Effect of copper toxicity on the hemolymph factors of the Indian edible oyster, *Crassostrea madrasensis* (Preston)

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ABSTRACT

The Indian edible oyster, *Crassostrea madrasensis* collected from the backwaters of Kochi, India were exposed to three sub-lethal concentrations of copper *viz.* 0.1, 0.5 and 1 ppm at a salinity of 12 ppt. Modulations in different heamolymph factors such as total hemocyte count, differential hemocyte count, phagocytosis, serum protein, serum acid phosphatase, serum phenol oxidase and serum lysozyme were studied. It was found that, the lowest concentration of copper had a stimulating effect on all the parameters studied. However, at higher concentrations except serum lysozyme all the hemolymph parameters studied showed significant reduction. Although copper could act as an immunostimulant at lower doses, it significantly reduced the disease resistance of the species at concentrations above 0.5 ppm in mesohaline waters.

Keywords: Copper toxicity, *Crassostrea madrasensis, Hemolymph factors, Indian edible oyster*

Introduction

Blood parameters have been recognized as valuable tools in assessing the physiological conditions of organisms and their response to physicochemical changes in the environment (Jyothirmayi and Rao, 1987). Among invertebrates, the defense systems of molluscs and insects have been studied extensively in order to understand the basics of invertebrate immune system. Immunity of molluscs is provided by cellular and humoral mechanisms. Cell-mediated immunity is the function of hemocytes, while humoral immunity is provided by the serum factors. There are many evidences, which suggest that these two are interrelated. In molluscs, fluctuation in environmental conditions and presence of additives in the environment compromise both their cell-mediated and humoral immune mechanisms (Cheng, 1990).

Copper is an ingredient in many fungicides that are extensively used in highlands of India for plantation crops. These pollutants get washed off during monsoon months and are commonly encountered in coastal waters. The presence of these xenobiotic compounds would affect the health of all animals in these systems and oysters being sedentary, would be more susceptible to the action of these compounds. In the present study, an attempt was made to find out the changes that occur in some of the hemolymph factors in response to copper exposure in the Indian edible oyster, *Crassostrea madrasensis*, which is an important species used for farming in coastal waters of India.

Materials and methods

Maintenance of experimental animals

The edible oyster, *C. madrasensis* (mean size 6.4 ± 1.2 cm x 4.3 ± 0.8 cm and mean weight 85.5 ± 2.3g) were collected from the backwaters of Kochi around Vypeen Island. The experiments were carried out in tanks of 50 l capacity and throughout the course of study, the animals were fed *Chaetoceros* sp. *ad libitum*. The salinity was maintained at 12 ppt based on preliminary studies by the same authors (George *et al.,* 2001). Other water quality parameters were maintained at optimum by adequate water exchange.

Exposure to copper

The lethal concentration of copper (LC_{50}) was found to be 5 ppm at a salinity of 12 ppt for *C. madrasensis* (Gijo Ittoop *et al.,* 2005). Thus for the experiment, three sub-lethal doses *viz.,* 0.1 ppm, 0.5 ppm and 1.0 ppm were selected along with the control. The amount of copper in the water used for the experiment was nil. Appropriate amounts of $CuSO_4.5H_2O$ was dissolved in water of 12 ppt salinity to get the required concentrations of copper ion. Each treatment had three replicates of 15 animals each. The animals were exposed to copper at different concentrations for four weeks before taking the hemolymph for the study.

Hemolymph collection

The hemolymph was collected from adductor muscle sinuses by the method followed by Chen (1996). A notch was filed on the dorsal side of the shell valve, adjacent to adductor muscle. About 0.5 ml to 2 ml of hemolymph was collected aseptically from the adductor muscle of each animal using a 27-gauge needle attached to a 5 ml sterile syringe and it was immediately stored at 4° C.

Total and differential hemocyte count

The total and differential hemocyte counts were estimated according to the method of Nakayama *et al.* (1997) using May-Gruenwald's eosin-methylene blue stain in a hemocytometer using phase contrast microscope. For differential count, a total of 200 cells were counted and the percentage of each type of cells such as granulocytes, semigranulocytes and hyalinocytes was calculated.

