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Black carbon accumulation in extrapulmonary human tissues

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Black carbon is a product of incomplete combustion and is associated with a number of adverse health outcomes in epidemiologic studies. To date, anatomic studies investigating the pathogenic mechanisms of black carbon in humans have confirmed black carbon particles accumulate in the lung and in the placenta of pregnant women, and are excreted in urine. In this feasibility study, the presence of black carbon particles was demonstrated in post-mortem spleen, myocardium, and hilar lymph node samples obtained from three de-identified sources. A protocol validated for extraction of chemically inert particles from fish tissue was utilized to extract black carbon particles from human tissue. Visual examination and micro-Raman scattering spectroscopy were used to identify recovered black carbon particles. Recovered particles ranged from 2 to 45 μm in greatest dimension. The highest concentrations of black carbon particles were recovered from hilar lymph nodes, followed by the myocardium, with lowest concentrations recovered from the spleen. Particles extracted from the spleen were, on average, larger than particles extracted from the heart or hilar lymph nodes. These findings confirm black carbon particles accumulate in human extrapulmonary organs. Based on the size and concentrations of recovered particles, it is suggested that black carbon particles are transported to extrapulmonary sites via the lymphatic system. Furthermore, the noted concentration differentials suggest reduced black carbon particle clearance from the myocardium compared with the spleen.

Key words: Black carbon, soot, human, heart, spleen.

INTRODUCTION

Black carbon pollution (BCP) is generated as a product of incomplete combustion. Globally, BCP is produced primarily by the burning of transportation-associated fossil fuels, residential wood and coal, oil and coal from power stations, and agricultural waste (Janssen et al., 2012; United Nations Environment Program, World Meteorological Organization, 2011). In the atmosphere, BCP particles coagulate, aggregate and associate with other chemicals of varying toxicities, including metals, metalloids, polyaromatic hydrocarbons, and bacterial endotoxin (D'Anna, 2009; Bølling et al., 2009; Janssen et al., 2012; Lindner et al., 2017). Epidemiologic studies

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independently associate BCP with all-cause mortality, mortality from pulmonary and cardiovascular causes, and morbidity resulting from disturbance of several physiologic systems. In the United States, 14,000 deaths and hundreds of thousands of illness cases per year are estimated to be attributable BCP exposure (Li et al., 2016).

Pulmonary morbidity associated with BCP includes hospitalizations for respiratory illnesses, increased asthma prevalence and severity, and decreased overall pulmonary function in all age groups (Jung et al., 2017; Hua et al., 2014; Kulkarni et al., 2006; Franco Suglia et al., 2008a). Specific cardiovascular morbidity associated with BCP exposure includes ischemic heart disease events, hospitalizations for cardiac illness, autonomic dysfunction and arrhythmias, and increased blood pressure (Samoli et al., 2016; Gan et al., 2011; Cole-Hunter et al., 2016; Zanobetti et al., 2014; Mordukhovich et al., 2009).

BCP is also associated with impaired cognitive development in children, increased cognitive decline in the elderly, and biologic aging (Franco Suglia et al., 2008b; Colicino et al., 2017). Prenatal exposure to BCP is independently associated with reduced birth weight and increased systolic blood pressure at birth (Lakshmanan et al., 2015; van Rossem et al., 2015).

Pathogenic mechanisms associated with BCP include inflammation, endothelial dysfunction, platelet activation, and plaque formation, but distribution of particles in the human body has not yet been fully described (Lovinsky-Desir et al., 2016; Fang et al., 2012; Garshick et al., 2018; Solomon et al., 2013; Guidetti et al., 2012; Wilker et al., 2013). Laboratory evidence indicates particles 2.5 μm in greatest dimension or smaller can be inhaled into lung alveoli, while larger particles are more likely to distribute to more proximal bronchi, bronchioles, and upper airways (Lippmann and Albert, 1969; Stahlhofen et al., 1986; Churg and Brauer, 1997). Inhaled particles 100 nm or smaller are able to translocate through alveoli into the bloodstream (Nemmar et al., 2002).

