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Gene expression of zebrafish embryos exposed to titanium dioxide nanoparticles and hydroxylated fullerenes

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ABSTRACT

Increased release of engineered nanoparticles to the environment suggests a rising need for the monitoring and evaluation of potential toxicity. Zebrafish frequently have been used as a model species in human and aquatic toxicology studies. In this study, zebrafish embryos were microinjected in the otic vesicle with a sublethal dose of engineered nanoparticles (titanium dioxide/TiO_2 and hydroxylated fullerenes/$C_{60}(OH)_{24}$). A gene microarray analysis was performed on injected and control embryos to determine the potential for nanoparticles to change the expression of genes involved in cross talk of the nervous and immune systems. The exposure to TiO_2 and hydroxylated fullerenes caused shifts in gene regulation response patterns that were similar for downregulated genes but different for upregulated genes. Significant effects on gene regulation were observed on genes involved in circadian rhythm, kinase activity, vesicular transport and immune response. This is the first report of circadian rhythm gene deregulation by nanoparticles in aquatic animals, indicating the potential for broad physiological and behavioral effects controlled by the circadian system.

1. Introduction

Titanium dioxide nanoparticles (nano-TiO_2) have the most industrial applications compared to any other nanoparticles. They are a major component of sunscreens, soaps, shampoos, toothpastes and other cosmetics (Melquiades et al., 2008), and regulations in the USA allow TiO_2 as the only inorganic UV filter, besides zinc-oxide, to be used in the cosmetic industry at maximum product content of 25% (FDA, 1999). Worldwide, four million tons of TiO_2 are consumed annually in uses as a pigment (Ortlieb, 2010). Nano-TiO_2 is also used as an additive in paint and building materials (Chen and Poon, 2009), from which it can leach and run off into aquatic ecosystems (Kaegi et al., 2008). It is found frequently in paper products, plastics and ink, and is used commonly as a food additive (E171 ingredient) for the whitening of skim milk (Ortlieb, 2010). Synthetic vitamin tablets, over-the-counter pills, and prescription drugs, including antidepressant and antibiotic capsulated products, contain TiO_2 in various quantities (Luft et al., 2010). Estimated environmental concentrations of nano-TiO_2 in aquatic ecosystems range from 0.0007 to 0.0245 $\mu g\ mL^{-1}$ (Mueller and Nowack, 2008; Pérez et al., 2009). Recently, the International Agency of Research on Cancer (IARC) classified TiO_2 as a possible group 2B human carcinogen (IARC, 2006) due to the possibility of it causing lung cancer by exposure through inhalation. Applications of TiO_2 in cosmetics, pharmaceutics and the food industry were debated by IARC, but the final report was inconclusive, as outlined by Jacobs and colleagues (2010) in the journal *NanoEthics*.

Hydroxylated fullerenes (fullerenols; $C_{60}(OH)_{24}$), as well as other water soluble fullerene species, are increasingly used in biomedical research and appear to have therapeutic and diagnostic potential (Cataldo and da Ros, 2008). They are readily absorbed at the organismal and cellular level (Handy et al., 2008; Moore, 2006), can act as a potent inhibitor of cytochrom P450-dependent monooxygenases (Ueng et al., 1997) and are strong antioxidants (Markovic and Trajkovic, 2008). Due to their suppression of Reactive Oxygen Species (ROS) production, hydroxylated fullerenes are being investigated as experimental drugs in the treatment of Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis (Cai et al., 2008; Cataldo and da Ros, 2008; Dugan et al., 2001). Research performed on zebrafish models indicate that water soluble fullerenes can be used as effective cytoprotective and antiapoptotic drugs (Beuerle et al., 2007). Other fullerene species are considered as photosensitizer agents in photodynamic therapy of bacterial, viral and neoplastic diseases (Cataldo and da Ros, 2008).

