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Evaluation of Antioxidant Circulatory Lipid-Soluble Vitamins and Sodium as Non-invasive Indicators of Chronic Copper Exposure and Toxicity in Rainbow Trout, *Oncorhynchus mykiss*

Collins Kamunde · Ruth MacPhail · Darlene Mahar · Bryan Grimmelt

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Abstract Measurement of circulatory indicators of copper (Cu) exposure and toxicity in rainbow trout revealed elevated Cu concurrent with reduced sodium (Na) concentrations in plasma of Cu-exposed fish. Using a new normal phase high performance liquid chromatography (HPLC) method developed and validated for simultaneous extraction of lipid-soluble antioxidant vitamins we found that, contrary to our original hypothesis, plasma antioxidant status was enhanced as evidenced by a linear increase in vitamin E concentration. This suggests that vitamin E was mobilized from other metabolic pools to enhance circulatory antioxidant status possibly for delivery to Cu-sensitive locales. On the other hand, plasma vitamin A was not affected by the Cu exposure although its level decreased with time concurrent with an increase in fish size suggesting increased demand for growth. Thus circulatory Cu, Na, and vitamin E, but not vitamin A, can be used as non-lethal biomarkers of chronic Cu exposure and toxicity in fish.

Keywords Copper · Chronic toxicity · Non-invasive circulatory biomarkers · HPLC

Copper is a component of many enzymes and proteins critical for numerous biochemical reactions in all organisms (Linder and Hazegh-Azam 1996). However when present in excess, labile Cu ions have the potential to mediate Haber–Weiss reactions involving the formation of highly reactive hydroxyl radicals with oxidative cellular damage (Stohs and Bagachi 1995; Halliwell and Gutteridge 1999; Matés 2000). The typical response to exposure of organisms or cells to redox active agents, such as Cu, is a shift in pro- and antioxidant balance in favor of the former, a condition referred to as oxidative stress. Although previous studies have demonstrated this typical response during exposure to redox active metals in fish (Baker et al. 1997; Payne 1998; Sanchez et al. 2005), these responses are often evanescent suggesting adaptive physiological mechanisms. Generally, protection against oxidative stress entails preventing formation of reactive oxygen species, intercepting the formed reactive oxygen or repairing the inflicted damage through the concerted action of enzymatic and non-enzymatic defense systems (Lopez-Torres et al. 1993; Sies 1997).

In fish an additional and probably the primary mechanism of acute toxicity of Cu is the impairment of Na homeostatic mechanisms through the inhibition of Na^+–K^+–ATPase associated with both reduced uptake and increased loss of Na (Wood 2001). During chronic exposure however, the initial disturbance of ionoregulatory mechanisms recovers as fish acclimate to the presence of elevated levels of Cu in their environment in what is termed the damage–repair paradigm (McGeer et al. 2000). Conceivably because the toxicological responses associated with the two key mechanisms of action of Cu in fish appear to be transitory in nature, a stable biomarker of chronic Cu toxicity remains to be identified.

The aim of this study therefore was to evaluate the effect of chronic waterborne Cu exposure on circulatory indices of toxicity in fish with a view to identifying a stable biomarker of chronic Cu exposure and toxicity that can be measured following minimally invasive sample collection. We selected potential indices based on the current knowledge of the mechanisms of acute toxicity of Cu -oxidative stress and Na

C. Kamunde (✉) · R. MacPhail · D. Mahar · B. Grimmelt
Department of Biomedical Sciences, Atlantic Veterinary College, University of Prince Edward Island, 550 University Avenue, Charlottetown, PE, Canada C1A 4P3
e-mail: ckamunde@upei.ca

loss- with the assumption that they would apply during chronic exposure. Specifically, we measured levels of antioxidant lipid-soluble vitamins (A and E), Na and Cu in rainbow trout plasma during chronic exposure to waterborne Cu. Our hypothesis was that chronic exposure to Cu would not only deplete antioxidant vitamins in blood circulation but also induce Na loss.

