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Characterization of serum metabolites as biomarkers of carbon black nanoparticles-induced subchronic toxicity in rats by hybrid triple quadrupole time-of-flight mass spectrometry with non-targeted metabolomics strategy

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ABSTRACT

Carbon black nanoparticles (CBNPs) are one of atmospheric particles components and have been closely related with a series of lung diseases. It can reach the depths of the respiratory tract or even alveolar more easily than those micro-particles. Although some of its toxicities have been confirmed in animals or human bodies, the subchronic toxicity mechanism of CBNPs has been uncertain so far. Therefore, it is very necessary to establish a novel method and clarify the mechanism of subchronic toxicity caused by concentration adjustments of small molecule metabolites in *vivo*. In animal experiments, CB exposure, recovery and control group were set up. The concentration of CBNPs in chamber was 30.06 ± 4.42 mg/m^3. We developed a UHPLC-Q-TOF-MS/MS-based non-targeted metabolomic analysis strategy to analyze serum samples of rats. Then, differential metabolites in serum were found by multivariate data analysis and 39 potential biomarkers were identified. It was showed that main metabolic pathways associated with CBNPs exposure were hormones metabolism, amino acid metabolism, nucleotide metabolism and lipid metabolism. It is worth noting that long-term exposure to CBNPs had the greatest impact on steroid hormones biosynthesis so that the risk of infertility could increase. The results provided a new mechanistic insight into the metabolic alterations owing to CBNPs induced subchronic toxicity.

1. Introduction

Air pollution causes health hazards to receive more and more attention. Generally, particulate matters less than 2.5 μm in aerodynamic diameter can be inhaled into the alveolar space without being detached from the respiratory tract by the mucociliary escalator, especially nanoparticles (Chu et al., 2017; Buonanno et al., 2013). Carbon black nanoparticles (CBNPs) are one of the primary pollutants of atmospheric fine particles and mostly formed by the incomplete combustion of diesel fuels, coal, biofuels and outdoor biomass burning (Chen et al., 2014). As a result of the small size, particulate matters can pass through the barrier at all levels into the deep respiratory tract, or even reach the alveoli, and then induce lung injury (Jin et al., 2016; Deja et al., 2014). In addition, a great deal of pathological evidences has suggested that the CBNPs could also induce a variety of diseases, including ultrastructural changes of the air-blood barrier (Inoue et al., 2009), endothelial dysfunction (Yan et al., 2017), memory damage (Ma et al., 2015), bronchial injury (Vuong et al., 2016; Husain et al., 2015). And International Agency for Research on Cancer (IARC) classifies CBNPs as a possible carcinogen to humans. And in our previous study, it has been confirmed that CBNPs could be responsible for the lung injury and pro-inflammatory cytokines secretion in male workers and the rats exposed to CBNPs (Zhang et al., 2014; Chu et al., 2019). At present, the studies on CBNPs in *vivo* mainly focused on the biochemical indicators and the histopathology. For example, Yang et al. found that red blood cell count, hemoglobin concentration and hematocrit increased slightly in mice after CB exposure (Yang et al., 2016). CBNPs could induce acute inflammation and apoptosis of the liver, damage the central vein and structure of hepatocytes; and increase lymphocytes and eosinophils in alveolar lavage fluid (Zhang et al., 2019; Jiang et al., 2018). Although these studies had contributed to the description of the toxicity of CBNPs, they hadn't been able to describe the effects of CBNPs on the

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complex physiological processes of animal. But the metabolic mechanism of CBNPs has remained obscure. Therefore, it is required to elucidate the details of toxic mechanisms induced by CBNPs.

Metabolomics is considered a robust tool that allowed identification and quantitation of broad low-molecular-weight metabolites (MW < 1 kDa) in a given biological sample and plays a significant role in studying the endogenous metabolic substances changes in organisms caused by internal or external factors (Calderón-Santiago et al., 2017; Cui et al., 2016). The advantage of metabolomics is to evaluate the dynamic multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification. Currently, a variety of high-throughput technology platforms such as nuclear magnetic resonance (NMR), mass spectrometry (Zhang et al., 2017; Wei et al., 2017) are common tools in metabolomics. Compared with NMR, UHPLC-MS/MS has fewer requirements for sample preparation, high sensitivity, wide dynamic range, and can detect metabolites with large difference in concentration in samples. As a result, UHPLC-Q-TOF-MS/MS has become an increasingly technical platform for metabolomics research (Chen et al., 2016a,b; Liu et al., 2017; Wang et al., 2017a,b,c). Meanwhile, information provided by metabolomics studies by aid of several chemometrics tools may help us understand the toxicity effects of nanomaterials more comprehensively.