Medicinal and therapeutic applications of hydroxylated fullerenes have been investigated so far using a set of studies that focus on desirable effects and efficacy, with limited or no

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information about potential off-target effects and safety concerns. Furthermore, the increase of industrial manufacturing of hydroxylated fullerenes may pose an environmental threat to aquatic ecosystems due to their water solubility of up to 100 g L^{-1} (Hirsch, 2003) and stability in solution for at least nine months (Deguchi et al., 2001). In 2008, the estimated concentration of fullerenes in aquatic environments was 0.00031 μg mL^{-1} (Pérez et al., 2009). Recently, it was shown that the administration of hydroxylated fullerenes leads to rapid uptake by immune granulocytic cells and significant reduction of the innate immune response in a fish model (Jovanović et al., 2011), suggesting further studies are needed to better estimate the potential risk of hydroxylated fullerenes for biomedical use as well as environmental effects.

This study examined the potential of zebrafish (*Danio rerio* Hamilton, 1822) embryo microarray analysis in nanotoxicology. Zebrafish is widely used as a model in biomedical research because of its rapid reproduction rate and the abundant genetic information from recent sequencing of the zebrafish genome. These characteristics make the zebrafish an excellent model for studies in developmental biology, genetics and toxicology (Berman et al., 2003; Chen et al., 2010). Zebrafish are also increasingly used in immunological research (Trede et al., 2004) and offer a unique opportunity to discover and study novel genes required for the control of normal vertebrate hematopoiesis and functioning of adult blood cells in health and disease. The immune system of zebrafish has many features in common with the mammalian innate immune responses (Palić et al., 2007; Renshaw et al., 2006; Traver et al., 2003). Similarities of inflammatory processes are used in the zebrafish model to study inflammation resolution related to lung pathology (Renshaw et al., 2007). The optical clarity, ease of maintenance, chemical administration and sensitivity make zebrafish embryos an irreplaceable tool in drug screening and toxicity testing (Parng, 2005; Parng et al., 2002; Rubinstein, 2006).

Gene microarrays are used frequently in large scale quantitative studies of mechanisms of disease, disease progression prediction, predicting activities of new compounds and grouping genes into functional pathways (Stoughton, 2005). Gene microarrays of zebrafish embryos have been used increasingly in place of adult zebrafish genomic studies (de Jong et al., 2010; Mathavan et al., 2005). However, most gene microarray nanoparticle studies are based solely on adult zebrafish, and limited attempts have been made to adapt zebrafish embryo microarrays to study the mechanisms of nanotoxicology. The few exceptions (Usenko et al., 2008; Yeo and Kim, 2010) have exposed the embryos through immersion in nanoparticle solutions without the ability to measure the concentration of nanoparticles that was actually entering the embryo and was responsible for observed transcriptomic changes. Although fish embryos can uptake nanoparticles from a water column (Kashiwada, 2006), the primary mechanism of nanoparticle uptake by adult fish is not through their skin or gills, but through the gut (Handy et al., 2008) and after the uptake nanoparticles are transported to target organs. Nanoparticles also have a limited ability to penetrate through mammalian skin as well (Baroli et al., 2007; Kokura et al., 2010; Xia et al., 2010). Due to the tender nature of the embryo epidermal layer, nanoparticle immersions can cause mechanical abrasions on embryo integument and hinder the detection of important molecular pathways. Such abrasions may explain the unusual expression of genes for keratin and β-actin in zebrafish microarray embryo studies (Usenko et al., 2008). Therefore, these changes in gene expression might not be the result of intrinsic toxicity feature of nanoparticles, but rather reflect mechanical damage and tissue regeneration. The zebrafish embryo microinjection technique is used to precisely deliver a known concentration of chemical to the target organ or developmental area (Xu, 1999), therefore, bypassing the fragile integument exposure through immersion. Injections are also a standard model for evaluating toxicology and hematology parameters not only in fish models but also in mammals, including humans (Janeway et al., 2008).

Due to the rising concerns about nanoparticle effects on the immune and nervous systems (Dobrovolskaia and McNeil, 2007; Yang et al., 2010) the aim of this study was to investigate transcriptomic pathways at the level of the neuro-immunological interface of zebrafish embryos microinjected with nano-TiO_2 and hydroxylated fullerenes in the otic vesicle which is in the close proximity of the brain.

