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Effects of Dietary Manganese Supplementation on Antioxidant Enzyme Activity in the Shrimp (*Neocaridina heteropoda*)

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Abstract

The effects of the manganese (Mn) supplemented diets on the level of superoxide anions (O^-_2) in the hemolymph, and the activity of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) in the muscle of *Neocaridina heteropoda* were investigated. Manganese sulfate ($MnSO_4$) was added to a basal diet at 0, 20, 40, 60, 80, or 100 µg/g. Diets were fed to triplicate groups of shrimp (0.30±0.11 g, 1.4±0.1 cm) in a recirculating freshwater rearing system for 30 days. The Mn concentration in the rearing water, monitored during feeding, was 1.00±0.02 µg/l. O^-_2 was lower and antioxidant enzyme activity was higher ($p<0.05$) in shrimps fed Mn-supplemented diets than in shrimps fed the control. Antioxidant enzyme activity reached a maximum when the Mn concentration was 60 µg/g diet.

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Introduction

Neocaridina heteropoda (also known as cherry shrimp) is a freshwater shrimp commonly kept in aquaria. It is also used for food and is a particularly good aquatic organism for study. Full-grown cherry shrimp reach about 4 cm. They prefer clean water, with a pH of 6.5-8.0, and a temperature of 18-30°C. *Neocaridina heteropoda* are omnivores that may live 1-2 years.

When an organism is subjected to chemical, physical, or biological (e.g., pathogenic infection) stress and a sudden shortage of oxygen, abnormal oxidative reactions in the aerobic metabolic pathway result in the cellular production of reactive oxygen species (ROS). ROS include the superoxide anion (O^{-}_{2}), hydrogen peroxide (H_2O_2), hydroxide ions (OH^-), and singlet oxygen ($^1O_2$). ROS are able to attack almost all bio-molecules in their vicinity causing protein modification, lipid peroxidation (LPX), and DNA strand breaks in cells, which can ultimately lead to pathological states including apoptosis (Buttke and Sandstorm, 1994). The effective control and rapid elimination of ROS is essential to the proper functioning and survival of organisms.

In a normal physiological state, harmful effects of ROS are neutralized by antioxidant defense systems that combat *in vivo* oxidation, maintain health, and prevent oxidation-induced lesions (Jacob, 1995). The antioxidant defense systems include radical scavenging enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX). SOD is involved in protective mechanisms in tissue injury following oxidation and phagocytosis. CAT is a major primary antioxidant defense component that works to catalyze the decomposition of H_2O_2 to H_2O, sharing this function with GPX. In the presence of low H_2O_2 levels, organic peroxides are the preferred substrate for GPX. At high H_2O_2 concentrations, they are metabolized by CAT (Yu, 1994).

Manganese (Mn) is a transition metal, essential for normal growth, reproduction, and prevention of skeletal abnormalities in terrestrial animals and fish. It is an important component of many key enzymes involved in the elimination of ROS, amino acid synthesis (glutamine synthetase), the urea cycle (arginase), and energy production (pyruvate carboxylase) (Erikson and Aschner, 2003). The best known manganese-containing polypeptide is Mn-containing superoxide dismutase (Mn-SOD).

Earlier studies of Mn and aquatic organisms focused on deficiencies (Davis and Lawrence, 1992), minimal requirements (Ogino and Yang, 1980; Gatlin and Wilson, 1984), toxicity (Stubblefield et al., 1997), and bioaccumulation concentration and distribution (Yilmaz et al., 2007). The aim of the present study was to examine the effects of dietary Mn on antioxidant enzyme activities in *N. heteropoda*.

Materials and Methods

Cherry shrimps, *N. heteropoda*, were collected from Baiyangdian Lake, Hebei Province, China, in March 2008 and acclimated in a holding tank for one week.

Nine hundred shrimps (0.30±0.11 g, 1.4±0.1 cm) were randomly selected for the experiment and stocked at 50 per tank (50 × 50 × 20 cm) in a

freshwater recirculating system. Tanks were aerated with an air pump attached to an aeration stone. The tanks were part of a closed recirculating system with a common freshwater reservoir maintained at 25±1°C and pH 8.0. The water was circulated at 2 l/min through two separate biofilters to remove impurities and reduce the ammonia concentration. Half the water volume was replaced every two weeks. The photoperiod was 12 h light (08:00-20:00), 12 h dark. The Mn concentration in the rearing water was monitored regularly and ranged 1.0±0.02 μg/g.

Ingredients of the basal diet are given in Table 1. Mn was added as manganese sulfate ($MnSO_4$) which is better utilized than manganese carbonate ($MnCO_3$) or manganese oxide (MnO) in common carp (*Cyprinus carpio*; Satoh et al., 1987). The dietary Mn concentration in the six experimental diets, analyzed by flame atomic absorption photometry after wet ashing (Rutkowska and Trzebska-Jeske, 1978), was 2.79 (0 supplementation), 22.57 (20 μg/g supplementation), 42.55 (40 μg/g), 62.69 (60 μg/g), 82.59 (80 μg/g), and 102.45 (100 μg/g). Prepared diets were stored at -20°C in sealed plastic bags. Each diet was fed to three groups of shrimps according to a completely randomized design. Shrimp were fed in the morning and at night at a daily rate of 5% of their body weight.

