Nanoparticulate Carbon Black Decreases Cellular Proliferation and Alters Mitochondrial Morphology

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ABSTRACT

Nanoparticulates of pure carbon, carbon black (CB), are a common atmospheric pollutant in industrialized and heavily populated areas. It is produced primarily via combustion of fossil fuels, and represents a significant health hazard. It is known to worsen asthma and bronchitis when inhaled and to cause inflammation, heart dysfunctions, and oxidative stress when incorporated into other organs. The key focal point of this work is to examine markers of stress signaling and cellular dysfunction when human bronchial epithelial cells (16HBE14o-) are exposed to CB particles ranging upward in size from 70 nm and averaging 130 nm in diameter. BrdU incorporation studies revealed CB exposure to reduce rates of cell division. Additionally cells were shown to induce apoptosis at significantly higher rates. Within 12 hours of CB exposure, reactive oxygen species (ROS) were shown to rise significantly, a clear marker of cellular stress signaling. Chronic exposure (24 days) of cells to low doses of CB revealed a clear impact upon cell division and survival. Sirtuin 1, a stress regulated protein deacetylase in the cytosol, was assessed for stability following CB exposure. No significant changes were observed by immunoblot. When using microscopy to evaluate cellular morphological changes in response to CB exposure, mitochondria were shown to display abnormal morphology. HBE cells were treated with varying doses of CB, fixed, and prepared for immunocytochemistry of the mitochondria (Tom20-Alexa488), microtubules (phalloidin-Alexa594), and the nucleus (DAPI). Using software developed in MatLab, mitochondria were analyzed for changes in mitochondrial size and localization. Significant changes were identified with regard to an increase in mitochondrial size, and strong trends were observed in an increased localization preference for the perinuclear region. A discussion of the link between elevated ROS levels and mitochondrial behavior will be discussed.

INTRODUCTION

The effects of air contaminants on human health are a critical topic of study. Air pollution consists of various types of particles; those ranging from 20 to 100 nm in diameter are termed nanoparticles (NPs) and are of particular concern because their minute size causes uniquely harmful effects on human health [1]. One such substance, carbon black (CB), consists mainly of elemental carbon released in industrial combustion and is one of the most common atmospheric pollutants [4]. CB is known to exacerbate respiratory diseases, decrease lung capacity and cause lung inflammation, and induce heart defects [1]. It is important to gain an understanding of how CB affects the body in order to determine how to safely prevent its effects.

It is well-established that CB exposure induces apoptosis in lung cells [4]. Unfortunately, not much is understood about the mechanism responsible for initiating this response. However, it has been demonstrated that CB induces dramatic changes in mitochondrial morphology, which indicates that the mitochondria play an important role [5]. It is known that this apoptotic pathway begins when a trigger signals Bax, a cytosolic protein, to translocate to the mitochondria. Bax forms pores in the mitochondrial outer membrane, allowing the release of cytochrome c (cty c). Cyt C activates the protein-degrading enzymes caspase-3 and caspase-7 (cas-3/7), which then induce apoptosis (see Figure 1)[8].

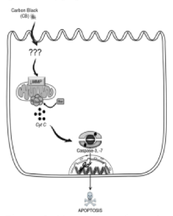
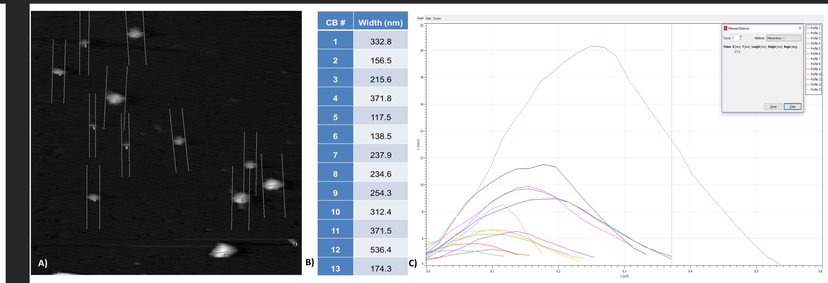


Figure 1: *Mechanism of apoptosis induced by CB in cells* (Modified from Boland et. al [4])

At the cellular level, CB induces apoptosis along a mitochondrial pathway, increasing levels of reactive oxygen species (ROS), subsequently activating BAX, a proapoptotic factor, to release cytochrome C and activate caspases which participate in nuclear degradation [4]. Low dose CB exhibits less cytotoxicity [4], yet both current and chronic exposure to these nanoparticles have been shown to reduce lung capacity and function [2]. A separate study has shown gene expression alterations persisting 42 days after a single CB exposure and 21% nanoparticle retention 26-27 days post exposure [3]. Though no studies were found focusing on long-term, chronic CB exposure on the cellular level, intuition suggests that reduced lung function and capacity results from reduced cell proliferation and increased apoptosis as incidence of exposure and retention of particles increases.