Phagocytosis

Phagocytic and endocytic indices were determined by following the method of Bayne *et al.* (1979) with modifications, using formalin inactivated yeast as the substrate. Hemocyte monolayers prepared on glass slides were incubated with yeast suspension in 2% seawater for 60 min at 25° C. The slide was rinsed with 2% seawater, fixed in 10% methanol, air dried and stained with Giemsa for 20 min. It was then differentiated in acetone and mounted using DPX. The slides were observed using oil immersion objective in a phase contrast microscope.

Total serum protein

The cells were separated from serum by centrifuging at 2600 *g* at 4°C. The total protein concentration of serum was estimated following the method of Lowry *et al.* (1951) with bovine serum albumin as standard. The result was expressed as $\mu g\ ml^{-1}$ of serum.

Serum acid phosphatase

The acid phosphatase in the serum was treated with 0.01 M disodium phenyl phosphate and the phenol released was allowed to react with 0.6% of amino-antipyrene in 2.4% ferric cyanide solution. Optical density (OD) was recorded at 510 nm. The amount of phenol released per 100 ml serum was determined from a standard curve constructed using known amount of phenol (Varley, 1980). The results were expressed in KA units (mg phenol released per 100 ml serum per hour).

Serum phenol oxidase

The method of Preston and Taylor (1970) was modified for the purpose. The test serum (0.3 ml) was added to 2.7 ml of 0.01 M L-dopa in 0.05 M tris - HCl buffer at pH 7.5. Sodium dodecyl sulphate was added to the serum

(1mg ml^{-1}). The increase in OD of the sample in the next one minute was noted at 420 nm. The phenol oxidase activity was calculated as increase in serum protein (Δ OD min^{-1} mg^{-1} of serum protein).

Serum lysozyme

The method of Parry *et al.* (1965) was employed with modification for estimating serum lysozyme. Lyophilised *Micrococcus lysodeikticus,* 0.2 mg was suspended per ml of 0.05 M sodium phosphate buffer of pH 6.2 to give an O D of 0.6 at 530 nm. Fifty microlitre of serum was taken in 5 ml cuvette and the above suspension was added to give a final volume of 5 ml. Same amount of 0.05 M buffer added to the culture suspension, served as control. The decrease in the absorbance of the solution between 0.5 min and 4.5 min was read at 530 nm. The unit of lysozyme activity is defined as the amount of sample causing a decrease in absorbance of 0.001 per minute (Lysozyme unit).

Results

Total hemocyte count

The mean hemocyte counts in different groups of animals treated with varying concentrations of copper are shown in Fig. 1. In all treatment groups, the hemocyte count was significantly (p<0.5) decreased compared to the control groups.
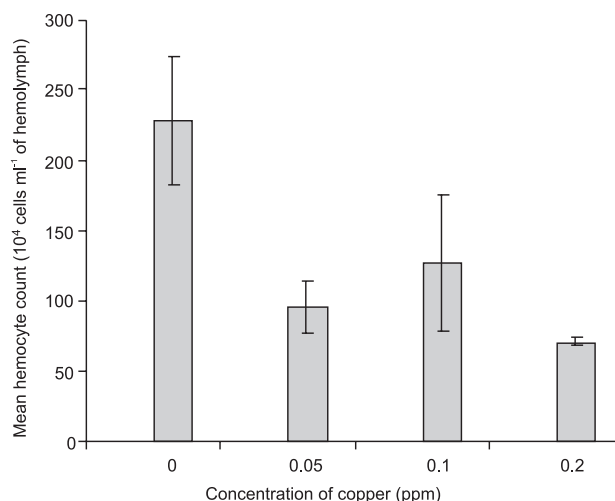

Fig. 1. Mean hemocyte count of *C. madrasensis* exposed to different concentrations of copper