Materials and Methods

Juvenile rainbow trout (20–30 g) were exposed to 40 μg/L waterborne Cu (as $CuSO_4$ $5H_2O$, Sigma-Aldrich, Oakville, ON, Canada) in Atlantic Veterinary College (AVC) well water. The AVC well water had a pH of 7.5 and contained (in mg/L): Na 47.1; Ca 58.8; Mg 27.6; K 2.26; Cl 137.3; sulfate 17.2; hardness 260; and total alkalinity 145. The measured Cu concentrations in the control and Cu-exposure water were 0.6 ± 0.1 and 41.06 ± 1.53 (μg/L, mean ± SEM; $n = 34$), respectively. The water temperature and dissolved oxygen as measured during the exposure were 12.70 ± 0.02°C ($n = 136$) and 10.59 ± 0.05 mg/L ($n = 19$), respectively. Fish were fed 2% bw daily ration of commercial granulated 3.0 grade trout chow (Corey Feed Mills Ltd, Fredericton, NB) containing crude protein 46% (minimum), crude fat 26% (minimum), crude fiber 1.7% (maximum), Ca^{2+} 1.3% (actual), phosphorous 1.0% (actual), Na^{+} 0.6% (actual), vitamin A 4,400 i.u. kg^{-1} (minimum), vitamin D3 3,200 i.u. kg^{-1} (minimum), and vitamin E 2,000 i.u. kg^{-1} (minimum). Measured concentrations (mg kg^{-1}, $n = 6$), of Cu and vitamins A and E in the feed were 26 ± 1; 1.46 ± 0.09 and 1.73 ± 0.08, respectively. Blood was collected weekly ($n = 9$ for controls and Cu-exposed fish) in heparinized 1 mL syringes from the caudal vein and centrifuged at 10,000 × g, 4°C for 4 min to separate plasma. The plasma samples were then stored at (−80°C till analyzed for Cu, Na, and vitamins A and E.

Copper and Na were analyzed by atomic absorption spectrometry (AAnalyst 800; Perkin-Elmer, Foster City, CA, USA) in furnace and flame modes, respectively, upon appropriate dilution of the plasma with 0.2% HNO_3. For quality control (QC), certified reference material (TMDA 54.3, National Water Research Institute, Burlington, ON, Canada), spiked samples and blanks were analyzed alongside the plasma samples.

Vitamins A and E were simultaneously extracted and quantified using a new, simple and reproducible normal phase high performance liquid chromatography-florescence detector (HPLC-FLD) method developed and validated for fish plasma. This method uses the same solvent for extraction and injection thereby eliminating evaporation step, and is rapid with retention times being 2.4 min for α-tocopherol and 3.8 min for retinol (Fig. 1). In this method, the vitamins were co-extracted from plasma with isooctane and quantified using a Waters 600E HPLC pump (Waters Corp., Milford, MA, USA) equipped with a photodiode array (model 996) and fluorescence (model 2475) detectors in tandem, a 717 autosampler and Millenium32 chromatography manager. Briefly, to 200 μL of freshly made 0.1% Na-ascorbate were added 250 μL of a cocktail containing 2 ppm Tocol, 2 ppm butylated hydroxyanisole (BHA) and 0.01% butylated hydroxytoluene (BHT) in ethanol, and 50 μL of plasma. After mixing for 30 s, 2 mL of isooctane were added and the extraction mixture was vigorously mixed for 5 min. Note that

