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Myo-inositol prevents copper-induced oxidative damage and changes in antioxidant capacity in various organs and the enterocytes of juvenile Jian carp (*Cyprinus carpio* var. Jian)

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ABSTRACT

Although oxidative stress has been demonstrated to be involved in copper (Cu)-induced toxicity, information regarding the effect of antioxidants on Cu toxicity is still scarce. This study assessed the possible protective effects of *myo*-inositol (MI) against subsequent Cu exposure in juvenile Jian carp (*Cyprinus carpio* var. Jian) *in vivo* and in their enterocytes *in vitro*. First, oxidative stress was established by exposing fish to different concentrations of Cu (0–7.2 mg Cu/L water) for 4 days. Next, the protective effects of MI (administered as a dietary supplement for 60 days) against subsequent Cu exposure (0.6 mg Cu/L water for 4 days) were studied in fish. The third trial determined the effects of Cu exposure (0–6.0 mg Cu/L of medium for 24 h) on enterocytes *in vitro*. Finally, enterocytes were pre-incubated with graded levels of MI (0–75 mg MI/L of medium) for 72 h and exposed to 6.0 mg Cu/L of medium for 24 h. The results indicated that ≥0.6 mg Cu/L water could induce oxidative stress in fish ($P < 0.05$). Cu exposure significantly induced increases in lipid peroxidation and protein oxidation in the gill, hepatopancreas and intestine in fish. However, these oxidative effects were prevented by MI pre-supplementation. MI also prevented the toxic effects of Cu on anti-superoxide anion (ASA), anti-hydroxyl radical (AHR), superoxide dismutase (SOD), catalase (CAT), glutathione-*S*-transferase (GST), glutathione peroxidase (GPx) and glutathione reductase (GR) activities and glutathione (GSH) content in these organs. *In vitro*, enterocytes exposed to Cu displayed a dose-dependent injury. Moreover, cell viability, protein retention (PR), alkaline phosphatase, total-SOD (T-SOD) and Cu/ZnSOD activities were all depressed by Cu ($P < 0.05$). Interestingly, the final experiment showed that MI pre-supplementation could block the toxic effects of Cu on the antioxidant system, and thus protect enterocytes from Cu-induced oxidative damage. All of these results indicated that the induction of key antioxidant defenses by MI pre-supplementation, including SOD, CAT, GPx, GST and GSH, may play an important role in the protection of fish against oxidative stress.

1. Introduction

The pollution of aquatic ecosystems by heavy metals is of concern due to their toxicity and bioaccumulation (Jain, 2004). Copper (Cu) is one of the most important pollution-causing metals (Bopp et al., 2008). It is released into the environment through anthropogenic activities, including the use of pesticides and fungicides and from industrial wastes (Yruela, 2005). In aquaculture systems, Cu is regularly used in the form of copper sulfate ($CuSO_4$) to control algal blooms and aquatic macrophyte infestations (Sampaio et al., 2008). Thus, aquatic organisms may suffer from exposure to Cu concentrations that might be 10–50 times higher than the required concentrations (Bopp et al., 2008). Elevated Cu concentrations in water can be toxic (Sandrini et al., 2009). They can induce the overproduction of reactive oxygen species (ROS) (Upadhyay and Panda, 2010). These ROS can react at a high rate with most of the molecules in the cell and thus damage proteins, amino acids and nucleic acids (Srivastava et al., 2006).

Cu accumulates mainly in the gill, liver and gut of fish (Handy et al., 2002). The gill is the first organ to be exposed to waterborne contaminants (Sancho et al., 1997). Cu toxicity primarily affects the liver, because it is the first site of Cu deposition after

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entering the blood (Gaetke and Chow, 2003). Exposure to Cu caused an increase in lipid peroxidation in the liver of an estuarine fish (*Pomatoschistus microps*) (Vieira et al., 2009). Considering the Cu-induced oxidative damage induced in the liver, it is possible that similar effects also occur in other organs (Vieira et al., 2009). In agreement with this hypothesis, an increased level of lipid peroxidation was shown in the brain of sea bream (*Sparus aurata*) fingerlings following Cu exposure (Varo et al., 2007). The intestine is an important site for nutrient digestion and absorption in fish (Pedersen and Sissons, 1984). However, the intestine is very sensitive to a wide range of stressors (Olsen et al., 2005). The intestinal epithelium is a site of nutrient uptake and is important for protecting the organism against harmful agents (Al-Hussaini, 1949a). It consists mainly of absorptive columnar cells (enterocytes) (Al-Hussaini, 1949b). Intestinal transport physiology was shown to be directly influenced by copper exposure in gulf toadfish (*Opsanus beta*) (Grosell et al., 2004). However, little attention has been given to the effects of Cu exposure on oxidative status and antioxidant response in the intestine and enterocytes of fish.

Given the increasing release of Cu into the environment and its potentially harmful effects on biota, it is important to expand our knowledge of how to protect biota against Cu toxicity (Jamers et al., 2006). Recently, Upadhyay and Panda (2010) showed that zinc could reduce copper-induced oxidative stress in the aquatic plant, duckweed (*Spirodela polyrhiza* L.). However, very little information is available to date on the effect of potent antioxidants on Cu toxicity in fish (Trevisan et al., 2011). *Myo*-inositol (MI) is a cyclitol and an important nutrient for fish (Shiau and Su, 2005). It can be beneficial by scavenging hydroxyl radicals ($OH^{\cdot}$) induced by Fenton-like reactions (Hu et al., 1995). Our previous studies showed that MI could improve the growth and increase the antioxidant enzyme activity in Jian carp (Jiang et al., 2009b,c, 2010). Hence, it is reasonable to hypothesize that MI could mitigate Cu-induced oxidative stress in fish.

