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Oxidative DNA damage may not mediate Ni-induced genotoxicity in marine mussels: Assessment of genotoxic biomarkers and transcriptional responses of key stress genes

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ABSTRACT

Nickel (Ni) is a known carcinogenic and mutagenic compound and an important contaminant of aquatic environments. Ni toxicity and its potential impact on aquatic organisms are, however, not well understood. This study used an integrated approach to evaluate genotoxic effects, tissue-specific accumulation and transcriptional profiles of key genes in mussels, *Mytilus galloprovincialis*, exposed to a range of concentrations of Ni. The genotoxic effects assessed were total and oxidative DNA damage (DNA strand breaks measured using the enzyme modified comet assay), and induction of micronuclei (MN; clastogenic and/or aneugenic effects) using haemocytes as the target cells. Six genes (*pgp*, *mt10*, *mt20*, *sod*, *hsp70* and *gst*) were selected for transcriptional analysis in the gills based on their key role in the stress response. Following exposure to sublethal concentrations of Ni (0–3600 $\mu g\,L^{-1}$) for 5 days, mussel haemocytes showed significant genotoxicity at >1800 $\mu g\,L^{-1}$ (4-fold increase for DNA strand breaks and 3-fold increase for MN induction). There was no significant difference between buffer (control) and enzyme treatments which target oxidised DNA bases (formamidopyrimidine glycosylase or endonuclease IIII). This suggested that, in haemocytes, oxidative DNA damage is not a major mechanism for Ni-induced genotoxicity. The expression of *mt20* and *gst* genes in gill was up-regulated at genotoxic concentrations, whilst *pgp* expression was markedly up-regulated, particularly at 18 $\mu g\,L^{-1}$ Ni (19-fold increase). Pearson's correlation analysis revealed significant associations between % tail DNA and MN induction in haemocytes ($r = 0.88$, $p < 0.05$), and between Ni accumulation in foot ($r = 0.47$, $p < 0.05$) and digestive gland ($r = 0.41$, $p < 0.05$) and induction of MN in the haemocytes. Our results are the first to suggest that Ni-induced genotoxicity in mussel haemocytes may not be a result of oxidative DNA damage, and that multixenobiotic resistance (MXR) may play an important role in Ni detoxification in this species.

1. Introduction

Nickel (Ni) is a naturally occurring metal of commercial importance, used in a variety of industries including coin production, engineering and the manufacture of stainless steel [1]. It is released into the atmosphere during its mining, smelting and refining, and after disposal of Ni-containing products. As a result, although Ni concentrations are typically low in the open ocean (0.12–0.70 $\mu g\,L^{-1}$; [2]), in rain (<1 $\mu g\,L^{-1}$; [1]) and in rivers and lakes (<10 $\mu g\,L^{-1}$; [1]), it is greatly enriched in freshwater environments close to mining establishments. For example, in lakes near the Sudbury nickel refinery, Canada, dissolved concentrations of 180,000 $\mu g\,L^{-1}$ have been reported [1]. Nickel concentrations in coastal marine waters can also be several orders of magnitude higher than in the open ocean. For example, Ratekevicius et al. [3] report coastal seawater Ni concentrations of 380 $\mu g\,L^{-1}$ in the Cachagua area of Chile, and dissolved Ni concentrations > 70 $\mu g\,L^{-1}$ have been reported in English estuaries (the Tyne, Wear and Tees; [4]). Data for Ni concentrations in coastal, marine areas close to Ni mines are not well reported. For example, it is well accepted that the coral lagoon surrounding the islands of New Caledonia (the third largest producer of Ni globally; [5]) has an extremely high heavy metal input [6], however Ni concentrations are yet to be quantified.

Although Ni is an essential trace metal [7,8], it is also a known carcinogen [9,10] and genotoxic agent for mammalian cells under in vitro *conditions* [11–14,98]. In relation to the aquatic environment there is a growing scientific and regulatory concern about carcinogenic, mutagenic or reproductive (CMR) toxicants [15,48]. In this context, Ni and its compounds have been classified as priority pollutants in the EU Water Framework Directive (WFD) and in the

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Priority Substances Directive amending the WFD [16,17]. Despite this, there is still only limited information on the potential toxic and genotoxic effects of Ni to aquatic organisms as a whole and invertebrates in particular. Recently, Ni-induced genetic damage has been quantified for mussels in the field [18] and under laboratory conditions [19]. These studies however considered only a biomarker of exposure (i.e. DNA strand breaks as measured by the comet assay) and did not quantify any biomarkers of genotoxic effects (e.g. micronuclei [MN] induction). Despite the fact that generation of free radicals or reactive oxygen species (ROS) is one of the mechanisms thought to induce the genotoxic effects of metals [98], neither of these studies determined the potential induction of oxidative damage to the DNA in response to Ni exposure.