Nanotoxicology is a new discipline in the study of the interaction between matter and organism, as well as the biological or health effects produced at the nanoscale. Nowadays, metabolomics has been applied to study toxicity mechanism of tiny particles or nanomaterials. For instance, a HPLC-Q-ToF-MS-based metabolomics was employed to investigate rat's urinary metabolic profile altered by oral administration of nanoparticulate silver and ionic silver. It was found that uric acid and its degradation product allantoin increased after administration of nanoparticulate silver while only allantoin increased after administration of ionic silver (Hadrup et al., 2012). A GC-MS-based metabolomics method was used to evaluate the dose and size-dependent toxicity of SiO_2 by analyzing histopathological examinations and serum biochemical profile. There were metabolic profiling changes which indicated disorder of energy, amino acid, lipid and nucleotide metabolism (Lu et al., 2011). In addition, a metabolomics method was developed to analyze changes of endogenous metabolites in mouse fibroblast cells under the influence of TiO_2 nanoparticles and it was found that cellular carbo hydrate metabolism was disturbed with a concentration of TiO_2 nanoparticles higher than 100 μg/mL and indicted that energy metabolism perturbation, pentose phosphate pathway inhibition, nicotinamide metabolism block, mitochondria damage and oxidative stress activation were disordering (Jin et al., 2013). These have been proved that metabolomics strategy would be a useful tool to elucidate the molecular mechanism of nanoparticle-induced toxicity. Therefore, the potential biomarkers of system abnormalities discovered by metabolomics which prior to the apparent phenotypic changes will be helpful for comprehensive understanding and early diagnosis of subchronic toxicity induced by CBNPs.

In the present study, a comprehensive non-targeted metabolomics strategy based on UHPLC-Q-TOF-MS/MS platform was first established to analysis the metabolic profiles of serum. We aimed to characterize the metabolic alterations among CBNPs exposure group, recovery group and control group to identify the potential biomarkers and pathways, then to illustrate the mechanisms of CBNPs-induced toxicity.

2. Materials and methods

2.1. Reagents and materials

Acetonitrile were acquired from TEDIA Company (USA) for UHPLC use. Pure Wahaha water was obtained from Wahaha Group Co. Ltd (Hangzhou, China). Formic acid was purchased from Dikma Technologies Co. Ltd (China) and other chemicals were analytical grade and got from Tianjin Chemical Corporation (China).

CBNPs (≥99.8%) were purchased from Jiaozuo Hexing Chemical Industry Co., Ltd. China. In our previous studies, the size, morphology and surface morphology of CBNPs were measured by transmission electron microscope (TEM) and scanning electron microscope (SEM) and the pore structure and specific surface characteristics were analyzed by Brunauer-Emmett-Teller (BET) method (Zhang et al., 2014).

CBNPs was baked at 80 °C for 12 h and loaded into the aerosol generator sample injection column before exposure. The output air of the aerosol generator and the dilution air were connected to the inhalation chamber in proportion, and the consistency of the carbon black aerosol in the inhalation chamber was controlled by adjusting the parameters of the generator. The HRH-002 rat inhalation exposure system including aerosol generator, inhalation chamber and mass concentration real-time monitor were purchased from Beijing Huironghe Technology Co., Ltd. According to our previous study, the concentration of carbon could reach 15 mg/m^3 in occupational environment (Zhang et al., 2014). And the concentration of 30 mg/m^3 has been applied in the literature to study the neurotoxicity of CBNPs (Umezawa et al., 2018). Therefore, 30 mg/m^3 was used as the rat exposure concentration in the present study. In our previous study, environmental parameters of the exposure chamber including concentration of CBNPs, temperature, humidity, concentration of CO_2 and O_2 were monitored in real time (Chu et al., 2019). The concentration of CBNPs aerosol in the exposed chamber was 22.50–42.50 mg/m^3 by filter weighing method and the average was 30.06 ± 4.42 mg/m^3.

2.2. Animal study design

Eighteen male pathogen-free Sprague-Dawley rats (6 weeks old) were acclimated for a week before the experiment. During the acclimation and inhalation exposure, these rats were housed in polycarbonate cages and provided food and tap water in a controlled humidity and temperature environment with a 12 h light/dark cycle. Rats were divided by stratified randomization into CBNPs exposure group (n = 12) and control group (n = 6). In exposure group, twelve rats have been exposed to CBNPs for 90 days in the nose-only inhalation chamber with 30 mg/m^3 concentration lasting for 6 h a day. The six control animals were exposed to filter air for 24 h/day. In order to explore whether the damages might partly recover or not after remove CBNPs exposure in a short time, six rats randomly selected from exposure group after 90 days of exposure were regarded as the recovery group. In the recovery group, rats recovered for another 14 days under filtered air. Rats were anesthetized by pentobarbital sodium and sacrificed at the endpoint of treatment. Blood samples were collected in a heparin vacutainer from abdominal aorta. The animal use protocol has been reviewed and approved by the Laboratory Animal Ethical and Welfare Committee Hebei Medical University (No. IACUC-Hebmu-20160047).

2.3. Samples collection and handling

Serum samples were obtained by separating the supernatant from the blood after a 1 h coagulation period at room temperature and then centrifugation at 3000 rpm for 10 min.

The serum samples were taken out of 100 μL after thawing in the temperature of 4 °C. And the serum samples were vortex-mixed with three times the amount of acetonitrile for 3 min. After that, the samples were centrifuged at 13,000 rpm for 10 min. The supernatant was transferred to the other EP tube and dried by nitrogen blowing instrument (Tianjin Auto-science Instrument Co. Ltd., China). Then the solid samples were re-dissolved with 200 μL methanol and centrifuged at 14,000 rpm for 20 min. For each sample, an aliquot of 5 μL was analyzed by UHPLC-Q-TOF-MS/MS.

2.4. Instrumentation and measurement conditions

The metabolic samples were performed on a Shimadzu Nexera X2