

RESEARCH ARTICLE

Cadmium Nanoparticle Induced Histological and Biochemical changes in Hepatopancreas of Mud Crab *Scylla olivacea* (Herbst, 1796)

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Abstract

Toxic effects of Cadmium nanoparticle (CdNP) (100 nm) on the hepatopancreas of mud crab *Scylla olivacea* was evaluated in this study. Crabs were exposed to different concentrations of CdNP (from 0 to 120 ppm/kg) for 8 d. The toxicity study revealed that LD₅₀ values of both male and female of *S. olivacea* were 40 and 60 ppm/kg of crab on day 6. CdNP induced abnormal structural changes in the hepatopancreas such as extensive vacuolation, necrotic lamellar lesion formation and haemocytic infiltration. In addition, CdNP also induced antioxidant enzymes such as Superoxide dismutase (SOD), Glutathione Peroxidase (GPx) and Catalase (CAT) which initially increased and subsequently decreased with increasing day of CdNP exposure.

Keywords: Cadmium nanoparticle, mud crab, hepatopancreas, toxicity study, antioxidant enzymes.

Introduction

Ecotoxicology is the evaluation of risk for an ecosystem exposed to environmental stress including contamination. Although physico-chemical parameters are essential for risk determination, the results of biological response to chemical stress have been used as references to determine the expected biological damage (Axiak, 1991). Estuaries and coastal zones receive pollutant inputs from both specific and non-specific sources, especially such ecosystems as seaports, cities or other industrialized coastal areas that receive chronic inputs of metals. Since, many species of crustaceans inhabit estuaries; numerous studies have aimed at examining the bioaccumulation and effects of various toxicants in these animals (Weis *et al.*, 1992; Weis and Weis, 1994). Mud crab farming is a recent activity practiced in Bangladesh, China, Indonesia, Malaysia, Singapore, Taiwan, Philippines and Vietnam. In India, crab farming is mainly carried out in West Bengal, Orissa, Andhra Pradesh, Tamil Nadu, Kerala, Karnataka, Goa, Maharashtra and Andaman islands. The mud crabs belonging to the genus *Scylla* are large, fast growing portunids with high commercial value in terms of domestic markets and exports by virtue of their delicacy. Of the three *Scylla* spp. occurring in Indian waters, *S. oceanica*, *S. serrata* and *S. olivacea* are commonly caught. It has been observed that *S. oceanica* is synonymous to *S. tranquebarica*, based on the presence of two spines on the border of the carpus of the cheliped. *Scylla paramamosain* has been identified as another variety of mud crab (Taylor, 1984). Cadmium (Cd), a toxic and non-essential element frequently used in electroplating, pigments, paints, welding and batteries, which results in both biotic and abiotic environments (Ayres, 1992).

Unlike organic compounds, Cd is not biodegradable and has a very long biological half-life (Sugita and Tsuchiya, 1995). Cd has been found to produce wide ranges of biochemical and physiological dysfunctions in humans and laboratory animals (Santos *et al.*, 2004). Many mammalian organs are adversely affected by Cd, which include kidney, liver, testis, lung, pancreas, prostate, ovary, and placenta (Waisberg *et al.*, 2003; Bridges and Zalups, 2005) and several studies have illustrated that the testis is exceedingly sensitive to Cd toxicity (Thompson and Bannigan, 2008; Ji *et al.*, 2010). During the last two decades, there has been an enormous interest in nanomaterials/nanoparticles due to their novel physical and chemical properties that differ markedly from those of bulk materials. Nanoscale materials find use in a variety of different areas such as electronic, biomedical, pharmaceutical, cosmetic, energy, environmental, catalytic and material application even though the current use and production of NP are sparse and often conflicting (Maynard, 2006). The forecasted huge increase in the manufacture and use of NP makes it likely that increasing human and environmental exposure to NP will occur. Most attention has thus far been devoted to the toxicology and health implications of NP (Oberdorster *et al.*, 2006; Kreyling *et al.*, 2006; Lam *et al.*, 2006; Nel *et al.*, 2006), while the behavior of NP in the environment (Biswas and Wu, 2005; Wiesner *et al.*, 2006) and their ecotoxicology (Colvin, 2003; Moore, 2006) have been less often studied. However, no systematic description of the effect of NP on living organisms is yet available. Hence, the present study was aimed to study the CdNP induced histological and biochemical changes in hepatopancreas of *S. olivacea*.

