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Endocrine activities and cellular stress responses in the marsh frog *Pelophylax ridibundus* exposed to cobalt, zinc and their organic nanocomplexes

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ABSTRACT

Metal-containing materials are extensively used in industry, personal care products and medicine, and their release in the environment causes concern for the potential impacts on aquatic organisms. We assessed endocrine disrupting potential of *N*-vinyl-2-pyrrolidone-based nanoparticles (Me-PSs) containing cobalt (Co^{2+}) or zinc (Zn^{2+}), using the marsh frog *Pelophylax ridibundus* as a model. Adult males were exposed for 14 days to waterborne Co^{2+} (50 μg/L), Zn^{2+} (100 μg/L) or corresponding concentrations of Co-PS, Zn-PS, or parental polymeric compound (PS). The indices of thyroid activity, vitellogenesis, cytochrome P450-dependent monooxygenases activity (EROD) and cytotoxicity markers were evaluated. Exposure to Co^{2+} led to the elevation of serum thyrotropin (TSH) and hepatic deiodinase activities accompanied by the up-regulation of EROD activity. In contrast, the action of the polymer-containing substances (Co-PS, Zn-PS and PS) as well as free Zn^{2+} caused a prominent decrease of EROD activity and a decrease in serum cortisol and TSH concentrations. Exposures to Zn^{2+}, Zn-PS and PS upregulated vitellogenesis in males. All exposures except Co^{2+} caused neurotoxicity as indicated by the depletion of cholinesterase. These results demonstrate toxicity of Co- and Zn-containing Me-PSs and their parental compounds (Zn^{2+} and PS) in frogs and indicate distinct mechanisms of Co^{2+} action. Broad disruption of the hormonal pathways and reduced capacity for organic xenobiotic detoxification may have deleterious impacts on amphibian populations from habitats exposed to metallorganic pollution.

1. Introduction

Amphibians are excellent bioindicators of aquatic pollution and ecosystem health due to susceptibility of all life stages to dermal absorption of toxicants in water, and useful models for environmental and biomedical research (Hermes-Lima and Storey, 1998). The endocrine-disrupting effects of environmental pollutants are of special concern in amphibians (Hayes et al., 2006; Kloas et al., 2009). Frogs are sensitive to environmental xenoestrogens such as the herbicide, atrazine, and estrogenic compounds in sewage effluents that could interfere with vitellogenin production, gonadal development and reproduction in males (Rouhani Rankouhi et al., 2005; Hayes et al., 2006; Falfushinska et al., 2008). During metamorphosis, frogs are also highly sensitive to thyroid-disrupting chemicals with wide-ranging consequences for development and growth (Veldhoen et al., 2006). Less is known about the effects of pollutants on the endocrine functions of adult amphibians and the potential interplay between the adverse effects of pollutants on different endocrine functions including reproductive hormones, thyroid and corticosterone systems.

Rapidly expanding production and application of engineered metal complexes of organic polymeric substances (Me-PSs) in industry and medicine increase the potential exposures and associated risks for humans and wildlife. The nanoscale size of these complexes as well as their biological uptake and biodegradation highlight the necessity to understand their biological fate and effects in an organism (Colognato et al., 2008; Lowry et al., 2012).

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Nanoparticles and their breakdown products (including the constituent metals) have a potential to cause cellular toxicity as well as to disrupt the endocrine system (Meeker et al., 2009; Iavicoli et al., 2013). However, the toxicity of these substances and their constituents has not been well studied, particularly in amphibians (Nations et al., 2011; Zhang et al., 2012a; Hammond et al., 2013).

N-vinyl-2-pyrrolidone (NVP) is an organic polymer-forming molecule with low toxicity, excellent film forming capacity and adhesive properties that is extensively used for the development of the metal-containing nanomaterials in industry, as well as in personal care products and pharmaceuticals (Xiong et al., 2006; Luther et al., 2012; Zhang et al., 2012b). Nanoparticles commonly contain zinc (Zn) and cobalt (Co) and may pose significant environmental and health concerns due to the important biological roles and established toxicity of Zn and Co. Zn is involved in molecular stabilization, catalysis, DNA replication, and cell signaling (Outten and O'Halloran, 2001; Krezel and Maret, 2007). In oviparous vertebrates including frogs, Zn is also involved in the oogenesis (Falchuk and Montorzi, 2001). Excess of Zn may cause reproductive failure due to the inhibition of the pentose phosphate pathway in frog oocytes (Naab et al., 2001). Co plays a crucial role in regulation of vital functions as a cofactor of multiple cyanocobalamin- and noncorrin-Co-containing enzymes (Kobayashi and Shimizu, 1999). However, excess of Co may induce toxicity due to the redox-active biochemistry of this metal (namely, the ability to promote hydroxyl and carbon monooxide production) and its interactions with heme-related prosthetic groups (Oyekan et al., 1999; Valko et al., 2005; Kubrak et al., 2012; Simonsen et al., 2012). In frogs, the effects of Co have not been well studied (Gaitanaki et al., 2007). Physiological and toxic effects of trace metals such as Zn and Co may be strongly modified in engineered nanoparticles derived from *N*-vinyl-2-pyrrolidone (NVP) due to the direct toxic effects of NVP, cellular disturbances induced by the nanosized particles, and the molecular mimicry or potential catalytic ability of NVP-based polymer-metal complexes that structurally resemble metalloenzymes (Anasuya et al., 2015).