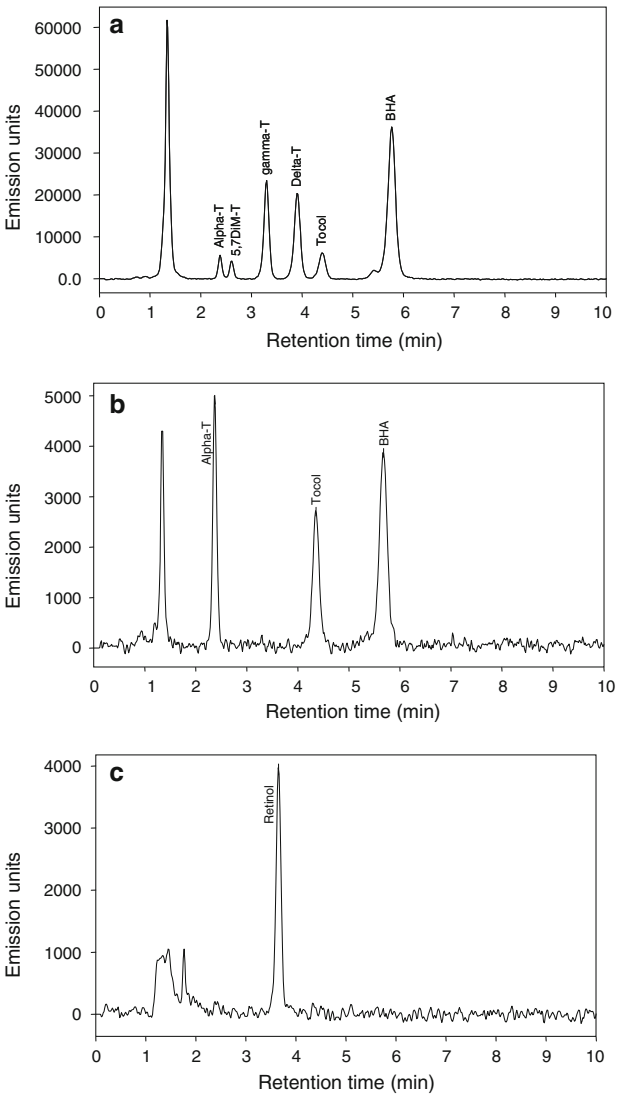

Fig. 1 Representative chromatographs obtained using normal phase HPLC-FLD for the simultaneous determination of vitamin A and E in fish plasma. (**a**), system suitability test; (**b**), vitamin E (α-tocopherol) separation and determination on channel 1 with excitation at 295 and emission at 330 nm; (**c**), vitamin A (retinol) separation and determination on channel 2 with excitation at 325 and emission at 480 nm. Typical retention times were 2.4 min for α-tocopherol and 3.8 min for retinol

the extraction volumes can be reduced to a minimum of 10 μL for plasma and 400 μL for isooctane. The mixture was then centrifuged for 5 min at 13,000 rpm and the top phase was transferred to HPLC vials for analysis. For QC a blank (50 μL of MilliQ water) and equine serum intra-laboratory QC sample were extracted and analyzed alongside the experimental samples. Analysis was accomplished by injecting a 25 μL-sample onto a Luna LC-NH_2 250 × 3.0 mm column (Phenomenex, Torrance, CA, USA) operated at ambient temperature, with isooctane as the injection solvent. System suitability was tested using a standard mixture containing BHT (0.1%), BHA, retinol, α-tocopherol, α-tocopherol-acetate, δ-tocopherol, tocol, and 5,7-dimethyl tocol (Fig. 1a). The vitamins were subsequently eluted with 4.0% isopropanol in hexane at a flow rate of 1.2 mL/minute and detected by dual fluorescence, i.e., channel 1 for α-tocopherol with excitation at 295 and emission at 330 nm, and channel 2 for retinol with excitation at 325 and emission at 480 nm. Typical chromatographs for α-tocopherol and retinol are shown in Fig. 1b and c, respectively.

The Cu, Na, and vitamin data were analyzed statistically using ANOVA (Statistical, StatSoft Inc., Tulsa, OK, USA). Tukey's honest significant difference pos hoc test was used to delineate differences among the means at $p < 0.05$.

Results and Dicussion

We measured circulatory indices of exposure and toxicity in rainbow trout following chronic sublethal waterborne Cu exposure. The exposure elevated plasma Cu concentration and concurrently reduced plasma Na concentration (Fig. 2a, b), suggesting that circulatory Na loss is a potential non-invasive indicator of chronic Cu toxicity. This is consistent with our earlier study (Kamunde et al. 2005) in which a decline in plasma Na was not only associated with elevated Cu but also with increased mortality and reduced growth. In the current study mortality and growth inhibition did not occur (data not shown) possibly because of the lower Cu exposure concentration (40 vs. 55 μg/L) and different water chemistries. There was, however, no correlation between plasma Cu and Na probably because of the fact that the majority of the plasma Cu content [e.g., 90–95% in humans, Fox et al. (1995)] is bound by ceruloplasmin with very little existing in the "free" toxic species, which is deemed more responsive to the level of Cu exposure.

