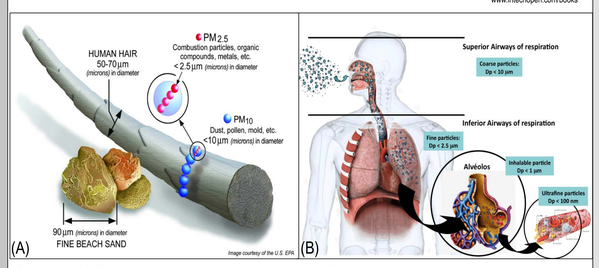

Figure 1. UFPs size relative to human hair (A) and infiltration into respiratory airways (B).

***PURPOSE:* To optimize an experimental *in vitro* model focusing on concentration and duration of BC exposure to determine a dose-dependent induction of pulmonary epithelial/ macrophage death. The model will delineate a chronic study to better address my overarching dissertation goal, which is to analyze BC UFPs and their impact on pulmonary function and determine their potential effects and contributions to pulmonary pathology.**

METHODS

Human epithelial lung cells (A549) seeded at 600 cells/mL and murine macrophages (RAW264.7) seeded at 900 cells/mL were treated with BC and assessed for metabolic activity after chronic exposure. The cell viability experiments included a chronic study at 7, 10, and 14 days of UFP exposure at six different concentrations of BC: **100, 300, 600, 1,250, 2,500, and 5,000 µM.**

The following assays were performed: Trypan blue exclusion test, Calcein-AM viability assay, and CellTiter- Glo viability assay. The trypan blue exclusion test determines the number of viable cells based on the principle that live cells have membranes that remain intact and exclude dyes like trypan blue, whereas dead cells do not. Calcein-AM is a cell-permeant dye that undergoes hydrolysis by live cells via mitochondrial enzymes, yielding a fluorescent product. CellTiter-Glo luminescent assay calculates metabolically active cells by quantitating the ATP.

The Trypan Blue Exclusion Assay was carried out using the Cellometer Auto T4 Cell Counter with bright field imaging and software by Nexcelom Bioscience. The Calcein-AM Assay was completed using the Fluoroskan Ascent Microplate Fluorometer by Thermo Scientific and Fluoroskan Ascent software. The Cell Titer- Glo Assay was completed using the Luminoskan Ascent Microplate Luminometer by Thermo Scientific and Luminoskan Ascent software.

Statistical Analysis was carried out using R studio. Non-parametric tests were performed on non-continuous, skewed data. Kruskal-Wallis test and kruskalmc post-hoc test were done upon installation of the {pgirmess} package

TEM IMAGES FOR BLACK CARBON

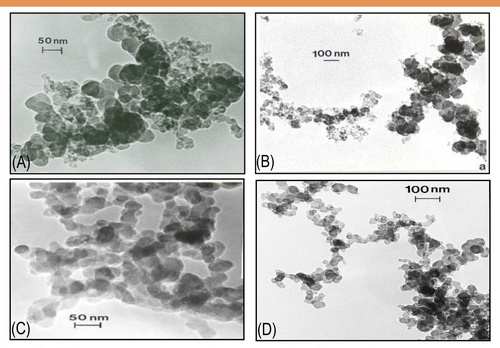

Figure 2. TEM image of commercially produced (A & B) and anthropogenic/combustion generated (C & D) Black Carbon containing spherule and branched aggregates. (A) Murr *et al.*, 2005; (B) Soto *et al.*, 2008; (C) Soto *et al.*, 2008; and (D) Murr and Garza, 2009.