Materials and methods

Experimental animals: Fresh samples of both male and female species of *Scylla olivacea* was collected from Pulicate Lake, Pulicate, Tamil Nadu, India. The male and female crabs were maintained separately in tanks with aerator which was (capacity of 1000 L) filled with filtered (0.45 mm pore) sea water. The sea water was changed periodically and crabs were fed with commercial fish feed. The morphological identification and authentication of species was done by a Scientist from Central Institute of Brackishwater Aquaculture (CIBA), Santhome, Chennai, India. Water conditions during acclimatization and the experimental period were at temperature of 25°C, a salinity of 30 ppt, dissolved oxygen (DO) of 5.8-6.5 mg L⁻¹ and a pH of 7.15-7.87, under a 12:12-h light-dark regime with continuous aeration and filtration.

Toxicity tests: The acute semistatic toxicity test was carried out according to the standard methodology described by Food and Agriculture Organization (FAO) (Ward and Parrish, 1982; Reish and Oshida, 1987) and the American Public Health Association (APHA, 1992). Semistatic toxicological bioassays were carried out for 120 h. Different concentrations such as 20, 40, 60, 80, 100 and 120 ppm of CdNP suspension of 100 nm in size (Sigma and Co., Bangalore, India) was injected intraperitoneally per kg of crab weight. Three replicates of at least 10 animals were exposed to the above stated concentrations. One group without CdNP treatment was maintained as control in both male and female separately. The criteria to determine death was the complete absence of movement once the animals were gently touched with a glass rod. Mortality was recorded every 24 h, a period of time after which dead crabs were removed. The experimental conditions (temperature, salinity and pH) of the toxicity test were similar to those found in the environment during the period. A probit analysis was used to estimate the concentration and 95% confidence limits of CdNP that kills 50% of the exposed crab (LD₅₀).

Histology: Experimental crabs were sacrificed and hepatopancreas tissue samples were taken after 2, 4, 6 and 8 d of exposure of 20 ppm/kg of CdNP. Hepatopancreas was carefully dissected out and fixed in 4% buffered formalin, embedded in paraffin, sectioned (8 mm thickness) on a microtome (Microm, HM330, Heidelberg, Germany), stained with hematoxylin and eosin (H and E) and examined with an Olympus microscope (Tokyo, Japan).

Protein extraction and quantification: A known quantity of hepatopancreas of both male and female *S. olivacea* was ground in a pre-chilled mortar and pestle at 4°C with 50 mM phosphate buffer (pH 7.2) amended with 0.01% polyvinyl poly pyrrolidone and 0.001% ascorbic acid in a ratio of 1:3, filtered and centrifuged (6000 x g) to obtain a clear supernatant.

The cell-free supernatant was used as a protein source. Protein content was determined by the method of Bradford (1976) using bovine serum albumin fraction V (Sigma Chemical Co., Bangalore) as a standard.

Catalase (CAT) activity: Catalase activity was determined in hepatopancreas colorimetrically according to Beers and Sizer (1952). The rate of disappearance of H₂O₂ is followed by observing the rate of decrease in the absorbance at 240 nm. The CAT activity was calculated as μM of H₂O₂ consumed/min/mg protein and the result were expressed as Units/mg. protein.

Superoxide dismutase (SOD) activity: The SOD activity was estimated by the method of McCord and Fridovich (1969). Cyt-c reduction was measured for 3 min at 4°C in a 1.5 mL assay mix containing SOD buffer 1 (50 mM KH₂PO₄ and 0.1 mM EDTA at pH 7.8), 10 μM Cyt-c (Sigma), 50 mM xanthine (Sigma, Steinheim, Germany) and XOD (Sigma, Steinheim, Germany) at 550 nm on a Cary 3E UV/Vis double beam spectrophotometer (Varian, Middelburg, Netherlands) equipped with a temperature controlled cell attached to a water bath. The SOD activity was expressed as Units/mg. protein.

