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From the lung to the knee joint: Toxicity evaluation of carbon black nanoparticles on macrophages and chondrocytes

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ABSTRACT

Carbon black (CB), a core elemental carbon component of airborne particles, has been used as a model material to study environmental safety and health impacts of airborne particles. Although potential adverse effects of CB have been reported, limited knowledge is available regarding CB-induced metabolic disorders and secondary effects distant from primary target organs, such as the effects on joints. The knee cavity is a relatively closed space along the peripheral circulation route with a slow rate of interchange of nutrition with blood. While epidemiologic studies have indicated that airborne particle exposure may affect the occurrence and severity of inflammatory knee diseases, no research has been performed to understand the potential hazardous direct/indirect interactions between particles and knee cells. Herein, we have scrutinized the toxicity of four commercial nano-sized CB samples in the lung and a distant site: knee joint. Our results indicated that CB triggered pulmonary and systemic inflammation upon inhalation exposure, and, more strikingly, CB also elicited injuries of the knee joint, as demonstrated by thickened synovial membrane, suggesting disordered cellular metabolism within the knee joint. Our data recognized the CB toxicity profiles to macrophages as characterized by pro-inflammatory reactions, and also defined an activated metabolic state of chondrocytes, as evidenced by metalloproteinase (MMP) induction. Of note, remarkable variations were also found for these changes induced by these four CB samples, due to their distinct physicochemical properties. Collectively, our results uncovered a significant toxicity of CB inhalation exposure to the knee joint, as reflected by metabolic activation of chondrocytes, and, more importantly, these findings unearthed CB-induced metabolic disorders and secondary effects owing to systemic pro-inflammatory conditions upon CB exposure, in addition to the likelihood of direct toxicity to knee cells.

1. Introduction

The potential influence of airborne particles on the environment and human health attracts great concern, especially in recent years. Carbon-based airborne particles exhibit a huge impact on the regional and global climate similar to carbon dioxide in absorbing solar radiation and heating the atmosphere, hence they have been considered as the second most important climate forcing emission [1]. Regarding the human health impact, the majority of the health data were primarily concluded from epidemiological surveys [2], revealing that inhalation of airborne particles could extensively induce lung inflammation and even deep lung tissue injuries. Plus, other studies have also reported that air pollution increases the risk of cardiovascular, cerebrovascular, and gastrointestinal disorders [3–5]. However, little research has been performed to look at metabolic disorders and the secondary effects of airborne particle exposure, particularly for off-target effects distant from primary target organs, such as the effects on joints. The knee cavity is a relatively closed space along the peripheral circulation route with a slow rate of interchange of nutrition with blood. Although some studies have suggested that the frequency and severity of pain in patients who suffered from osteoarthritis arthritis (OA), juvenile idiopathic arthritis (JIA), rheumatoid arthritis (RA) or osteoporosis were worsened upon prolonged exposure to air pollution [6–8], these studies did not obtain sufficient insights into the spread route of detrimental factors from the primary site to joint sites, and may not be able to offer the underlying molecular mechanisms.

The chemical composition of airborne particles is comprised of inorganic carbon, organic carbon, and some non-carbon components

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dependent on the complex environmental sources. However, considering the uncontrolled physiochemical properties and complicated composition of airborne particles [9,10], it is difficult to determine the weighting coefficient of each component to the overall toxicity under different settings. Therefore, determination of individual components within airborne particles is an optional strategy to decipher the synergistic toxic effects of air pollution. Carbon black (CB), a manufactured product that contains nearly 97% pure elemental carbon, has been wildly used in commercial and consumer products [11]. In contrast to other components, the chemical composition of CB is clear and simple, and nano CB thus could represent carbonous nanoparticles under air pollution. In spite of similarities in morphology and physicochemical properties, there is no answer to address whether nano-sized CB might exhibit similar toxicity profiles to that of synthesized carbon nanoparticles [12,13]. With continuous efforts from our research group, we have previously demonstrated that exposure to carbonous nanomaterials (*e.g.* carbon nanotubes) evoked the activation of inflammatory cells in the initial exposure site, such as the lung and peritoneal cavity [14,15], and also triggered systemic inflammation with an enhanced content of inflammatory cytokines and activated inflammatory cells in circulation [14,15]. To this end, our previous studies depicted the novel mechanisms responsible for indirect effects induced by carbon nanotubes, which involved disordered iron homeostasis, anemia of inflammation and activation of knee cells through the inflammatory pathway [15,16]. However, it is still a pending question for CB in this regard.

In this work, we attempted to shed light on the likely adverse effects on the knee joint in animals challenged with CB exposure through inhalation, and to look for the molecular mechanisms underlying lung inflammation and abnormal activation of knee cells. We here demonstrated that all four selected CB samples could alter the metabolic state of chondrocytes, in addition to lung inflammation and macrophagic activation. Meanwhile, these CB samples with different physicochemical properties manifested varied effects. This study thus signifies the evaluation of toxicity at distant sites in addition to toxicity at the primary site upon exposure to airborne particles.