Approximately, half of particles that deposit in ciliated airways will undergo mucociliary clearance within 24 h, whereby particles are cleared from the lung, swallowed, and potentially encounter phagocytic cells in gut-associated lymphoid tissue (Lippmann and Albert, 1969; Pais Soares and Fernandes Borges, 2018). Particles that remain in airways can form chain-aggregates and may accumulate in airway and interstitial macrophages, bronchial epithelial cells, or pulmonary fibroblasts (Bai et al., 2018; Brauer et al., 2001; Belade et al., 2012; Geiser, 2002).

Although the upper limit of the size of particles phagocytosed by macrophages *in vivo* has not been defined, murine macrophages *in vitro* can phagocytose carbon particles up to 20 μm in greatest dimension (Brandwood et al., 1992). In addition, human macrophages have been observed to phagocytose more than one particle, in one case accumulating up to 72 microparticles before migrating from the airway (Lay et al., 1998).

To date, human anatomic studies have confirmed black carbon particles accumulate in the lung, circulate systemically to accumulate on the fetal side of the placenta, and are excreted in urine (Lewis et al., 1973; Bove et al., 2019; Saenen et al., 2017). However, the toxicokinetics of black carbon particles beyond the lung to other organs in the adult human body requires further exploration and a validated method for the study of contamination of the human body with BCP. In this study, we applied micro-Raman spectroscopy to demonstrate accumulation of BCP in adult human hilar lymph node, heart, and spleen tissue samples.

METHODOLOGY

This study postulated that particles are likely to accumulate in extrapulmonary organs, including the hilar lymph nodes, heart, and spleen. Within this framework, a protocol recently validated for extraction of chemmically inert microplastic particles from fish tissue was applied to investigate the characteristics of particles that could be recovered from post-mortem human tissue (Karami et al., 2017). This protocol was submitted to the Johns Hopkins Bloomberg School of Public Health Institutional Review Board Office and determined not to qualify as human subjects research and to not require IRB review or oversight. For this feasibility study, myocardial and splenic specimens were collected from three de-identified sources aged 30 to 60 years old, post-mortem. Hilar lymph nodes were also collected from two of these sources. Extraction of black carbon particles from the hilar lymph nodes, myocardium, and spleens from each source (referred to herein as Source #1, Source #2, and Source #3) was confirmed.

Chemicals and other materials

Materials included ACS grade potassium hydroxide (KOH) and sodium iodide (NaI). Solutions of KOH (10% *w/v*) and 4.4 M NaI were prepared by dissolving powder/pellets in reverse osmosis-grade water. Filter papers were supplied by Whatman Inc. (Grade 1 and 540 hardened ashless, Florham Park, MI).

Tissue preparation and digestion

Post-mortem tissue samples were obtained from the Johns Hopkins Department of Pathology. Samples were received in polypropylene tubes containing 10% neutral buffered formalin. Samples were removed from formalin with forceps and rinsed three times with reverse osmosis-grade water before being transferred to a glass plate to be weighed on a Mettler analytical scale. Tissue was manually cut with razor blades to fragments less than 0.5 cm in greatest dimension to enhance homogenization. 10% *w/v* KOH was added to the tissue at 10 mL/g tissue. KOH solutions containing tissue samples were then homogenized with a Polytron homogenizer at medium speed for 5 min. The homogenate was covered with paraffin wax and transferred to a 40°C water bath for 48 to 72 h.

