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Heart developmental toxicity by carbon black waste generated from oil refinery on zebrafish embryos (*Danio rerio*): Combined toxicity on heart function by nickel and vanadium

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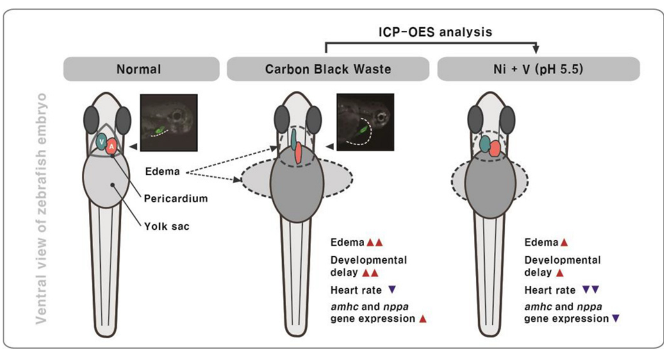
GRAPHICAL ABSTRACT



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ABSTRACT

This study assessed the developmental toxicities of water-soluble carbon black wastes (CBW) extract (1:5, w/v) in zebrafish embryos (*Danio rerio*). Acute embryonic toxicity was performed following OECD guideline 236. Analysis using ICP-OES revealed that nickel (Ni) and vanadium (V) were predominant in CBW. Embryos exposed to CBW exhibited developmental delay, along with pericardial and yolk sac edemas. Malformed heart chambers were found in the CBW-exposed embryos and heart rates were significantly reduced since 48 h post fertilization (hpf). After RT-qPCR analysis, two cardiac forming-related genes, *amhc* and *nppa* responsible for atrial cardiac myofibril assembly and cardiac muscle cell proliferation, were up-regulated after 96 hpf. The increased mortality and delayed yolk-sac development appeared related to CBW-induced decrease in pH to about 5.5. Individual treatments of Ni and V did not cause identical toxic effects as CBW showed. At 100 ppm, V had a pH of approximately 5.5, causing developmental delay and pericardial edema in zebrafish embryos. At the same pH, combined Ni and V induced morphological anomalies and reduced heart rates similar to CBW-exposed embryos. Conclusively, this study demonstrates that environmental runoff is a serious concern, and thus, CBW incineration bottom ash should be treated carefully before disposal in landfills.

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1. Introduction

Carbon black (CB) is a substance resulting from incomplete combustion of oil refinery byproducts (thus also called carbon black waste, CBW) that has been highlighted for its industry applications in the formulation of printing inks, as well as new rubber and plastic products, including tires [1,2]. Used tires are frequently recycled into activated carbons [3]. Recently, CBs have also become relevant as a means to improve water and soil quality through contaminant removal and as a land filler [4,5]. Carbon-rich materials synthesized through different processes such as CBs and biochar have effective absorption properties of organic materials due to their large surface area, micro-porosity and hydrophobicity [6]. Using this adsorption property, nanomaterials using CBs were used to mitigate toxicity of the aqueous dispersed polycyclic aromatic hydrocarbons [7]. However, CBs are considered a risk to human health because their small size allows them to be airborne and inhalable [8,9]. Furthermore, *in vitro* studies using human cell lines (e.g., HepG2, MRC-5, and MDA-MB-231) have examined dose- and time-dependent toxicity of CBWs, revealing that the presence of heavy metal ions, specifically vanadium, molybdenum, and nickel, are a major mechanism underlying toxic effects [10].

The negative effect of heavy metal ions on human and animals has been extensively studied [11]. Toxic heavy metals with high occurrence in the environment include cadmium, chromium, lead, and mercury; all are major pollutants with considerable public health significance [12]. Even low concentrations of a single heavy metal can cause behavioral abnormality and impaired cognitive functions (lead) or osteoporosis (cadmium). Toxicity investigations of multiple heavy-metal combinations in rats have shown that M4B (Cu + Zn + Mn + Cr) is the most toxic, disturbing both electrolyte and lipid balance [11]. Therefore, it is a public-health priority to monitor CBW release of residual heavy metal ions, while verifying their levels of individual and mixture toxicities. A rapid toxicity screening method has been developed to evaluate gasification ash toxicity, using *Drosophila melanogaster*, *Daphnia magna*, as well as human cell lines (MRC-5 lung fibroblasts and liver hepatocellular carcinoma HepG2) [13]. After 24 h exposure, compounds with acute toxicity can be divided into three sub-categories based on IC_{50} values [13]. However, this technique does not provide any insight on the mechanisms underlying toxic effects in living organisms. Likewise, the previous study that identified CBW heavy-metal composition determined only individual toxic effects on the tested cell lines [10].

