

RESEARCH ARTICLE

Histological and Biochemical Changes in Reproductive Organs of Mud Crab *Scylla olivacea* (Herbst, 1796) Exposed to Cadmium Nanoparticle

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Abstract

Cadmium (Cd) is one of the most toxic environmental and industrial pollutants, known to exert gonadotoxic and spermiotoxic effects. In this study, we have investigated the toxic effect of Cadmium Nanoparticle (CdNP) (100 nm) on testis and ovary of mud crab *Scylla olivacea*. Crabs were exposed to different concentrations of CdNP (0-120 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. Oxidative stress and apoptotic changes in the testes were detected. Histopathology of ovary showed pycnosis of nutritive cells and shrinkage of oocyte, destruction of epithelial layer, degeneration of oocytes and vacuolization was also observed in the periphery of the oocyte cells upon CdNP exposure. The activities of SOD, GPx and CAT were increased in both testis and ovary.

Keywords: Cadmium, *Scylla olivacea*, toxicity study, oxidative stress, apoptotic changes, CdNP exposure.

Introduction

Cadmium (Cd) is one of the most toxic heavy metals for humans. The main source of non-occupational exposure to Cd includes smoking and air by which food and water are contaminated by Cd (Nagata *et al.*, 2005). In addition, herbal medicine is another source of Cd. World Health Organization (WHO) estimates that 4 billion people or 80% of the world population presently use herbal medicine (Naithani *et al.*, 2010). In addition, Cd is a common inorganic contaminant of coastal sediments and waters due to anthropogenic pollution and natural sources (Sokolova *et al.*, 2004; Ivanina *et al.*, 2010). It can be accumulated in aquatic animals (e.g. crabs, shrimps, oysters and mussels) after entering through different ways such as respiratory tract, digestive tract, surface penetration etc. (Dailianis and Kaloyianni, 2004; Li *et al.*, 2008). It is seriously harmful to the growth of aquatic life and survival resulting in decline of their populations. At the same time, aquatic food products and animals exposed to Cd might threaten human health. Ecotoxicology is the evaluation of risk for an ecosystem exposed to environmental stress including contamination. Although physico-chemical parameters are essential for risk determination, during the past decade, the results of biological response to chemical stress have been used as references to determine the expected biological damage (Axiak, 1991). Mud crab farming is a recent activity and is practiced in south Asian countries including India. In India, crab farming is being mainly carried out in West Bengal, Orissa, Andhra Pradesh, Tamil Nadu, Kerala, Karnataka, Goa, Maharashtra and Andaman and Nicobar islands.

The mud crabs belonging to the genus *Scylla* are large, fast growing portunids with high commercial value in terms of domestic markets and export by virtue of their delicacy. Of the three *Scylla* species occurring in Indian waters, *Scylla oceanica*, *S. serrata* and *S. olivacea* are commonly caught. During the last two decades, there has been an enormous interest in nanoparticles (NP) due to their novel physical and chemical properties that differ markedly from those of bulk materials. Nanoscale materials find use in a variety of 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 effects of NP on living organisms is yet to be investigated. Hence, the present study was designed to investigate the biochemical and morphological changes on the testis and ovary of *Scylla olivacea* after CdNP exposure.

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 tissue samples of ovary and testis were taken after 2, 4, 6 and 8 d of exposure of 20 ppm/kg of CdNP. The ovary and testis were 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 ovary and testis of *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 of ovary and testis were determined 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.