Removal of digestion-resistant particles

After 48 to 72 h, the homogenates were gravity filtered over a

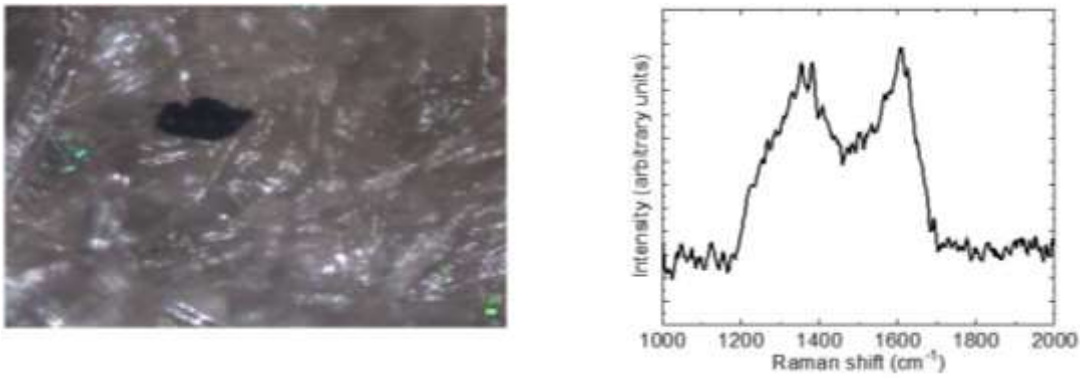

Figure 1. Microscopic image and associated micro-Raman spectrum of carbon particle extracted from the hilar lymph node of Source #1

Whatman Grade 540 membrane (8 µm pore size). Filter membranes with remaining residue were transferred to beakers containing 10 mL 4.4 M NaI solution. To dislodge residue bound to the filter, beakers were sonicated at 50 Hz for 5 min and agitated on an orbital shaker (200 rpm) for 5 min. Particles were extracted from residue by centrifugation at 500 × g for 2 min. The supernatant containing extracted particles was collected in a collection bottle, and remaining pellets were re-suspended in the NaI solution, sonicated, agitated, and centrifuged to extract any particles remaining in the pellets. This re-suspension procedure was repeated twice. The final volumes of collected supernatants were gravity filtered over Whatman Grade 540 membranes. Filter membranes containing residual particles were transferred to glass Petri dishes with lids. Covered Petri dishes containing filter membranes with residual particles were vacuum-dried at 25 to 30 mmHg at 40°C for 45 to 60 min. After drying, covered Petri dishes were sealed with paraffin wax for transport for microscopic examination and micro-Raman analysis (Karami et al., 2017).

Microscopy and micro-Raman analysis

An Olympus Upright BX63 microscope (a standard component of micro-Raman set-up) equipped with a Thorlabs XY stage with 0.05 µm minimum incremental movement was used to scan filter papers for evidence of accumulation of inert particles, including black carbon. The focus of this study was identification and characterization of recovered black carbon particles. Once identified, the greatest dimensions of suspected black carbon particles were measured and recorded. The Olympus microscope was attached to the micro-Raman spectrometer T64000 Horiba Jobin Yvon equipped with a liquid nitrogen cooled CCD detector. To evaluate the chemical composition of isolated particles, micro-Raman scattering spectra were measured in the spectral range of 100 to 2000 cm^{-1}. A 514.5 nm line of Ar+-Kr+ laser was used for excitation. A 2 µm laser probe allowed for probing of particles 2 µm in greatest dimension or larger. Spectra of carbon particles were identified by comparison to literature on micro-Raman spectra of various forms of carbon (Chu and Li, 2006). To minimize bias, researchers conducting microscopy and micro-Raman analysis were blinded as to the source of the particles being characterized.

Prevention of contamination and procedural controls

To prevent contamination, working surface areas were covered with aluminium foil thoroughly cleaned with KIMWIPES (Kimberly-Clark Worldwide, Inc., Irving, TX) soaked in distilled water prior to work. All the solvents were filtered over a Whatman Grade 1 filter membrane (11 µm pore size) prior to use. All glassware was cleaned with commercial dishwashing liquid and rinsed with reverse osmosis-grade water. Procedures were carried out under a flow cabinet to prevent potential contamination with airborne pollutants. When not under a flow cabinet, solvent containers were covered with paraffin wax and Petri dishes were covered with glass lids and paraffin wax.

Between processing of samples, all equipment was cleaned with commercial dishwashing liquid and rinsed three times with reverse osmosis-grade water. The procedural control was a 40 mL KOH blank processed in parallel with tissue samples. The 40 mL volume. of the KOH blank was comparable to the volume of most of the experimental samples processed

RESULTS

Recovery and confirmation of black carbon particles

Petri dishes containing samples were prepared according to the procedure described earlier. These samples were examined visually using an Olympus microscope with 10 and 50x objective. Raman scattering spectra were measured *in-situ* for the visually identified black particles to confirm their content. All black particles found in the samples were of amorphous carbon or soot origin, according to spectra identified based on carbon spectra published in Paul et al. (2006). An example of a visual image of a carbon particle extracted from the hilar lymph node of Source #1 and the Raman scattering spectrum of this particle allowing for its identification as carbon are presented in Figure 1.