Differential hemocyte count

The differential hemocyte counts recorded for different copper concentrations are presented in Fig. 2. The percentage granulocyte values obtained for all the copper treated groups were significantly (p<0.05) lower compared to the control. The percentage of semigranulocytes was

significantly (p<0.05) high in all the treatments with copper. The copper exposure resulted in a reduction in the percentage of granulocytes and increase in the percentage of semigranulocytes and hyalinocytes.
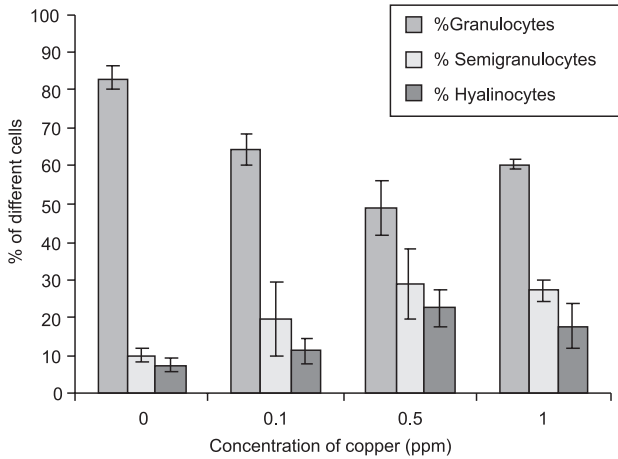

Fig. 2. Mean percentage of different cells in the hemolymph of *C. madrasensis* exposed to different concentrations of copper

Phagocytosis

The values obtained for phagocytic index are given in Fig. 3. The phagocytic index showed a slight increase at the lowest concentration of 0.1 ppm of copper. But at 0.5 ppm and at 1 ppm of copper, there was significant reduction (p< 0.05) in the phagocytic index. It was noted that 0.1 ppm of copper had a stimulating effect on the endocytic index. The value obtained at this concentration was significantly high (p< 0.05) compared to that at 0 ppm copper. When the concentration increased to 0.5 ppm, the mean endocytic index was significantly (p< 0.05) reduced (Fig. 4).
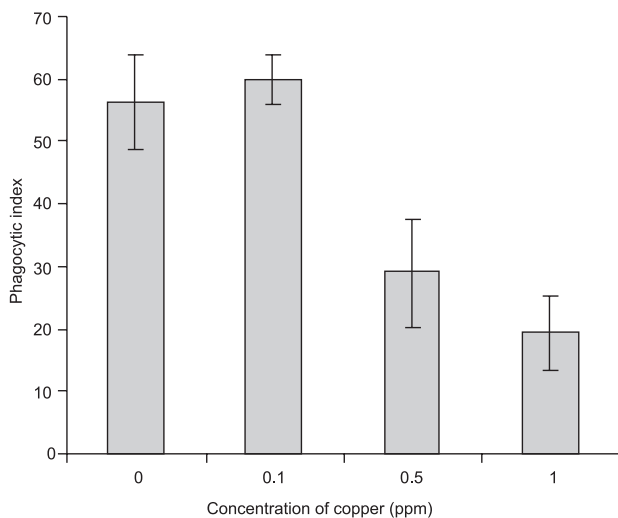

Fig. 3. Mean phagocytic index of hemocytes of *C. madrasensis* exposed to different concentrations of copper
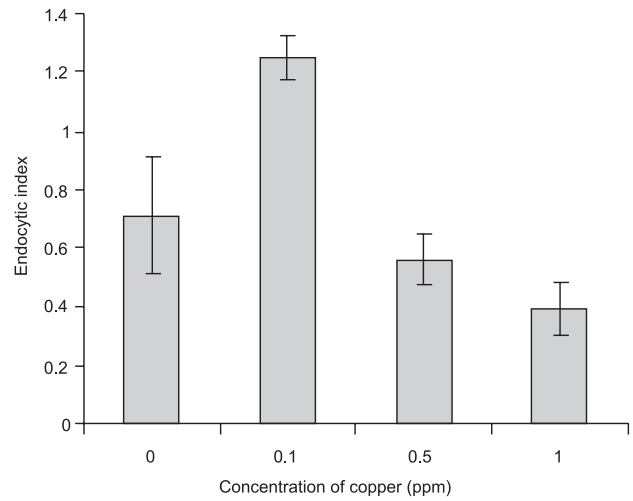

Fig. 4. Mean endocytic index of hemocytes of *C. madrasensis* exposed to different concentrations of copper