Glutathione Peroxidase (GPx) activity: GPx was assayed by method of Rotruck *et al.* (1973). The reaction consisting of 0.2 mL of EDTA, 0.1 mL sodium azide, 0.1 mL of H₂O₂, 0.2 mL of GSH, 0.4 mL of phosphate buffer and 0.5 mL of homogenate was incubated at 37°C for 10 min, the reaction was arrested by the addition of 0.5 mL of TCA and the tubes were centrifuged at 2000 rpm. To the 0.2 mL of supernatant, 3 mL of disodium hydrogen phosphate and 1.0 mL of DTNB were added and the color was read at 420 nm immediately. The activity of GPx was calculated as μM of glutathione oxidize/min/mg protein and the result expressed as Units/mg. protein.

Results and discussion

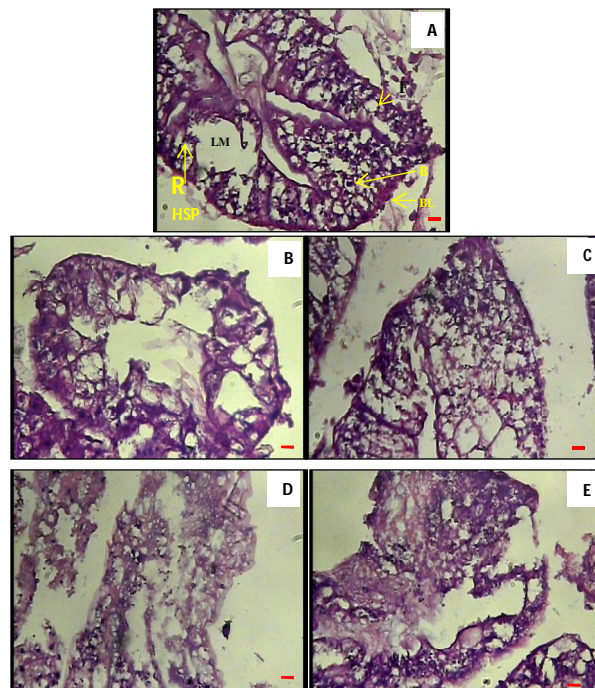
The toxicity study revealed that LD₅₀ values of both male and female of *S. olivacea* were 40 and 60 ppm/kg of crab on day 6. Increasing concentration of CdNP such as 80, 100 and 120 ppm/kg resulted in death of all the crabs after 120 h. Based on this toxicity test, 20 ppm/kg of CdNP was chosen for further experiments. The designer of LD test in 1927 acknowledged its serious inadequacies intending it only for certain narrow medical purposes (Trevan, 1927). Inadequacies was determined by continuous changes in different factors affected Cd toxicity, so it has been well documented that species, age, weight, sex, temperature, pH, animal susceptibility, food in addition to method of by which chemical administrated have marked effect on LD₅₀ results (Wiehe, 1973). Nevertheless, use of the LD test has become widespread as general measure of chemical toxicity and has been challenged for decades as both unreliable and informative criteria.

Thereby, it is useful to reconsider the repeat determination of the LD₅₀ before carrying out laboratory experiments. The hepatopancreas due to its critical role in the digestion of food is richly supplied with hemolymph through main blood vessels that branch out into small capillaries to nourish each individual hepatopancreatic tubule. The whole organ, composed of numerous blind tubules, is ensheathed in a membrane or “tunica propria”, which covers the tubules as well, maintaining the unity of the organ and fastened to other surrounding structures (Gibson and Barker, 1979).

