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Insights into the toxicity of iron oxides nanoparticles in land snails

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ABSTRACT

The use of manufactured nanoparticles (NPs) is spreading rapidly across technology and medicine fields, posing concerns about their consequence on ecosystems and human health. The present study aims to assess the biological responses triggered by iron oxide NPs (IONPs) and iron oxide NPs incorporated into zeolite (IONPZ) in relation to oxidative stress on the land snail *Helix aspersa* in order to investigate its use as a biomarker for terrestrial environments. Morphology and structure of both NPs were characterized. Snail food was supplemented with a range of concentrations of IONPs and IONPZ and values of the hemocyte lysosomal membranes' destabilization by 50% were estimated by the neutral red retention (NRRT50) assay. Subsequently, snails were fed with NPs concentrations equal to half of the NRRT50 values, 0.05 mg L^{-1} for IONPs and 1 mg L^{-1} for IONPZ, for 1, 5, 10 and 20 days. Both effectors induced oxidative stress in snails' hemocytes compared to untreated animals. The latter was detected by NRRT changes, reactive oxygen species (ROS) production, lipid peroxidation estimation, DNA integrity loss, measurement of protein carbonyl content by an enzyme-linked immunoabsorbent assay (ELISA), determination of ubiquitin conjugates and cleaved caspases conjugates levels. The results showed that the simultaneous use of the parameters tested could constitute possible reliable biomarkers for the evaluation of NPs toxicity. However, more research is required in order to enlighten the disposal and toxic impact of iron oxide NPs on the environment to ensure their safe use in the future.

1. Introduction

Owing to the rapidly emerging breakthroughs in the field of nanotechnology, iron oxide nanoparticles (IONPs) have gained great potential in many applications in medicine, magnetic printing and media recording, as catalysts (Lorentzou et al., 2008), in heavy metal and waste water treatment and in the removal of pharmaceutical products from aqueous solutions (Gallios and Vaclavikova, 2008; Teja and Koh, 2009). For biocompatibility and biodistribution enhancement purposes, iron oxide NPs are manufactured with surface coating modifications (Singh et al., 2010; Mahmoudi et al., 2011) such as incorporation into zeolite, a biologically safe, highly porous material (Misaelides, 2011). Recently, the production of engineered nanoparticles (NPs) has increased, resulting to their inevitable release to the environment. Although recent reviews have shed light into the environmental concentrations of engineered nanomaterials such as TiO_2, ZnO, and Ag (Gottschalk et al., 2013; Maurer-Jones et al., 2013), to our knowledge data on the levels of IONPs released into aquatic and terrestrial ecosystems are not available.

The harmful effects of iron oxide NPs have been demonstrated in mussels *Mytilus galloprovincialis* (Taze et al., 2016), in murine macrophages (Naqvi et al., 2010) and in mouse fibroblasts (Mahmoudi et al., 2009). Moreover, the effects of iron oxide NPs have been investigated in human lung epithelial cells (Karlsson et al., 2008) and fibroblasts (Gupta and Curtis, 2004). In a recent review on the carcinogenicity of inhaled iron oxide particulates, it is suggested that the larger surface

Abbreviations: NPs, nanoparticles; IONPs, iron oxide NPs; IONPZ, iron oxide NPs incorporated into zeolite; NRRT, neutral red retention time; ROS, reactive oxygen species; $DCFH_2$-DA, 2′,7′-dichlorodihydrofluorescin diacetate; DCFH, 2′,7′-dichlorofluorescin; DCF, 2′,7′-dichlorofluorescein; 4-HNE, 4-hydroxynonenal; RLU, relative luminescence units; MDA, malondialdehyde; PUFAs, polyunsaturated fatty acids; DNPH, 2,4-dinitrophenylhydrazine; BSA, bovine serum albumin; PMSF, phenyl methyl sulfonyl fluoride; HRP, horseradish peroxidase-linked; ANOVA, two-way analysis of variance; TEM, transmission electron microscopy; SEM, scanning electron microscopy; FTIR, Fourier transform infrared spectroscopy; PCC, protein carbonyl content; Cas, caspase

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area of the "nano" iron oxide particulates in comparison to the "bulk" iron oxide particulates could lead to generation of reactive oxygen species (ROS) in the cell and subsequently to toxicity (Pease et al., 2016). It has been reported that after uptake and intracellular internalization, iron oxide NPs induce oxidative stress via three primary routes: direct ROS generation on the NPs surface, production of ROS due to leaching of iron ions by surface enzymatic degradation and modification of organelle functions including mitochondria leading to cell signaling pathway induction (Liu et al., 2012; Mahmoudi et al., 2012). The determination of ROS levels in hemocytes has been used to assess oxidative stress in marine (Patetsini et al., 2013; Taze et al., 2016) and terrestrial environments (Itziou and Dimitriadis, 2011). ROS generation can cause protein carbonylation (Mohanty et al., 2010), DNA breakage that can be detected by the comet assay (McKelvey-Martin et al., 1993), lipid peroxidation (Kaloyianni et al., 2009; Taze et al., 2016), proteolysis through the ubiquitin pathway (Tedesco et al., 2008) and apoptosis through the activation of proteins of the caspase family (Gomes et al., 2014).

