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Ecotoxicological assessment of cobalt using *Hydra* model: ROS, oxidative stress, DNA damage, cell cycle arrest, and apoptosis as mechanisms of toxicity☆

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ABSTRACT

The mechanisms underlying cobalt toxicity in aquatic species in general and cnidarians in particular remain poorly understood. Herein we investigated cobalt toxicity in a *Hydra* model from morphological, histological, developmental, and molecular biological perspectives. *Hydra*, exposed to cobalt (0–60 mg/L), were altered in morphology, histology, and regeneration. Exposure to standardized sublethal doses of cobalt impaired feeding by affecting nematocytes, which in turn affected reproduction. At the cellular level, excessive ROS generation, as the principal mechanism of action, primarily occurred in the lysosomes, which was accompanied by the upregulation of expression of the antioxidant genes SOD, GST, GPx, and G6PD. The number of Hsp70 and FoxO transcripts also increased. Interestingly, the upregulations were higher in the 24-h than in the 48-h time-point group, indicating that ROS overwhelmed the cellular defense mechanisms at the latter time-point. Comet assay revealed DNA damage. Cell cycle analysis indicated the induction of apoptosis accompanied or not by cell cycle arrest. Immunoblot analyses revealed that cobalt treatment triggered mitochondria-mediated apoptosis as inferred from the modulation of the key proteins Bax, Bcl-2, and caspase-3. From this data, we suggest the use of *Hydra* as a model organism for the risk assessment of heavy metal pollution in aquatic ecosystems.

1. Introduction

Cobalt is an oligometal required in trace amount by plants and animals for the synthesis of vitamin B12 and other cobalamins. The biological role of cobalt is linked to neuronal activity, cell division, and hemopoiesis (Simonsen et al., 2012). The sources of cobalt in the environment are both natural and anthropogenic. Anthropogenic sources include fossil fuel combustion, mining industries, and agricultural run-offs. Exposure to cobalt at concentrations above certain levels can inflict toxic effects in humans and animals (Kim et al., 2006), but unlike other divalent metals (copper, iron, and cadmium), only limited data are available for cobalt, based on which the International Agency for Research on Cancer (IARC) classified it as a possible carcinogen (IARC, 2006). Hence, studies on the potential adverse effects of cobalt in the biological systems and the environment are pertinent.

Cobalt toxicity has been investigated mostly in cell lines with emphasis on hypoxia-linked neuronal damages. Cobalt stabilizes hypoxia-inducible factor-1 alpha protein by blocking its ubiquitination and proteasomal degradation, thereby mimicking hypoxia-like condition (Simonsen et al., 2012). Studies also indicate that cobalt is cytotoxic and genotoxic to cells (Gault et al., 2010; Tsuchida et al., 2014; Uboldi et al., 2016), leading to apoptosis by the modulation of Bcl-2, Bax, and caspases and disturbance of ubiquitin-proteasomal pathway (Araya et al., 2002; Zou et al., 2002; Petit et al., 2004). Exposure of aquatic species to cobalt affects viability, development, and behavior (Kim et al., 2006; Norwood et al., 2007; Howe et al., 2014a,b), but there is little information

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regarding changes at molecular level. Therefore, the mechanism of cobalt toxicity in aquatic species remains elusive. Moreover, *in vitro* systems lack *in vivo* complexities, and data generated from *in vitro* studies limit the accuracy of *in vivo* predictions. In more recent times, invertebrates, among other aquatic species, are considered as the organisms of choice for investigating heavy metal toxicity (Burger, 2006). Therefore, an appropriate organism from among the invertebrates that can help elucidate both acute and chronic toxicities of heavy metals with reproducible results, unravel the underlying mechanism of toxicity at various levels of biological organization (i.e., molecular, cellular, whole organism and population), and ultimately facilitate efficient environmental health risk assessment is worth investigating.

Hydra is a popular animal in many areas of research including developmental biology, stem cell biology, and environmental toxicology. *Hydra* is a simple diploblastic animal with a remarkable capacity for regeneration, practically immortal, and a majority of its genes and peptides are conserved (Bosch, 2007; Galliott, 2012). Among the several environmental pollutants so far tested, *Hydra* is highly sensitive to heavy metals in bulk (Quinn et al., 2012; Zeeshan et al., 2016) and nano forms (Murugadas et al., 2016). The present study aimed to perform a comprehensive risk assessment of cobalt in *Hydra* because cobalt has hardly been tested in a cnidarian (Howe et al., 2014a,b). Herein we report the effects of cobalt in *Hydra magnipapillata* with regard to morphology, histology, physiology, development, and reproduction, where the generation of ROS in the lysosomes is linked to the upregulation of antioxidant genes, DNA damage, cell cycle arrest, and apoptotic cell death.