Total serum protein

The mean total serum protein concentrations of hemolymph at different concentrations of copper are given in Fig. 5. The values increased significantly (p< 0.05) at 0.1 ppm compared to the control. At 0.5 ppm, the value decreased significantly (p< 0.05) and lowest value was obtained at the highest concentration of copper (1 ppm).
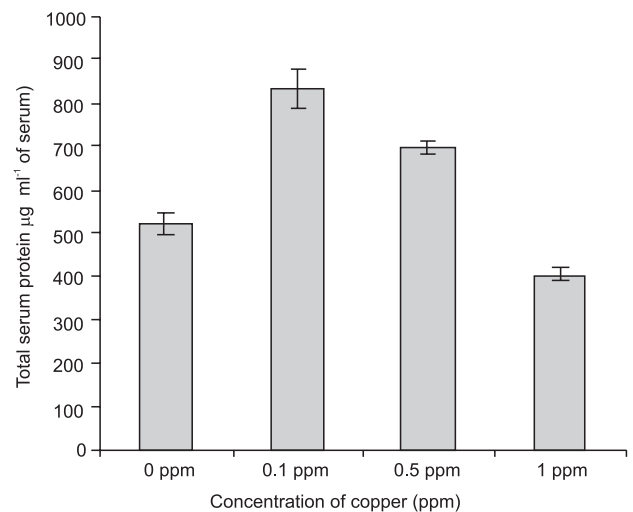

Fig. 5. Mean serum protein of *C. madrasensis* exposed to different concentrations of copper

Serum acid phosphatase

The mean serum acid phosphatase values of *C. madrasensis* exposed to different concentrations of copper are presented in Fig. 6. As the copper concentration increased, the amount of serum acid phosphatase was significantly (p< 0.05) decreased.
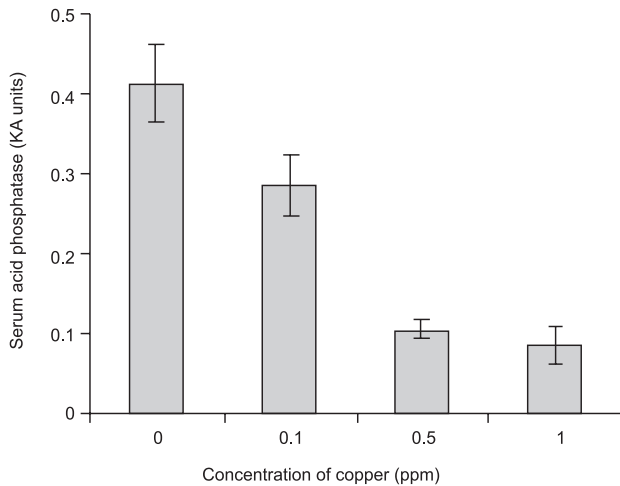
Fig. 6. Mean serum acid phosphatase of *C. madrasensis* exposed to different concentrations of copper

Serum phenol oxidase

The serum phenol oxidase in all the treatments with copper was significantly (p< 0.05) low compared to that of the control (Fig. 7).
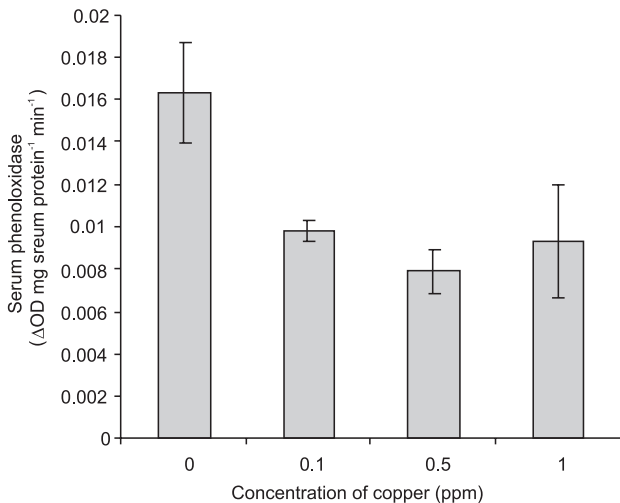

Fig. 7. Mean serum phenoloxidase of *C. madrasensis* exposed to different concentrations of copper

Serum lysozyme

At a concentration of 0.1 ppm, the amount of lysozyme in the serum of the *C. madrasensis* showed an increase followed by a fall in the values at 0.5 ppm and 1ppm concentrations of copper. However, the result was not statistically significant at 5% level.