Black carbon particles were confirmed on all filter membranes exposed to products of digested tissue samples and on the KOH procedural blank.

Variability of black carbon particle concentrations

Tissue samples ranged from 3.3 g to 8.3 g in weight. To evaluate the variability of the concentrations of black

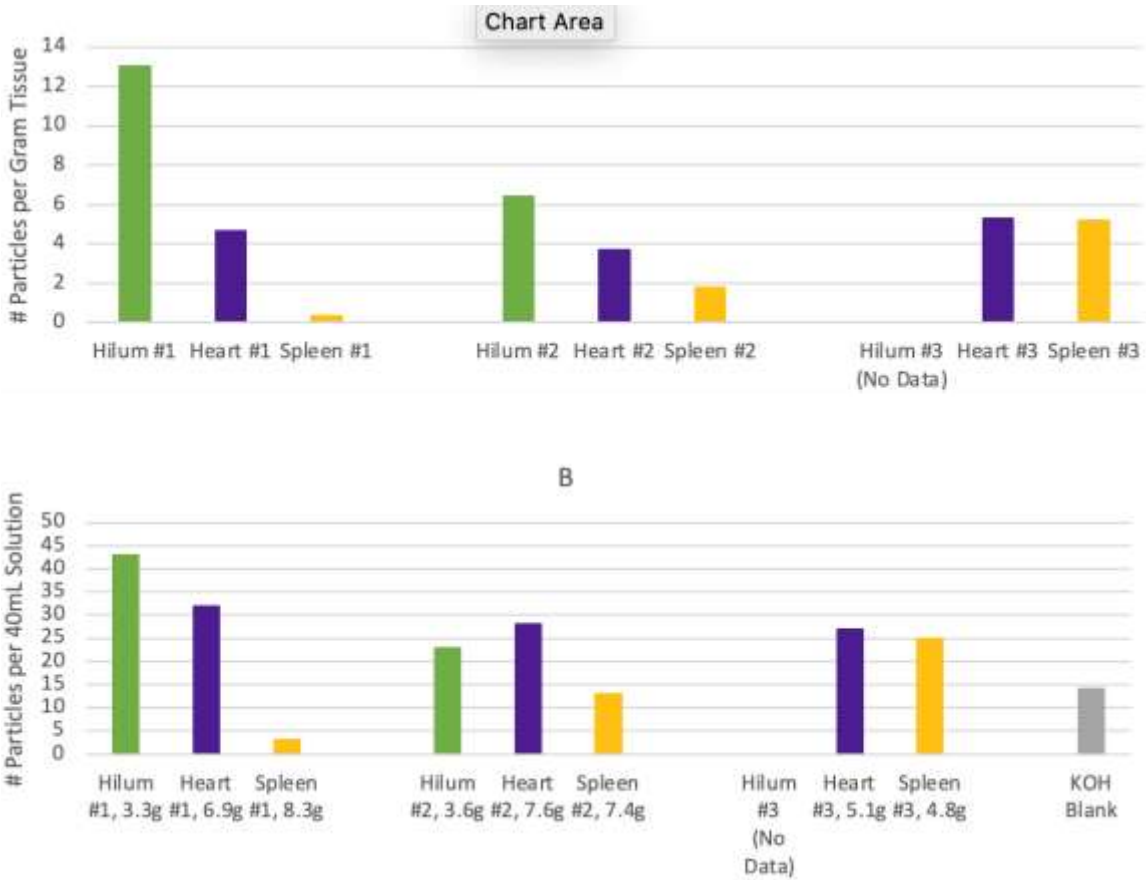

Figure 2. Black carbon loads of tissue specimens and procedural blank. A) Number of accumulated black carbon particles per gram of tissue by organ and source individual. B) Total number of carbon particles per 40 mL solution comparing experimental samples with the procedural blank