2. Materials and methods

2.1. CB particle preparation and physiochemical characterization

Four commercial CB powders were commercially purchased, and were referred to SB4A, Printex U, C1864, and C824455 according to the product labels. SB4A and Printex U were purchased from Degussa lnc. C1864 was purchased from Spectrum Chemical Mfg. Corp. C824455 was obtained from Macklin Inc. The CB solution concentrations were adjusted to 1 mg/mL in sterile water, followed by bath sonication for 2 h with 100 Hz, 200 W (Ultrasonic Cleaner, KunShan, JiangSu, China) with water change every hour. The morphology of CB samples was visualized using scanning electron microscope (SEM, Buruker, MM3D Germany) and transmission electron microscope (TEM, Hitachi, H7650, Japan). The surface charge, the polymer dispersity index (PDI) and the hydrodynamic size of the samples suspended either in Milli-Q water or in cell culture medium containing 10% fetal bovine serum (FBS) were measured using a Zeta-sizer (Malvern Nano ZS, Nalvem, UK) [17]. The CB samples were subjected to X-ray photoelectron spectroscopy (XPS) to identify and calculate the presence of surface functional groups. The software XPSPEAK41 was applied to analyze the type of functional groups.

2.2. Animal experiments

Male BALB/c mice were purchased from the Vital River Laboratories based in Beijing, China. Mice used for experiments were 7 weeks old with body weight of approximately 25 g. Mice were bred at a specific pathogen-free (SPF) facility. Prior to experiments, all animal experimental protocols were approved by the Animal Ethics Committee at the Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences. Pre-sonicated CB samples were administrated through intratracheal *(I.T.)* injection at 2.0 mg/kg body weight (in 50 μL and with 1 injection only), and the exposure period lasted for 2 or 7 days. Sera were extracted from peripheral blood for the cytokine assay, when mice were sacrificed at the end of experiments. Lung tissue samples were collected and fixed in 4% phosphate buffer saline (PBS)-buffered formaldehyde for future histological examination. The joint knees were separated from mice, and then fixed in 4% PBS-buffered formaldehyde. Thereafter, fixed knee tissues were subjected to decalcification with fresh decalcifying solution containing 10% ethylene diamine tetraacetic (EDTA), followed by embedding and sectioning.

2.3. Histological and immunohistochemistry analyses

Hematoxylin-eosin (H&E) staining of various tissues was carried out following the standard protocol [15,16]. Meanwhile, lung sections were subjected to immunohistochemical staining with an antibody (Ab) against F4/80 [15]. Full-range scanning images of each lung section were collected by a tissue section scanner (Pannoramic MIDI. 3D HISTECH, Hungary). The percentage of positive immunostaining and the staining intensity of each pixels ("0" denotes negative; "1 +" denotes weak; "2 +" means moderate; and "3 +" indicates strong) were recorded using the software Quant Center. Histochemistry score (H-score) was calculated using the following formula: H-SCORE = Σ(PI × I) = (percentage of cells with weak intensity × 1) + (percentage of cells wtih moderate intensity × 2) + percentage of cells with strong intensity × 3), with PI indicative the percentage of positive cells, and I indicative of the staining intensity of each pixels, as previously described [18,19].

2.4. Complete blood count (CBC) analysis

For CBC analysis, 20 μL fresh blood was diluted into 2 mL of standard dilution buffer purchased from the manufacturer. The diluted samples were then subjected to cell count analysis on a hematology analyzer (Nihon Kohden, MEK-7222 K, Japan).

2.5. Cell culture and cellular treatment with CBs

The mouse macrophage cell line (J774.A1) and the human chondrocyte cell line (SW-1353) were purchased from the Shanghai Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Cells were cultured following the standard protocol [15–17]. Stock CBs were further diluted in PBS for cellular treatment at various concentrations. Untreated cells received the same volume of PBS only.

2.6. Cytokine and metalloproteinase (MMP) -3 determination

The cytokine concentration in sera or culture medium were assayed using ELISA kits for TNF-alpha (TNF-α) Ab and interleukin-6 (IL-6) [16]. In order to assess MMP-3 that was secreted by SW-1353 cells into cell culture media upon CB treatment, SW-1353 cells were first cultured in serum-free medium and then supernatants were collected 24 h post incubation with 10 μg/mL CBs. MMP-3 content was then measured using an ELISA kit according to the manufacturer's instructions (QnDSystem^{TM}, Xinqidi Biological Technology CO, Ltd. Wuhan, China).

2.7. Cell viability assay

Cells were treated with CBs at various concentrations for 24 h, followed by cell viability determination using a LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen, Thermo Fisher Scientific Inc., USA). In the meantime, according phase-contrast morphology of cells was imaged under a microscope.