In this study, we evaluated CBW toxicity in zebrafish (*Danio rerio*) embryos, specifically examining effects on development, heart formation, and heart rates. The expression of genes involved in heart formation was measured in CBW-exposed embryos to verify developmental delays. Our ICP-OES analysis confirmed the presence of nickel and vanadium in CBW before determining their individual toxicities in zebrafish embryos. Additionally, we tested whether a combination of nickel and vanadium alone (at the same concentrations as measured in CBW) would exhibit mixture toxicity comparable to CBW toxicity.

2. Materials and methods

2.1. Animal ethics

All experiments were approved by the institutional ethics committee of Kyungpook National University, Republic of Korea. In accordance with animal welfare regulations, toxicity assessments were performed only on embryos at unprotected and non-regulated stages (i.e., under 120 h post-fertilization, hpf) [14].

2.2. Fish care and breeding conditions

Wild-type (WT) and *Tg* (*cmlc2:EGFP*) transgenic zebrafish were provided by Professor Tae-Lin Huh from the Korean Zebrafish Resource Bank and School of Life Science and Biotechnology, Kyungpook National University, Daegu, Republic of Korea. Zebrafishes were maintained in the laboratory (26 °C ± 1 °C, 16:8 h light-dark cycle) from embryo to adult, and fed brine shrimp three times a day. To obtain embryos, 10 pairs each of adult male and female zebrafish were placed in a breeding chamber overnight. Embryos were primarily selected within 3 hpf, and only clutches with a total fertilization rate of ≥80% were used. To exclude individuals that would have died naturally, 6 hpf embryos (shield stage) were randomly selected based on existing morphological indicators of developmental stages [15].

2.3. Reagents

Carbon black waste (CBW), which is the carbonaceous solid powder type industrial waste from gasification of crude oil, was obtained from oil refinery in Singapore. Nickel (II) acetate tetrahydrate (98% purity) and vanadium (IV) oxide sulfate hydrate (97% purity) were purchased from Sigma-Aldrich (St Louis, MO). DEPC-treated water was purchased from Biosesang (Seongnam, Republic of Korea). TRIzol Reagent and Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). Rotor-Gene SYBR® Green PCR Kit was purchased from Qiagen (Dusseldorf, Germany).

2.4. Preparation of water-soluble CBW extract

The solution used in all experiments was 'egg water' containing 60 μg/mL sea-salt (80% salinity, Dong Kwang Inc., Daegu, Republic of Korea) to add a minimum salts and minerals to distilled water [16]. Carbon black waste powder was mixed with egg water (1:5, W/V), vortexed for 10 min, and shaken for 12 h at 26 °C ± 1 °C. The mixture was centrifuged at 12,000 rpm for 10 min to remove precipitate. The supernatant was then centrifuged under identical conditions and passed through 0.2 μm Whatman ® syringe filters (Sigma-Aldrich Co., St Louis, MO) to prevent microbial contamination. The pH of CBW aqueous extracts was measured before and after experiments. Elemental components of water-soluble CBW extract were quantified using inductively coupled plasma optical emission spectroscopy (Optima 7300 V ICP-OES, PerkinElmer, Waltham, MA).

2.5. Acute toxicity of CBW extract on zebrafish embryos

The acute exposure procedure was based on OECD guideline No. 236 (Fish Embryo Test, FET) [17] and we slightly modified exposure time to exclude selection errors of observer bias at the early development stage of the zebrafish embryo. Twenty wild-type embryos of shield stage (6 hpf) randomly selected and used for each treatment group in 96 well-plate containing 200 μL of each control solution (egg water only) and the water-soluble CBW extract. For a comparison of how chorions modulate the effects of CBW, individuals that retained chorions were defined as the "indirect-exposure group" and the group that removed chorion at 24 hpf was called "the direct-exposed groups". Mortality, hatching rate, and morphological malformation were recorded observed every 24 h at 26 °C ± 1 °C until 96 hpf. All experiments were performed in triplicate.

2.6. Quantifying cardiac functions & imaging

Wild-type adult male and *Tg* (*cmlc2:EGFP*) female zebrafish were crossed at a 1: 1 ratio. Fifty embryos of shield stage (6 hpf) randomly selected and treated with each 20 mL solution in glass-plate. The tested embryos were exposed for 24–28 hpf until GFP was detected and were randomly selected twenty embryos of *Tg* (*cmlc2:EGFP*) only, transferring individual embryo to single well in 96 well plate with 200 μL tested solutions to observe heart development and heart rate every 24 h until 96 hpf at 26 °C ± 1 °C. The heart rate was video-recorded for 15 s and counted heart beat with a time course at 24, 48, 72, and 96 hpf. The