carbon particles extracted from specific tissue types and tissue sources, we counted the number of particles visually identified on each filter membrane. The results comparing tissue specimens are reported as the total number of black carbon particles per gram of tissue processed (Figure 2A). Because this calculation was not possible for the procedural blank, experimental samples were compared to the procedural blank by total particle count per mL volume of KOH (Figure 2B). The comparison of the per volume concentration of number of particles with the blank demonstrates that, at the exception of the spleen #1 and #2 samples, the number of the black carbon particles in the experimental samples exceeded that of the procedural control.

Overall, specimens from Source #3 exhibited a greater carbon concentration compared with like specimens from Sources #2 and #1. Due to the lower concentration of particles extracted from the spleen of Source #1, comparisons of like concentrations were not consistent between Sources #2 and #1. Within individuals, the carbon concentrations of hilar lymph nodes consistently exceeded myocardial and splenic concentrations, and myocardial concentrations exceeded those of particles extracted from the spleen.

Variability of carbon particle size

From the size determination of identified particles, it is clear that large black carbon particles can be found in the human heart, spleen, and hilar lymph nodes (Figure 3). Recovered particles were measured visually and ranged from 2 to 45 μm in greatest dimension. Particle statistics were analyzed by boxplot to compare size trends across sources and organ types.

Particles recovered from the spleen were on average larger than those recovered from the myocardium. No other discernable pattern could be appreciated within or between sources regarding particle size distribution.

DISCUSSION

First, this work demonstrates a successful application of a protocol developed originally for extraction of microplastics from fish tissue to extract black carbon

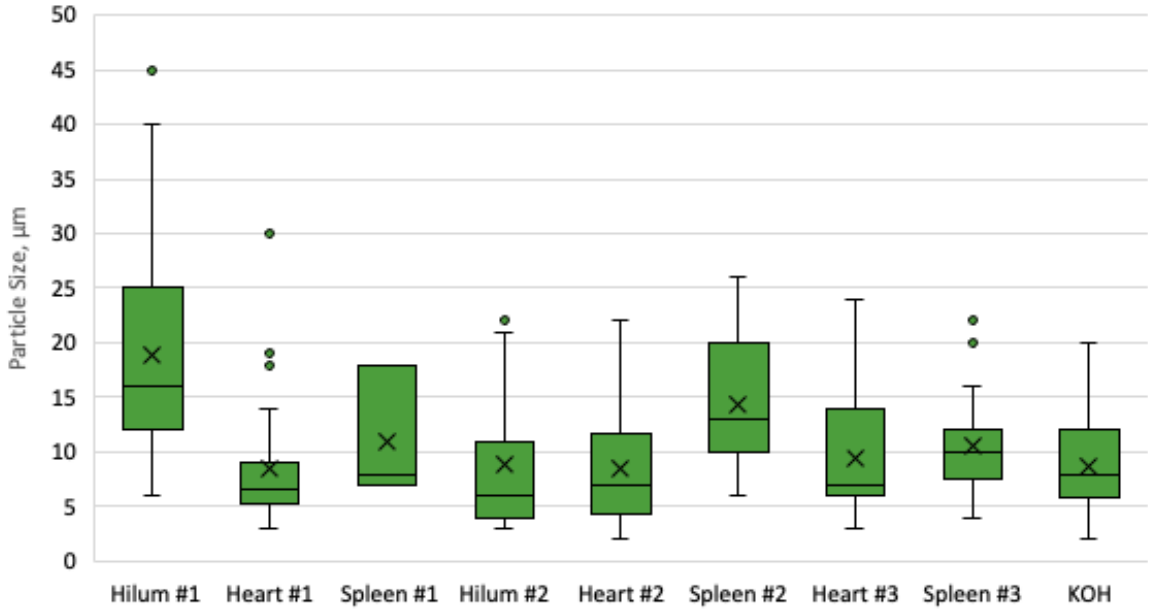

Figure 3. Black carbon particle size in tissue specimens and KOH procedural blank. Data representing 208 particle measurements is visualized in box plot format with X representing mean measurements of particle greatest dimension and horizontal lines within each box representing median values.

particles from human tissue. The patterns of relative concentrations of black carbon microparticles between sources and organ tissues are validating for this technique. The size of recovered particles and their patterns of distribution were analyzed to generate hypotheses to explain how black carbon migrates to extrapulmonary sites in the human body.