EXPOSURE REGIMEN

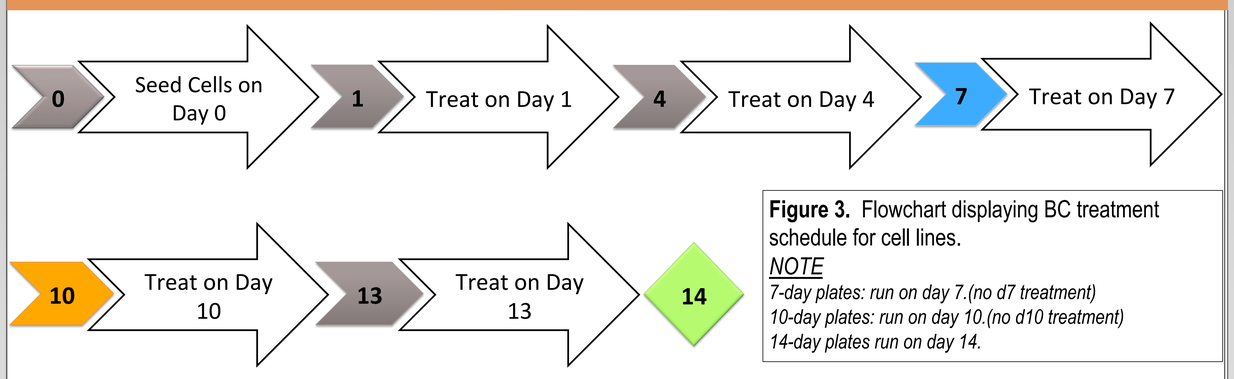

Figure 3. Flowchart displaying BC treatment schedule for cell lines.
NOTE
7-day plates: run on day 7.(no d7 treatment)
10-day plates: run on day 10.(no d10 treatment)
14-day plates run on day 14.

VIABILITY DATA FOR HUMAN EPITHELIAL LUNG CELLS (A549)

