Aquatic Toxicology 142–143 (2013) 17–25

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Zn-stimulated mucus secretion in the rainbow trout (*Oncorhynchus mykiss*) intestine inhibits Cd accumulation and Cd-induced lipid peroxidation

Farhan R. Khan*, James C. McGeer

Department of Biology, Wilfrid Laurier University, 75 University Avenue W, Waterloo, ON N2L 3C5, Canada

ARTICLE INFO

Article history:
Received 3 June 2013
Received in revised form 15 July 2013
Accepted 20 July 2013

Keywords:
Dietary metals
Mucus
Oxidative stress
Gastro-intestinal tract

ABSTRACT

Interest in the interactions between dietary constituents in the gut is increasing, but information remains sparse. In this study rainbow trout were fed non-enriched (186.7 ± 19.0 μg Zn g^{-1} (dw)), enriched (20% increase) and hyper-enriched Zn (200% increase) diet for 21 d followed by a single meal of Cd-spiked food (188.6 ± 9.9 μg Cd g^{-1} (dw)). Intestinal, hepatic and renal Zn burdens were measured on Days 7, 14 and 21 and Cd concentrations in the same tissue were measured 48 h-post Cd exposure. Oxidative stress was measured as lipid peroxidation in dissected tissues and intestinal mucus was quantified as sialic acid using the thiobarbituric acid assay. Rainbow trout maintained on the hyper-enriched Zn diet experienced significantly increased intestinal mucus secretion ($p < 0.01$), were the only treatment group not to accumulate Cd in the intestine, and there was also no increase in intestinal oxidative damage. Conversely, fish fed the non-enriched and enriched Zn diets did not produce greater than basal levels of intestinal mucus and accumulated significantly greater concentrations of Cd in the intestine ($p < 0.01$) leading to significant localised Cd-induced lipid peroxidation ($p < 0.01$). High levels of mucus production correlated to lower incidences of lipid peroxidation ($r^2 = 0.54$, $p < 0.05$). These results demonstrate that mucus production stimulated by a high Zn diet have an inhibitory effect on Cd accumulation in the intestine and on Cd-induced lipid peroxidation. Mechanistically, it is likely that the elevated mucus production provides a barrier to Cd uptake. This study describes how one dietary constituent directly modifies the gut environment which indirectly influences the fate of another ingested cation.

1. Introduction

The focus of aquatic toxicology has traditionally been on uptake from solution, but for metals, there is an increased concern for diet as a potentially important route of uptake (Dallinger et al., 1987; Clearwater et al., 2002; Meyer et al., 2005). The influence of water chemistry in modifying the uptake of metals at the gill is well-understood (Pagenkopf, 1983), parameterised (through models such as the BLM, Di Toro et al., 2001; Santore et al., 2002) and has been recently incorporated into assessments of environmental risk (US EPA, 1990), but research on similar influences within the digestive tract that may modify dietary bioavailability is only starting to emerge. For instance, in rainbow trout chronic Ca supplementation reduced the uptake of Cd through the gut (Baldisserotto et al., 2004, 2005; Franklin et al., 2005; Klinck et al., 2007), while dietary Na was actually shown to increase Cu uptake in the gut (Kjoss et al., 2005).

When two (or more) metals are present in the exposure scenario interactions may occur between the metals that affect their individual bioavailability. Again, there is more research on mixed metal exposures in water and metal–metal interactions at the gill, more specifically at the binding sites of transporter proteins, have been characterised as additive, antagonistic or synergistic (Playle, 2004). Although there have been studies in which fish have been fed diets of field-collected invertebrates enriched in more than one metal (Farag et al., 1999), the interactions between the metals was not investigated. Thus information on the potential metal–metal interactions within the intestinal lumen is scarce. In one of the few studies to deliberately expose fish (rainbow trout) to feeds spiked with multiple metals (Cd, Cu and Zn), the interactions were not consistent (Kamunde and MacPhail, 2011). Zn appeared not to accumulate, while early increases in whole body Cu concentrations correlated to reduced Cd concentrations, indicating an antagonistic relationship, but later Cd accumulation corresponded to elevated Cu, suggesting a change to synergism potentially via interactions at variable metal uptake sites (Kamunde and MacPhail, 2011). Even rarer are studies which investigate how one metal may alter the gastrointestinal environment and the effect on the uptake of

* Corresponding author. Present address: School of Life Sciences, Heriot-Watt University, Edinburgh, EH14 4AS, United Kingdom. Tel.: +44 131 451 3462.
E-mail addresses: f.khan@hw.ac.uk, farhan.khan@gmx.com (F.R. Khan).