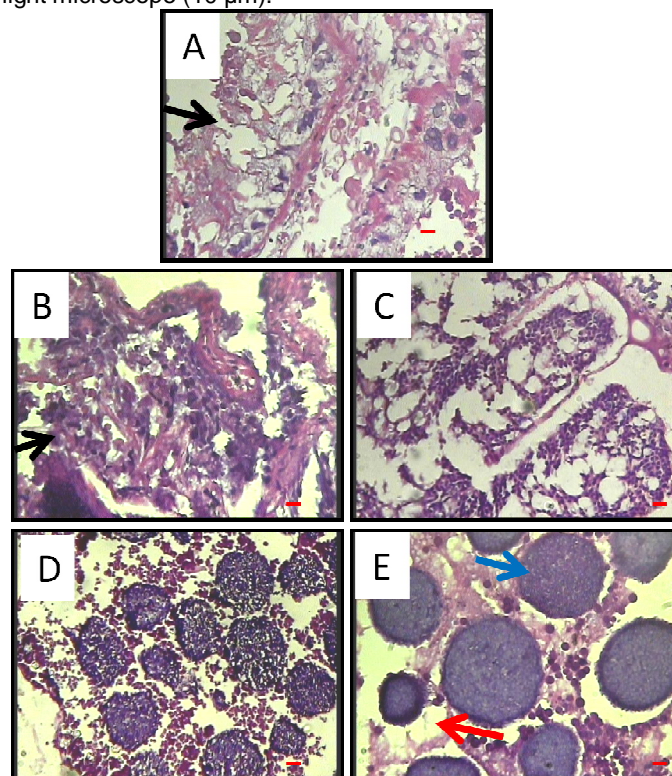
Results and discussion

In the present study, an attempt was made to study the effect of CdNP on the toxicity, histology and antioxidant activity in *S. olivacea*. The toxicity study revealed that LD₅₀ values of both male and female of *S. olivacea* were 40 and 60 ppm/kg of crab respectively on day 5 (Kavitha *et al.*, 2013). Based on this toxicity test, 20 ppm/kg of CdNP was chosen for further experiments. Cadmium exerts adverse effects on structures and functions of reproductive organs directly at the testis level or by altering post-testis events such as sperm progress motility and/or function (viability), all of which may culminate in hypogonadism and infertility (Koizumi and Li, 1992; Shaikh *et al.*, 1999; Xu *et al.*, 2001; Santos *et al.*, 2004; Thompson and Bannigan, 2008). Oxidative stress, which is induced by reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide and hydroxyl radical is known to play a critical role in testis injury (Stohs *et al.*, 2000; Aydilek *et al.*, 2004; Murugesan *et al.*, 2005).

In the present study, our investigation demonstrated that exposure to CdNP induced histopathological changes of testis in a time-dependent manner. The light microscopy examination of the testis of the control crabs showed normal structure as evidenced by well-organized distribution of cells in the seminiferous epithelium (Fig. 1A). Cd treatment resulted in vacuolar degeneration appeared in the spermatogenic epithelium and as the day progresses there was an extensive degeneration of testis. On day 6, the seminiferous tubules were decreased and the seminiferous epithelia were atrophied compared to control. On day 8, the testis exhibited extensive necrosis associated with impaired spermatogenesis as well as edema in the interstitial space (Fig. 1B-E). Machale *et al.* (1990) studied that cuprous oxide exposure induced significant alternations in the ovary of the crab *Barytelphusa guerini*. Reddy *et al.* (1982) reported rupturing of oocyte, vacuolization, irregular arrangement of oocyte and disappearance of nucleus were observed in freshwater crab *Barytelphusa cunicularis* during sub-lethal exposure of heavy metal. In this study, ovaries of control crabs were covered with outer thin epithelium and inner germinative epithelial layer from which the oocytes proliferate. The oocytes are covered with a layer of follicle cell. The ooplasm is compactly arranged with thick yolk granules. Ovarian follicles are filled with different types of maturing oocytes (Fig. 2A). On day 2 after CdNP exposure, pycnosis of nutritive cells and nucleus of oocyte was observed due to shrinkage of oocyte. Destruction of epithelial layer and degeneration of oocytes were seen, epidermal layer was loosely arranged and vacuolization was also observed in the periphery of the oocyte (Fig. 2B, C). As the day progressed, it brought damage to ovarian layer, destruction of follicular epithelial layer and vacuolization towards the periphery of oocytes. On day 8, ovary showed irregular shape of oocytes, mixing of ooplasmic material due to disintegration of follicular epithelium, maximum nature of degenerating oocytes with disintegrated nuclei was observed (Fig. 2D, E).

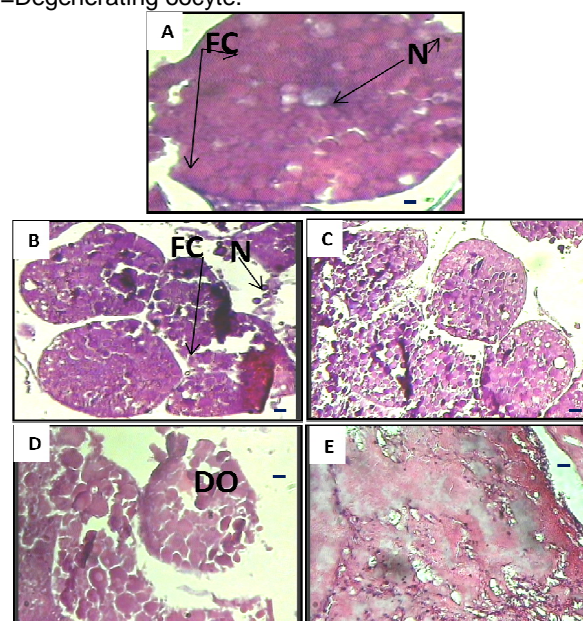
SOD and CAT are the two primary enzymes for radical scavenging which are involved in protective mechanisms within tissue injury following oxidative process and phagocytosis and their activities are related to the status of the organisms affected by different factors including dietary nutrition, environmental factors etc. Usually, higher SOD and CAT activities indicate that there are more radicals need to be reacted. In the present study, the differential response of catalase by ovary and testis to CdNP was observed in *S. olivacea*. In ovary, CdNP exposure resulted in a time-dependent increase in the activity of catalase up to day 6. A maximum of three fold increase in activity was observed on day 6 compared to control (Fig. 3) whereas, in the case of testis, maximum of two fold increase was recorded on day 8. But, testis recorded a time-dependent increase in catalase activity up to day 8 unlike ovary (Fig. 4).