A monotonic response of plasma α-tocopherol to the Cu exposure was observed but contrary to our expectation, there was a linear increase of this vitamin during the exposure (Fig. 3a). Moreover, there was a significant positive correlation ($r = 0.28$, $p = 0.02$) between plasma Cu and α-tocopherol concentrations (Fig. 3b). Although

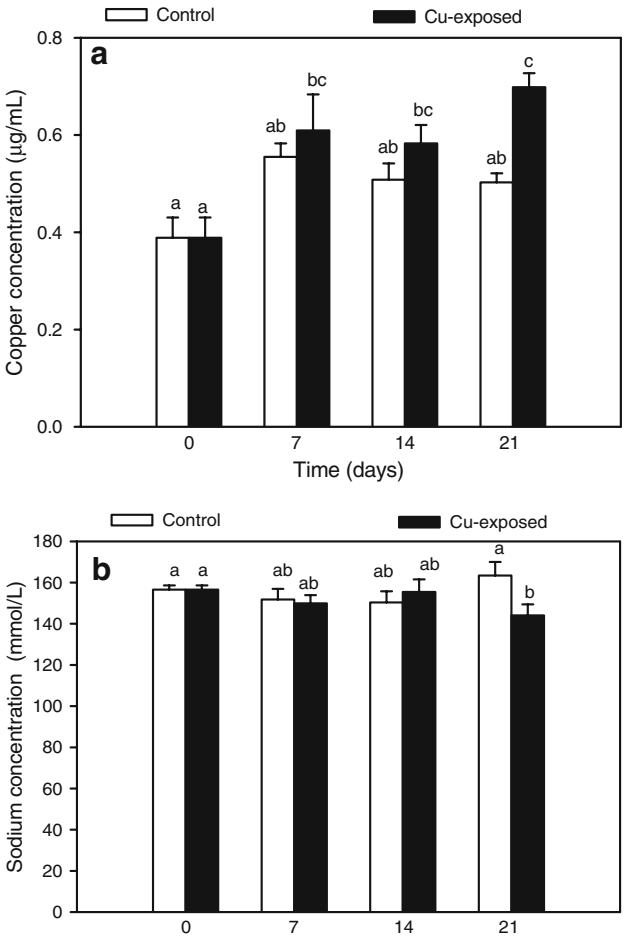

Fig. 2 Effect of exposure of juvenile rainbow trout to 40 μg/L waterborne Cu for 21 days on plasma Cu (**a**) and Na (**b**) concentrations. Open bars: controls; solid bars: Cu-exposed fish. Bars with different letters are significantly different (ANOVA, $p < 0.05$, n = 9 per group)

this is not a strong relationship, the clean linear response (Fig. 3a) suggests that vitamin E was mobilized from other metabolic pools, such as the hepatic pool and/or that its absorption from dietary sources was increased. Moreover, protection against Cu toxicity involves several mechanisms including but not limited to the antioxidant system, internal sequestration and physiological responses, such as reduced uptake and increased excretion. Our data suggest that when measured individually, each of these mechanisms would account for a small proportion of the overall protection against Cu toxicity. As well, the enhancement of antioxidant vitamin status during oxidative stress observed in this study is not an uncommon phenomenon. For example, humans had improved antioxidant status including elevation of vitamin E concentration during exercise (which is associated with oxidative stress) (Brites 1999; Cazzola et al. 2003). In fish, Palace et al. (1994) reported that exposure of lake char to low dose polychlorinated biphenyl congener 126 increased the concentration of α-tocopherol