This study aims to elucidate the toxic and endocrine disrupting mechanisms of the organic NVP-based nanoscale composites of Co and Zn in frogs and determine whether these effects are mediated by biodegradation of the metal-containing polymer nanoparticles (Me-PS) releasing toxic metals. In order to do this, we assessed cellular, molecular and endocrine effects of exposures to Me-PSs in frogs and compared them to the effects of the free ionic forms of Co^{2+} and Zn^{2+} and the parent organic polymeric compound, *N*-vinyl-2-pyrrolidone (PS). We focused on the effects of Me-PSs and their constituents on three major hormonal systems—the thyroid axis involved in the regulation of metabolism (using concentration of thyrotropin (TSH) as a marker of the thyroid function), the stress axis (assessed by changes in the cortisol concentration) and reproductive axis assessed by the induction of vitellogenin (Vtg) in males as a biomarker for xenooestrogen exposure. At the cellular level, stress biomarkers were assessed including activity of the Phase I enzymes (hepatic ethoxyresorufin-*O*-deethylase (EROD) involved in biotransformation and detoxification of organic xenobiotics), oxidative stress assessed by oxyradical formation, cytotoxicity (lysosomal membrane stability) and neurotoxicity (cholinesterase activity). This study provides an integrative assessment of the potential health effects of the NVP-based metalloparticles that are common pollutants in freshwater ecosystems and offers new insights into the toxic and endocrine disrupting mechanisms of the NVP-metal nanoparticles.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin, phenylmethylsulfonyl fluoride (PMSF), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), ethylenediaminetetraacetic acid (EDTA), dihydrorhodamine, β-NADPH, 7-ethoxyresorufin, Human TSH (thyroid stimulating hormone) ELISA (Enzyme-Linked Immunosorbent Assay) kit, Cortisol ELISA Kit, and Mouse/Rat Triiodothyronine (T3) ELISA Kit were purchased from Sigma–Aldrich, USA. Monoclonal antibody-goat antimouse horseradish peroxidase (MAb GAM-HRP) was purchased from Biosense, Norway. All other chemicals were of analytical grade and purchased from Merck (Synbias, Donetsk, Ukraine). The PS from N-vinyl pyrrolidone (NVP), 5-(*tert*butylperoxy)-5-methyl-1-hexene-3-yne (VEP) and dimethyl aminoethyl methacrylate (DMAEM) and its coordinated Me-PSs nanoparticles were synthesized as described elsewhere (Zaichenko et al., 1998; Falfushynska et al., 2014). Average molecular mass of the synthesized Me-NC was 1.5 kDa (Supplement 1). Co and Zn comprised 6.5% and 16.5% by mass of the corresponding Me-PSs.

2.2. Animal collection and experimental exposures

The experiments were carried out in mid-September of 2013. Adult males of *Pelophylax ridibundus* (*Rana ridibunda*) (8–10 cm long, 45–65 g body mass) were collected from a rural site in the upstream portion of river Seret (near the village Ivachiv, 49°49′ N, 25°23′ E). This site was not exposed to industrial contamination as was shown by low environmental concentrations of pollutants and levels of biomarkers in frogs that corresponded to pristine conditions (Stolyar et al., 2008; Falfushinska et al., 2008; Falfushynska et al., 2010). The animals were out of the breeding season. Frogs were transported to the laboratory in 60 L cages with aerated native water (dissolved oxygen concentration was 8.67 ± 0.51 mg L^{-1}). Experiments were performed in accordance with the national and institutional guidelines for the protection of animal welfare with permission of the Ministry of Ecology and Natural Resources of Ukraine, No 466/17.04.2013 and approval of the Committee on the Bio-Ethics at Ternopil National Pedagogical University (No 2/10.06.2013).

Frogs were acclimated in plastic containers (6 animals per container) with 40 L of aerated, softened tap water and fed throughout the experiment with commercial sticks "Turtle menu" (21% of protein, Aquarius, Ukraine) placed in a tray inside the container. After seven days of preliminary acclimation, frogs were randomly distributed into six groups (10 individuals per group). One group was used as a control and five other groups were exposed to one of the five experimental conditions: 833 μg L^{-1} of Co-PS; 883 μg L^{-1} Zn-PS; 50 μg L^{-1} (0.85 μmoles L^{-1}) Co as $CoCl_2$; 100 μg L^{-1} (1.54 μmoles L^{-1}) of Zn as $ZnCl_2$, or 783 μg L^{-1} of the corresponding polymeric substance (PS). A stock colloidal solution of the Me-PS nanoparticles or PS was prepared by dispersing the nanoparticle powder in distilled deionized water, followed by vigorous vortexing for 180 min at room temperature. The exposure concentrations of Co, Zn and the polymeric substance were selected to correspond to the concentrations of these components in Me-PS nanocomposites. Co concentration was chosen based on the limits found in fresh water (4–110 μg L^{-1}), with the lower concentration considered not harmful for the freshwater organisms, while the higher concentrations correspond to the acute toxicity (US EPA). Similarly, selected concentration of Zn corresponded to those found in natural ponds (67.3 ± 6.8 μg L^{-1}) (Falfushynska et al., 2010). Concentrations of Zn and Co in the water at the sampling sites were 20.2 μg L^{-1} and 0.7 μg L^{-1}, respectively. In general, Co and Zn have low toxicity to frogs compared with other trace met-