Our histological study revealed each tubule of the hepatopancreas of both male and female *S. olivacea* was surrounded by haemal spaces that contained haemocytes and apparent blood vessels (Fig. 1A and 2A). The hepatopancreatic tubules were enclosed by a basal lamina with central lumen. Three types of epithelial cells have previously been recognized in these tubules: R-cells, F-cells and B-cells (Ceccaldi, 1998) (Fig. 1 and 2 A). R-cells, the most common cell type observed, were characterized by variable numbers of cytoplasmic vacuoles. The F-cells were distinguished by their deeply staining basophilic cytoplasm. Unlike the other cells of the tubules, mature B-cells bordered the lumen with a vacuolar apical complex and had no apparent connection to the basal lamina. Injection of CdNP resulted in extensive vacuolation, necrotic lamellar lesion formation and haemocytic infiltration of the hepatopancreas in both male and female of *S. olivacea* compared to control (Fig. 1B-E and 2B-E). Similar results have been reported in response to chemical (Anderson *et al.*, 1997; Bhavan and Geraldine 2000), bacterial exposure (Bowser *et al.*, 1981) and sub-lethal levels of cadmium (Victor 1993, Soegianto *et al.*, 1999a), lead (Victor, 1994) and copper (Soegianto *et al.*, 1999b).

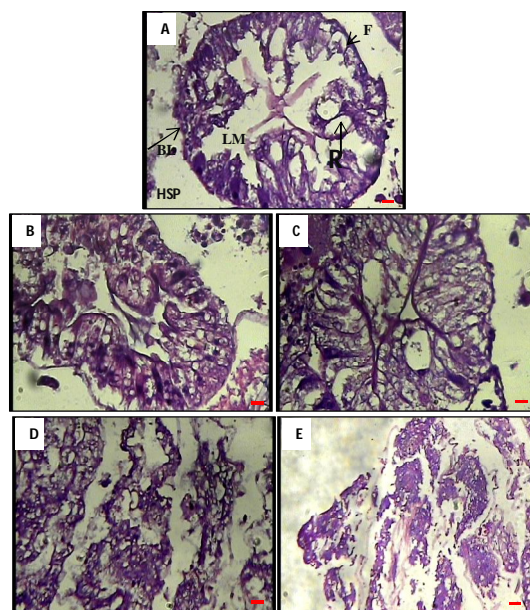
SOD and CAT are the two primary enzymes for radical scavenging, involved in protective mechanisms within tissue injury following oxidative process and phagocytosis and their activities are related to the status of the organisms affected by different factors including dietary nutrition, environmental factors etc. Usually, higher SOD and CAT activities indicate that there are more radicals need to be reacted. In the present study, the response of catalase to CdNP is varied from male to female *S. olivacea*. In male, CdNP resulted in a exposure-dependent increase in the activity of catalase and a maximum increase of 20% was observed on day 2 compared to control whereas, maximum catalase activity up to 50% was recorded on day 6. In female, CdNP resulted in up to 30% increase in catalase activity was observed on day 2 compared to control, whereas maximum catalase activity up to 55% was recorded on day 6. There was a decrease in catalase activity on day 8 in both male and female crabs exposed to 20 ppm/kg of CdNP but still above control levels (Fig. 3 and 4).

Fig. 1. Effects of CdNP on the ultrastructure of hepatopancreas in male *S. olivacea* by light microscope. Tubules containing R-cells (R), F-cells (F) and B-cells (B) with underlying basal lamina (BL), lumen of tubules (lm) and inter-tubular (haemal) spaces (hsp). Scale bar-10 µm.



A. Control; B. 2nd d; C. 4th d; D. 6th d; E. 8th d after CdNP exposure.

Fig. 2. Effects of CdNP on the ultrastructure of hepatopancreas in female *S. olivacea* by light microscope. Tubules containing R-cells (R), F-cells (F) and B-cells (B) with underlying basal lamina (BL), lumen of tubules (lm) and inter-tubular (haemal) spaces (hsp). Scale bar-10 µm.



A. Control; B. 2nd d; C. 4th d; D. 6th d; E. 8th d after CdNP exposure.

Fig. 3. Catalase activity in hepatopancreas of male *S. olivacea* after exposure of CdNP.