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http://dx.doi.org/10.1016/j.aquatox.2013.07.015

a subsequently ingested metal. Zebrafish fed a Fe poor diet for 10 weeks followed by a single bolus of Cd accumulated significantly more Cd in their livers via the gastrointestinal tract compared to fish fed a normal diet (Cooper et al., 2006). The gastrointestinal tract of fish fed the Fe poor diet was subject to an increase at the transcriptional level of genes encoding Fe transporters DMT1 and ferroportin1 suggesting that dietary Cd uptake may be achieved by iron transporters, but also that a diet deficient in an essential trace metal may lead to change in the intestine that promotes the uptake of a non-essential and potentially toxic metal.

Here we investigate whether dietary Zn enrichment can influence the uptake and oxidative damage that is often attributed to dietary Cd exposure (Berntssen et al., 2000; Khan et al., 2010a). Cd does not directly produce free radicals through Haber–Weiss or Fenton type reactions (as Cu and Fe do), but instead provokes oxidative stress (more specifically lipid peroxidation) by reducing the effectiveness of antioxidant enzymes (e.g. superoxide dismutase and glutathione peroxidase) which scavenge reactive oxygen species (ROS) produced by normal oxygen metabolism (Zikic et al., 1998; Ognjanovic et al., 2008). Zn(II) is generally regarded as inert to oxidoreduction, a property that has given rise to its biological ubiquity (Vallee and Auld, 1990). *In vitro* Zn has been shown to reduce the oxidative damage caused by free radicals (Chung et al., 2005). Although Zn did not act as an antioxidant per se, the mobilisation of antioxidant defence mechanisms appears to be a Zn dependent process (Chung et al., 2006). However, Loro et al. (2012) recently demonstrated that sublethal waterborne Zn exposure was an oxidative stressor to the teleost, *Fundulus heterocliuts*, resulting in elevated lipid peroxidation and antioxidant enzyme levels. Thus, the potential of Zn to act as part of the anti-oxidant system or induce oxidative damage may depend on the level of exposure.

The dietary zinc requirements for rainbow trout range from 15 to 30 mg kg^{-1} (Ogino and Yang, 1978), but supplementations of up to 600 mg kg^{-1} have been shown not to cause toxicity nor result in impact upon the growth of juvenile rainbow trout (Wantabe et al., 1997). Mechanistically, this may be explained by the regulation of Zn uptake by intestinal mucus which serves to promote uptake at low luminal bioavailabilities and provides a barrier to exposure at excessive concentrations (Glover and Hogstrand, 2002; Bury et al., 2003). The buffering capability of Zn-induced mucus secretion is also influenced by the presence of other hydrominerals (Ca, Cu, Cd and Mg, Glover and Hogstrand, 2003). As mucus secretions vary with intestinal Zn concentrations, it may be reasonable to presume that the quantity of Zn in the diet will affect the uptake other metals, such as Cd. Perhaps more significantly, Zn-stimulated mucus could also offer some protection against the cytotoxicity of other ingested trace metals.

In this study we ask whether feeding rainbow trout (*Oncorhynchus mykiss*) Zn rich diets would modify the environment of the intestinal lumen so as to influence the biological responses induced by a subsequent single dose of Cd-spiked feed. Based on the values of Zn dietary requirements described by Wantabe et al. (1997), rainbow trout were fed for 21 days with a commercial fish food with no additional Zn enrichment and the same feed spiked at 20% or 200% above the base concentration, termed 'non-enriched', 'enriched' and 'hyper-enriched' feeds, respectively. After this diet, rainbow trout were fed a single meal of Cd enriched food (200 μg Cd g^{-1} (dw)). Rainbow trout whole body and tissue Zn burdens (intestine, liver and kidney) were determined at Days 7, 14 and 21 and Cd tissue burdens were measured following ingestion of Cd spiked food. At each time point, lipid peroxidation in the intestine, liver and kidney was determined by TBARs (thiobarbituric acid reactive substances) assay and intestinal mucus secretion was quantified by thiobarbituric acid assay of sialic acid, a monosaccharide which is found within glycoproteins that has been utilised as a marker for mucus production (Warren, 1959; Eddy and Fraser, 1982).