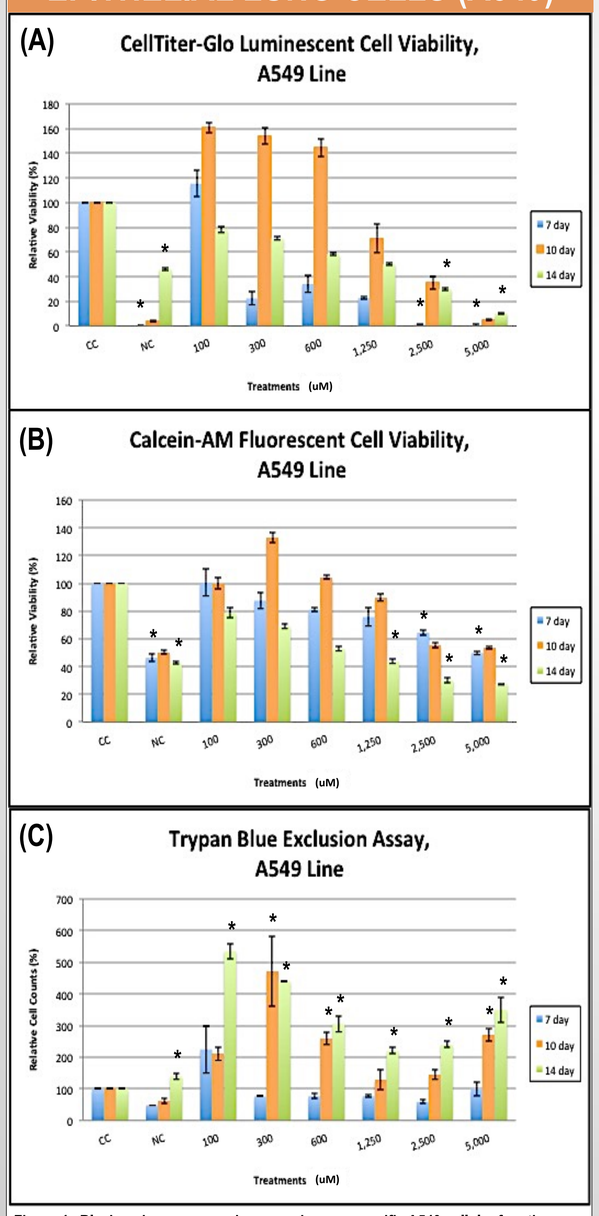

Figure 4. Black carbon compromises or enhances specific A549 cellular functions depending on dose/length of exposure. A549s were seeded, then treated with BC as illustrated in the "Exposure Regimen". On day 7, 10, or 14, the cells were harvested and assessed for viability based on: (A) mitochondrial enzyme activity (CellTiter-Glo Assay); (B) cytoplasmic enzyme activity (Calcein-AM Assay); or (C) cell membrane integrity (Trypan Blue Exclusion Assay). The data is presented as the mean of duplicate wells ± SE. *p ≤ 0.05 (in comparison to day-matched cellular control).

TB Viability A549	7 Day		10 Day		14 Day	
Treatments uM	*Avg (%)*	*SE*	*Avg (%)*	*SE*	*Avg (%)*	*SE*
Cell Control	55	11	72	9	63	2
Neg Control	47	13	24	13	63	3
100	42	5	48	6	62	2
300	19	0	68	3	58	4
600	20	3	59	2	44	6
1,250	27	2	42	12	37	1
2,500	28	3	32	2	32	1
5,000	28	7	38	3	37	8

VIABILITY DATA FOR MURINE MACROPHAGES (RAW264.7)

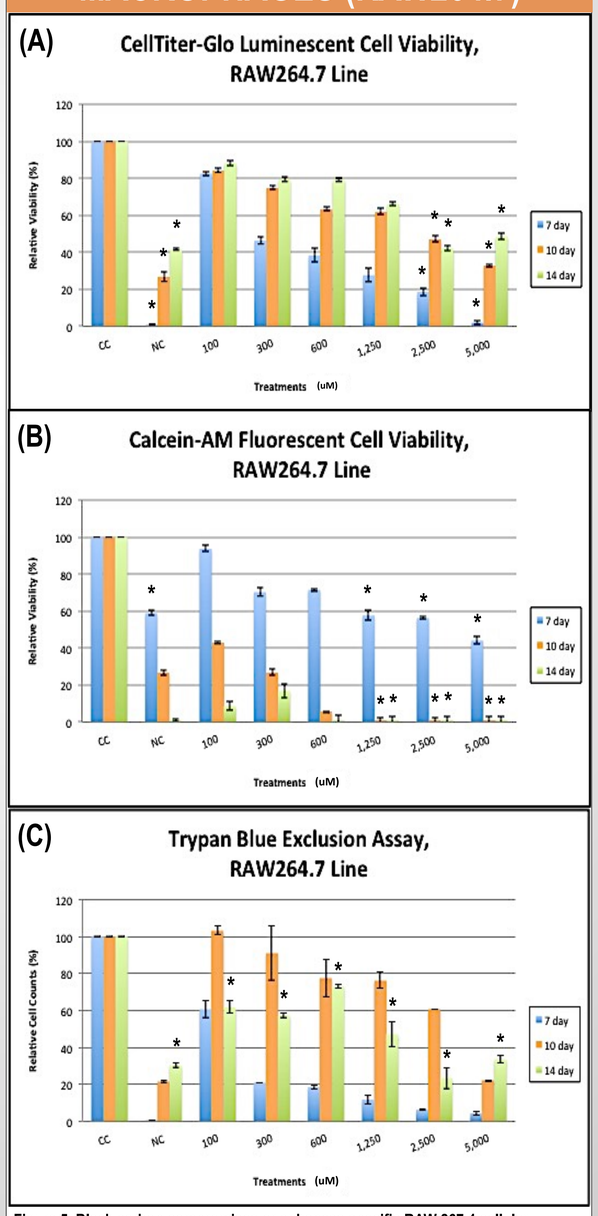

Figure 5. Black carbon compromises or enhances specific RAW 267.4 cellular functions depending on dose/length of exposure. RAW264.7s were seeded, then treated with BC as illustrated in the "Exposure Regimen". On day 7, 10, or 14, the cells were harvested and assessed for viability based on: (A) mitochondrial enzyme activity (CellTiter-Glo Assay); (B) cytoplasmic enzyme activity (Calcein-AM Assay); or (C) cell membrane integrity (Trypan Blue Exclusion Assay). The data is presented as the mean of duplicate wells ± SE. *p ≤ 0.05 (in comparison to day-matched cellular control).