Fig. 1. Effects of CdNP on the testis of *Scylla olivacea* by light microscope (10 μ m).



(A) Testis of the control group showing normal structure, (B) Treated with 20 ppm/kg CdNP on day 2, vacuolar degeneration appeared in the spermatogenic epithelium (arrow), (C, D) day 4 and 6 CdNP group, the number of sperms in the lumina of the seminiferous tubules was decreased, (E) Day 8 CdNP group, the germinal layer in the seminiferous tubules exhibited extensive necrosis (blue arrow) as well as edema in the interstitial space (red arrow).

Fig. 2. TS of ovary of *Scylla olivacea* after exposed to CdNP by light microscope (10 μ m). FC=Follicular cells; N=Nucleus; DO=Degenerating oocyte.



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

Fig. 3. Catalase activity (CAT) activity in ovary of *Scylla olivacea* after CdNP exposure.

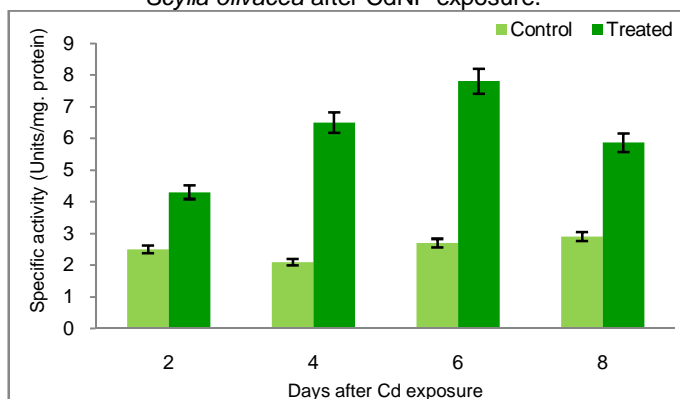


Fig. 6. Superoxide dismutase (SOD) activity in testis of *Scylla olivacea* after CdNP exposure.

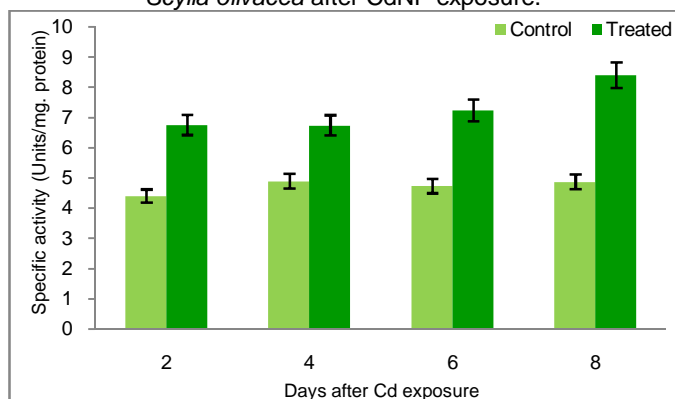


Fig. 4. Catalase activity (CAT) activity in testis of *Scylla olivacea* after CdNP exposure.

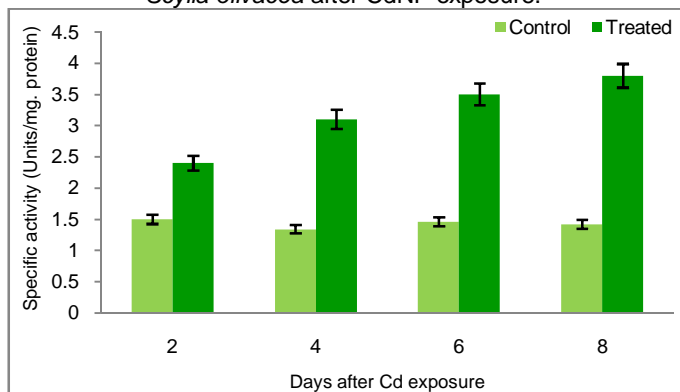


Fig. 7. Glutathione peroxidase (GPx) activity in ovary of *Scylla olivacea* after CdNP exposure.