The present study's purpose is to evaluate iron oxide NPs' toxicity and to elucidate the underlying nanotoxicity mechanisms with respect to *Helix aspersa* cellular redox state. For this reason, the animals' food was supplemented with the particular NPs for various periods and the impact of short and long term exposure to iron oxide NPs (IONPs) and iron oxide NPs incorporated into zeolite (IONPZ) was determined by measuring parameters related to oxidative stress in *Helix aspersa* hemocytes and hemolymph. Among terrestrial invertebrates, snail species *H. aspersa* is a metal organotrophic organism that is considered a bioindicator of great significance in the ecotoxicology field (Viard et al., 2004) and therefore it is used extensively in biomonitoring surveys (Swaileh and Ezzughayyar, 2000; Regoli et al., 2006). To our knowledge this is the first report to investigate the influence of the particular nanoparticles on terrestrial snails' biomonitoring studies. The biomarkers applied include the established biomarker Neutral Red Retention Time (NRRT50) used broadly for the evaluation of the stability of lysosomal membranes, ROS measurement as indicative of immune system activation, DNA damage, lipid peroxidation, protein carbonylation, quantification of ubiquitin conjugates and caspase activity. Thus, this battery of biomarkers is suggested in order to better evaluate the impact of the increasing versatile use and the eventual disposal and accumulation of iron oxide NPs on the terrestrial environment.

2. Materials and methods

2.1. Animal maintenance, exposure and sample collection

Snails *H. aspersa* with 27.06 ± 2.1 mm mean shell length and 34.2 ± 1.65 mm mean shell width were obtained from a snail breeding farm and placed in aerated transparent polystyrene containers (30 × 21 × 17 cm, 7 L) under the following conditions: 14:10 h light:-dark photoperiod, 20–22 °C temperature, 80–90% relative humidity. A layer of absorbent paper was placed on the bottom of the containers and food was supplied daily in Petri dishes. The paper was changed daily and the containers were cleaned 3 times per week. Snail food was prepared as following: 4 g Cerelac baby food (Nestle, Belgium), 1 g Agar (Sigma), 0.3% v/v fungicide (methylparaben) (Swaileh and Ezzughayyar, 2000) mixed with the required concentrations of NPs in distilled water to 100 mL final volume and then dispersed in petri dishes (25 mL/dish).

Animals received proper care in compliance with the "Guidelines for the Care and Use of Laboratory Animals" published by US National Institutes of Health (NIH publication No 85–23, revised 1996) and the Greek Presidential Degree No 56/2013 Official Journal of the Greek Government No. 106/30 April 2013 enforcing the EU Directive No2010-63/2010-EU on the protection of animals used for scientific purposes.

2.2. NPs preparation

Pure synthetic IONPs and IONPZ were prepared according to Stefusova (2010) and Vaclavikova et al. (2004, 2010) by adding equimolar solutions of Fe(III) and Fe(II) salts in a beaker. The pH was adjusted by adding NaOH solution and iron (II) and (III) oxides co-precipitated forming magnetite. The original NPs sizes were ~20 nm, however they are able to form clusters ranging from 20 to 900 nm (Vaclavikova et al., 2004). Their point of zero charge was at pH 5.5. Their charge was negative at pH values over 5.5 reaching around −25 mV at pH 7.0 (Stefusova, 2010). Magnetic zeolite was prepared with a similar procedure. Zeolite particles [clinoptinolite type from NiznyHrabovec (Slovakia)] ranging 400–500 μm were added in the beaker together with the iron salts and the same precipitation procedure was applied after 1 h constantly mixing at 30 rpm and 80 °C temperature. The magnetite to zeolite ratio was 1:2 and the zeolite produced had a surface area of 113 $m^2 g^{-1}$. The point of zero charge was at pH 5.0 with a negative charge (approximately −20 mV) at pH 7.0. The average load of iron oxide NPs encountered in zeolite structure was 6% (Vaclavikova et al., 2010).

2.3. NPs characterization

IONPs and IONPZ were characterized for their morphology by Transmission Electron Microscopy (TEM, JEOL JEM-2010) and Scanning Electron Microscopy (SEM JEOL JSM-6300); for their structure by X-Ray Diffraction (XRD Siemens D500/501); their particle size distribution was obtained by an aerodynamic particle size analyzer (API, Aerosizer-Aerodisperser, TSI 3603); their surface area was measured by nitrogen adsorption by BET method (Autosorb-1) and their Raman spectra was recorded in the 100–3200 cm^{-1} region using a micro-Raman (Renishaw in Via Reflex) Spectrometer equipped with an air-cooled Argon ion laser, a CCD detector and an automatic X-Y-Z stage. A 514.5 nm (delivering ~7.5 mW) laser line was used as an excitation source.

2.4. Experimental and exposure procedure

Prior to the administration of NPs, the snails were kept without food supply for 4 days in order to acclimatize to laboratory conditions. The control group was fed with artificial food lacking NPs whereas two groups of snails were fed with the same food containing concentrations of 0.05 mg L^{-1} IONPs and 1 mg L^{-1} IONPZ, respectively for 20 days. NPs concentration in the present study was selected following an initial estimation of the NRRT50 values for both NPs. At 4 time points during the treatment period (1st, 5th, 10th and 20th day) hemolymph from 15 snails per group was pooled and estimation of destabilization of the hemocyte's lysosomal membranes, ROS levels, lipid peroxidation, protein carbonylation, DNA damage, ubiquitin conjugates and cleaved caspases conjugates levels were measured in isolated hemocytes. The present results are depicted as mean values ± SD of 3 separate assays. During the treatment no mortality incidents were observed in control or exposed groups.

2.5. Hemolymph and hemocytes collection and handling

At each time point, hemolymph of 15 snails per group was collected from the heart cavity with a sterile syringe (18G1/2′ needle). Pooled hemolymph aliquots (1 mL) were centrifuged at 3000 rpm, for 10 min at 4 °C and the hemocytes were used for the biochemical analysis. For the NRRT assay, hemolymph from 10 snails was extracted from the heart cavity and was mixed with snail physiological saline buffer (pH 7.3) containing 20 mM Hepes, 436 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$ and 53 mM $MgSO_4$.

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