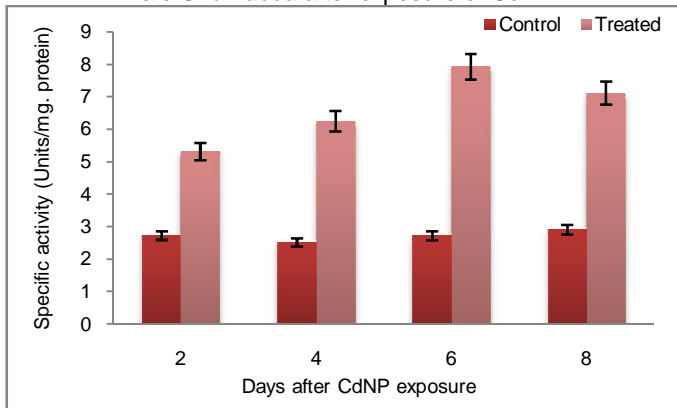


Fig. 6. Superoxide dismutase (SOD) activity in hepatopancreas of female *S. olivacea* after exposure of CdNP.

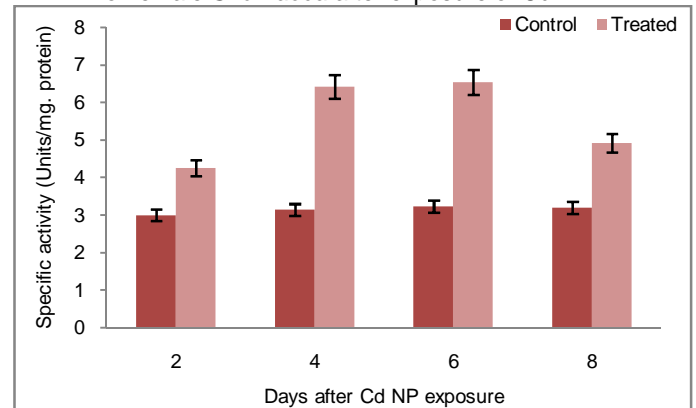


Fig. 4. Catalase activity in hepatopancreas of female *S. olivacea* after exposure of CdNP.

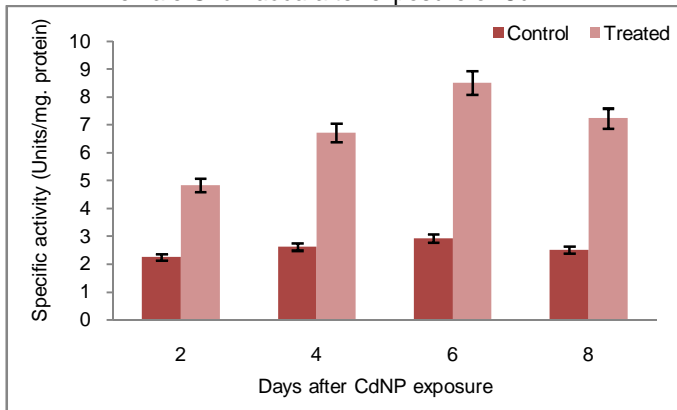


Fig. 7. Glutathione peroxidase (GPx) activity in hepatopancreas of male *S. olivacea* after exposure of CdNP.

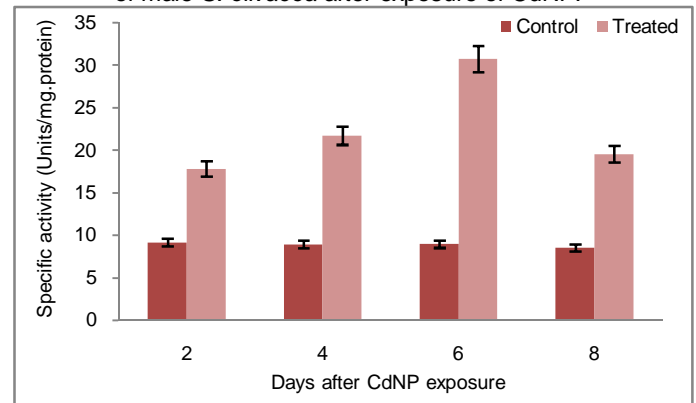


Fig. 5. Superoxide dismutase (SOD) activity in hepatopancreas of male *S. olivacea* after exposure of CdNP.