Feces and excess feed were removed in the morning before feeding. Shrimps were weighed at the beginning and end of the experiment. After 30 d, shrimps at the intermolt stage, as determined by Peebles (1977), were randomly selected for examination of antioxidant enzyme activities.

The muscle was rapidly excised from living shrimps, frozen in liquid nitrogen, and homogenized in 9 volumes of 20 mmol/l phosphate buffer pH 7.4, 1 mmol/l EDTA, and 0.1% triton X-100 at a temperature of 0-4°C. The homogenates were centrifuged at 600 × *g* (relative centrifugal force) at 0°C to remove debris. The resultant supernatant fluids were immediately used for SOD, CAT, and GPX analysis**.**

Hemolymph was drawn from the heart with a 250-Al glass syringe and placed in plastic tubes on ice. The hemolymph of shrimps from each exposure was pooled to measure reactive oxygen intermediate production in hemocytes according to Munoz et al. (2000). Before measuring, the hemolymph was homogenated in 30 volumes of 50 mmol/l phosphate buffer, pH 7.2, and the homogenates were centrifuged at 9960 × *g*. The resultant supernatant was used for the O^-_2 assay**.**

Fifty μl of the culture medium, modified Hank's balanced salt solution (MHBSS) with 6 mmol/l Ca^{2+} and 13 mmol/l Mg^{2+}, were placed in the wells of a 96-well microtiter plate and incubated in humid conditions at room temperature. After 30 min, the supernatant was eliminated and replaced with 50 μl medium, then 50 μl of 0.3% nitroblue tetrazolium (NBT) diluted in the appropriate medium was immediately distributed to each well. Following 2 h incubation, the supernatant was removed. The hemocytes were fixed by adding 200 μl absolute methanol and the supernatant was washed twice with 70% methanol, then dried. The formazan precipitates were solubilized in 120

Table 1. Composition of the basal diet.

Ingredient	per kg
Bean cake	412 g
Soybean meal	237 g
Wheat starch	227 g
Corn starch	52 g
Soybean oil	31 ml
Vitamin premix[a]	21 g
Mineral premix[b]	20 g

[a] Supplied (per kg diet): vitamin A 300,000 IU; riboflavin 480 mg; pyridoxine 360 mg; cyanocobalamin 1.2mg; thiamin 20.0 mg; menadione 20 mg; folic acid 170 mg; biotin 10 mg; a-tocopherol 3000 IU; myo-inositol8000 mg; calcium pantothenate 800 mg; nicotinic acid 200 mg; choline chloride 8000 mg; vitamin D 40,000 IU.

[b] Supplied (per kg diet): $ZnSO_4 \cdot 7H_2O$, 0.817 g; $CaCO_3$, 3.28 g; NaH_2PO_4,2.96 g; KH_2PO_4, 6.752 g; $CaCl_2$,1.3328 g; $MgSO_4 \cdot 7H_2O$, 1.6 g; KCl, 0.448 g; $AlCl_3 \cdot 6H_2O$, 0.0192 g; $MnSO_4 \cdot (4–6)H_2O$, 0.229 g; $CuCl_2$, 0.52 g; $FeSO_4 \cdot 7H_2O$,1.8 g; $CoCl_2$, 0.0282 g; KI, 0.036 g.

µl 2 mmol/l KOH and 140 µl dimethyl sulfoxide (DMSO, Sigma). After homogenization of the contents in the wells, the extinction was read spectrophotometrically at 620 nm**.**

SOD (EC1.15.1.1) activity was determined using the method of Marklund and Marklund (1974) based on the autoxidation of pyrogallol**.** GPX (EC 1.11.1.9) activity was assayed by following the rate of nicotinamide adenine dinucleotide phosphate (NADPH) oxidation at 340 nm by coupled reaction with glutathione reductase (Bell et al., 1985). CAT (EC 1.11.1.6) activity was measured by ultraviolet spectrophotometer (Ball et al., 2000). A 10-µl sample was added to 3.0 ml of H_2O_2 phosphate buffer pH 7.0 (0.16 ml 30% H_2O_2 to 100 ml 67 mmol/l phosphate buffer) and the variation of H_2O_2 absorbance in 60 s was measured with a UV-2100 spectrophotometer at 250 nm. One unit of enzyme activity was defined as the amount of enzyme that lowers the concentration of H_2O_2 by 50% in 100 s at 25°C**.** Protein concentrations of the muscle homogenates were determined using the coomassie brilliant blue dye-binding technique (Bradford, 1976) with bovine serum albumin (BSA) as the standard. Absorbance of samples was detected spectrophotometrically at 595 nm**.**

Differences between mean values were analyzed by one-way analysis of variance followed, when pertinent, by a Tukey multiple comparison test. The results were considered significant at $p<0.05$.