2. Materials and methods

2.1. Animal care

Adult zebrafish were maintained in the Iowa State University College of Veterinary Medicine Laboratory Animal Resources Facility, Ames, Iowa, USA. Fish were housed in an AHAB Benchtop system from Aquatic Habitats (Apopka, FL, USA) supplied with dechlorinated tap water at 28 °C. Fish were cared for in accordance with standard zebrafish protocol (Westerfield, 2000), approved by the Animal Care and Use Committee of Iowa State University.

2.2. Nanoparticle characterization

Nano-TiO_2 (anatase, nanopowder, <25 nm, 99.7% metals basis; Sigma-Aldrich Corp, St. Louis, MO, USA) and hydroxylated fullerenes $C_{60}(OH)_{24}$ (MER Corporation, Tucson, AZ, USA, cat# MR16, 98% purity) were suspended in Hanks Balanced Salt Solution without Ca, Mg, no Phenol Red, (HBSS; Mediatech—CellGro, Manassas, VA, USA). Full characterization of both particles' suspension in HBSS and suspension preparation is described in our previous research (Jovanović et al., 2011, In Press). Briefly, nano-TiO_2 and hydroxylated fullerenes with mean particle diameters of 86 and 409 nm and zeta potential of −8.87 and −19.1 mV, respectively, were used in the experiments.

2.3. Fish mating and embryo microinjections

Zebrafish mating and embryo collection was performed daily according to (Levraud et al., 2007; Westerfield, 2000). Several mating tanks were set up each day with 3 females and 2 males. After successful mating, embryos from three random mating tanks were pooled together, rinsed with fish egg water (60 μg of sea salt [Instant Ocean, Kingman, AZ, USA] per mL of deionized water) (Westerfield, 2000), transferred to a Petri dish with egg water and incubated for 48 h at 28.5 °C. At 48 h post-fertilization (hpf) 25 dechorionated embryos were randomly collected, weighed (average weight of an embryo was 0.2 mg), anesthetized (0.25 mg mL^{-1} buffered [sodium bicarbonate, pH 7.2] solution of the tricaine methane sulfonate [MS-222, Argent Laboratories, Redmond WA, USA] in egg water and microinjected in the otic vesicle with 10 nL of nanoparticle suspension (Levraud et al., 2007). Control group embryos were injected with 10 nL of HBSS. In order to deliver 10 nL PLI-90 Pico-Injector (Harvard Apparatus, Holliston, MA, USA) was used with the following set up: pressure at 30 psi, time at 2 s and diameter of syringe at 1 μm, according to manufacturer's protocol.

Nanoparticle injection suspensions were made fresh daily, with a concentration of 40 μg mL^{-1} of hydroxylated fullerenes and 170 ng mL^{-1} of nano-TiO_2. Nano-TiO_2 was previously filtered through 220 nm filter and the concentration of 170 ng mL^{-1} was determined after filtration by Inductively Coupled Plasma Mass Spectroscopy (ICP–MS). After the administration of 10 nL to 0.2 mg embryos, the final concentration inside the embryo was 2 μg g^{-1} bodyweight for hydroxylated fullerenes and 8.5 ng g^{-1} bodyweight for nano-TiO_2. These concentrations were chosen based on previous findings for sublethal intraperitoneal injections in fathead minnow that were affecting the innate immune response (Jovanović et al., 2011, In Press). After the microinjections, embryos were incubated for 48 h at 28.5 °C. At 96 hpf embryos were collected and sacrificed with quick asphyxia, and total RNA was immediately isolated. The whole process was repeated to obtain a total of 18 samples, each consisting of 25 pooled embryos ($n=6$ samples per each treatment, and $n=6$ samples per control).

2.4. RNA isolation and microarray hybridization

Total RNA extraction was performed according to the acid guanidinium thiocyanate-phenol-chloroform extraction protocol (Chomczynski and Sacchi, 2006) using TRI Reagent® (Molecular Research Center, Inc. Cincinnati, OH, USA). Embryos were ground in a 1.5 mL centrifuge tube containing 1 mL of Tri-Reagent.