2. Materials and methods

2.1. Test organisms and culture methods

H. magnipapillata strain 105 was cultured in *Hydra* medium (Muscatine and Lenhoff, 1965). Experiments were conducted on polyps starved for 24 h. All experiments were performed in triplicate.

2.2. Acute exposure and morphological study

Cobalt stock solution (500 mg/L) was prepared in *Hydra* medium using analytical grade cobaltous chloride ($CoCl_2$). Experiments were performed in glass petri dishes (50 × 17 mm). In each run, groups of 15 polyps were exposed to cobalt (0–60 mg/L), and the toxicant (6 mL each well) was renewed daily. Control animals were maintained in *Hydra* medium without $CoCl_2$. Morphological changes, if any, were observed and recorded at 24-h intervals until 96 h, and scores for structural changes were assigned [score 10, healthy animal; scores 9–6, grades of morphological changes that are not lethal; and scores 5 and down, lethality (Table S1, supplementary information)] (Wilby, 1988). Using the median scores, LC_{50} was calculated for 24, 48, 72, and 96 h. Two sublethal doses, 8 and 16 mg/L, were selected from the data for 48-h exposure and denoted as T1 and T2, respectively. T1 induced little or no visible morphological change, whereas T2 treatment induced moderate morphological changes. All further experiments were performed with T1 and T2.

2.3. Tissue uptake of cobalt and histological changes

To investigate the accumulation of cobalt, animals were exposed to T1, T2, or *Hydra* medium without cobalt (control) for 24 and 48 h. Each group consisted of five *Hydra*. At the end of the experiment, the animals were anesthetized in 2% urethane in *Hydra* medium, washed in PBS, fixed in Lavdowsky's fixative, and mounted in 50% glycerol in PBS (Marchesano et al., 2015). The animals were observed under a bright-field microscope.

In a similar experiment, the cobalt-exposed animals were relaxed in urethane, fixed in 4% paraformaldehyde, washed in PBS, and processed for paraffin embedding. Sections of 5-μm thickness were stained in hematoxylin and eosin and mounted in DPX mountant for observation under a bright-field microscope.

2.4. Assay of regeneration

Adult polyps were cut below the hypostome and above the bud to separate the gastric region. The latter was placed in 3 mL of T1, T2, or control (12 gastric regions each), and the medium was changed daily. The regeneration was recorded at every 24-h interval until 96 h. Score of 10 indicates complete regeneration and 0 represents mortality [Table S1] (Wilby, 1988). From the scoring, the median score was calculated for 24, 48, 72 and 96 h.

2.5. Assay of asexual reproduction

Growth and population study was performed according to Ambrosone et al. (2012). Individual *Hydra*, each with a bud, were exposed to T1, T2, or control for 24 or 48 h. The test was continued for 14 days, and the animals were fed daily with five *Artemia* nauplii each; the medium was changed after each feeding. The buds produced were counted at every 24 h interval, and the growth rate constant (K) of exponentially growing animals was determined according to Bosch and David (1984) using the equation $\ln(n/n_0) = kt$, where n is the number of animals at time t and n_0 is the number of animals at t_0. Population doubling time (t_2) was determined when the number of animals (n_0) at time T doubled to that at the beginning of the experiment (n), i.e., when $n/n_0 = 2$, $t = t_2$ (population doubling time).

2.6. Study of the tentacle structure

Alterations of tentacle structure and battery cell complex (BCC) were observed using toluidine blue O, which specifically stains nematocytes. After 48 h of incubation, the untreated and treated *Hydra* were relaxed in 2% urethane, fixed in 70% ethanol, stained with toluidine blue O in 10 mM Tris-HCl (pH 7.5), dehydrated in graded series of ethanol, cleared in xylene, and mounted in DPX, and the tentacles were observed under a bright-field microscope (Ambrosone et al., 2014).

2.7. Assay of feeding

Animals exposed to T1, T2, or control were placed in 6-well plates, and each well contained 3 mL of the *Hydra* medium. Live *Artemia* nauplii were released near to the polyps, and the feeding behavior was recorded using a stereo zoom microscope (Carl Zeiss, Jena, Germany) equipped with AxioCam ERc5s (Zeiss) camera. The polyps were examined for the ability to catch, kill, or ingest the prey. The duration of the observation was 10 min, wherein recording was performed during the first 3 min, and the animals were only observed during the next 7 min.

2.8. Intracellular ROS generation

Intracellular ROS generation in treated and untreated polyps was studied using 2,7-dichlorodihydrofluorescein diacetate

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