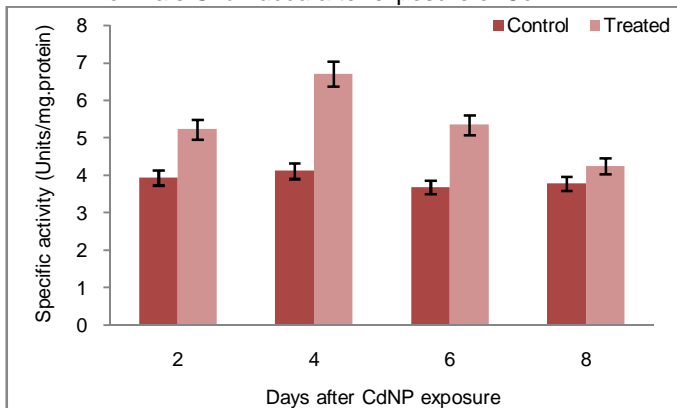
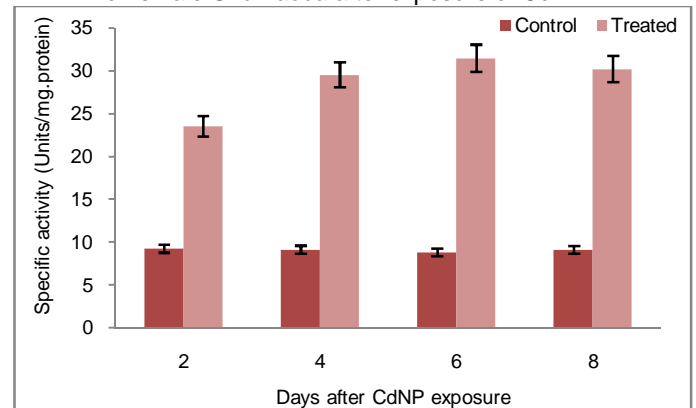


Fig. 8. Glutathione peroxidase (GPx) activity in hepatopancreas of female *S. olivacea* after exposure of CdNP.



It was noticed that CdNP increased the level of SOD gradually in hepatopancreas of both male and female crabs as the days of exposure up to day 4 and day 6 respectively (Fig. 5 and 6). In male, the SOD activity increased up to 5% and 10% on day 2 and day 4 respectively, whereas, in female it was 10% and 20% on day 2 and day 6 respectively. Therefore, significantly higher SOD and CAT activities might indicate that the stress resulted in an accumulation of radicals to a higher level in crustaceans (Winston and Giulio, 1991).

Therefore, the enhanced activities of both SOD and CAT at may enable crabs to maintain health by scavenging the radicals produced. The adaptive mechanism may be partially explained by the increasing activities of SOD and CAT for scavenging the radicals produced in a certain extent (Messaudi *et al.*, 2010). In male crabs, glutathione peroxidase activity was 10% higher on day 2 compared to control and the maximum of 40% increase was recorded on day 6 compared to their respective control after CdNP exposure (Fig. 7).

On the contrary, female crabs showed 40% increase in enzyme activity even on day 2 and maximum of 43% activity was recorded on day 4 and was maintained up to day 8 (Fig. 8). However, our study also revealed that longer exposure of CdNP i.e., 8 d resulted in decreased activities of SOD, CAT and GPx indicating that the scavenging function of antioxidant activities was impaired under prolonged exposure of Cd (Blanco, 2007). The present study clearly demonstrated that acute exposure to CdNP led to cell death in the hepatopancreas of mud crab, which may lend strong support to the conclusion that acute exposure to CdNP results in a cumulative and/or progressive hepatopancreas injury and induce antioxidant defence.

Conclusion

In the present study, we made an attempt to study CdNP induced structural and biochemical changes in hepatopancreas of mud crab *Scylla olivacea*. Based on the results obtained, we can conclude that CdNP alters both structural integrity and biochemical defence in hepatopancreas of the mud crab.

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