Currently, CB-induced morphological changes in the mitochondria are well documented but have not yet been quantitatively characterized. This study quantitatively examines mitochondrial morphological traits compared between cells without stress and those exposed to CB. Additionally, this study examines the effects of chronic, long-term CB exposure on HBE cells, specifically with regards to cellular viability and proliferation.

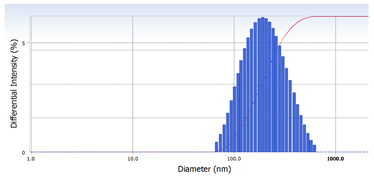
RESULTS



CB #	Width (nm)
1	332.8
2	156.5
3	215.6
4	371.8
5	117.5
6	138.5
7	237.9
8	234.6
9	254.3
10	312.4
11	371.5
12	536.4
13	174.3

Figure 2: Atomic Force Microscopy of Carbon Black Nanoparticles. AFM was used to image CB nanoparticles and size was analyzed using Gwyddion software. Average size is 358.03 nm with a standard deviation of 139.8. The range of sizes are from 100.0 nm to 674.0 nm. A 5 µm AFM scan with CB100 µg/mL on mica substrate and shown with size bars from Gwyddion software (a) with values shown in table (b). Size was quantitatively analyzed using Gwyddion software (c) and graphed based on frequency.

Figure 3: Probe sonication techniques distribute carbon black in solution on the nanoparticle scale as tested by Micromeritics Analytical Services (Norcross, GA). A mixture of particles at 1 mg/mL, bovine serum albumin (BSA) in PBS at 6.5 mg/mL, & distilled water was sonicated for 5 minutes at 20% amplitude of a 500 W ultrasonic probe. CB particles exhibited a range of diameters from 70 - 660 nm with an average size of 220 nm.



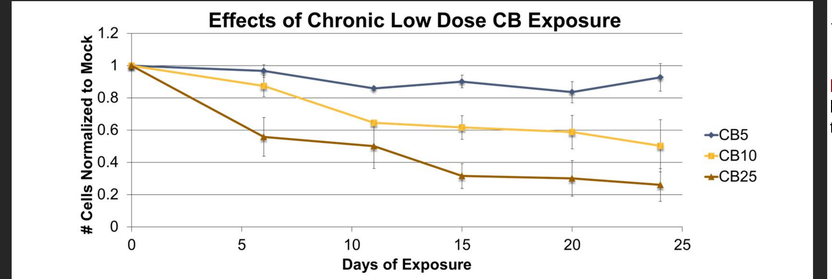


Figure 4: Chronic low dose CB exposure decreases cellular viability as treatment duration increases. Cells were seeded at 25,000 cells/cm^2 and grown in the following treatments: mock, 5 µg/mL CB, 10 µg/mL CB, and 25 µg/mL CB. At each passaging, 20% of cells were passaged and 70% fixed to determine cellular density (10% remained to avoid pipetman volume loss). Cellular densities for each treatment and time point were normalized to mock treatment. Values represented with ±SE.

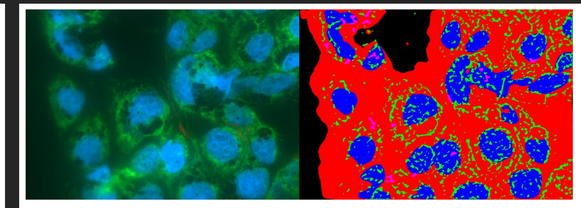
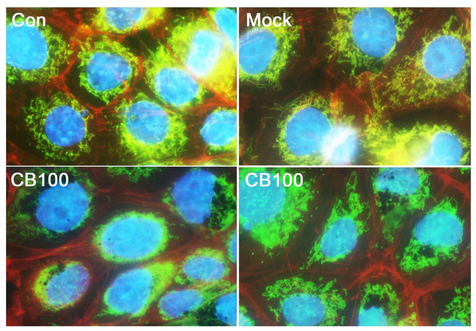
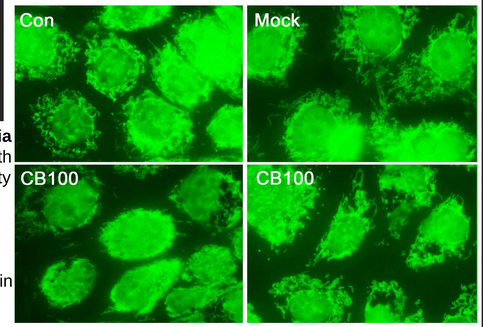


Figure 6: Mitochondrial imaging For imaging assays, HBE cells were seeded at 25,000 cells/cm^2 on glass coverslips in 24-well plates and treated with mock, CB25µg/ml, and CB100µg/ml. Cells were fixed with 0.5mL of 4.4% PFA after 24 hours. For fluorescence imaging, all coverslips were soaked in 300 µl of Block solution and followed by 300µl of primary antibody solution (primary antibody stock solution, 1:1000 dilution Phalloidin, and 1:150 dilution Tom20). Coverslips were mounted onto microscope slides in Prolong Gold Antifade with DAPI. One slide per treatment for each replicate was imaged, and 10 pictures of each coverslip were captured using a Nikon TI Eclipse microscope at 100X. Analysis was performed using software developed by McClatchey et. al at University of Colorado [6].