Recovered BCP particles measured up to 45 μm in greatest dimension. This finding was interpreted by considering that recovered particles may represent primary particles or aggregate particles comprising primary particles compacted by crystallization (Walter, 2013). Moreover, the increased average size of recovered particles may in part be due to tissue fixation with formalin strengthening aggregate crystals prior to sample preparation or smaller particles escaping the 8 μm membrane pores during the extraction process.

Because recovered particles are similar in size to those known to be phagocytosed by macrophages but up to over 200 times larger than those known to translocate directly through alveoli into the bloodstream, recovered particles are more likely to have been extracted from extrapulmonary organs after transport by phagocytic cells originating in the airway or gut rather than by the bloodstream after translocation through alveoli.

Within each source, the concentration of particles recovered from hilar lymph nodes exceeded that of particles recovered from the myocardium, which exceeded the concentration of particles from the spleen. These findings suggest the possibility that particles phagocytosed by macrophages are more likely to become trapped in the myocardium compared with the spleen after draining through the lymphatic system, the thoracic duct, and the venous circulation. The increased average size of particles recovered from the spleen compared with the heart may be a result of increased particle aggregation in macrophages prior to deposition in the spleen.

Although the results of this study offer new insights on the toxicokinetics of black carbon particles in the human body, several limitations of this study were noted which can be addressed by further investigation of contamination of human tissues with black carbon particles. Most importantly, the number of particles recovered from the procedural KOH blank was higher than expected and likely indicates the need for a more powerful hood or stronger environmental controls during microscopy or Raman analysis during future investigations. Second, due to the difficulties associated with manually slicing fixed tissue to the appropriate dimensions, the tissue samples from which BCP particles were extracted were relatively small. This limited the data that were analyzed to support the conclusions of this study. In addition, the lower-than-expected concentrations of particles derived from the spleens of sources #1 and #2 suggest the possibility the filter membranes may have been compromised or had a pore size that allowed a significant portion of recovered particles to escape. There was also no standardization of tissue samples chosen for analysis to account for possible regional differences of particle concentrations within organs. Lastly, although spectra indicate recovered particles were of soot or

amorphous carbon origin, this technique did not allow for determination of specific particle source or timeline of particle entry into analyzed samples.

Based on this work, a number of opportunities exist for future study. First, an experimental protocol that recovers black carbon particles placed *in vivo* would be useful for validating the use of these methods for the recovery of black carbon. Also, histologic examination of tissue pathology slides will be important to verify reported findings and to offer additional detail regarding intracellular and extracellular distribution of black carbon particles at extrapulmonary sites. Conducting studies using larger samples, samples from additional extrapulmonary sites, and comparing results between fixed and unfixed tissue could also be a next step. Because the efficiency of short- and long-term clearance of inhaled particles is known to be affected by a number of pulmonary diseases and smoking history, conducting a study using tissue from sources with known medical and tobacco histories in addition to known geographic and tattoo histories would be useful (Smaldone et al., 1988). Lastly, conducting a study using filter membranes with a smaller pore size might yield results that more accurately reflect *in vivo* particle accumulation and allow for more robust statistical analyses.

Conclusions

This study demonstrates black carbon particles accumulate in adult human organs (namely the hilar lymph node, heart, and spleen) beyond the lungs. The size and relative concentrations of recovered black carbon particles suggest they are transported to extrapulmonary sites via the lymphatic system and reduced particle clearance from the myocardium compared with the spleen. Furthermore, the described technique of sample preparation and micro-Raman spectroscopy are suitable tools for identification and quantification of black carbon particles extracted from human tissue. While a study with larger statistics is necessary to enhance our knowledge of black carbon distribution in the human body, this study is the first to describe the presence of black carbon particles in extrapulmonary organs of human adults.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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