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Toxicity of single walled carbon nanotubes to rainbow trout, (*Oncorhynchus mykiss*): Respiratory toxicity, organ pathologies, and other physiological effects

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Abstract

Mammalian studies have raised concerns about the toxicity of carbon nanotubes (CNTs), but there is very limited data on ecotoxicity to aquatic life. We describe the first detailed report on the toxicity of single walled carbon nanotubes (SWCNT) to rainbow trout, using a body systems approach. Stock solutions of dispersed SWCNT were prepared using a combination of solvent (sodium dodecyl sulphate, SDS) and sonication. A semi-static test system was used to expose rainbow trout to either a freshwater control, solvent control, 0.1, 0.25 or 0.5 mg l^{-1} SWCNT for up to 10 days. SWCNT exposure caused a dose-dependent rise in ventilation rate, gill pathologies (oedema, altered mucocytes, hyperplasia), and mucus secretion with SWCNT precipitation on the gill mucus. No major haematological or blood disturbances were observed in terms of red and white blood cell counts, haematocrits, whole blood haemoglobin, and plasma Na^+ or K^+. Tissue metal levels (Na^+, K^+, Ca^{2+}, Cu, Zn and Co) were generally unaffected. However some dose-dependent changes in brain and gill Zn or Cu were observed (but not tissue Ca^{2+}), that were also partly attributed to the solvent. SWCNT exposure caused statistically significant increases in Na^+K^+-ATPase activity in the gills and intestine, but not in the brain. Thiobarbituric acid reactive substances (TBARS) showed dose-dependent and statistically significant decreases especially in the gill, brain and liver during SWCNT exposure compared to controls. SWCNT exposure caused statistically significant increases in the total glutathione levels in the gills (28%) and livers (18%), compared to the solvent control. Total glutathione in the brain and intestine remained stable in all treatments. Pathologies in the brain included possible aneurisms or swellings on the ventral surface of the cerebellum. Liver cells exposed to SWCNT showed condensed nuclear bodies (apoptotic bodies) and cells in abnormal nuclear division. Overt fatty change or wide spread lipidosis was absent in the liver. Fish ingested water containing SWCNT during exposure (presumably stress-induced drinking) which resulted in precipitated SWCNT in the gut lumen and intestinal pathology. Aggressive behaviour and fin nipping caused some mortalities at the end of the experiment, which may be associated with the gill irritation and brain injury, although the solvent may also partly contributed to aggression. Overall we conclude that SWCNTs are a respiratory toxicant in trout, the fish are able to manage oxidative stress and osmoregulatory disturbances, but other cellular pathologies raise concerns about cell cycle defects, neurotoxicity, and as yet unidentified blood borne factors that possibly mediate systemic pathologies.

Keywords: Single walled carbon nanotubes; SWCNT; Gill; Respiration; Brain pathology; Liver pathology; Haematology; Na^+K^+-ATPase; TBARS; Glutathione; Rainbow trout; Copper; Zinc

1. Introduction

Nanotechnology has been defined as using materials and structures with nanoscale dimensions, usually in the range 1–100 nm (Masciangioli and Zhang, 2003; Roco, 2003). However from the view point of toxicology, this definition is not absolute and studies have included aggregates of nanomaterials, as well as individual particles (Handy and Shaw, 2007). Manufactured nanomaterials have numerous industrial applications including electronics, optics, and textiles, as well as applications in medical devices, drug delivery systems, chemical sensors, biosensors, and in environmental remediation (Kong et al., 2000; Masciangioli and Zhang, 2003; Freitas, 2005; Aitken et al., 2006). The materials are often custom made for the particular application, and it is therefore no surprise that there are a wide variety of nanomaterials and nanoparticles. Toxicological research on nanomaterials has currently focused on two major groups of materials. These include the effects of carbon-

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based nanomaterials such as carbon nanotubes (CNTs) or carbon nanospheres ("fullerenes") (Lam et al., 2004; Oberdörster, 2004; Cui et al., 2005), and the effects of metal or metal oxide nanoparticles (Bermudez et al., 2004; Chen et al., 2006; Sayes et al., 2006). There are many variations in structure within each of these major types of engineered nanomaterials, and CNTs are commercially available as single walled (SWCNT) or multi-walled carbon nanotubes (MWCNTs) of different sizes (Roco, 2003).

Most of the emerging literature on the toxicity of nanoparticles has focused on respiratory exposure in mammalian models and the implications for human health; and these studies confirm that nanoparticles can have toxic effects (review, Handy and Shaw, 2007). Some toxic effects in mammals have been attributed to CNTs. For example, mice exposed to a single intratracheal instillation (i.t.) dose of 0.1 or 0.5 mg SWCNT, experienced over 55% mortalities within 7 days of exposure, with a dose-dependent incidence of lung pathologies (epithelioid granulomas, inflammation injuries, necrosis) presenting during the 90-day post-exposure follow up (Lam et al., 2004). Warheit et al. (2004) made similar post-exposure observations with SWCNT, and it is now recognised that CNTs are not easily cleared from the lung (e.g. MWCNT in rat lung 60 days after exposure, Muller et al., 2005). There have also been a range of *in vitro* studies using human or mammalian cell lines to investigate toxic mechanisms (see Handy and Shaw, 2007), which suggest that oxidative stress, inflammation reactions, and immunotoxicity may be key features of nanoparticle toxicity. For example, Barlow et al. (2005) exposed bovine serum to ultrafine carbon black particles and demonstrated that chemical anti-oxidants delayed macrophage aggregation responses. Shvedova et al. (2003) showed that SWCNT caused antioxidant depletion, free radical formation, and the accumulation of peroxide products with a loss of cell viability in human epidermal keratinocytes (HEK) cells.