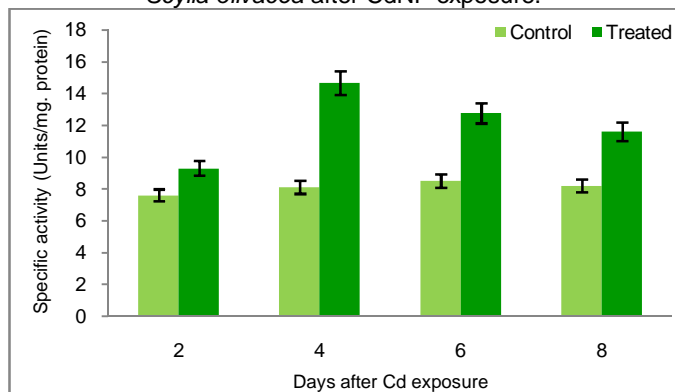


Fig. 5. Superoxide dismutase (SOD) activity in ovary of *Scylla olivacea* after CdNP exposure.

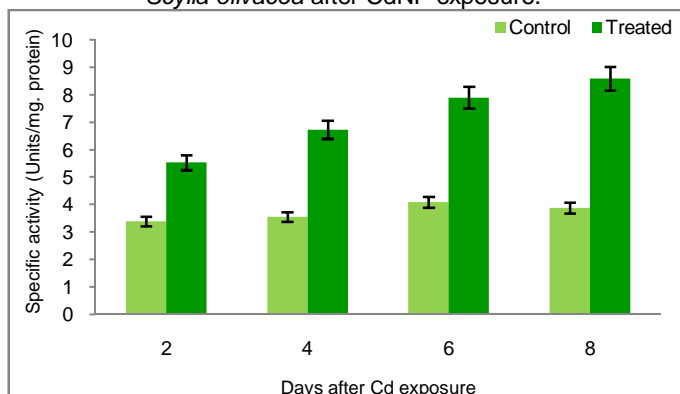
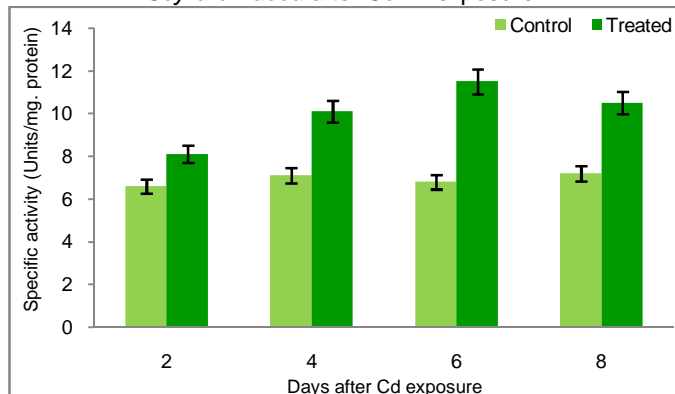


Fig. 8. Glutathione peroxidase (GPx) activity in testis of *Scylla olivacea* after CdNP exposure.



There was a decrease in catalase activity on day 8 in ovary of female crabs exposed to 20 ppm/kg of CdNP but still above control levels. It was noticed that CdNP gradually increased the level of SOD in both ovary and testis up to day 8. In ovary, the SOD activity was increased two and three fold on day 6 and day 8 respectively (Fig. 5) whereas, in testis, the maximum of 20% increase was observed on day 8 (Fig. 6). 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 may enable crabs to maintain the reproductive organs 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 at a certain extent (Messaoudi *et al.*, 2010). In ovary, Glutathione peroxidase (GPx) activity was only 5% higher on day 8 compared to control and time-dependent increase was recorded throughout the study period compared to their respective control after CdNP exposure (Fig. 7).

On the contrary, testis showed 12% increase in enzyme activity even on day 6 and maximum of 15% activity was recorded on day 8 (Fig. 8). However, our study also revealed that longer exposure of CdNP i.e., 8 d resulted in decreased activities of CAT and GPx in ovary and testis indicating that the scavenging function of antioxidant enzymes was impaired under prolonged exposure of Cd (Blanco, 2007).

Conclusion

We made an attempt to study CdNP induced structural and biochemical changes in both ovary and testis of mud crab *Scylla olivacea*. Based on the results obtained, we noticed that CdNP alters both structural integrity and biochemical defence in reproductive organs of the mud crab. To conclude, this study clearly demonstrated that acute exposure to CdNP led to cell death in the ovary and testis of mud crab, which may lend strong support to the conclusion that acute exposure to CdNP results in a cumulative and/or progressive injury of both testis and ovary and also induce antioxidant defence.

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