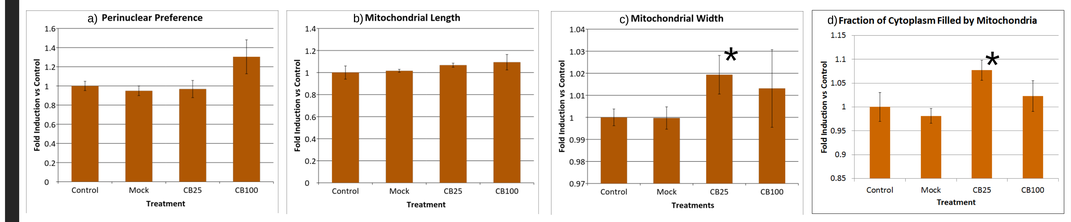


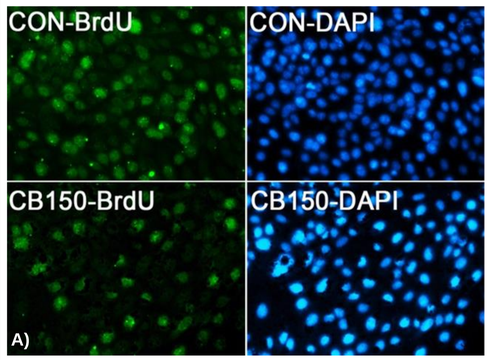
e) Healthy v. CB-poisoned cells. Mitochondria (green), nuclei (blue), tubulin (red)



f) Healthy v. CB-poisoned cells. Only mitochondria shown

Figure 7: CB induces morphological changes in lung cell mitochondria Average perinuclear preference and average mitochondrial length and width were measured using software developed by McClatchey et al. at University of Colorado [6]. Average mitochondrial measurements for each treatment were compared to a control. **A increasing trend in mitochondrial perinuclear preference** was observed in cells exposed to higher dosages of CB compared to mock cells (a) . Perinculear preference is defined by McClatchey et al. as "the natural log of the ratio of the mitochondrial mass in the 30% of the cytoplasm closest to the nearest nucleus to the mitochondrial mass in the 30% of the cytoplasm furthest from the nearest nucleus" [5]. Additionally, **a slight increasing trend in mitochondrial length** was seen as CB exposure increased (b). **A slight increasing trend in average mitochondrial width** was also observed at higher dosages of CB (c). **No consistent trend was noted in the average fraction of the cytoplasm filled by mitochondria** between treatments (d). *Denotes significance from mock with $p < 0.05$ using a paired t-test. Values represented with ±SE.





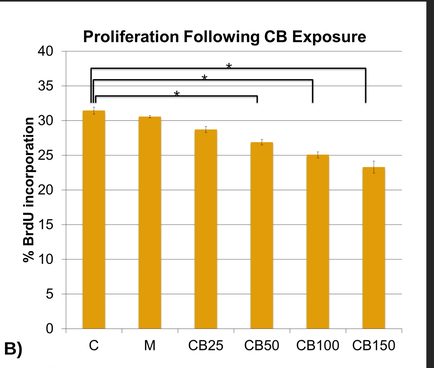


Figure 5: Carbon black reduces levels of proliferation at 24 hours. Cellular proliferation was determined through 8 hours of BrdU incorporation followed by fixation at 24 hours post-CB poisoning. A) Side-by-side comparison of total nuclei (DAPI) and BrdU incorporated nuclei for control and 150 µg/mL CB treatments. B) Percent representation of BrdU incorporated nuclei divided by total nuclei for the following treatments: control (31.4%), mock (30.6%), 25 µg/mL CB (28.7%), 50 µg/mL CB (26.9%), 100 µg/mL CB (25.1%), and 150 µg/mL CB (23.3%). Cells were seeded at 30,000 cells/cm^2 and allowed 24 hours rest before poisoning with respective treatment. A 50 µM solution of BrdU (ThermoFisher) in cell culture media flooded cells for 8 hours of incorporation before fixation at 24 hours of poisoning. * Denotes significance of $p<0.05$ based on a paired t-test with an alpha value of 0.05. Values represented with ±SE.

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CONCLUSIONS

- CB exposure decreases rates of proliferation at 24 hours.
- Chronic exposure to low dose CB reduces number of cells as duration of exposure increases.
- CB induces a trending increase in perinuclear preference.
- CB causes a significant increase in average mitochondrial length.
- CB induces a slight increasing trend in mitochondrial width.
- CB does not alter the fraction of the cytoplasm filled by mitochondria.

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