TB Viability RAW264.7	7 Day		10 Day		14 Day	
Treatments uM	*Avg (%)*	*SE*	*Avg (%)*	*SE*	*Avg (%)*	*SE*
Cell Control	89	0	90	2	87	8
Neg Control	5	3	42	0	47	3
100	84	1	93	1	87	4
300	77	1	92	2	82	4
600	71	2	89	1	87	0
1,250	63	0	91	0	80	0
2,500	61	1	83	0	62	5
5,000	20	6	72	5	70	0

DISCUSSIONS & CONCLUSIONS

Luminescent Assay (indicator of cytoplasmic enzyme activity):

- The 7-day exposure time in both cell lines displayed higher sensitivity to BC; an induction of rapid cytotoxicity upon BC treatments across all concentrations was observed.
- The 10- and 14-day exposure time for RAW264.7 presented a gradual dose-dependent induction of killing by BC.
- The 10-day exposure time for A549 resulted in an increase in viability at the lower BC concentrations, which converted to dose-dependent killing at the higher BC concentrations.
- The 14-day exposure time for A549 presented a gradual dose-dependent induction of killing by BC.

The data for A549 cells may indicate that the cell line is adapting or shifting its sensitivity as the exposure time is increased from 7 days to 10 days. The cells may be more vulnerable in early exposure times and possibly develop a resistance to the BC as the exposure time increases and thus show higher viability. Experiments investigating two possible subpopulations of cells need to be performed to test this idea.

Fluorescent Assay (indicator of mitochondrial enzyme activity):

- The 7,10, and 14-day exposure time for A549 shows a gradual dose-dependent killing by BC.
- The 7-day exposure time for RAW264.7 also shows a gradual dose-dependent killing by BC.
- The 10- and 14-day exposure time for RAW264.7 displayed extreme sensitivity to BC; an induction of rapid cytotoxicity upon BC treatments across all concentrations was observed.

The data may indicate that BC is compromising certain cellular activities at different components of the cell. The RAW264.7 (macrophages) show a higher susceptibility to BC exposure due to their nature of internalizing foreign materials and causing stress responses, demonstrating higher cellular death (diminished cellular activity) in general.

Colorimetric Assay (indicator of cell membrane integrity):

- The 7-day exposure time in both cell lines displayed higher sensitivity to BC; rapid loss of membrane integrity upon BC treatments across all concentrations was observed.
- The A549 cells showed bimodal behavior of viability, where an inverted bell curve presented enhanced cell survival at low and high concentrations of BC for all three exposure times.
- The 10- and 14-day exposure time for RAW264.7 presented a gradual dose-dependent induction of killing by BC.

The bimodal behavior of A549 cells could potentially indicate modification-inducing effects from BC thus causing a transformation in the cells' activity and ability to function.

FUTURE WORK

My doctoral project will continue to investigate important components of my research in order to produce competitive manuscripts and broadening scientific knowledge in my area of interest. These future goals will be achieved by developing further methods towards the improvement of my techniques.

1. Conduct apoptosis vs. necrosis studies to distinguish between sensitive versus resistant cell line upon BC treatments via Apoptosis/ Necrosis Detection Assay
2. Conduct proliferation studies to determine the proliferation of cells versus enhanced survival of cells upon BC exposure via Tritium-labeled Thymidine Uptake
3. Conduct additional cytotoxicity/ viability studies to delineate the effects of BC exposure time versus the cumulative number of BC treatments utilizing the same chronic exposures

ACKNOWLEDGEMENTS

NIH-NIMHD-RCMI. Grant No. 2G12MD007592