2. Materials and methods

2.1. Experimental animals

Juvenile rainbow trout (approximately 5–7 g body mass) were purchased from Rainbow Springs Trout Hatchery (Thamesford, Ontario, Canada) and housed within the aquatic facility at Wilfrid Laurier University (Waterloo, Ontario, Canada). Fish were initially maintained a 200 L tank receiving flowing well water (conductivity 740 μS cm^{-1}, 12 ± 1 °C) and fed at daily ration of 2.5% body weight (bw) with Biovita Starter fish feed. Three weeks prior to experimentation, 144 individuals were randomly distributed between 6 replicate 40 L tanks receiving a mixture of 50% well water and 50% dechlorinated Waterloo City tap water. Over the next 7 days fish were acclimated to 100% Waterloo City water. The conductivity and water temperature of each tank were monitored throughout the experimental period and remained within ±10% of 690 μS cm^{-1} and 16 °C, respectively. The flow rate of each tank remained constant at 250 mL min^{-1} and the feeding regime was continued at 2.5% bw daily. Food was withheld for a period of 72 h prior to the start of all experiments.

2.2. Diet preparation

All diets were prepared with Biovita Starter fish feed (manufacturers specifications: [P] = 1.1%, [Na] = 0.3%, crude protein = 52%, crude fat = 20%, crude fibre = 0.6%). Zn concentration of the feed was measured as 186.7 ± 19.0 μg Zn g^{-1} (mean ± SD for the non-enriched diet on a dry weight basis, $n = 4$) and Cd concentration was below detection limit of the instrumentation used (see Section 2.5). To prepare Zn or Cd spiked feed we adopted the method outlined by Baldisserotto et al. (2005). Ground food was hydrated with 40% deionised water (v/w) containing the volume of 1000 μg L^{-1} Zn or Cd stock (1000 ppm certified standards for metal analyses, Fisher Scientific, Canada) to yield nominal experimental diets at 10, 25, 50, 100 and 200 μg Cd g^{-1} (dw), or 30 and 600 μg Zn g^{-1} (dw). The resulting feed paste was mixed, extruded through a pasta maker and air dried. Once dry, feeds were broken into smaller pellets by hand. The control diets (i.e. no added metal) were similarly prepared with deionised water only.

Measured concentrations in the Cd feeds were 20.8 ± 8.5, 28.3 ± 4.4, 56.8 ± 8.1, 89.0 ± 5.6 and 188.6 ± 9.9 μg Cd g^{-1} (dw) (mean ± SD, $n = 4$). For the Zn feeds nominally spiked at 20% (enriched diet) and 200% (hyper-enriched diet) above the base concentration the measured Zn concentrations were 228.1 ± 18.9 and 645.3 ± 51.3 μg Zn g^{-1} (mean ± SD, $n = 4$), respectively. Actual Zn enrichment was therefore 22% and 245% above baseline.

2.3. Pilot study

A pilot study was conducted to determine the concentration at which dietary Cd provokes an increase in intestinal lipid peroxidation. Six fish were removed from each tank (36 fish in total) and randomly assigned to individually aerated 0.5 L beakers containing City water and fitted with lids. The pilot study design followed the methodology of a pulse-chase experiment (Wang and Fisher, 1999), in which fish are fed a single dose of spiked food followed by unlabeled food, typically, 8–12 h later. Here, each fish was fed at 2.5% bw (approximately 150–200 mg) with Cd-spiked feed (nominal concentrations were 0, 10, 25, 50, 100 and 200 μg Cd g^{-1} (dw), measured concentration are stated in the previous section, $n = 6$ fish per concentration). Fish were allowed to feed for 30 min after which time water in each beaker was renewed to remove uneaten food. At