Discussion

There are several studies which indicate that salinity plays a major role in the toxicity of copper to molluscs. These studies point to the fact that the copper toxicity has less impact at higher salinity. According to Cheng (1988a), an exposure to 1 ppm copper does not alter hemocyte number at 30 ppt in *Crassostrea virginica.* In another study by Suresh and Mohandas (1990), the hemocyte count decreases significantly in *Villorita cyprinoides* var. *cochinensis* at 15 ppt, when exposed to 1 ppm copper. Philips (1977) and Elfing and Tedegren (2002) have also reported high toxicity of copper at low salinity. Thus within the same group of organisms there was difference in sensitivity to different metals in different species (Sauvé *et al.,* 2002). This difference may be because of the ability of the low saline water to maintain the metals in solution or suspension. Because of the lower uptake of copper at high salinity (30 ppt) and higher uptake of copper at low salinity (15 ppt) the effect of copper would be more pronounced at low salinity (Cheng, 1988a). Since the present study was conducted at a salinity of 12 ppt, the effect of copper on almost all of the parameters studied were highly significant.

In most of the earlier studies, total hemocyte count has been reported to increase by heavy metal pollution (Pickwell and Steinert, 1984; Pipe *et al.,* 1995, 1999; Fisher *et al.,* 2000). A significant increase in the percentage of granulocytes (Pickwell and Steinert, 1984) and a significant decrease in the percentage of hyalinocytes (Cheng, 1988a) are reported in bivalves exposed to copper. These results are contrary to the results obtained in the present experiment. It may be that, since the present set of experiments were carried out at a lower salinity of 12 ppt, the uptake of copper by the animal tissue would have been more, thereby resulting in increased toxic effect on the animal. According to Cheng (1988a), copper is lethal to the hemocytes. This may be the reason for the decreased hemocyte count and decreased percentage of granulocytes observed in the present experiment. George *et al*. (1983) also have reported a decrease in hemocyte count in cadmium exposed *Ostrea edulis*. According to Cheng (1990), since granulocytes are more actively phagocytic, a reduction in the percentage of granulocytes in the hemolymph would affect the animals' immunity.

The effect of exposure to heavy metal on phagocytosis in molluscs depends on the species on which the study is conducted (Sauvé *et al.,* 2002) and also on the metal used and its concentrations (Cheng and Sullivan, 1984; Cheng, 1988b). The result obtained in the present experiment is similar to that reported by Cheng (1988b) in *C. virginica.* According to him, copper inhibited phagocytosis, but stimulated endocytosis. In the present experiment, the endocytic index has increased at low concentration of copper, while it decreased at higher concentration of copper. The stimulation of phagocytosis at low concentration of heavy metals and its suppression at higher concentration are reported (Cheng and Sullivan, 1984). The reduction in

the percentage of granulocytes may be one of the reasons for reduced phagocytosis (Balquet and Poder, 1985).

The exposure to copper resulted in a significant decrease in the acid phosphatase concentration in *C. madrasensis*. In contrast, increased serum acid phosphatase is reported in copper exposed bivalves by Cheng and Mohandas (1985) and Suresh and Mohandas (1990). This may be because of the difference in the concentrations of copper used and also the difference in the salinity of the water used. Inhibition of the release of acid phosphatase from granulocytes in copper stressed bivalves is also reported (Cheng 1989; 1990). During the present investigations, since the percentage of granulocytes was significantly reduced at higher concentrations of copper, it has to be assumed that the ability of the hemocytes to synthesize the enzyme has reduced, which subsequently resulted in the decreased amount of acid phosphatase in the serum. The exposure to copper resulted in a significant reduction in the amount of serum phenol oxidase in all the treatments. An increase in the percentage of cells containing phenol oxidase is reported in *M. edulis* (Coles *et al.*, 1994). The decrease in phenol oxidase observed in the present study may be because of decreased synthesis of the enzyme in the hemocytes or inhibition of its release into serum. In copper exposed animals, there was no significant change in the serum lysozyme. The result is in agreement with the report of Cheng (1989) in *C. virginica*. This indicates that copper is not having any effect on lysozyme synthesis and its release into the serum.

Thus the present study revealed that copper at a concentration higher than 0.5 ppm could significantly reduce the immune response of the animals leaving it highly susceptible to diseases. The findings of the present study emphasize the need for screening of the culture environments for copper levels so as to reduce the occurrence of diseases which would in turn result in better production.

Acknowledgements

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