These mammalian studies, and the apparent persistence of CNTs in tissues, raises concerns that nanomaterials may also be toxic to wildlife (Owen and Depledge, 2005). This has at least been partially confirmed in a few ecotoxicity studies on fish and invertebrates using carbon-based nanomaterials. Oberdörster (2004) showed that juvenile largemouth bass (*Micropterus salmoides*) exposed to 0.5 or 1 mg l^{-1} C_{60} fullerenes for up to 48 h (dissolved in tetrahydrofuran, THF) showed elevated lipid peroxidation products in the brain (but not the gill or liver) and a small reduction in the total glutathione pool of the gills. In a subsequent study on C_{60} fullerenes (without THF dispersion), Oberdörster et al. (2006) showed that 2.5–5 mg l^{-1} of C_{60} delayed moulting in *Daphnia*. Lovern and Klaper (2006) estimate the lethal concentrations of C_{60} fullerenes (48 h LC_{50}) to *Daphnia* was between 460 μg l^{-1} and 7.9 mg l^{-1} depending on the method of preparation of the nanomaterial. Although these examples raise concerns about ecotoxicity, there are few ecotoxicological studies on CNTs, and there have been no systematic investigations of the toxic effects of CNTs to rainbow trout.

In this study we used SWCNTs and similar dosimetry to that used in mammalian studies, and adopt a body systems approach to give the first detailed overview of the toxic effects of CNT in trout. Our aim was to measure functional responses in key areas of physiology (e.g. ventilation, osmoregulation, haematology) as well as documenting organ pathologies and biochemical responses during aqueous exposure. We therefore measured a range of end points including behaviours, gill ventilation rates, haematology and plasma ions, trace element profiles in the major organs, a suite of histopathology, as well as biochemical measurements relating to physiological fuction (e.g. Na^+K^+-ATPase activity) or oxidative stress (TBARS, glutathione content).

2. Materials and methods

2.1. Experimental design

Juvenile rainbow trout ($n = 180$) were obtained from Hatchlands Trout Farm, Rattery, Devon, and held for 4 weeks in stock aquaria with flowing, aerated, dechlorinated Plymouth tap water (see below). Stock animals were fed to satiation on a commercial trout food. Fish weighing 30.0 g ± 5.0 (mean ± S.E.M., $n = 180$) were then graded into fifteen experimental glass aquaria (12 fish/tank), in a triplicate design (3 tanks/treatment), and allowed to rest for 24 h prior to the commencement of the experiment. Fish were exposed in triplicate to one of the following treatments for 10 days using a semi-static exposure regime (80% water change every 12 h with re-dosing after each change): control (freshwater only, no CNT or solvent), solvent control (0.15 mg l^{-1} sodium dodecyl sulphate, SDS), 0.1, 0.25 or 0.5 mg l^{-1} SWCNT (see below for stock solutions). These SWCNT concentrations were selected after considering the doses used to produce epithelial injury in rat lung (Lam et al., 2004), and the sub-lethal effects of low mg amounts of fullerenes in largemouth bass and fathead minnows (Oberdörster, 2004; Oberdörster et al., 2006). In this experiment, the 0.15 mg l^{-1} concentration of SDS (also performed in triplicate tanks) represented the highest amount of solvent added to the highest SWCNT concentration. However, the 0.1 and 0.25 mg l^{-1} SWCNT contained less solvent (see stock solutions below) and we therefore also performed an additional solvent control experiment with the range of SDS concentrations used in triplicate (see stock solutions below) to verify that there was no dose-effect that could be attributed to the solvent (none was observed, data not shown).

Fish were not fed 24 h prior to, or during the experiment in order to minimise the risk of the CNT absorbing to food or faecal material, and to help maintain water quality. Water samples were collected immediately before and after each water change for pH (YSI 63 pH meter), total ammonia (HI 95715, Hanna Instruments), dissolved oxygen (YSI 85 D.O. meter). There were no treatment differences in water quality between tanks (ANOVA, $P > 0.05$). Values were (means ± S.E.M., $n = 253$-261 samples); total ammonia, 0.83 ± 0.18 mg l^{-1}; pH, 7.16 ± 0.01; oxygen saturation, 83 ± 0.13 %; temperature, 15.5 ± 0.3 °C. Photoperiod was 12 h light: 12 h dark. The electrolyte composition of the dechlorinated Plymouth tap water used was 0.3, 0.1, and 0.4 mmol l^{-1} for Na^+, K^+ and Ca^{2+} respectively. Fish were randomly sampled on day 0 (initial fish from the stock), day 4, and day 10 for haematology, plasma ions, tissue electrolytes,