Against the backdrop of the above information, the present study aimed to: (a) use the comet assay (a biomarker of exposure) to determine DNA strand breakage in response to Ni exposure, and in particular to use the enzyme-modified comet assay to determine oxidative damage to DNA bases; (b) use the MN assay (a biomarker of effect) to determine the incidence of clastogenic and/or aneugenic effects; and (c) evaluate the transcription profile of key genes involved in stress response (i.e. *pgp*, *mt10*, *mt20*, *sod*, *hsp70* and *gst*) to elucidate any cascading effects of the observed genotoxicity, or mechanisms of defence. We also determined tissue-specific Ni accumulation, to complement the potential biological responses, compare our results with those published previously (e.g. [19]) and assess any relationship between genotoxicity and accumulation, where appropriate. This integrated approach was adopted to obtain scientifically robust data and allowed the relative evaluation of biomarkers at different levels of organisation, alone and in combination with each other, producing a holistic picture of biological responses in this ecologically relevant organism.

2. Materials and methods

All chemicals used in the experiments were supplied from Sigma–Aldrich, St Louis, MO, USA unless otherwise stated.

2.1. Experimental design and mussel exposure conditions

Two identical exposures were set up during February–March 2011. The first was used to determine DNA strand breaks using the enzyme modified comet assay, and the second to quantify tissue-specific Ni accumulation, induction of micronuclei and transcriptional expression in gill tissue.

Adult *Mytilus galloprovincialis* (shell length 46.5 ± 2.04 mm) were collected from Trebarwith Strand, Cornwall, UK (50 38′ 40″ N, 4 45′ 44″ W), and transported immediately to the laboratory in a cool box. Mussels were transferred to a 75 L aquarium, filled with approximately 55 L of filtered (<10 μm), aerated seawater at 15 °C (salinity 36.16 ± 0.70, pH 7.92 ± 0.07, dissolved oxygen 89.75 ± 6.72%). A minimum 2-week depuration period was allowed prior to moving mussels to the exposure vessels. During this holding period, mussels were fed twice weekly with a suspension of *Isochrysis* algae (Reed Mariculture, Campbell, CA, USA) and a complete water change was performed 24 h after feeding. Throughout depuration, acclimatisation and exposure mussels were under a 12:12 h photoperiod with artificial lighting and continuous aeration. After depuration mussels were transferred to 15 glass beakers containing 2 L filtered (<10 μm) seawater, at a density of 1.5 mussels L^{-1}, and allowed to acclimatise for 48 h prior to exposure.

Beakers were randomly assigned to one of 5 treatment groups: 0 (control), 18, 180, 1800 or 3600 μg L^{-1} Ni; with three replicates per treatment. This concentration range was selected based on the published literature and prior work in our laboratory [19–21]. Adsorption of Ni to beakers was quantified (by ICP-MS analysis of nitric acid washes) as <0.10% of the concentrations added. According to established thermodynamic data, the speciation of inorganic Ni in seawater is as follows; free ion 47%, hydroxides 1%, chlorides 34%, sulphides 4% and carbonates 14% [22]. However, it is important to bear in mind that in seawater Ni is also known to bind strongly to organic ligands [23], who's binding characteristics were not quantified during this study.

Nickel exposure lasted 5 days, during which mussels were not fed and a complete water change was performed daily. This exposure duration and replacement of water matched that used in the earlier studies using mussels exposed to metals and reference genotoxic agents [24–27]. Water samples were taken immediately following the water change each day, and processed for inductively-coupled plasma mass spectroscopy (ICP-MS) analysis, as detailed in Section 2.2.

After the 5 days exposure period, haemolymph samples were extracted from each mussel via a 21 gauge hypodermic needle into a 1 mL syringe pre-filled with 0.10 mL physiological saline (20 mM HEPES, 435 mM NaCl, 100 mM $MgSO_4$, 10 mM KCl, 10 mM $CaCl_2$, pH = 7.36) and stored on ice until use. Following haemolymph extraction, mussels were dissected into their individual organs for analysis of their Ni content by ICP-MS (n = 6). A small (~5 mm) section of gill was also removed (n = 6), preserved in RNALater, and stored at −20 °C for transcriptional analysis of target genes.