This study is in line with our previous investigations, and its principal purpose is to test the hypothesis that MI could mitigate the toxic effects of Cu on oxidative status and the antioxidant system in the gill, hepatopancreas, intestine and enterocytes of fish.

2. Materials and methods

2.1. Chemicals

Copper sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$), MI, insulin, transferrin, collagenase, dispase, collagen, benzyl penicillin, streptomycin sulfate, and triton X-100 were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Media (DMEM) were purchased from Hyclone (Logan, UT, USA). MI-free DMEM was ordered from Beijing Tsing Skywing Bio-Tech. Co. Ltd. (Beijing, China). Hank's balanced salt solution (HBSS) was prepared in our laboratory.

2.2. Animal collection and acclimation conditions

Juvenile Jian carp were purchased from the Tong Wei Hatchery (Sichuan, China) and acclimated to the following laboratory conditions: $23.5 \pm 1\,^{\circ}C$, constant aeration, daily dechlorinated water change, natural photoperiod and feeding with a commercial food. The acclimation conditions were similar to those used by Trevisan et al. (2011).

2.3. Cu-induced oxidative stress in carp (Experiment 1)

A total of 144 fish (mean initial weight 50.6 ± 1.2 g) were collected from the acclimatization aquaria and distributed into each of 18 aquaria (90 cm × 30 cm × 40 cm). Cu exposure was performed following the general Organization for Economic Co-operation and Development (OECD) guidelines for fish acute bioassays (OECD, 1993). Food was withheld for 24 h prior to the experiment. A stock solution was prepared by dissolving $CuSO_4{\cdot}5H_2O$ in distilled water to achieve a final concentration of 1 g Cu/L; this was used to prepare the test solutions by diluting it in the water of the experimental aquaria to attain the desired concentrations (0, 0.6, 1.2, 2.4, 4.8 and 7.2 mg Cu/L water). Twenty-four fish were randomly assigned to each concentration for 4 days, with 3 aquaria per concentration and 8 fish per aquarium. During the test, the photoperiod and aeration conditions were the same as during the acclimation period, but no food was provided and the water was not renewed. This bioassay was performed according as described by Sampaio et al. (2008) and Vieira et al. (2009). The mortality of the fish was monitored daily. The serum malondialdehyde (MDA) content in all surviving individuals was determined at the end of the exposure.

2.4. Protective effects of MI against a subsequent Cu exposure (Experiment 2)

To investigate the potential protective effects of MI against a subsequent Cu exposure, a second experiment was carried out. The formulation of the basal diet was the same as in our previous study (Jiang et al., 2009b). Briefly, it contained 327.6 g crude protein/kg diet and 49.1 g crude lipid/kg diet. The basal diet was the MI-unsupplemented control (Ctrl). MI premix was added to the basal diet to provide 518.0 mg MI/kg diet, which was the required MI concentration for optimal growth established by our previous study (Jiang et al., 2009b), and the amount of cellulose was reduced to compensate (MI group). Procedures for diet preparation and storage ($-20\,^{\circ}C$) were the same as those described by Shiau and Su (2005). The MI concentration in the experimental diets was determined by an enzymatic assay as described by Ashizawa et al. (2000).

A total of 300 fish (mean initial weight 7.93 ± 0.01 g) from the acclimatization aquarium were randomly distributed into 2 groups of 3 replicates each. The groups were fed either the Ctrl diet or the MI diet for 60 days. The experimental conditions were the same as in our previous study (Jiang et al., 2009b).

At the end of the feeding trial, the fish in each aquarium were weighed and collected for Cu exposure. Fish from both the Ctrl and MI groups were exposed to 0.6 mg Cu/L water for 4 days. The Ctrl/Ctrl (pre-treatment/exposure) treatment was performed by exposing fish from the Ctrl group to clean water. Hence, there were 3 different pre-treatment/exposure groups, Ctrl/Ctrl, Ctrl/Cu and MI/Cu, with 3 replicates per group and 12 fish per replicate (36 fish for each group). The procedure and conditions of Cu exposure were the same as those in Experiment 1. At the end of Cu exposure, the fish were collected. The gill, hepatopancreas and intestine were quickly removed, and stored at $-70\,^{\circ}C$ for subsequent analysis.

2.5. Cell isolation and culture

Cell isolation and culture were performed according to the methods of Jiang et al. (2009a) and Bicho et al. (1999), with slight modifications. Briefly, healthy Jian carp (50–80 g) were maintained for ~24 h without feeding before the experiment and were killed by decapitation. The intestines were rapidly removed from the carcass, opened and rinsed with HBSS containing 100 μg streptomycin sulfate/mL and 100 IU benzyl penicillin/mL. Enterocytes were isolated by collagenase and dispase digestion. Cells were suspended in DMEM and washed with the same medium 5 times to remove enzymes and blood cells. Isolated enterocytes were plated in DMEM containing 5% FBS, 0.02 mg transferrin/mL, 0.01 mg insulin/mL, 100 μg streptomycin sulfate/mL and 100 IU benzyl penicillin/mL. The cells were allowed to attach to plates for 72 h.