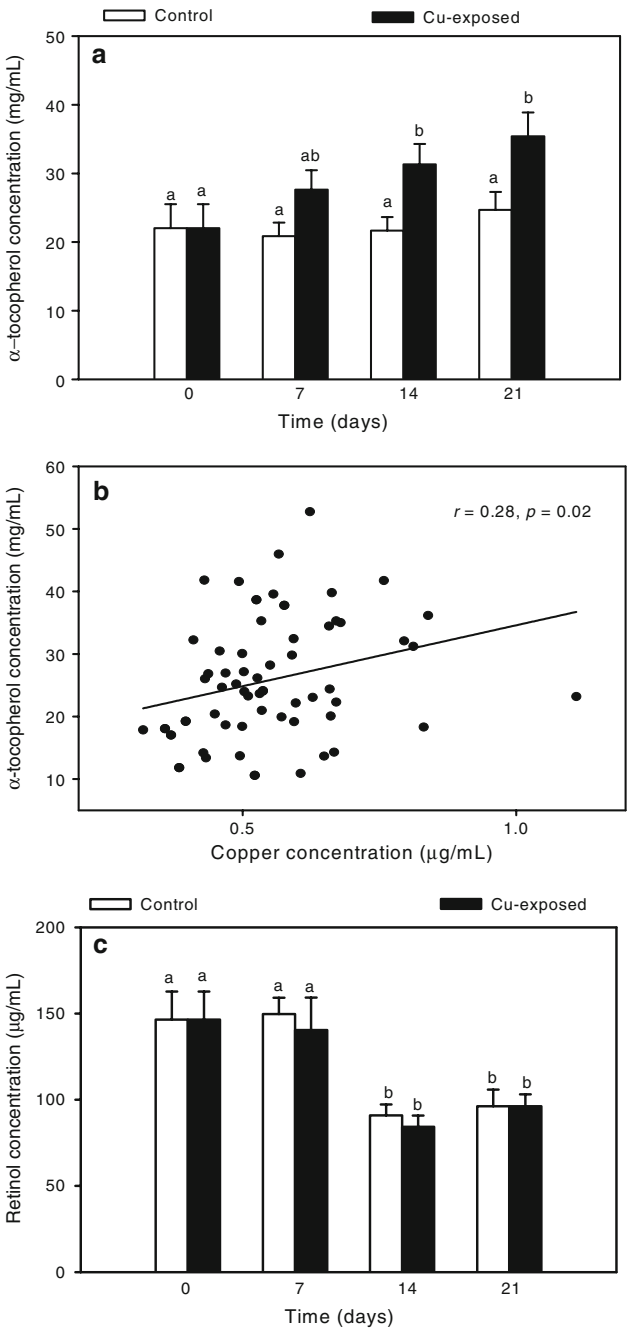

Fig. 3 Vitamin E concentration (**a**), correlation between plasma Cu and Vitamin E (**b**) and vitamin A concentration (**c**) in plasma of juvenile rainbow trout following exposure to 40 μg/L waterborne Cu for 21 days. Open bars: controls; solid bars: Cu-exposed fish. Bars with different letters are significantly different (ANOVA, $p < 0.05$, n = 9 per group). These results were subsequently reproduced (data not shown)

in the kidney but it had no effect on liver and plasma levels of α-tocopherol. Thus although the ultimate response to redox active toxicants may be a global depletion of antioxidants, redistribution among various metabolic pools may results in variable tissue-specific effects. Our results however contradict earlier studies on the effect of redox active metals exposure in aquatic organisms. For example, Baker et al. (1997) reported that exposure of the African catfish to dietary iron depleted hepatic stores of vitamin E. In another recent study in which zebra fish were exposed to dietary Cu for up to 260 days, whole body α-tocopherol concentration was not affected (Alsop et al. 2007).

Because vitamin A is a biologically relevant antioxidant (Livrea and Tesoriere 1998; Palace et al. 1999) we reasoned that Cu, a redox active metal, would interfere with its metabolism. Our data show that plasma vitamin A concentration was not sensitive to Cu exposure but it was significantly decreased in both the controls and Cu-exposed groups after 14 days of the experiment (Fig. 3c). We speculate that there was increased demand for vitamin A concurrent with growth dilution since it is an important mediator of growth and differentiation (Holder and Hill 1991). It is also possible that there was increased bioconversion of retinol to one or more of the other forms of vitamin A (e.g., retinoic acid, retinal or 3-hydroretinol), which were not measured in this study. Nonetheless, based on the fact that the normal plasma vitamin A levels have not yet been described for fish, the significantly lower concentrations measured on days 14 and 21 are likely within the physiological range for rainbow trout because growth was not impaired. This is reinforced by the fact that the measured in-feed vitamin A concentration was within the recommended range for normal metabolism in rainbow trout.

In conclusion, we believe that elevation of plasma α-tocopherol following chronic waterborne Cu exposure in rainbow trout has not been previously demonstrated. Enhancement of circulatory α-tocopherol level suggests that vitamin E was likely mobilized from other metabolic pools possibly to protect cellular elements in locales prone to oxidative damage. Future studies should measure concentrations of products of oxidative damage, such as malondialdehyde and protein carbonyls, and relate them to Cu exposure and oxidative status. On the other hand, vitamin A was unaffected by the Cu exposure but decreased with time (or fish size) suggesting increased demand for growth or conversion to other forms. Overall the utility of circulatory biomarkers should be considered for non-invasive monitoring of chronic exposure of aquatic biota to metals and other pollutants.

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