2.2. ICP-MS measurements to determine tissue-specific accumulation and water Ni concentrations

For determination of tissue-specific Ni concentration, each individually dissected tissue (gills, mantle, digestive gland, foot, adductor muscle and byssus) was washed with distilled water, blotted dry and transferred to a pre-weighed scintillation vial. Samples were dried overnight at 60 °C and re-weighed. Tissue digestion was achieved by addition of 1 mL concentrated nitric acid (trace analysis grade) and incubation for 2 h at 70 °C. Digested tissue samples were diluted to a final volume of 5 mL with Millipore Milli Q water and stored at room temperature until analysis. For analysis of water Ni concentration, water in exposure beakers was mixed thoroughly and a 2.50 mL water sample was removed and transferred to a scintillation vial pre-filled with 22.50 mL of 2% HNO_3, and stored at room temperature until analysis. An internal standard of 115-In was added to both tissue and water samples, to a final concentration of 10 μg L^{-1}. This verified that instrumental drift was not the cause of sample variation. Indium was selected based on its minimal occurrence in marine samples and low polyatomic interference with seawater. Samples were analysed using an X Series II ICP-MS (Thermo Fisher Scientific Inc., Waltham, MA, USA) with PlasmaLab software (Thermo Fisher Scientific Inc., Waltham, MA, USA). Six replicate analyses of Milli-Q water were used to determine the limit of detection (LOD; three standard deviations) of 0.67 μg L^{-1} and limit of quantification (LOQ; ten standard deviations) of 2.20 μg L^{-1} for Ni using this apparatus.

2.3. Enzyme-modified comet assay to determine oxidative DNA damage

DNA strand breaks were detected using the enzyme-modified comet assay. Although assessment of these breaks may be influenced by many factors (e.g. cytotoxicity, excision repair) and the fact that the tail region could also contain DNA loops [28,29], for the purposes of this study, we will refer to the outcome of the comet assay as DNA damage. The comet assay was performed using the haemocytes of mussels as described in detail by our research group [25], modified to include the use of bacterial enzymes to target oxidised purine and pyrimidine bases [30]. Prior to performance of the comet and micronucleus assays, total cell counts were taken and cell viability was checked using Eosin Y stain and was found be >90% for all treatments (data not shown).

Prior to Ni exposure, two separate experiments were carried out to validate the enzyme-modified comet assay, using mussel haemocytes after in vitro exposure to various concentrations of hydrogen peroxide. In the first, the bacterial enzyme, formamidopyrimidine DNA glycosylase (Fpg) was used to convert oxidised purines to strand breaks, whilst in the second experiment endonuclease III (Endo III) was used to target oxidised pyrimidines [30]. Briefly, haemolymph from 18 mussels was pooled, and exposed in triplicate to concentrations of 0 (control), 10, 50 and 500 μM hydrogen peroxide. After 1 h exposure in the dark at 4 °C, cells were collected by centrifugation at 350 × g for 3 min and the hydrogen peroxide was removed. The enzyme-modified comet assay was then performed as detailed below.

Slides were pre-coated with normal melting point agarose (NMPA; 1.5% in Milli-Q water). Haemolymph-saline suspension (200 μL) was centrifuged at 350 × g for 3 min at 4 °C. The resulting pellet was resuspended in 150 μL of low melting point agarose (LMPA; 0.75% in PBS) and two 75 μL drops were dispensed onto each slide and coverslipped (to produce two replicate microgels). Slides were refrigerated at 4 °C for 1 h to allow gels to set. Coverslips were gently removed and slides stored at 4 °C overnight, before transfer to lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% N-lauryl-sarcosine, 1% Triton X-100, 10% DMSO, pH adjusted to 10 with NaOH) and incubation for 1 h at 4 °C.

Three slides were prepared per sample, one enzyme buffer control, one with Fpg and one with Endo III. After lysis, slides were washed three times (5 min) in enzyme buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.20 mg mL^{-1} BSA, pH 8.0), drained and one unit of Fpg or Endo III (diluted in 50 μL buffer) was added to each microgel. Enzyme buffer control slides had 50 μL of buffer only added at this stage. Slides were then transferred to a humidity chamber and incubated at 37 °C for 45 min. All enzymes were used at a final protein concentration of 1500 μg L^{-1}.

Following the enzyme incubation step, slides were transferred to an electrophoresis chamber containing chilled electrophoresis buffer (1 mM EDTA, 0.3 M NaOH, pH 13). DNA was allowed to unwind for 20 min at 4 °C then electrophoresis was carried out for 20 min (1.25 V/cm). Following electrophoresis, slides were transferred to neutralisation buffer (0.4 M Tris, adjusted to pH 7 with HCl) for 10 min, rinsed (3×) with distilled water and allowed to air dry. Slides were usually stained and scored within 24 h, and always within 2 weeks. Each replicate microgel was stained with 20 μL of 20,000 μg L^{-1} ethidium bromide, and 50 cells per microgel (100 per slide) were scored using an epifluorescence microscope (DMR; Leica Microsystems, Milton Keynes, UK) and Komet 5.0 imaging system (Kinetic Imaging,