Results

Mean length and weight gains ranged 16.0-42.0% and 14.1-38.9%, respectively, and were significantly affected by dietary treatment (Table 2). The specific growth rate was greatest when Mn supplementation was 60 µg/g.

The O^-_2 value in supplemented groups was lower than that of shrimps in the control with the lowest O^-_2 level in the 60 µg/g group (Fig. 1). The O^-_2 value dropped from 0 to 60 µg/g Mn, then increased from 60 to 100 µg/g Mn. Total SOD, GPX, and CAT activity were higher in supplemented groups than in the control. Activity of all three enzymes rose from 0 to 60 µg/g, dropped from 60 to 100 µg/g, and significantly differed from the control. SOD, GPX,

and CAT activity in the 60 μg/g group was 114%, 133%, and 91% higher, respectively, than in the control.

Table 2. Mean growth rates of *Neocaridina heteropoda* fed dietary manganese for 1 month.

	Mn (μg/g)					
	0 (control)	*20*	*40*	*60*	*80*	*100*
Length (%)	16.0±2.5	21.0±2.6*	36.8±3.8*	42.0±4.5*	32.6±2.4*	26.7±1.9*
Weight (%)	14.1±1.2	23.1±1.3*	30.3±2.7*	38.9±2.3*	33.4±1.7*	23.3±1.3*

* Significantly different from the control at $p<0.05$.

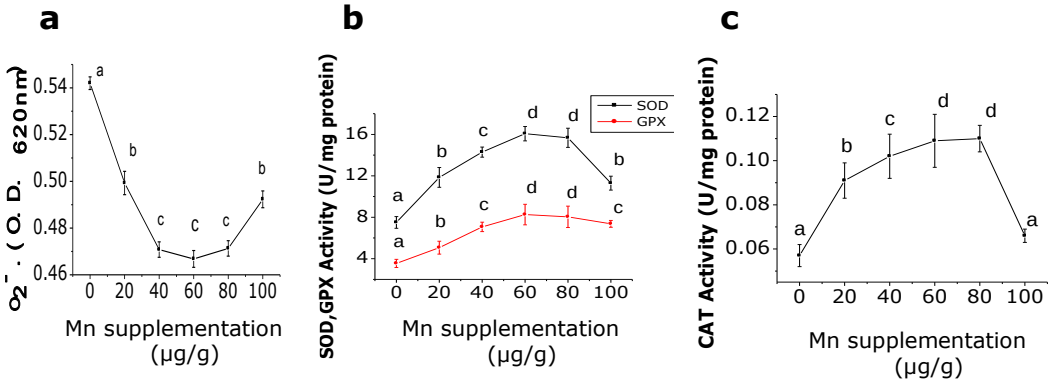

Fig. 1. The effect of diets supplemented with different concentrations of Mn on (a) O^-_2 value, (b) SOD and GPX activity, and (c) CAT activity of cherry shrimp (*Neocaridina heteropoda*). Different superscripts indicate significant differences at $p<0.05$ (means±SD, n = 6).

Discussion

After 30 d, Mn supplementation caused a significant increase in specific growth rate of shrimps compared to the control. Greater growth was observed in common carp (Ogino and Yang, 1980) and gibel carp (Pan et al., 2008) that received Mn-supplemented diets than in fish that received a diet without such supplementation.

In our study, the reduction of O^-_2 and increased activity of SOD, GPX, and CAT indicate that adequate protection against oxidative stress can be achieved by a moderate dose of Mn. Similarly, total hepatic SOD activity increased significantly with increased dietary manganese ($p<0.05$) while the condition factor (body wt/body length) decreased significantly ($p<0.05$) in juvenile gibel carp (Pan et al., 2008). However, excessive amounts of Mn promote the production of high levels of O^-_2. Mn-SOD activity in the tissue can be used as a specific sensitive indicator of the condition of body Mn nutrition

and Mn nutrition requirements (Zidenbergcherr et al., 1983). However, the exact mechanism of effects of dietary Mn supplementation on Mn-SOD should be further investigated.

In our study, the adequate dietary Mn requirement for maximum growth and improvement of antioxidant enzyme activities for *N. heteropoda* was 60 μg/g. The minimum Mn requirement for common carp and rainbow trout is 13 μg/g (Ogino and Yang, 1980). The optimum Mn requirement is 70-140 μg/g for shrimp (*Penaeus vannamei*) over 1 g, but Mn supplementation is unnecessary (Liu and Lawrence, 1997). The requirements for dietary trace elements in fish or shrimp is dependent upon their availability and utilization, the dietary source and form of the element ingested, the adequacy of stores within the body, interactions with other mineral elements present in the gastro-intestinal tract and body tissues, and finally by element interactions with other dietary ingredients or their metabolites.

In summary, it is necessary to supply Mn in the diet of *N. heteropoda*. We found that Mn treatment was effective in enhancing the activities of SOD, CAT, and GPX. Our work suggests that the adequate dietary Mn requirement for growth and improvement of antioxidant enzyme activities for *N. heteropoda* is 60 μg/g. Further research is needed to investigate the molecular mechanism of Mn effects on the antioxidant enzyme response of *N. heteropoda*.

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